# Heat shock protein 70 and bovine paratuberculosis

## Heat shock eiwit 70 en bovine paratuberculose

*(met een samenvatting in het Nederlands)*

## Proefschrift

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***Aan wijlen mijn grootouders,***
*voorbeelden van wijsheid en ruimdenkendheid*

***Aan mijn vader en in liefdevolle herinnering aan mijn moeder,***
*m'n nature en nurture*

***Aan Henk, Annebelle en Marieke,***
*m'n alles*

ABBREVIATIONS

| | |
|---|---|
| **APC** | Antigen Presenting Cell(s) |
| **BCA** | BiCinchoninic Acid |
| **Bomac** | Bovine macrophages |
| **BSA** | Bovine Serum Albumin |
| **CD** | Cluster of Differentiation (*e.g.* CD8) |
| **Con A** | Concanavalin A |
| **CTL** | Cytotoxic T Lymphocyte |
| **DC** | Dendritic Cells(s) |
| **ER** | Endoplasmatic Reticulum |
| **FACS** | Fluorescence Activated Cell Sorter |
| **FITC** | Fluorescein IsoThioCyanate |
| **GFP** | Green Fluorescent Protein |
| **GM-CSF** | Granulocyte Monocyte-Colony Stimulating Factor |
| **HEYM** | Herrold's Egg Yolk Media |
| **Hsp** | Heat shock protein(s) |
| **IFN**–$\gamma$ | Interferon gamma |
| **IL-** | Interleukin- |
| **IPTG** | Isopropyl-Beta-D-ThioGalactopyranoside |
| **IU** | International Unit |
| **LAL** | Limulus Amebocyte Lysate |
| **LB** | Luria-Bertani |
| **LOX-1** | Lectin-like Oxidized LDL receptor |
| **LPS** | LipoPolySaccharide |
| **LST** | Lymphocyte Stimulation Test |
| **M.O.I.** | Multiplicity Of Infection |
| **Map** | *Mycobacterium avium subspecies paratuberculosis* |
| **MDM** | Monocyte Derived Macrophage |
| **MFI** | Mean Fluorescence Intensity |
| **MHC** | Major Histocompatibility Complex |
| **NF-$\kappa$B** | Nuclear Factor- $\kappa$B |
| **Ni-NTA** | Nickel Nitrilo-Triacetic Acid |
| **NO** | Nitric Oxide |
| **OVA** | Ovalbumin (chicken egg white) |
| **PAMP** | Pathogen Associated Recognition Pattern |
| **PBMC** | Peripheral Blood Mononuclear Cell |
| **PBS** | Phosphate Buffered Saline |
| **PCR** | Polymerase Chain Reaction |
| **PP** | Peyer's Patches |
| **PPD** | Purified Protein Derivative |
| **R:T** | responder to target ratio |
| **rbd** | receptor binding domain |
| **RT-PCR** | Reverse Transciptase-Polymerase Chain Reaction |
| **sbd** | substrate binding domain |
| **SD** | Standard Deviation |
| **SDS-PAGE** | Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis |
| **SI** | Stimulation Index |
| **TACO** | Tryptophan Aspartate Containing Coat |
| **TAP** | Transporter associated with Antigen Processing |
| **Taq** | Thermus aquaticus |
| **TCR** | T Cell Receptor |
| **TGF-$\beta$** | Transforming Growth Factor-$\beta$ |
| **TLR** | Toll-like Receptor |
| **TNF-$\alpha$** | Tumor Necrosis Factor-$\alpha$ |

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## General Introduction

HSP MAP

More than a century has passed since the first case of paratuberculosis (or Johne's disease) in cattle was described but the ins and outs of the disease are still far from being elucidated (Valentin-Weigand and Goethe, 1999). Despite the seriousness of economic losses for cattle industry (Ott et al., 1999), exceeding the costs of other veterinary relevant diseases like bovine virus diarrhoea (BVD) (Chi et al., 2002) and despite intensive efforts to eradicate the disease in The Netherlands (Benedictus et al., 2000), paratuberculosis remains a serious problem (Stabel, 1998).

Different approaches to investigate such a disease are imaginable. Epidemiological studies, economic cost/benefit studies of prevention programmes, clinical case descriptions of diseased animals, etc.. In this study, paratuberculosis is investigated from an immunological point of view. For a better understanding of the experimental work, three main items are highlighted in this general introduction: paratuberculosis, cytotoxic $CD8^+$ T cells (CTL) and heat shock proteins (Hsp).

# Paratuberculosis

## The pathogen

Paratuberculosis is caused by *Mycobacterium avium subspecies paratuberculosis* (*M.a.p.*), a pathogenic acid-fast bacterium that grows slowly under laboratory circumstances, belonging to the *Mycobacterium avium (M.avium)* complex (Stahl and Urbance, 1990). The bacterium is particularly resistant to environmental influences and survives long periods in soil, food and water (Sweeney, 1996; Whittington et al., 2004). Depending on the methods applied, it was also possible to culture the bacterium from pasteurized milk (Lund et al., 2002).

Compared to other mycobacteria, there is a 99.9% homology to *M.avium*, 98.1% homology to *M.bovis*, and more than 95% homology to other mycobacteria, based on 16S rRNA sequences (Stahl and Urbance, 1990).

*Mycobacterium a. paratuberculosis* is pathogenic for ruminants in general, although some strain-specificity has been demonstrated for different ruminant species (Motiwala et al., 2003). Laboratory animals can be infected with *M.a.p.* when administered by injection (Cocito et al., 1994), but although the bacteria might replicate in these species (Cocito et al., 1994), disease manifestations are not consistent (Harris and Barletta, 2001). Attempts to orally infect Lewis rats did not lead to disease nor to shedding of the bacteria, although immunological responses were measured (Koets et al., 2000b). The possibility to orally infect rabbits with *M.a.p.*, albeit with high doses of bacteria compared to infection of calves (Harris and Barletta, 2001), raised the question as to whether wild rabbits would represent a reservoir for infection of domesticated ruminants. As indeed rabbits were demonstrated to excrete *M.a.p.* on pasture and grazing cows did not avoid contaminated areas (Beard et al., 2001; Greig et al., 1999), this finding gave rise to general concern (Dixon, 2002). However, these studies are far from proving the ingestion of *M.a.p.* during pasture of adult cows as an important route of transmission of the pathogen.

## The disease

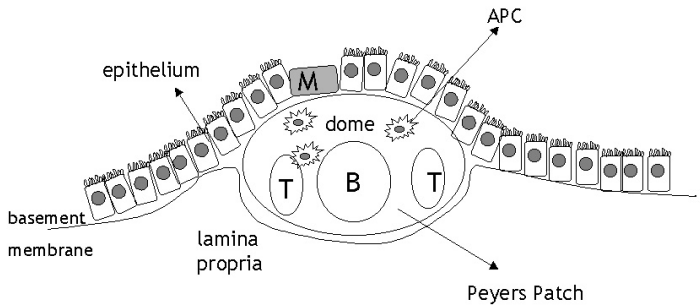
***Clinical aspects and transmission of paratuberculosis***

Paratuberculosis (Johne's disease) of cattle is the result of the infection of an animal with *M.a.p.* that succesfully invades the host gut, where it resides and replicates, leading to chronic inflammation of the intestine and thereby to impaired function, resulting in protein loss leading to cachexia and ultimately death of the host (Clarke, 1997; Cocito et al., 1994; Stabel, 1998).

The most important way of transmission is the fecal-oral route, young calves being the most sensitive to infection (Cocito et al., 1994). Ingestion of the bacteria occurs by drinking infected milk or by suckling or licking the contaminated environment (Sweeney, 1996). After infection a period without clinical signs follows, the duration of which is inversely related to the dose of infection (Whitlock and Buergelt, 1996; Whittington and Sergeant, 2001). When finally the animal starts to demonstrate clinical signs of disease, this has been preceded by a period of shedding bacteria by apperently healthy animals (Cocito et al., 1994). Although not all animals develop clinical disease (Cocito et al., 1994), harm is done by those asymptomatic shedders that contribute to the vicious circle of infection and contamination of the environment. Animals with overt signs of paratuberculosis suffer from chronic diarrhoea, weight loss, decreased milk production, edema, anemia and related symptoms of a generalised infectious process (Clarke, 1997; Cocito et al., 1994; Stabel, 1998).

***Establishment of disease at a microscopic level***

Once ingested, the bacteria pass through the gastro-intestinal tract to the ileum (Whitlock and Buergelt, 1996). The common theory is that *M.a.p.* bacteria are taken up in the ileum by M cells and transported to the underlying macrophages (Whitlock and Buergelt, 1996). M cells are cells of an as yet uncompletely determined ontogeny that serve as antigen sampling cells (Sigurethoardottir et al., 2004). They may develop from undifferentiated dome-associated crypt cells under the influence of lymphocytes, or from fully differentiated enterocytes (Clark and Jepson, 2003). The main task of these specialized cells of the follicle-associated epithelium (FAE) that covers the Peyer's patches (PP) is to transport antigen from the gut lumen to the subepithelial and intraepithelial phagocytosing cells without degrading it (Momotani et al., 1988). The more specific targeting of *M.a.p.* to M cells might occur via opsonization with fibronectin (Sigurethoardottir et al., 2004).




M cells, in contrast to epithelial cells, have a high density of integrins at their apical surface, which bind fibronectin. *M.a.p.* on its turn is also described to bind fibronectin and the variable pH of the gastro-intestinal tract might activate the bacteria to bind the fibronectin that is present in bile secretions in the duodenum (Sigurethoardottir et al., 2004). In this way, fibronectin would serve as a bridge to facilitate uptake of *M.a.p.*. However, alternate routes of uptake, not via M cells, might exist. Invasion of damaged areas of intestinal epithelium by *M.a.p.*, or uptake by migrating dendritic cells (Clark and Jepson, 2003; Granucci and Ricciardi-Castagnoli, 2003) are as yet undetermined modes of entry for paratuberculosis. A study by Bannantine (Bannantine et al., 2003) suggests the 35 kD major membrane protein (MMP) of *M.a.p.* as a virulence factor of the bacteria, involved in invasion of intestinal epithelial cells.

Whatever way of entrance, once across the epithelium, the bacteria are taken up by macrophages. Macrophages are the host cells for *M.a.p.*, were the bacteria reside and replicate (Clarke, 1997; Harris and Barletta, 2001; Sigurethoardottir et al., 2004; Valentin-Weigand and Goethe, 1999; Whitlock and Buergelt, 1996), despite the function of those cells to serve as a line of defense in host immunity against invading microbes (Janeway CA, 1999; Roitt, 1991). This is the first scene for the cat and mouse play between host immune system and pathogen.

***M.a.p. mechanisms to deviate the macrophage immune machinery***

Typically, pattern recognition receptors (PRR) on macrophages sense microbes, after which activation of the macrophage membrane occurs. Pseudopods form around the microbe, leading to ingestion (phagocytosis) by sequestration of a part of the membrane surrounding the particle into the cytoplasma of the cell. The so-called phagosome fuses with late endosomes and lysozomes that lower the pH of the resulting phago-lysozome and contain numerous bactericidal factors. Hydrolytic enzymes finally digest the killed microbes. The

process of phagocytosis by itself also provokes an oxygen burst, that leads to the production of oxygen derived bactericidal compounds, like superoxide and nitric oxide. Finally, bacterial degredation products are released to the extracellular environment. At the same time, antigenic peptides are presented at the surface of the macrophage by major histo-compatibility complex (MHC) class II molecules, together with co-stimulatory molecules of which expression is induced by the same receptors that recognized the microbes. Presentation of antigenic peptides and co-stimulation activates specific T cells, that in turn further activate the macrophage. By then, the a-specific uptake of microbes, an aspect of the innate immunity, has provoked a specific, adaptive, T cell reaction. Besides by direct cell-cell contact of the macrophage and the T cell, the macrophage is also influenced by cytokines, that are produced by both the macrophage itself upon triggering through phagocytosis, and the activated T cell (Janeway CA, 1999; Pieters, 2001; Roitt, 1991).

*M.a.p.* obviously found ways to resist this cascade of detrimental events. Compared to the phagocytosis of *E.coli* or latex beads, different genes are expressed upon *M.a.p.* phagocytosis by bovine macrophages (Tooker et al., 2002). The following section describes what is known about immune evasion of *M.a.p.* at the different steps of the macrophage reaction. Only few evasive mechanisms have been shown to be specifically related to *M.a.p.*, others are a matter of extrapolation of what is known from other mycobacteria or other intracelullar bacteria (Pieters, 2001; Rosenberger and Finlay, 2003; Russell, 1995; Valentin-Weigand and Goethe, 1999).

*Receptors for entry*

The first gap in knowledge on *M.a.p.*-macrophage interaction is two-fold; how does *M.a.p.* specifically into macrophages and which receptors are used to avoid subsequent degradation? *M.tuberculosis* was demonstrated to use complement receptors, mannose receptors, scavenger receptors and surfactant protein A receptors (Ernst, 1998), but not CD14 for invasion of human macrophages (Shams et al., 2003). Preincubation with serum increased uptake of *M.a.p.* by bovine macrophages (Zurbrick and Czuprynski, 1987), indicating that in *M.a.p.* infection at least opsonizing receptors are involved. Interaction with Toll-like receptors (TLR) on the macrophage via pathogen-associated recognition patterns (PAMP) on the mycobacteria is most likely to occur via TLR2 and TLR4 (Werling et al., 2004), and leads to the induction of reactive oxygen and nitrogen intermediates, production of pro-inflammatory cytokines like IL-1β, IL-6, IL-12, GM-CSF, TNF-α, and IFN–γ, and upregulation of co-stimulatory molecules (Werling et al., 2004). The TLR however are not indispensable for phagocytosis (Means et al., 2001).

*Phagosomes*

Impairment of phagosome maturation and phagosome-lysozome fusion is a well known evasion mechanism of mycobacteria (Hope et al., 2004). Live mycobacteria are able to retain tryptophan-aspartate-containing coat protein (TACO), a protein present on the phagosome coat, that is normally released before fusion with lysozomes (Pieters, 2001). Prevention of phagosome-lysozome fusion not only hides the bacteria from direct bactericidal mechanisms, but also from degradation and subsequent presentation to cells of the adaptive immune system (Ferrari et al., 1999; Pieters and Gatfield, 2002).

Lipids seem to play an important role in phagocytosis and phagosome maturation. Cholesterol in the cell membrane is indispensable for the internalisation of mycobacteria (Gatfield and Pieters, 2000; Pieters, 2001; Pieters and Gatfield, 2002), while several other lipids influence, both positively and negatively, phagosomal function and mycobacterial growth (Anes et al., 2003). The widely recommended addition of polyunsaturated fatty acids like σ-3 lipids to the human diet has even been questioned, as it seems to enhance mycobacterial growth (Anes et al., 2003). The role of lipids on phagosomes is related to

activation or inhibition of actin assembly (Anes et al., 2003).

The process of phagosome maturation inhibition was shown for live bacteria, but not for (heat-)killed bacteria. Therefore, the bacterial excretion of substances that actively inhibit the maturation process is suspected (Anes et al., 2003; Pieters, 2001; Pieters and Gatfield, 2002). However, the involvement of TLR as shown by Blanders et al.(Blander and Medzhitov, 2004) could shed new light on this presumption. They demonstrated that only phagosomes that contained cargo that was engaged by TLR were subject to inducible maturation, and that this TLR engagement involved the Myd88- dependent activation of p38 mitogen-activated protein kinase (MAPK). Phagosome-lysozome fusion inhibition would thus not represent an active process, but a passive consequence of the receptors involved during phagocytosis of the bacteria.

*Apoptosis?*

Is macrophage apoptosis helpful for *M.a.p.* survival, or in contrast beneficial to the host? Conflicting lines of thought about the consequences of apoptosis have been exposed in the literature on other mycobacteria and other pathogenic intracellular bacteria.

A generally accepted idea is that virulence of mycobacteria is inversely related to apoptosis induction (Greenwell-Wild et al., 2002; Means et al., 2001). Indeed, uptake of the avirulent *M.avium* induced expression of apoptosis regulatory genes in human macrophages (Greenwell-Wild et al., 2002), while more virulent mycobacteria like *M.tuberculosis* would prevent apoptosis, by that means maintaining the infected habitat. Apoptosis would be more advantageous to the host, as it would trap bacteria in apoptotic bodies, thereby reducing dissemination from infected macrophages, and apoptotic macrophages containing *M.tuberculosis* would be rapidly phagocytosed and destroyed by uninfected macrophages (Means et al., 2001). On the other hand, a study by Rojas et al. (Rojas et al., 2002) provided evidence for the activation of cell signaling events by virulent *M.tuberculosis* leading to apoptosis. Induction of apoptosis by other virulent intra-cellular pathogens like *Salmonella typhimurium* was also shown and explained as advantageous for the pathogen, as it leads to a silent death, with uptake by other phagocytic cells without the risk of a subsequent inflammatory response (Yrlid and Wick, 2000). Thus, apoptotic death of the host cell would promote bacterial spreading to other cells. However, these data on *Salmonella*, together with data on *M.tuberculosis* (Schaible et al., 2003) provide evidence for yet another mechanism of host defence: apoptotic cells and vesicles thereof are an important source of antigen for another type of phagocytic cell, the dendritic cell (DC). DC are able to take up antigen contained in apoptotic bodies and present this in the context of their MHC class I molecules to cytotoxic $CD8^+$ T cells (CTL), a process called cross-priming or cross-presentation (Albert et al., 1998; Boes and Ploegh, 2004; Fadok and Chimini, 2001; Green and Beere, 2000). Via this detour mechanism antigen specific T cells are activated, that in the first place will produce interferon-γ (IFN–γ), important for the activation of macrophages (Schaible et al., 2003). Summarizing the different studies, one would conclude that macrophage apoptosis is a host defence mechanism to be escaped by *M.a.p.*, if infection is to be continued.

*Antigen presenting and co-stimulatory surface molecules*

A next step in immune evasion is the influence of the mycobacteria on the antigen presenting and co-stimulatory molecules on the macrophage surface. *M.tuberculosis* infected macrophages lose the capacity to present mycobacterial antigens and to activate $CD8^+$ T cells (Schaible et al., 2003). Bovine macrophages infected with *M.a.p.*, in contrast to *M.a.avium*, have impaired capacity to stimulate T cells, because of the down-regulation of MHC class I and MHC class II molecules (Weiss et al., 2001). The mechanism by which *M.a.p.* suppresses MHC presentation was not elucidated, but was presumably not mediated by a suppressive factor excreted by the bacteria. Cell wall components of the bacteria might be involved and MHC class II down-regulation was therefore expected to be a non-specific event related to

the phagocytosis of mycobacteria (Weiss et al., 2001). Furthermore it was demonstrated that addition of exogenous IFN–γ could not upregulate MHC class I and class II expression on infected macrophages, nor were infected macrophages killed by primed autologous lymphocytes (Weiss et al., 2001). The impaired T cell stimulating capacity of macrophages infected with *M.a.p.* but not with *M.a.avium* was also demonstrated when the murine macrophage J774 cell line was used (Zur Lage et al., 2003). Stimulation of a specific $CD4^+$ T cell line was significantly inhibited, although MHC class II molecule expression in these experiments was not altered. Differences in the expression of co-stimulatory molecules could not account for the impaired antigen presentation either, at least for the molecules measured in those experiments no differences in expression were found (Zur Lage et al., 2003). However, changes might be subtle, as was shown for the impaired capacity of DC to stimulate T cells, despite unaffected MHC-peptide molecules, when cell membrane organisation was perturbed (Kropshofer et al., 2002).

*Cytokines*

Immunoregulatory cytokines play a key role in linking innate and adaptive immunity and the immune response to a given antigen is not only inherent to the antigen but also determined by the environment in which the antigen is encountered (Murtaugh and Foss, 2002). The major decision to react to invading organisms lies with the cells of the innate immune system, such as the macrophages (Janeway and Medzhitov, 2002). It is also believed that the (dys)balance of regulatory cytokines is of central importance to the outcome of certain diseases (Lucey et al., 1996). Therefore, the mechanism of immune evasion of *M.a.p.* will likely also involve the cytokines produced upon macrophage invasion.

In response to cell wall components of *M.a.p.*, freshly isolated monocytes produce TNF-α, interleukin (IL)1-β and IL-6 (Adams and Czuprynski, 1994). Upon phagocytosis of both *M.a.p.* and *M.avium*, the murine macrophage cell line J774 produced TNF-α, IL-6 and IL-12 in comparable amounts after two days of culture, and no differences between viable or killed *M.a.p.* bacteria were observed with regard to cytokine production (Zur Lage et al., 2003). However, bovine monocyte derived macrophages (MDM) infected with either *M.a.p.* or *M.avium* showed different patterns of gene expression for several cytokines when measured at different time-points up to 96 hours *post infectionem* (*p.i.*)(Weiss et al., 2002). MDM infected with *M.a.p.* were not able to sustain IL-12 expression, IL-10 expression was consistently higher and TNF-α expression was lower in comparison to *M.avium* infected macrophages (Weiss et al., 2002). Moreover, TNF-α bioactivity was lower. As IL-12 plays a central role in the induction of a type 1 response, required for effective anti-mycobacterial immunity (Demangel and Britton, 2000), failure to sustain IL-12 expression might have important consequences for the induction of specific acquired immunity (Weiss et al., 2002). Overexpression of IL-10 could be responsible for the repression of TNF-α and IFN–γ expression observed in the same study, while decreased TNF-α activity would lower inflammatory and immune responses (Elenkov and Chrousos, 2002). At the same time, the TNF-α/IL-10 balance is important for macrophage apoptosis or survival (Rojas et al., 1999). A more recent study by Weiss et al. (Weiss et al., 2004) demonstrated increased induction of TGF–β mRNA expression upon infection with *M.a.p.* compared to *M.avium* phagocytosis, consistent with anti-inflammatory capacities of this cytokine and again inferior killing of *M.a.p.* by bovine macrophages compared to killing of *M.avium* (Weiss et al., 2004). These results were confirmed by a study by Khalifeh and Stabel (Khalifeh and Stabel, 2004) for bovine cells, were killing of *M.a.p.* was less efficient when TGF–β and/or IL-10 were added to a six days culture period, even in the presence of IFN–γ producing cells. The production of the pro-inflammatory cytokine IL-6 reported by different groups (Adams and Czuprynski, 1994; Weiss et al., 2004; Zur Lage et al., 2003) might not in all cases lead to enhanced microbicidal activity of infected macrophages, as IL-6 was also demonstrated to interfere with TNF-α

activation of *M.avium* infected macrophages, by down-regulating the receptors for TNF-α (Bermudez et al., 1992).

Although at first glance macrophages seem to react with a pro-inflammatory cytokine profile, the more detailed studies on bovine macrophages and *M.a.p.* phagocytosis point more towards a deviation of host reaction by the bacteria, via production of IL-10 and TGF–β, that hamper IL-12 induced anti-mycobacterial immunity, thereby creating a more friendly environment for bacterial life inside the host cell. These observations are in agreement with data on other pathogenic mycobacteria infecting human macrophages (Greenwell-Wild et al., 2002; Nau et al., 2002). However, as stated in the beginning of this paragraph, the environment in which the antigen is encountered plays a decisive role. The observations described here rely on *in vitro* studies, where only limited variation in cytokine balance is possible. It is imaginable that the local cytokine environment at the level of the intestine in the natural infection will have major influences on macrophage function, impossible to mimic under laboratory circumstances.

*Of cat and mouse; the chicken and the egg; in vivo and in vitro*

Intriguing are the mechanisms by which pathogens are capable to escape their host's immune reactions, be it viruses (Ploegh, 1998) or bacteria (Rosenberger and Finlay, 2003). The question arises as to where variability comes from. Do different hosts react in the same way or differently to different pathogens? Is this difference a difference between individuals or between species of host or pathogen, or maybe between genera of the pathogen, or even the host? Is virulence related to pathogen or to host characteristics? If differences in pathogenicity of different mycobacterial species are related to macrophage capacity of bacterial elimination, then it would be unlogic to extrapolate results of unnatural hosts to natural disease outcome. After all, if phenotypic make-up of the pathogen is so important to the host, why then would the host cell be unimportant to the pathogen in an *in vitro* system that aims at mimicking disease? From an evolutionary point of view, adaptation of microbes to the host species is only of interest in the natural host, because other species are a dead end for them (Merrell and Falkow, 2004). Immunological reactions to an unnatural pathogen might therefore be irrelevant. To better understand host-pathogen interactions, a completely different approach to looking at this interplay has been proposed by Casadevall and Pirofski (Casadevall and Pirofski, 2003) who seek at bringing together microbiology and immunology in the damage-response framework theory to ultimately better understand infectious diseases. In their view, host damage is the relevant parameter by which pathogen virulence should be assessed. More from the host's point of view, others emphasize the importance of investigating and understanding T cell activation *in vivo* to develop strategies for disease prevention (Boes and Ploegh, 2004). For human mycobacterial disease, 'the human model' has been proposed, that investigates the different genetic disorders that lead to vulnerability to mycobacterial disease as a starting point to understand the relation between pathogen and host (Casanova and Abel, 2002). By this model, the crucial role of T cell immunity, NF-κB signalling and the IL-12-IFN–γ axis in human immunity to mycobacterial disease was confirmed.

Although information on what happens at the local level in paratuberculosis is still largely unknown, studies describing the first encounter of *M.a.p.* with the bovine intestine (Sigurethoardottir et al., 2004) together with *in vitro* work on peripheral blood lymphocytes (Bassey and Collins, 1997; Olsen and Storset, 2001; Stabel, 2000) and especially recent microarray experiments comparing gene expression profiles of clinical and subclinical animals with controls (Coussens et al., 2003; Coussens et al., 2002; Coussens et al., 2004), provide more and more insight in the immune responsiveness of cattle against paratuberculosis.

***Immunological responses***

The time-frame of development from *M.a.p.* infection to active bacterial shedding and clinical disease is not known (Clarke, 1997; Cocito et al., 1994; Whitlock and Buergelt, 1996), although exacerbation of disease was demonstrated to be related to altered immunological reaction patterns of the host (Clarke, 1997). Knowledge on immunological reactions against paratuberculosis is based for the larger part on peripheral lymphocyte activity, despite the fact that this might not always accurately reflect disease manifestation at the local level (Chiodini, 1996).

Of the different T cell populations involved in *M.a.p.* infection, specific $CD4^+$ T cells play a major role, mainly by producing IFN–γ, important for the activation of macrophages to kill *M.a.p.* (Bassey and Collins, 1997; Stabel, 2000; Waters et al., 2003a). Probably, in bovine macrophages this killing occurs via another pathway than via nitric oxide (NO) production, as bovine monocytes and macrophages have been shown to be relatively refractory to NO production in response to IFN–γ, compared to cells of other species (Jungi et al., 1996). $CD4^+$ T cells were shown to be the dominant cell population at the site of infection in subclinically infected animals (Stabel, 2000c). Disease progression is characterised by decreased cell mediated responses, related to a loss of $CD4^+$ T cells (Koets et al., 2002) specific for PPD-P and whole bacteria, notably in the lesional ileum. However, a population of $CD4^+$ T cells reactive to *M.a.p.* Hsp70 remained in the ileal lamina propria lymphocytes (LPL) that reacted more vigorously in symptomatic than in asymptomatic animals (Koets et al., 2002).

Together with the loss of $CD4^+$ T cells, a relatively increased frequency of γδ T cells was observed in the ileal LPL (Koets et al., 2002). An immunomodulatory role for these cells was speculated, however, no definite conclusions on their role could be drawn. Chiodini demonstrated in earlier studies (Chiodini and Davis, 1992) that *M.a.p.* specific γδ T cells are cytotoxic for specific $CD4^+$ T cells, by that means causing the disappearance of $CD4^+$ T cells. Although it was concluded from this study that cytotoxicity could be the only mechanism by which the γδ T cells could specifically inhibit the $CD4^+$ T cell responses, figures of restauration of responsiveness by depletion of the γδ T cells are suggestive of recent studies in which the addition of anti-IL-10 monoclonal antibodies to *M.a.p.* reactive PBMC leads to impressively higher responses (Buza et al., 2004). The importance of IL-10 has also been demonstrated by others (Khalifeh and Stabel, 2004), but the source of IL-10 in natural infection has not been described. Combining these data, one could suspect a possible role for γδ T cells in producing IL-10. The γδ T cells themselves are influenced by another subset of lymphocytes, the $CD8^+$ T cells, as described by Chiodini in another paper (Chiodini and Davis, 1993). It demonstrates that the cytotoxic γδ T cells that kill specific $CD4^+$ T cells are under veto of immunomodulatory $CD8^+$ T cells. However, the mechanism by which the three cell types influence each other remains largely unknown.

Besides an immunomodulatory role for $CD8^+$ T cells as described by Chiodini (Chiodini and Davis, 1993), and the statement that cytotoxic $CD8^+$ T cells predominate at the site of *M.a.p.* colonization in later stages of infection (Stabel, 2000c), little is known about the role of specific $CD8^+$ T cells in bovine paratuberculosis. The contribution to IFN–γ production by $CD8^+$ T cells was inferior to that of $CD4^+$ T cells (Bassey and Collins, 1997), but despite the primordial role of cytokine production by T lymphocytes in protection against paratuberculosis (Stabel, 2000), $CD8^+$ T cells may still be relevant.

The waning of cell mediated responses as disease progresses, together with the detection of abundant amounts of specific antibodies, indicating humoral immunity becoming dominant, has been described as the classical Th1 to Th2 shift in immunity to paratuberculosis (Stabel, 2000). However, this dogma needs refinement, because the presence of antibodies was demonstrated to depend on the antigen specificity and the isotype of the antibody (Koets et al., 2001a). Waters et al. (Waters et al., 2003) were also able to detect antibodies as early as two weeks after experimental infection. Possibly the decrease in Th1 responses

predominates over the increase of Th2 responses (Koets et al., 2002b; Koets et al., 2001a). It has been shown that in general the Th1-Th2 dichotomy is less prominent in cattle than in mice (Estes and Brown, 2002).

A review article by Coussens (Coussens, 2004) stressed the importance of the subtle balance between different cytokines produced by the different cells involved. It also gives an explanation on the differences in immune reaction found at the lesional sites and in the periphery, and therefore in cultured PBMC. In his view, a proinflammatory T cell population is found in the lymph nodes draining the lesional sites in early infection. This is followed by the expansion of a noncytotoxic suppressor cell population, producing IL-10. The first population being $CD4^+$ T cells, the second either γδ or $CD4^+$ T cells. This suppressor T cell population helps in limiting tissue damage but at the same time permits the propagation of infection. Proinflammatory cells that are suppressed at the lesional site restore their capacity to produce proinflammatory cytokines once they go into the peripheral blood circulation, away from the IL-10 mediated suppression. In the very late stage of infection, a cytotoxic immunoregulatory cell type develops, that induces apoptosis of the proinflammatory cells. These cells would be type 2 $CD8^+$ T cells or γδ T cells (Coussens, 2004).

***Dendritic cells early in infection?***

A major shortcoming in the knowledge on paratuberculosis concerns the role of the dendritic cell (DC) population in phagocytosis of *M.a.p.* and development of anti-mycobacterial immunity. DC are known not to be just another type of phagocytosing cell, but play a pivotal role in the stimulation of T cells (Banchereau and Steinman, 1998; Lanzavecchia and Sallusto, 2000; Thery and Amigorena, 2001). In relation to mycobacteria, DC were shown to be capable to induce potent Th1 type T cell reactivity (Demangel and Britton, 2000). Cattle DC in response to mycobacteria were demonstrated to be efficient in stimulating T cell responses (Hope et al., 2004). It is also evident that bovine DC respond differently to infection with mycobacteria than macrophages (Hope et al., 2004; Werling et al., 2004). Especially in the lamina propria of the gut, two different types of DC might exist: one resident population that induces T regulatory cells, producing IL-10, in reaction to commensal bacteria, and a population of DC that is recruited to the site of infection during inflammation (Granucci and Ricciardi-Castagnoli, 2003).The latter population phagocytoses bacteria upon infection, migrates to the lymphnodes and activates T cells that help in destroying and eliminating the pathogens (Granucci and Ricciardi-Castagnoli, 2003). What if *M.a.p.* manages to enter the host gut dressed up as a commensal bacterium, thereby preventing the development of anti-mycobacterial immunity and inducing an anti-inflammatory response instead? And what happens with the DC that encounters *M.a.p.* during the chronic infection, when cytokine environment has been altered compared to the initial infection? To understand the different types of T cell reactions during the development of paratuberculosis insight in the reaction of DC at the site of infection is crucial.

***Crohn's disease?***

Whether or not a human chronic inflammatory bowel disease, Crohn's disease, is caused by *M.a.p.* is an ongoing debate (Acheson, 2001; Bernstein et al., 2004; Bull et al., 2003; El-Zaatari et al., 2001; Greenstein, 2003; Motiwala et al., 2003; Seldenrijk et al., 1990). *Mycobacterium a.paratuberculosis* DNA was detected by PCR in up to 90% of Crohn's disease cases (Greenstein and Collins, 2004), however, co-existence of *M.a.p.* and a defect in the human NOD2 gene, involved in resistance against intracellular bacteria, lead Behr et al. (Behr et al., 2004) to refine the idea of a causal connection between Johne's and Crohn's disease. Their idea is that '*M.a.p.* infection results in the Crohn's disease phenotype in a genetically susceptible host' (Behr et al., 2004). Where 'susceptible' has to be read as 'defective' and rare (Young, 2001). Adding to confusion was the finding that Crohn's disease

patients were less likely to have ingested unpasteurized milk in childhood (Bernstein et al., 2004). This might support the hypothesis (Bernstein et al., 2004) that access to various organisms (*e.g.* mycobacteria (Rook et al., 2004)) in childhood could lead to protection against later development of auto-immune diseases (*e.g.* Crohn's disease) (Bernstein et al., 2004). Describing Johne's disease without mentioning Crohn's disease would be a serious omission, however, conclusive data on the subject have not been published so far.

***Disease prevention?***

Vaccination against paratuberculosis, by using heat-killed bacteria in a water in oil emulsion, has been done at a limited scale in The Netherlands (Muskens et al., 2002). However, although clinical signs of disease in infected cattle herds were much less severe or even absent, vaccination does not prevent fecal shedding of the bacteria (Kormendy, 1994). Moreover, long lasting immune reactions interfere with diagnosis of both *M.a.p.* and *M.bovis* infection (Muskens et al., 2002). Potentiating the commercially available vaccine by adding recombinant IL-12 (rIL-12) does not sufficiently improve the vaccine to prevent infection of calves in all cases (Uzonna et al., 2003). At this moment, prevention of paratuberculosis depends on hygiene and management measures at the farm level, but in order to fully eradicate the disease there certainly is a need for vaccination (Benedictus et al., 2000), provided a vaccine can overcome the afore mentioned disadvantages.

# CD8+ T CELLS

The composition of a vaccine efficient in preventing mycobacterial disease is not known. As adressed before we hypothesize that at least one component should be able to induce cytotoxic T cells (CTL) (Feng et al., 2001; Lalvani et al., 1998; Lalvani and Hill, 1998; Silva et al., 2001; van Pinxteren et al., 2000).

Classically, CD8+ CTL confer protection against viral infections (Janeway CA, 1999; Roitt, 1991). Peptides, derived from viral antigen, are presented at the surface of infected cells in the context of MHC class I molecules and recognised by specific CTL. Upon recognition, the CTL causes death of the host cell by inducing apoptosis. This can be achieved by releasing effector molecules like granzymes or perforins, or by ligating to Fas, an apoptosis inducing receptor, at the host cell surface. At the same time, CTL can produce cytokines, *e.g.* IFN–γ. The effector CTL, that can kill several consecutive infected target cells, has been primed by an antigen presenting cell (APC). This APC presented the viral peptide in the context of the MHC class I molecule, together with a costimulatory signal, to naïve T cells, that then became effector cells. By definition, as virus replicates intracellularly, the viral antigen is derived from inside the cell, from the cytosol, and is loaded onto the MHC class I molecule. This is opposed to the induction of CD4+ effector T cells, that recognise antigen in the context of MHC class II molecules. This antigen is derived from foreign antigen (*e.g.* phagocytosed bacteria) that has been taken up by the APC and has been translocated into vesicles (endosomes, phagosomes) inside the cell. Upon degradation, peptide is loaded onto MHC class II molecules and presented to CD4+ T cells (Janeway CA, 1999; Roitt, 1991).

As a complication to the above, CTL were proven to be important cells in protection against intracellular pathogens (Harty and Bevan, 1999; Kaufmann, 1991a, b; Kerksiek and Pamer, 1999; Raupach and Kaufmann, 2001; Silva et al., 2001; Wong and Pamer, 2003). Moreover, not only has the existence been recognised of cytotoxic CD4+ T cells (Silva and Lowrie, 2000) and γδ T cells (Chiodini and Davis, 1992; Egan and Carding, 2000), also were CTL restricted to CD1, a MHC-like molecule, demonstrated (Stenger et al., 1997). CD8+ T cells with another role, suppressive and immunomodulatory, were also detected in mycobacterial infections (Bloom et al., 1992; Salgame et al., 1991).

## CD8+ CTL-MHC class I

Antigen processing and presentation in MHC class I by the APC finally leads to activation of the CD8+ T cell. This process not only determines the specificity of the CTL reaction, but also the phenotype (Ma and Kapp, 2000; Mutis et al., 1994). Different mechanisms have been proposed for bacterial antigen to be processed in the MHC class I presentation pathway. Escape of bacteria to the cytosol (Kerksiek and Pamer, 1999) after entrance in a vacuole, leakage or secretion of bacterial products out of the phagosome (Beatty and Russell, 2000; Harty and Bevan, 1999; Lewinsohn et al., 2001), or cross-priming (den Haan and Bevan, 2001) (see ad: CD8+ CTL-priming). Generally, antigen that is directly introduced into the cytosol, leads to MHC class I presentation (Kaufmann, 1991). Transporter associated with antigen processing (TAP)-dependent and TAP-independent mechanisms (Castellino et al., 2000; den Haan and Bevan, 2001) have been described, depending on the need for degredation prior to presentation (Wick and Ljunggren, 1999). It has been postulated that the number of peptides that is finally available for presentation in the MHC class I molecules (Badovinac and Harty, 2000; Montoya and Del Val, 1999), as well as the nature of the antigen and thereby the MHC binding affinity (Ma and Kapp, 2001) , can determine the strength and/or the phenotype of the following CTL reaction, by determining the avidity of the T cell – APC interaction, although it has also been doubted whether there is physiological evidence for this (Kapsenberg, 2003).

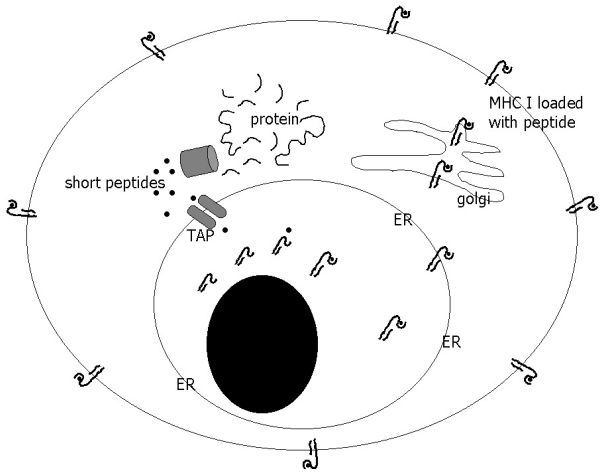



Figure depicting loading of MHC class I molecules. Proteins that are produced in the cytosol are degraded by the proteasome into peptides, that gain entrance to the ER via TAP. In the ER the peptides are loaded onto the MHC molecules and the complex travels, via the Golgi apparatus, to the cell surface where the peptides are presented to T cells.

## CD8⁺ CTL-effector mechanisms

A number of CTL effector mechanisms are available (Janeway CA, 1999); lytic granules, Fas-Fas ligand interaction and cytokine production. Lytic granules are released upon close contact of the CTL and the target cell. The T cell receptor (TCR) interacts with the specific antigen presented in MHC class I on the target cell, which leads to activation of the CTL and rearrangement of its cytoskeleton. This propulses the lytic granules to the site of cell-cell contact, were they are released into the intercellular synapse. Perforins then form pores in the target cell membrane, allowing granzymes to enter the cell. Granzymes, that are proteases, activate enzymes that are part of the caspase family of proteases, a process which finally leads to DNA fragmentation in apoptosis. *De novo* synthesis of lytic granules also occurs upon TCR triggering, which allows the CTL to kill several targets in succession. A second mechanism to induce cell death of the target is by the expression of Fas ligand, that interacts with the Fas receptor molecule at the target cell membrane. The Fas receptor contains a 'death domain' in its cytoplasmatic tail, that via a signalling pathway including caspases ultimately leads to apoptosis of the cell. Target cells like macrophages and DC are relatively resistant to Fas mediated apoptosis (Lewinsohn et al., 2001) and killing of these cells would therefore require killing by lytic granules. Cytokine production by CTL (Slifka and Whitton, 2000), like IFN–γ, TNF-α and TNF-β mainly assists in macrophage activation to kill intracellular microbes (Janeway CA, 1999).

Different opinions exist on what the CTL should do, once activated, to most effectively restrain mycobacterial infection. Some studies indicate the production of IFN–γ as the major role (Hope et al., 2000; Liebana et al., 1999; van Pinxteren et al., 2000), thereby helping CD4⁺ T cells and activating macrophages, although excessive IFN–γ production is related to immunopathology (Villarreal-Ramos et al., 2003; Wigginton and Kirschner, 2001). Others emphasize the importance of a cytotoxic effect on infected macrophages (Cho et al., 2000; Serbina et al., 2000; Silva and Lowrie, 2000; Skinner et al., 2003). A modulatory role has been attributed to regulatory or suppressor CTL, although this is in general related to immune unresponsiveness and a survival mechanism of the pathogen (Bloom et al., 1992; Coussens, 2004; Salgame et al., 1991; Sussman and Wadee, 1991). Eliminating infected macrophages would, like in viral diseases, eliminate the pathogen's home, while eliminating CD4⁺ T cells in some stages of disease would prevent damage to the host due to exaggerated inflammatory reactions. Moreover, a direct cytotoxic effect on the mycobacteria has been shown (Silva and Lowrie, 2000; Stenger et al., 1997) for CTL producing cytotoxic granules. In a mathematical tuber-

culosis model by Wigginton and Kirschner (Wigginton and Kirschner, 2001), both IFN–γ production and cytotoxic mechanisms, mainly directed against bacteria, were attributed to CTL as important input on the disease outcome of their equation.

## CD8⁺ CTL-priming

The priming of naïve T cells by professional APC, *in casu* DC, relies on three signals: firstly, ligation of the TCR with the specific peptide presented in the MHC class I molecule, secondly, co-stimulation by different molecules on the DC and thirdly, the cytokine environment (Kapsenberg, 2003). Cytokines "polarize" the DC to stimulate a "polarized" T cell, meaning that *e.g.* mycobacteria, via TLR2 triggering on the DC, induce production of IL-12 and IFN–γ, which in turn "polarizes" the DC to induce a Th1 type of T cells (Kapsenberg, 2003). For the presentation of MHC class I epitopes to CD8⁺ T cells, the above mentioned (ad: CD8⁺ CTL-MHC class I) routes could be involved when DC present phagocytosed mycobacteria. Another route, of antigen transport from the endosomes into the cytosol, has been described for DC (Rodriguez et al., 1999) only, while macrophages are not capable of directing antigen to the classical MHC class I pathway by that mechanism. As naïve T cells were primed by DC that were not infected by viruses of bacteria themselves, it was questioned how this could happen (den Haan and Bevan, 2001). It is now believed that an important mechanism of antigen presentation by DC occurs through a phenomenon called 'cross-priming' . Especially for bacterial infection this has been shown to be an important way of stimulating CD8⁺ T cells (Harty and Bevan, 1999). Cross-priming is the efficient uptake and presentation of exogenous antigen by DC (Harty and Bevan, 1999). By this definition cross-priming also involves presentation of antigen after

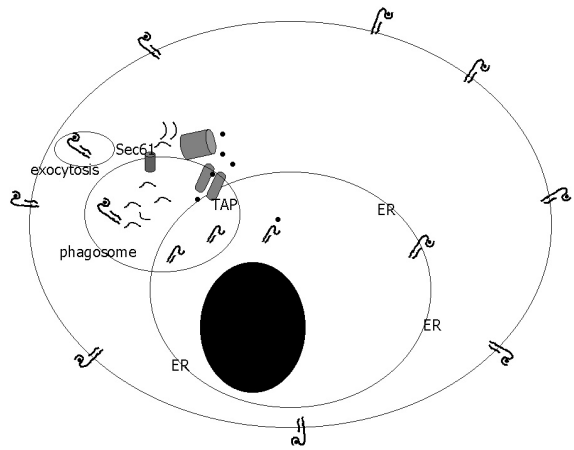



Figure depicting a mechanism of cross-priming. Proteins enclosed in the phagosome are transported to the cytosol via Sec61, degraded by the proteasome and redirected into the phagosome via TAP. The MHC class I loading machinery, including TAP, has been taken over by the phagosome by fusion with the ER. After reuptake of the degraded peptides, these are loaded onto the MHC class I molecules and then directed to the cell membrane.
Ref: Rock, Nature Immunology, 2003

phagocytosis, while cross-priming is sometimes also referred to as a mechanism that requires a 'donor' cell (Norbury et al., 2004). It has been demonstrated that phagosomes are self-sufficient organels for cross-priming, able to translocate proteins to the cytosol for degradation and then back into the phagosome lumen for MHC class I binding ((Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003) reviewed in:(Lehner and Cresswell, 2004)). The question is asked if this mechanism is restricted to DC or also applies to macrophages, and what it means for pathogens that reside in the phagosome. Will these pathogens interfere with cross-priming? (Lehner and Cresswell, 2004). Apart from phagocytosis, the DC can acquire antigen by the uptake of apoptotic or necrotic infected cells (Albert et

al., 1998; Fadok and Chimini, 2001; Green and Beere, 2000), uptake of apoptotic bodies or vesicles (Schaible et al., 2003), uptake of proteasome substrates (Norbury et al., 2004), or the uptake of chaperonins to which antigen is bound (Cho et al., 2000; Li et al., 2002; Schild et al., 1999; Silva, 1999), all leading to the cross-presentation of antigen. For some models, this exogenous antigen presentation system has even been proved dominant over the 'classical' endogenous system (Wolkers et al., 2004), provided that the cross-presented antigen consists of mature protein, rather than peptide (Norbury et al., 2004; Wolkers et al., 2004). However, the physiological relevance of this phenomenon has also been seriously doubted (Zinkernagel, 2002).

## CD8⁺ CTL-kinetics

Naïve CD8⁺ T cells are primed by DC (Janeway CA, 1999) in secondary lymphoid tissue near the site of infection, where the DC migrated to (Kapsenberg, 2003). First IL-2 driven proliferation and differentation takes place, whereafter CTL are released into the circulation, home to the site of infection, and exert their effects on infected target cells (Janeway CA, 1999). When a pathogen is encountered for the first time, this process (priming) takes several days (Janeway CA, 1999). A small population of memory cells is able to react directly upon secondary recognition of the same antigen, without the need for the time consuming priming in the secondary lymphoid tissue (Janeway CA, 1999).

What are memory cells and how do they arise? Although the development of effector CTL was shown without help of CD4⁺ T cells (Huang et al., 2000; Schuurhuis et al., 2000), it has now been demonstrated that help is needed for the installation of memory (Janssen et al., 2003; Kumaraguru et al., 2004; Rocha and Tanchot, 2004; Tanchot and Rocha, 2003). In a study of *Listeria monocytogenes* infected mice, CTL were induced without T cell help that were able to cure a primary infection, but unable to mount a rapid response against rechallenge with the same antigen, because of defective memory (Sun and Bevan, 2003). In addition it was demonstrated that T cell help is mainly needed at the time of the primary infection, but not at the time of reinfection and reexpansion of CTL (Janssen et al., 2003). CD4⁺ T cell help also functions in the opposite way: it down-modulates CD8⁺ T cell responses to prevent harmful consequences after eradication of the pathogen (Kursar et al., 2002).

How is memory sustained? Obviously, repetitive triggering of the memory T cell population is needed, but it is much less clear how often this should be, whether this requires constant reinfection with the same antigen or a cross-reactive related antigen; whether it is provided by cross reactive auto-antigens, or whether a constant treshold of antigen is needed (Janeway CA, 1999). Zinkernagel (Zinkernagel, 2003) introduced the term infection-immunity, that describes why a latent tuberculosis infection in fact might act as a natural vaccination to the host and thus might be advantageous to the human population. This idea is supported by experiments with IL-10 defective mice that effectively cleared *Leishmania major* infection, but thereby lost immunity to reinfection (Belkaid et al., 2002).

In the light of a chronic persistent infection, like paratuberculosis, many more questions arise, especially when this infection resides in macrophages. If memory cells are induced, what exactly do they do, and when do they become effector cells again? Are new effector and memory cells continously induced? What is the phenotype of these new effector and memory cells? One could argue that polarisation of the T cells by DC differs in the course of infection because of an altered cytokine environment, leading to a different phenotype and effector mechanism of the CD8⁺ T cell, with the same specificity. But it could also be argued that the specificity of the CD8⁺ T cells will alter, because different bacterial products will be released or because the abundancy of certain microbial products or degradation products changes. Another question arises as to what happens to the T cell upon contact with the infected macrophage. Will the CTL indeed kill its target cell, or will inversely the macrophage exert a modulating effect on the CTL? In a mouse model of chronic tuberculo-

sis, increased expression of FasL has been found on a subpopulation of infected macrophages, probably inducing apoptosis of CTL or Th1 type cells (Mustafa et al., 1999). To date, limited answers to these questions can be found in the literature. In *Listeria monocytogenes* (*L.monocytogenes*) infection, a feedback mechanism has been demonstrated in which primed $CD8^+$ T cells lyse infected DC, thereby preventing ongoing stimulation of effector cell populations (Wong and Pamer, 2003). A review article by Serbina and Pamer (Serbina and Pamer, 2003) summarizes recent knowledge on the course of $CD8^+$ T cell responses, indicating that the kinetics of memory T cell development may very well depend on the pathogen that is causing primary infection (Serbina and Pamer, 2003). Bevan (Bevan, 2004) argued that CTL can not revert to a population of memory cells, because the antigen has to be cleared before this can occur. Chronic antigen exposure also leads to a crippled $CD8^+$ T cell effector function. However, he mainly reviews acute infections and warns against comparing these with persistent infections (Bevan, 2004). For the time being, knowledge on $CD8^+$ T cell effector and memory cell kinetics is still marginal in mycobacterial disease, despite the general consensus on the importance of its contribution to disease prevention and resolution.

Considering CTL reactions as important reactions in preventing mycobacterial disease, how to induce such a reaction? Knowing that DC are professional APC, very well able to induce CTL, the first objective is to target DC in this respect. Activation of CTL by the presentation of exogenous antigen by DC could occur via the mechanism of cross-priming. Suitable carriers for cross-presentation of antigen are heat shock proteins (Silva, 1999).

# Heat Shock Proteins

Heat shock proteins (Hsp) are proteins of vital importance in prokaryotic and eukaryotic cellular organisms and are grouped in different families of related sequence, based on their molecular weights. Although, by definition, the levels of Hsp are elevated under conditions of cell stress, several Hsp are constitutively expressed under normal conditions. The Hsp, or stress proteins, also called molecular chaperones, are best known for their participation in protein folding, assembly and translocation. A unique degree of sequence homology exists, not only between Hsp familymembers of an organism, but even between those of different organisms, and even prokaryotic and eukaryotic cells (Rassow et al., 1995; van Eden et al., 1998; Welch and Feramisco, 1985; Young, 1990). The highly conserved nature of the Hsp is held responsible for immunological cross-reactivity between bacterial and mammalian Hsp, which has implications for control of autoimmunity (van Eden et al., 2003).

Despite their strong resemblance, Hsp are very immunogenic. Moreover, Hsp are endowed with immunological properties in interaction with certain cell types, like APC. By acting as a danger signal to the APC, they activate these cells, while at the same time they function as carriers to cross-prime APC with exogenous antigen, thus connecting the innate and the adaptive immune response (Gallucci and Matzinger, 2001; Robert et al., 2001; van Eden et al., 2003).

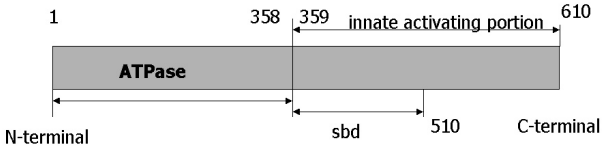
Of the four different families of Hsp (low molecular Hsp, Hsp60, Hsp70 and Hsp90), these paragraphs will mainly focus on Hsp70. The *E.coli* homolog of Hsp70 is called DnaK (Zhu et al., 1996).

## Hsp70-structure and substrate binding

Mycobacterial heat shock protein 70 has a length of approximately 620 amino acids (AA) (*M.a.p.* Hsp70: 623 AA (Stevenson et al., 1991)). It is composed of two functional domains: an ATPase domain (residues 1-387 in DnaK) and a substrate binding domain (sbd) (residues 388-638 in DnaK). At the $NH_2$-terminal ATPase domain of ~44 kD, ATP and ADP are exchanged, and ATP synthesis and hydrolysis takes place by intrinsic enzymatic activity.

Linear depiction of mycobacterial Hsp70.

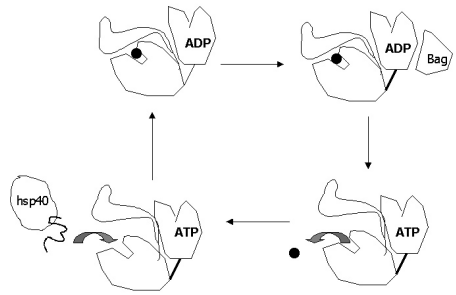
Ref: Todryk, Immunology, 2003




At the ~27 kD COOH-terminal part polypeptides are bound. In its ADP-bound state, Hsp70 has a high affinity for substrate, while in the ATP-bound state this substrate is released (Slepenkov and Witt, 2003; Wu et al., 2004; Zhu et al., 1996). Although not very stringent, there is a specificity for different peptides, which may vary in length from 8 to 26 AA (Grossmann et al., 2004). In a three dimensional structure, residues 507 to 638 form a helical lid over the substrate that is bound in the ADP bound state of the Hsp70 molecule (Zhu et al., 1996). The binding of ATP triggers a rapid release of the substrate, while conversely the binding of substrate stimulates ATP hydrolysis. Peptide binding leads to a conformational switch in the sbd and simultaneous conformational switch in the ATPase domain. Information on the activity on either side of the molecule communicates via the highly conserved linker group of residues between the two domains. Mutations in the linker abolish the domain-domain communication, resulting in uncoupled ATP hydrolysis and binding in the ATPase domain and substrate binding and release in the sbd (Wu et al., 2004).

Refolding of substrate proteins depends on repeated cycles of binding and release of the substrate, coupled to ATP hydrolysis and ATP-ADP exchange. Co-factors like Hsp40 (DnaJ in prokaryotes) and Bcl2-associated athanogene (Bag) protein (GrpE in prokaryotes) promote substrate binding and nucleotide exchange to drive the chaperone activities of Hsp70 efficiently (Sondermann et al., 2001; Wu et al., 2004). The co-factors selectively determine Hsp70 function, which explains that different Hsp70s (*e.g.* cytosolic and mitochondrial Hsp70) are not functionally exchangeable (Rassow et al., 1995).

The most conserved areas of the Hsp70 molecule are located in the ATPase domain and in the peptide binding groove of the sbd, while the helical lid is less conserved and the COOH-terminal 'tail' (residues 608-638) has very little communality (Flaherty et al., 1990; Zhu et al., 1996).




Hsp70, mechanistic function. Clockwise, from upper left:

Hsp70 in its ADP state has a substrate bound to it and the 'lid'closed. Bag stimulates ATP binding and subsequent opening of the lid and release of the substrate. Substrate binding, promoted by Hsp40, stimulates ATP hydrolysis and replacement of the lid. Ref: Zhu, Science , 1996

## Hsp-immunological properties

The Hsp that operate intracellularly might be liberated into the extracellular space, *e.g.* in case of cell necrosis (Basu et al., 2000). Antigen presenting cells will recognise the loss of cellular integrity and react with an inflammatory response to this danger signal, in a similar way as they would react to exogenous signals produced by invading organisms (Gallucci and Matzinger, 2001). Meanwhile, the Hsp maintain their function as (poly)peptide carrier. This means that the APC is not only activated, but also that the Hsp transport the captured protein over the cell membrane of the APC. In this way, the cargo of the Hsp will be released in the cytosol, ready for degredation and presentation. In other words, the APC will cross-present the antigenic cargo in the MHC class I molecule, thereby eliciting CTL reactions, the mechanism called cross-priming (Gullo and Teoh, 2004; Li et al., 2002; Moseley, 2000; Schild et al., 1999; Wallin et al., 2002). Thanks to the conserved nature of the Hsp, bacterial Hsp also interact with mammalian APC (Gallucci and Matzinger, 2001; Wallin et al., 2002), and may exert this function.

The above discussed immunological properties of Hsp make them ideal candidates for vaccine development, both in treatment of cancers and in prevention of infectious diseases (Hoos and Levey, 2003; Todryk et al., 2003). Methods have been described to make Hsp-peptide complexes (Li, 2004) or Hsp-antigen-fusion proteins (Udono et al., 2004). The latter offers good opportunities to mount CTL reactions without knowing the exact $CD8^+$ T cell epitope of the antigen (Suzue et al., 1997).

Under experimental conditions, CTL have succesfully been raised against ovalbumin (OVA) or OVA-peptides fused to mycobacterial Hsp (Cho et al., 2000a; Huang et al., 2000; Suzue et al., 1997), and fused or complexed to mammalian Hsp (Moroi et al., 2000; Udono et al., 2001). To date, different experimental studies have explored the potential of Hsp to elicit specific tumor immunity (Blachere et al., 1997; Ishii et al., 1999; Li, 1997; Noessner et al., 2002). Extraction of Hsp from tumors can provide for Hsp that is already loaded with

tumor antigen (Noessner et al., 2002). Induction of immunity to infectious disease has been proposed, so far a study on DNA-immunization (Rapp and Kaufmann, 2004) with a gp96 fusion product is the first to demonstrate protection against an otherwise lethal challenge with *L.monocytogenes* in mice.

## Hsp-activation of the innate immune system

Although many ideas exist about the effect of Hsp on the innate immune system, and the underlying mechanisms, this has not completely been elucidated. Adjuvanticity in eliciting CTL reactions has been described, without further explanation on the working mechanism (Harmala et al., 2002). Different reports demonstrate the release of nitric oxide (NO) (Panjwani et al., 2002; Wang et al., 2002), chemokines (Wang et al., 2002) or cytokines (Asea et al., 2000b; Breloer et al., 1999; Detanico et al., 2004; Galdiero et al., 1997; Todryk et al., 1999; Wang et al., 2002) after the interaction of Hsp70 with APC. Cytokines involved are mostly of the inflammatory type after activation of the nuclear factor (NF)-κB pathway, like TNF-α, IL1–β, IL6 (Asea et al., 2000b), IL-12 (Todryk et al., 1999; Wang et al., 2002), IFN–γ (Breloer et al., 1999) and GM-CSF (Galdiero et al., 1997), but also the anti-inflammatory IL-10 (Detanico et al., 2004). Maturation of DC, as expressed by the upregulation of antigen presenting- and costimulatory molecules, was also demonstrated (Basu et al., 2000; Kuppner et al., 2001). Some studies emphasize that these adjuvant effects of Hsp are properties of the Hsp molecule per se, independent of a substrate bound to the molecule (Asea et al., 2000a; Breloer et al., 1999; More et al., 1999). Using Hsp70-peptide binding mutants it was possible to completely separate antigen delivery from immunostimulation of DC (MacAry et al., 2004).

Despite the common features of different APC, clear differences exist between macrophages and DC, and also between the different maturation stages of DC. This is also reflected in the interaction with Hsp. It was shown that while Hsp70 matures DC, it inhibits the differentation of precursor cells, indicating that the time-point in the course of differentation and maturation at which the Hsp is added to the cells influences their reaction pattern (Kuppner et al., 2001). A study by Tobian (Tobian et al., 2004) demonstrates the different pathways used by macrophages and DC, vacuolar and cytosolic respectively, to cross-present antigen carried by Hsp70. This clearly demonstrates that experimental outcome of Hsp-APC interaction of one type of APC can not directly be extrapolated to another, similarly the conserved nature of the Hsp can not guarantee exactly the same working mechanism whether derived from prokaryotic or eukaryotic origin.

A paragraph on the adjuvanticity of Hsp can not ignore the debate on endotoxin contamination. As many Hsp for experimental studies are recombinant proteins produced in bacterial expression systems, LPS contamination is difficult to rule out. Moreover, many of the properties attributed to Hsp are very similar to the reaction patterns of APC activation by LPS (Suzuki et al., 2000). It was therefore that Bausinger et al. (Bausinger et al., 2002) expressed their concern on the eventual misinterpretation of data on Hsp, and indeed were able to demonstrate that endotoxin free solutions of Hsp70 failed to activate APC (Bausinger et al., 2002). However, eventhough another study attributed NO production and NF-κB signalling to LPS contamination, it leaves intact the cross-presentation capacities and other signalling pathways activated by Hsp (Reed et al., 2003).

## Hsp70- receptors

For both APC activation and cross-priming, Hsp have to bind to the cell surface via a specific receptor (Singh-Jasuja et al., 2000). Several studies described the existence of a receptor

for Hsp70 without being able to identify it (Arnold-Schild et al., 1999; Binder et al., 2000; Castellino et al., 2000; Guzhova et al., 1998; Lipsker et al., 2002; Sondermann et al., 2000). In other studies different receptors have now been identified: scavenger receptor lectin-like oxidized LDL receptor-1 (LOX-1) (Delneste et al., 2002), CD14 (Asea et al., 2000b), CD40 (Becker et al., 2002; Lazarevic et al., 2003; Wang et al., 2001), CD91, the α2-macroglobulin receptor/LDL receptor-related protein (LRP) (Basu et al., 2001; Binder et al., 2000a) and TLR2 and 4 (Asea et al., 2002; Vabulas et al., 2002).

**LOX-1** is a lipoprotein receptor recognised as a scavenger receptor on macrophages. The role of scavenger receptors is the neutralization and destruction of foreign pathogens by induction of endocytosis and phagocytosis (Yoshida et al., 1998). Expression of this receptor depends on the maturation stage of the cell, thus it was demonstrated on macrophages but not on monocytes (Yoshida et al., 1998). LOX-1 is also present on other cell types like endothelial cells and smooth muscle cells, and was shown to be involved in uptake of both Gram- positive and Gram-negative bacteria (Shimaoka et al., 2001). Receptor expression can be induced by several cytokines and LPS (Shimaoka et al., 2001). Delneste et al. (Delneste et al., 2002) demonstrated the presence of LOX-1 on immature DC that disappeared after maturation, and discovered that human Hsp70 was a ligand for this receptor. In their study they could show endocytosis and cross-priming by DC due to interaction with Hsp70, but were not able to activate the DC.

**CD14** is a cell surface protein without intracellular domain. It is present in different amounts on monocytes, macrophages and neutrophils and is a crucial receptor for lipopolysaccharides (LPS), constituants of the cell membrane of Gram-negative bacteria. The binding of LPS is augmented by the presence of LPS-binding protein (LBP), and signalling of this complex via CD14 is transmitted by other receptors on the cell surface that are members of the Toll-like receptor (TLR) family (Antal-Szalmas et al., 2004; Troelstra et al., 1997).

**CD40** is a cell surface receptor that belongs to the TNF-α receptor family (Kooten and Banchereau, 1997). First CD40 was described as a receptor on B-cells upon triggering of which resting B-cells are activated. It is now known to be present on many different cell types, including monocytes and dendritic cells. CD40 activation by its counterpart CD40-ligand (CD40L), that is mainly present on $CD4^+$ T cells, leads to numerous signalling events. Interaction of CD40-CD40L on APC and T cells results in activation of the APC as demonstrated by the production of NO and cytokines, and upregulation of costimulatory molecules. At the same time T cells are activated and, via IL-12 production, skewed towards a Th1 profile (Kooten and Banchereau, 1997). Via this APC activation mechanism $CD4^+$ T cells deliver their help to $CD8^+$ T cells (Bevan, 2004) for the promotion of effector and memory CTL, although a direct T cell- T cell interaction has also been described for $CD8^+$ T cells expressing CD40 (Rocha and Tanchot, 2004). Mycobacterial Hsp70 has been proposed as an alternative ligand for CD40 on DC (Lazarevic et al., 2003), providing protection against tuberculosis in mice by the production of IL-12 and subsequent priming of IFN–γ T cell responses. Wang et al. (Wang et al., 2001) stimulated human monocytes to produce chemokines with mycobacterial Hsp70 but failed to do so with human Hsp70. Moreover, it was the sbd of Hsp70 that induced chemokine production via receptor binding, which was in contrast with other studies that described the ATPase domain as the receptor binding part of the Hsp70 molecule (Huang et al., 2000; Udono et al., 2001). Becker et al.(Becker et al., 2002) showed binding of human Hsp70 to CD40 on macrophages, but only when Hsp70 was in the ADP bound state. Interaction of human Hsp70 and CD40 not only lead to signalling to NF-κB, leading to TNF-α production, but also to endocytosis of the Hsp70. Therefore CD40 was proposed as a receptor involved in cross-priming.

**CD91** is a receptor belonging to the LDL receptor family (Hussain et al., 1999) and is expressed on cells of many different organs, including liver, brain and lung. It is a constitutively recycling receptor, involved in endocytosis and signalling of several structurally dissimilar

ligands, including lipoproteins, proteases, albumin, lactoferrin, gentamicin and specifically $\alpha$2-macroglobulin. CD91 on macrophages was shown to bind several Hsp, including Hsp70 (Basu et al., 2001), leading to cross-presentation of chaperoned peptides. Ever since, CD91 has been referred to as 'the Hsp receptor'. However, not everybody agreed on CD91 being the one and only Hsp receptor, and besides the demonstration that uptake of the chaperone gp96 can occur independently of CD91 (Berwin et al., 2002), an alternative receptor has also been identified, being a scavenger receptor other than LOX-1 (Berwin et al., 2003). Whatever its role in Hsp binding may be, the CD91 molecule is certainly an important molecule in both innate and adaptive immunity (Stebbing et al., 2003).

**TLR** are signalling receptors that allow cells of the immune system to recognise microbes by their molecular patterns (Beutler, 2004; Janeway and Medzhitov, 2002; Werling and Jungi, 2003). Upon signalling through the TLR, APC are activated to produce reactive oxygen and nitrogen intermediates and cytokines, and to upregulate costimulatory molecules. Triggering of the TLR induce an immediate response and therefore TLR are very important molecules in the innate immune system. The production of cytokines by activated APC links the reaction to the adaptive arm of the immune system. In humans, ten TLR are known and many ligands have been described. Signalling through TLR leads via four different adaptor proteins to the activation of primary and secondary kinases and finally to the transcription of many genes (Beutler, 2004). Although this seems simple at a first glance, the use of different TLR by the same ligand, or of different adaptor proteins by the same TLR (Means et al., 2001), makes this system complex. Two studies on human Hsp70 demonstrated that Hsp70 induces cytokine production via TLR2 and TLR4 signalling, dependent on the Myd88 adaptor molecule (Asea et al., 2002; Vabulas et al., 2002). Apparently, the TLR have to interact with each other at some stage of the reaction, as TLR4 defective DC were unresponsive to Hsp70 (Vabulas et al., 2002).

In conclusion, the immunological properties of Hsp have been proven in many studies, but have also been subject to a lot of debate. In studies of interaction of Hsp with APC, it is crucial to clearly define the origin of the Hsp and the type and activation stage of the APC under investigation.

# Aim of the study and outline of the thesis

The general aim of this study was to gain insight in the role that CTL play in bovine paratuberculosis and to investigate if we could induce CTL reactivity by the use of heat shock proteins. We hypothesized that CTL contribute to protective immunity towards *M.a.p.* infection in cattle (1), and that we could induce CTL activity with a Hsp70 fusion protein consisting of recombinant Hsp70 of *M.a.p.* (rHsp70) fused to a model antigen (2).

Little is known about the occurrence and activity of $CD8^+$ T cells in the course of paratuberculosis. Therefore we conducted a longitudinal study of experimentally infected cattle that were followed during a five year period **(chapter 2)**. In this study lymphocyte subsets were determined in peripheral blood, together with lymphoproliferative responses specific for rHsp70, PPD-P and whole bacteria. In parallel, antibody responses and fecal shedding of bacteria were determined. To support our hypothesis that CTL can be induced with rHsp70, we analysed the interaction between rHsp70 and bovine APC *in vitro* **(chapter 3)**. Subsequently we generated a fusion protein consisting of the receptor binding domain of rHsp70 fused to a model antigen, *i.c.* enhanced green fluorescent protein (eGFP), known to contain CTL epitopes in several species **(chapter 4)**.To prove our second hypothesis we immunised cattle with the fusion protein (rbd-H70-GFP) and assessed cytotoxicity *in vitro* by the classical $^{51}Cr$ release assay, and a non-radioactive flow cytometric assay **(chapter 5)**. As DC are key players in the very early response against *M.a.p.* infection, we investigated differential cytokine gene expression of bovine immature DC in reaction to different antigens, by real time RT-PCR **(chapter 6)**. The findings of the different chapters are summarized and discussed in **chapter 7**.

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# APC 2 HSP MAP

**Host – pathogen interactions during experimental *Mycobacterium avium subspecies paratuberculosis* infection in cattle**

## Abstract

Paratuberculosis is a chronic infection of the small intestine of cattle caused by *Mycobacterium avium subspecies paratuberculosis* (*M.a.p.*), leading to an incurable form of protein loosing enteropathy. Immuno-pathogenesis of the disease is still poorly understood, in part as a result of the long time interval between infection and development of clinical symptoms, in average 4 to 6 years. In the current study 20 orally infected calves were followed for a period of five years. During this period bacterial shedding in the feces was measured, leading to the repartition of the animals in three different groups; of high (HFS), intermediate (IS) and low (LFS) frequent shedders. In parallel, immunological parameters in peripheral blood were determined. Together the data shed new light on several aspects of the immunology of paratuberculosis. The differences between bacterial shedding frequencies were only reflected in temporal differences in cellular immune reaction. Parturition and the periparturient period were the most important factors influencing both a shift in lymphocyte subset frequencies, as determined by flow cytometry (mainly a decrease in $CD4^+$ and γδ T cell subsets) and in antigen specific lymphocyte proliferation, as determined in lymphocyte stimulation tests (LST) (mainly reflected by the disappearance of higher responses of LFS in early infection). The observation was made that at the time of parturition the ratio of $CD4^+$ T cells as compared to γδ T cells was higher in the LFS group, a phenomenon that repeated itself at second and third parturition, while it disappeared between parturitions. In contrast to cellular immunological reactions, distinction between the groups could be made based upon serological data. High frequent shedders showed detectable levels of PPD-P specific antibodies from one year after infection onwards. We concluded that the early detection of antibodies related to faecal shedding implied that antibody production is an 'immunological phenotype' related to susceptibility to paratuberculosis, more than a loss of control represented by a Th1 to a Th2 shift of individual animals, as proposed in previous cross-sectional studies. Although the group of LFS contained *M.a.p.* infection for a much longer period, no true protection in terms of clearance of the bacteria could be observed.

***Keywords***
mycobacterium paratuberculosis, bovine, immunopathogenesis, heat shock protein, experimental infection

## Introduction

Paratuberculosis is a chronic mycobacterial infection of the small intestine of ruminants in general and cattle in particular, causing substantial economic losses at farm level (Johnson-Ifearulundu et al., 1999; Ott et al., 1999). Calves acquire the infection in the first months of life through oral uptake of *Mycobacterium avium subspecies paratuberculosis* (*M.a.p.*), present in colostrum or feces (Hagan, 1938). The bacteria enter the host most likely via the M-cells, abundantly present in the jejunum and particularly the distal ileum, to be taken up by macrophages underlying the M-cells in the dome of the Peyers Patches (Momotani et al., 1988). Comparable to other pathogenic species in the genus, *M.a.p.* is able to survive inside the macrophages. Both experimental infections as well as field data so far, indicate that calves either clear the infections rapidly, or become subclinically infected for life. The subclinically infected animals intermittently shed the bacteria in the feces, in increasing amounts as the infection progresses, thus contributing to the spread of the disease. Following an incubation period of on average 4-5 years, a proportion of the infected animals develops an incurable progressive form of protein loosing enteropathy, clinically recognized by chronic diarrhea and emaciation and ultimately leading to death (Chiodini et al., 1984; Clarke, 1997; Payne and Rankin, 1961).

Several aspects of the (immuno-)pathogenesis of this mycobacterial infection are poorly understood and basic knowledge on host-pathogen interactions that may explain the apparent inability of the host to mount a protective immune response to the infection with *M.a.p.* is still lacking (Valentin-Weigand and Goethe, 1999). In order to improve diagnostic methods and develop protective vaccines these issues have to be dealt with. In previous studies it was argued that the immune responses, measured in serum or using peripheral blood mononuclear cells (PBMC), are indicative of an early cell mediated response that switches to a non-protective antibody response during the course of the disease. Collectively these data have been used to argue that chronic progressive forms of paratuberculosis involve a switch in the host immune response from a protective Type I, cell mediated, immune response to a permissive Type II, antibody mediated, immune response (Koets et al., 1999; Stabel, 2000b; Sweeney et al., 1998), according to the murine Th1-Th2 paradigm (Mosmann and Sad, 1996). However, more recent studies indicate that both Type I and Type II responses may be impaired during later stages of disease (Koets et al., 2002; Koets et al., 2001). Most of the data leading to these conclusions have been generated using cross-sectional studies. Whether the observed changes in immune response occur within individual animals during disease progression is largely unknown, especially for later stages of infection (Waters et al., 2003).

To study immune-responsiveness longitudinally, we have completed a 5 year follow-up of 20 cows experimentally infected with *M.a.p.* under controlled conditions. Host responses were determined by measuring immunological responses to crude and recombinant antigens as well as bacterial excretion through fecal culture. Comparisons of immunological reactions related to fecal shedding frequency were made in an attempt to shed light on the question of what constitutes a protective immune response with regard to bovine paratuberculosis.

## Materials and methods

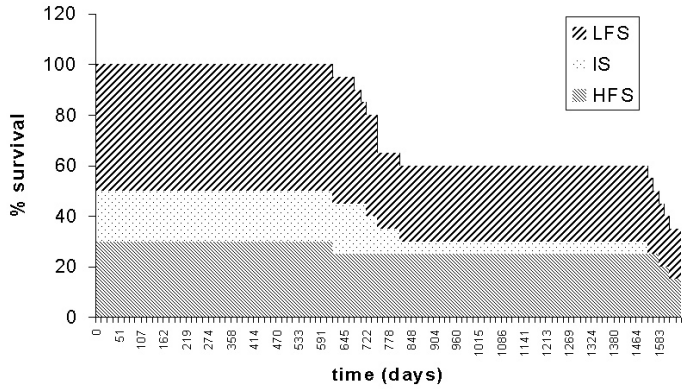
### *Animals*

Twenty Holstein-Friesian calves were purchased at birth from different commercial farms and housed at the SPF facilities of the CIDC in Lelystad, The Netherlands, throughout the experimental period. Animals were kept on a regular feed regimen according to their age and lactation status, but never received fresh grass. Experimental procedures were approved by the Ethical Committee of the CIDC.

During the course of the investigation period 7 out of the initial 20 cattle survived to the end. Figure 1 depicts the survival analysis of the animals, divided over the 3 groups (see: *Fecal culture*), during the longitudinal study. Post-mortem examinations of animals that died during the study showed a diverse number of causes of death, none of which were due to the experimental infection with *M.a.p.*

Figure 1.

Survival analysis. Of the 20 animals at the beginning of the study (100%), 7 survived to the end (35%). Times of death (for various easons) are indicated (x-axis) and repartition of high, intermediate an low frequent fecal shedders over te group of survivors is indicated




### *Experimental infection*

Calves were infected with a field isolate of *Mycobacterium avium subspecies paratuberculosis* (*M.a.p.*) by way of 20 grams of contaminated feces given orally, three times a week for a period of four weeks. Feces was obtained from an adult animal with clinical signs of paratuberculosis that constantly shed bacteria.

### *Fecal culture*

Rectal samples for fecal culture were taken every two weeks. Bacteria were cultured according to a modified method of Jorgenson (Jorgensen, 1982). Growth of *M.a.p.* was mycobactin dependent and checked every 4 weeks. If no growth was observed after 6 months of culture, the sample was considered negative. The presence of *M.a.p.* in positive cultures was confirmed by amplification of the *M.a.p.* specific IS900 by PCR (Vary et al., 1990).

For analysis an overall fecal shedding frequency was calculated per animal, *i.e.* the percentage of measurements in which a positive culture result was obtained during the entire test period. Animals were placed into 1 of 3 groups of shedding frequency intervals; high frequent shedders (HFS) containing animals with a shedding frequency of more than 60%, intermediate shedders (IS) containing animals with a shedding frequency of 40-60% or low frequent shedders (LFS) containing animals with a shedding frequency of less than 40%. To visualize the fecal shedding pattern a semi quantitative fecal score was attributed to each animal at every time-point of data sampling, calculated as follows:

$$FC_{score}(t) = C(t) + FC_{score}(t-1)$$

This is a cumulative score representing fecal shedding frequency which at timepoint (t) = culture result at timepoint (t) + cumulative score from the previous dates, in which:

C(t) = -1 (negative culture result), 0 (no result), +1 (positive culture result)

***Blood sampling***
Blood was collected every two weeks from the jugular vein into heparinised tubes and into serum tubes (BD Vacutainer, Becton, Dickinson, Europe). Heparinised blood was used for immediate clinical chemistry evaluation to determine total white blood cell counts (G/L) and leucocyte differentiation, and for the isolation of peripheral blood mononuclear cells (PBMC). Serum was stored at $-20^0$ C and processed at a later time-point.

***Isolation of PBMC***
Heparinised blood was diluted 1:1 in RPMI 1640 tissue culture medium supplemented with 2% FCS, 50 IU/ml penicillin, 50 µg/ml streptomycin (all from Gibco BRL, Paisley, UK) and 5 IU/ml heparin (Leo Pharmaceutical Products, Weesp, The Netherlands). This suspension was centrifuged (25 min, 1200 g) on a Ficoll-metrizoate density gradient (density 1.078; Pharmacia, Uppsala, Sweden). Blood mononuclear cells were isolated from the interphase, washed twice in RPMI, counted and diluted to a final concentration of $2 \times 10^6$ cells/ml in RPMI tissue culture medium supplemented with 10% FCS, 50 IU/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine and $5 \times 10^{-5}$ M β-mercaptoethanol (Flow Laboratories, Ivume, UK).

***Antigens***
In stimulations assays as assessed by both tritiated ($^3$H) thymidine incorporation and flow cytometry, recombinant heat shock proteins (Hsp):*M.a.p.* Hsp 65kD (Hsp60) and Hsp 70kD (Hsp70), *M.a.p.* strain 316F and PPDs were used. Recombinant proteins were produced according to methods described in detail earlier (Koets et al., 2001; Koets et al., 1999). *M. paratuberculosis* strain 316F was grown at the CIDC (Lelystad, The Netherlands). The PPDs were produced at CIDC, Lelystad, as previously described, from *M. paratuberculosis* strain 3+5/C (10 µg/ml) (PPD-P), *Mycobacterium bovis* strain AN5 (10µg/ml) (PPD-B), and *Mycobacterium avium* strain D4 (10 µg/ml) (PPD-A). (Gilmour and Wood, 1996).

***Flow cytometric analysis of lymphocyte phenotypes***
Dual colour flow cytometric analysis was performed using monoclonal antibodies specific for bovine lymphocyte markers that have been described earlier (see Table 1), according to procedures published previously (Koets et al., 1999). Briefly, PBMC were washed twice in Facsbuffer, incubated with the first antibody (table 1) for 15 minutes at 4ºC, washed twice, subsequently incubated with a PE labelled Goat-anti-Mouse Ig (H+L) antibody (Becton-Dickinson) for 30 minutes at 4ºC, and washed twice. Subsequently IL-A59-FITC (recognizing bovine B cells) (Brodersen et al., 1998) was added and incubated for 15 minutes at 4ºC. Finally cells were washed and collected in 200 µL Facsbuffer prior to analysis. Data were obtained on a flow cytometer (FACS-Calibur, Becton-Dickinson) and analysed using Cellquest software. A forward scatter (FSC) – side scatter (SSC) life-gate was used to measure $10^4$ lymphocytes per sample. Based on the fluorescence data of the lymphocytes, the results were expressed as the percentage of cells with positive staining relative to a sample stained with an isotype control antibody. The $CD4^+$ to γδ ratio was calculated by dividing the frequency (%) of $CD4^+$ T cells by the frequency (%) of γδ T cells.

*Table 1*
Monoclonal antibodies (mAb) used in flow cytometric analysis

| mAb | Isotype | Antigen identified | Refs. |
|---|---|---|---|
| ILA-42 | IgG2a | Bovine CD2 | (Davis et al., 1988) |
| ILA-12 | IgG2a | Bovine CD4 | (Baldwin et al., 1986) |
| ILA-51 | IgG1 | Bovine CD8 | (Ellis et al., 1986) |
| ILA-29 | IgG1 | Bovine WC1 (γδ T cells) | (Clevers et al., 1990) |
| CACT61A | IgM | Bovine TCR1-N12 γδ T cells) | (Davis et al., 1996) |
| MM61A | IgG1 | Bovine CD14 (monocytes) | (Brodersen et al., 1998; Sager et al., 1997) |
| GB25A | IgG1 | Bovine CD21-like (B-cell) | (Brodersen et al., 1998) |

***Lymphocyte Stimulation Test***
Lymphocyte Stimulation Tests (LST) were performed in 96 well microtitre plates (Costar, Cambridge, MA, USA) using 100 μl of the PBMC suspension and 100 μl of antigen per well, all tests were performed in triplicate. The mycobacterial antigens PPD-P, Hsp65, Hsp70 and bovine Hsp70 were used in predetermined optimal concentrations of 10 μg/ml each. Strain 316F bacteria were briefly sonicated, counted and used in a concentration of $1.10^7$ CFU/ml. Concanavalin A (ConA) was used as a positive control (2.5 μg/ml) and medium alone as a negative control.

Cells were cultured at 37 °C and 5 % $CO_2$ in a humidified incubator for 3 days. Then 0.4 μCi $^3$H thymidine (Amersham International) was added to each well and cells were cultured for an additional 18 hrs. Subsequently, cells were harvested onto glass fibre filters. Incorporation of $^3$H thymidine was measured by liquid scintillation counting and expressed as Stimulation Index (S.I.) which was calculated by dividing the cpm of a specific stimulation by the cpm of the medium control.

***Flow cytometric analysis of cellular activation***
In order to study the *in vitro* reaction of subsets of lymphocytes in response to relevant antigens, PBMC were labelled with carboxy-fluorescein diacetate succinimidyl ester (CFDASE) (Molecular Probes, Oregon, USA). The dye is equally distributed among daughter cells thus several generations of lymphocytes can be identified by flow cytometry (Lyons and Parish, 1994). For the proliferation assays (CFSE assays) the labeled PBMC were incubated with antigen as outlined above (see: *LST*). On the fourth day of the assay, following subset staining as outlined above (see: *Flow cytometric analysis of lymphocyte phenotypes*), proliferation was evaluated in relation to subset. A FSC- SSC life-gate was used to measure $10^5$ viable lymphocytes per sample.

***Serological responses***
For the detection of *M.a.p.* specific antibodies an ELISA kit of the Pourquier Institute (Couquet, 1999) was used. The technique used has been described, for the main part, in the O.*I.E.* recommandations 5B/009 ("Manual of recommended diagnostic techniques and requirements for biological products", vol.III-Paratuberculosis). Microplates coated with *M.a.p.* antigen were furnished in the kit. Serum samples were diluted in a dilution buffer containing *M.phlei* at 1:20 for one hour in order to eliminate cross-reactive antibodies. Subsequently, samples were incubated (for one hour or overnight) at room temperature (RT), divided both in one well coated with *M.a.p.* antigen, and one control well. After washing, a peroxidase (PO) labelled monoclonal anti-bovine IgG conjugate was added to each well, followed by a washing step. A Tetra-Methyl-Benzidine (TMB) buffer was then added,

which turns blue in the presence of PO labelled antibodies, after which the reaction is stopped with stop solution. The intensity of the colour, as determined spectrophotometrically, is a measure of the level of antibodies present in the sample. The corrected optical density is the difference between the optical density of the sample minus the optical density of the control well. The kit includes a positive control serum which is tested in each plate.

Per animal one microplate was used. Interplate variation was within the acceptable range.

Results are expressed as mean corrected optical densities (absorbance at 492 nm wavelength) per group of animals (high, intermediate or low frequent faecal shedders).

***Statistical analysis***

To better approximate a normal distribution of the data, all SI response variables (ConA, PPD-P, rHsp65, rHsp70 and *M.a.p.*) were transformed by using a logarithmic transformation. Subsequently, a generalized linear mixed effect (GLME) model was used to analyse the time effect, the group effect and their interaction. To take into account the dependence of data, a random cow effect was included. The time of sample collection was taken as a fixed effect. Because the residuals were heterogeneous, we included a variance structure into the model, which allowed different standard deviations for each time point. Then, to model any remaining dependence in the data we introduced an autoregressive order 1 (AR 1) correlation structure, which assumes the current residual to be only dependent on the residual at the previous time-point . In particular, the maximum likelihood (ML) method was used. Models were compared with a likelihood ratio test (LRT), with Bonferroni correction. For computations, we used R statistical software (Team, 2003).

## Results

***Fecal culture results***

The analysis of the fecal culture results indicated that three groups with different fecal shedding frequencies could be distinguished as outlined in material and methods. The cumulative fecal culture scores support a grouping of animals as high frequent shedders (HFS, n=6), low frequent shedders (LFS, n=10) and intermediate shedders (IS, n=4).

The cumulative frequency score of the fecal shedding of *M.a.p.* of the different groups is depicted in figure 2. Subsequent immune response data are presented according to this division in three groups.

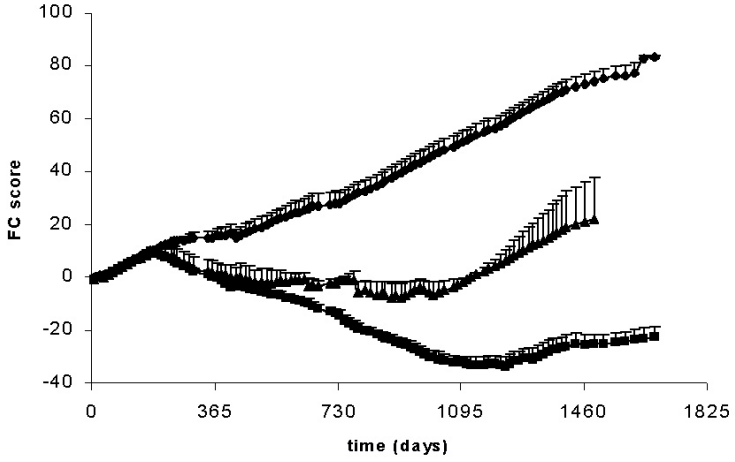
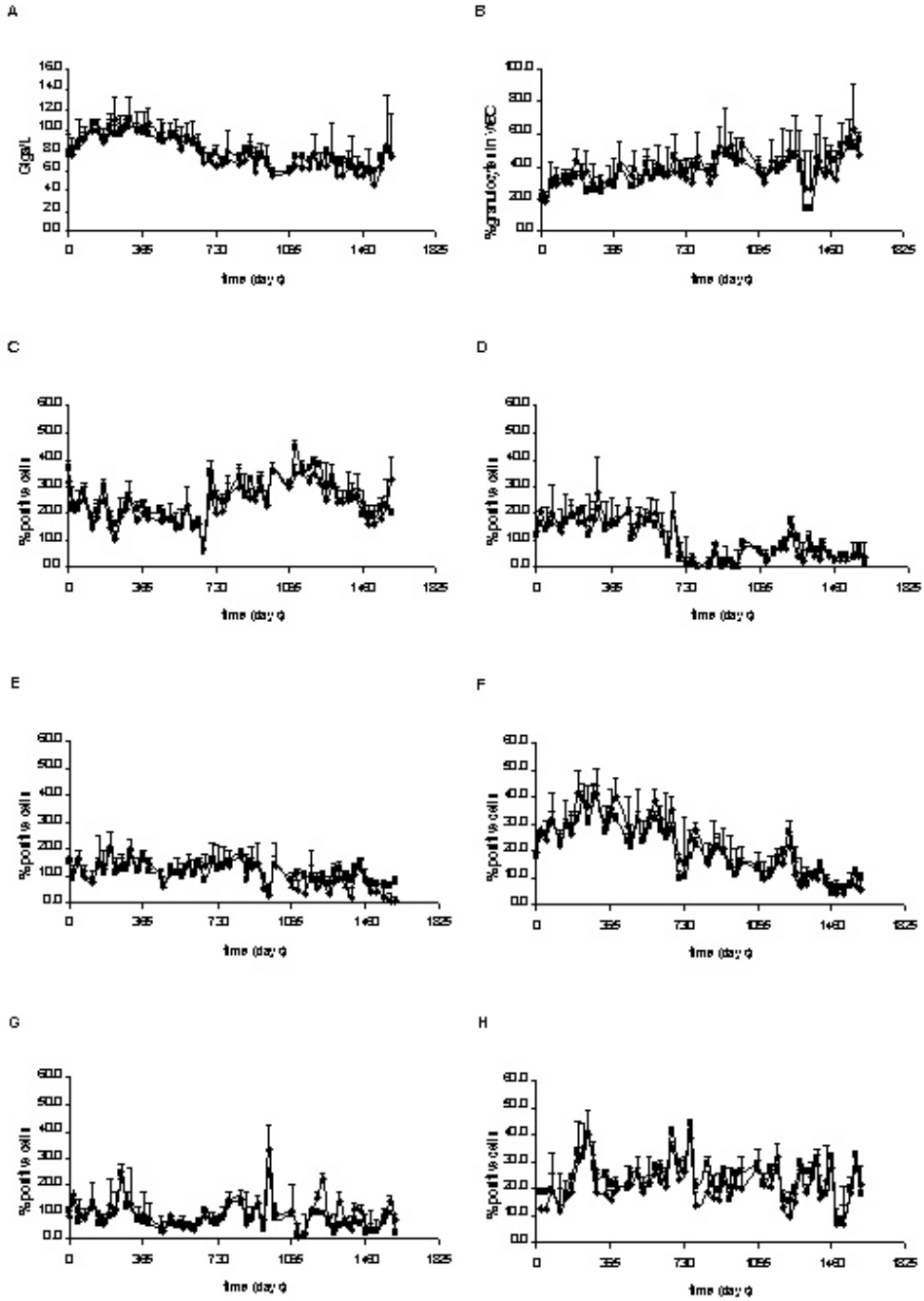



Figure 2.

Cumulative fecal score (y-axis) in time (x-axis), of the 3 different groups: HFS (triangles), IS (squares) and LFS (diamonds), based on fecal culture results.

Figure 3.
White blood cell counts, percentages granulocytes and mononuclear cell subsets of HFS and LFS.
During the investigation period (time, x-axis), blood samples were taken to determine the total WBC (in G/l, y-axis, panelA), the percentages of granulocytes (panel B), and mononuclear cell subsets (percentages of total PBMC, y-axis, panels C-H). C= $CD4^+$, D=$WC1^+$, E=$CD8^+$, F=$N12^+$ T cells, G=$CD14^+$ cells (monocytes), H=$CD21^+$ B cells. HFS=triangles, LFS=diamonds.

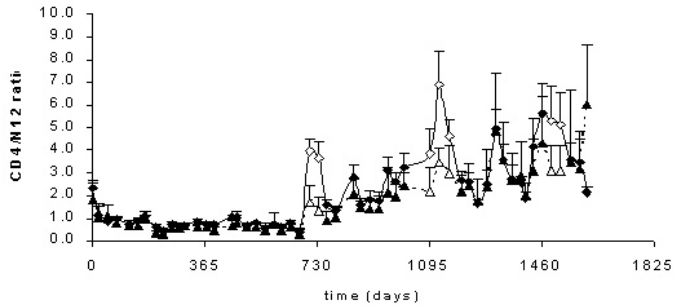


***Peripheral blood leukocytes***

Data on total white blood cell counts (WBC), percentage of granulocytes, and leukocyte subsets in the HFS and LFS group is depicted in figure 3. The data of the IS group was intermediate to the HFS and LFS group and was omitted from these graphs for clarity. The WBC (fig. 3A) stayed within reference values for the duration of the study. Similarly, the majority of granulocytes consisted of neutrophils, the minority consisted of eosinophils and basophils within reference value ranges. No differences between individual granulocyte types were observed between groups and the sum of neutrophils, basophils and eosinophils is presented (fig. 3B).

***Flow cytometric analysis of lymphocyte subsets***

No differences were observed between the HFS, IS and LFS group with respect to the leukocyte subsets as depicted in figure 3C-H. Subset data for HFS and LFS shows the characteristic decrease in the frequency of γδ T cells, both in γδ TCR$^+$ (fig. 3F) as well as in the WC1 subset (fig. 3D) of γδ T cells in the course of the first 2 years of life. In addition a significant decrease in the frequency of CD4$^+$ cells (fig. 3C) was observed from day 670 onwards in all animals. Between days 705 and 736, 11 out of 16 animals calved for the first time (3 of 6 HFS animals, 6 of 10 LFS, 2 of 4 IS). At that time the ratio of CD4$^+$ cells over N12$^+$ γδ T cells differed between LFS and HFS groups, to the extent that in the LFS group a higher CD4/N12 ratio was observed indicating a relative surplus of CD4$^+$ T cells over N12 γδ T cells in the peri-parturient period (fig. 4).



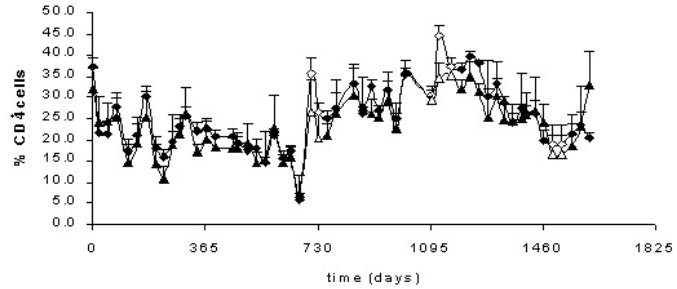




Figure 4.

Ratio CD4/N12 T cells. The ratio of CD4$^+$ T cells to γδ T cells (y-axis) during the study period (x-axis). HFS=triangles, LFS= diamonds. Open symbols represent the (peri-) parturient periods. In the first period 11/16 animals calved.

***Antigen specific activation of PBMC***

Figure 5A shows that unstimulated cells of young animals consistently showed high levels of background proliferation throughout the first 2 years of life, that gradually decreased to much lower background levels in the third year. The proliferation in response to PPD-P

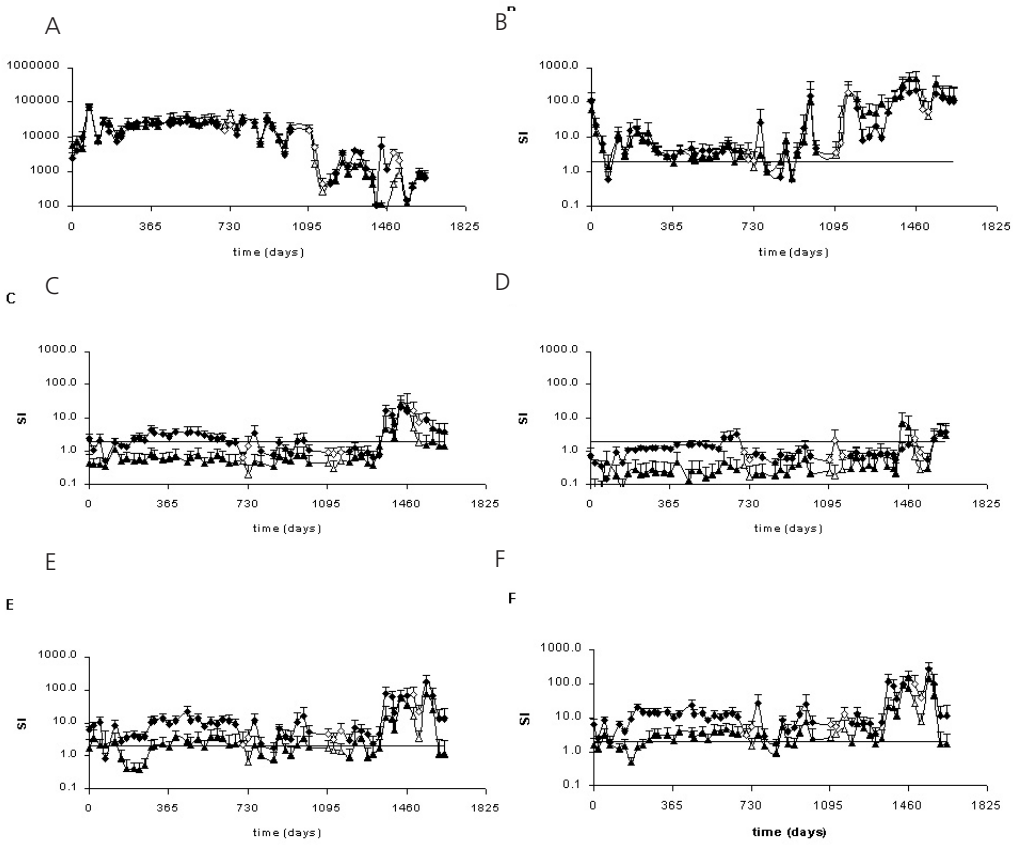



Figure 5.

Lymphocyte proliferation of HFS and LFS in LST. Panel A demonstrates the high background proliferation (cpm, y-axis) of both groups (HFS (triangles) and LFS (diamonds)), during the first two years (time, x-axis) when cells were cultured in medium only. Panel B represents the proliferation compared to the medium control (SI, y-axis) of cells in response to Con A. Panel C-F depict proliferation in response to specific antigens; recombinant Hsp70 (C), rHsp65 (D), whole bacteria, *M.a.p.*(E), and PPD-P (F) respectively.

as antigen (fig 5F) can be measured from 3 months post infection onwards. The shedding frequency groups differ most between ~200 and ~730 days post infection where the LFS group displays the highest proliferative responses. Values were statistically different from day 174 to day 283. Similar observations were made when using the recombinant Hsp70 antigen (panel C), and whole mycobacteria (panel E). These differences between the groups disappeared around the time of first calving (approximately 730 days post infection).

Although the difference between the two groups was similar in response to Hsp65, no net proliferative responses were measured when using this antigen (fig 5D). Animals from the groups with intermediate shedding frequency scores also had intermediate responses (data not shown).

***Antibody responses***

Antibodies in response to PPD-P (fig 6) were not detected in sera of LFS, but seroconversion of the HFS group was measured from one year after infection onwards. During the inves-

tigation period, antibody titers increased, with an inclination around day 730 and sharper inclination around day 1080 *p.i.*, the (peri-) parturient period. Seroconversion of the group of IS appeared after day 1100 *p.i.*.

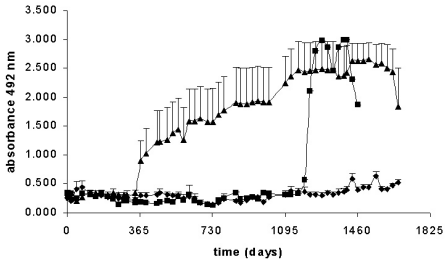



Figure 6.

Anti PPD-P antibodies. Antibody responses of HFS (triangles), IS (squares) and LFS (diamonds) measured in an ELISA specific for PPD-P. Levels of antibodies are represented by the OD measured at 492 wavelength (y-axis).

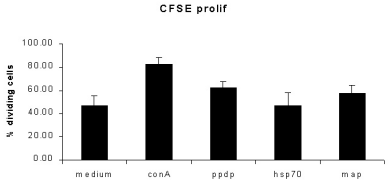
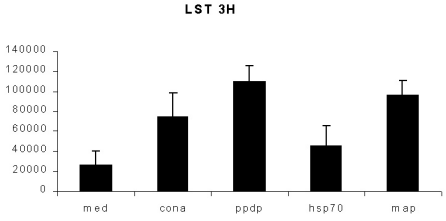
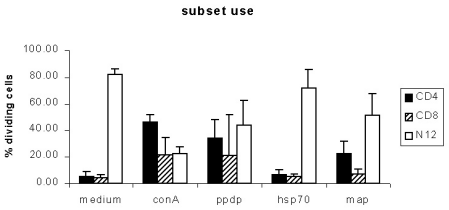



Figure 7.

Lymphocyte proliferation and lymphocyte subset use. Proliferation of PBMC in response to medium, Con A, PPD-P, rHsp70 and *M.a.p.* respectively, measured in a CFSE assay (panel A, depicted as percentage dividing cells) and in a LST (panel B, depicted as cpm). Proliferation as determined in the CFSE assay (panel A) is divided between $CD4^+$ (black bars), $CD8^+$ (striped bars) and γδ T cells (white bars) as shown in panel C. n=4 animals.




***Involvement of lymphocyte subsets in proliferation***

At selected time-points the involvement of individual T-cell subsets was evaluated using fluorescent labelled PBMC. Figure 7A shows proliferation of total PBMC using the CFSE assay at day 365. Proliferation of total PBMC in response to the antigens PPD-P, Hsp70 and whole *M.a.p.* corresponded to proliferative reponses of the same animals in the LST (fig.7B).

In panel 7C involvement of different lymphocyte subsets is depicted. In response to PPD-P, attribution to the response is almost equally distributed between the three subsets; CD4, CD8 and N12 positive T cells. In response to Hsp70 and *M.a.p.*, the major contribution to the response is delivered by the γδ T cells. The γδ T cells are similarly responsible for the high background proliferation in the assay.

## Discussion

The current experimental infection was established using a high dose inoculum, and from the pattern of bacterial secretion it was evident that infection was successful in all animals. In the first 200 days of the infection, all animals shed bacteria in the faeces. This will have contributed to constant reinfections, after the initial doses that were given experimentally, as is likely to occur in natural situations. From 200 days *p.i.* onwards, three groups of animals could be distinguished according to shedding frequency. High frequent shedders (HFS) retained this phenotype throughout the investigation period, while low (LFS) and intermediate frequent shedders (IS) gradually started to shed bacteria more often after the second and third calving.

It can be argued that shedding of bacteria and shedding frequency are related to the capacity of the animal to contain the disease. Immunological responses of high and low frequent shedders were compared to find explanations for apparent differences in this capacity. General clinical parameters such as total white blood cell counts and differentiation did not show differences between the groups and the alterations in time were all within the physiological range. Percentages of PBMC subsets, as determined by flow cytometry, were not different between the HFS and LFS either. However, an interesting observation was made that while γδ T cell populations decreased when the animals were around two years of age, which is a common observation made in cattle, there was also a sudden and significant decrease in the percentage of $CD4^+$ T cells around day 670 (~3 weeks before parturition) in both groups. After this decrease, the percentage of $CD4^+$ T cells gradually increased again. However, although the relative contribution to PBMC of both subsets of lymphocytes (gd and $CD4^+$ T cells) decreased, the ratio of $CD4^+$ T cells to γδ T cells was in favour of the $CD4^+$ subset. Moreover, this ratio was significantly higher in the group of LFS than in the group of HFS at every parturition, while between the calving periods the ratio was the same for both groups. This demonstrates that important shifts in lymphocyte populations can be observed in the peri-parturient period, as has been shown previously in other studies (Kimura et al., 1999), while at the same time it indicates that maintaining a surplus of $CD4^+$ T cells over γδ T cells at that critical time-point is of crucial importance to contain *M.a.p.* infection as this apparently occurred most efficiently in animals of the LFS group.

Antigen specific responses as measured by LST were similar for both groups, except for the period from 200 to around 700 days of infection, during which these responses were higher in the LFS than in the HFS group. Recombinant Hsp70, that has been shown to be an immunodominant antigen of *M.a.p.* in previous cross-sectional studies (Koets et al., 1999), in this study also induced lymphoproliferation, while rHsp65 did not significantly stimulate lymphocytes in LST. Again the (peri)-parturient period was the critical period, in this case levelling off the initial differences. Comparison of LST data to proliferation as measured by CFSE staining demonstrated similar proliferation of the total population of PBMC specific for PPD-P, rHsp70 and whole *M.a.p.*. High background proliferation was associated with near exclusive activity of the γδ T cell subset. Subset staining also revealed a major contribution of γδ T cells to the proliferation stimulated by rHsp70 and *M.a.p.*, while in reaction to PPD-P the three subsets of lymphocytes contributed equally. This would indicate a contribution of γδ T cells to cellular immunity, resulting in higher proliferative responses of LFS at this stage.

Antibody responses to PPD-P were high in the high frequency shedder group from one year *p.i.* onwards, while in the low frequency shedder group the responses remained at a very low level. The time of onset of antibody production could not be related to any of the cellular immune reactions determined, but steadily increased, with a boost around every calving. Antibody production was detectable relatively early in our study, probably as a result of the high dose experimental infection, and was related to parity as has been demonstrated in studies of natural infection (Nielsen et al., 2002; van Schaik et al., 2003). A longitudi-

nal study by Schaik et al. (van Schaik et al., 2003) demonstrated a relationship between the amount of faecal shedding and the onset of antibody production, which confirm our data. Heritability of the ability to mount an immune response by the production of antibodies has been demonstrated for paratuberculosis (Mortensen et al., 2004). As we show here that high frequent shedding of bacteria is correlated to early onset of antibody production, the latter being a trait with significant heritability, we might conclude that the incapacity to contain the bacteria and susceptibility to paratuberculosis is at least in part genetically determined. Genetic variation in the susceptibility to paratuberculosis has been demonstrated previously (Koets et al., 2000), although it has not been linked to specific genes as yet. Candidate genes could be related to innate resistance, as was suggested (Koets et al., 2000), but also to adaptive resistance, *e.g.* major histocompatibility complex (MHC) genes, as was demonstrated for susceptibility to mastitis (Sharif et al., 2000). This would imply that the presumed shift from a Th1 to a Th2 type of immunity that has mainly been suggested to occur based on data from cross-sectional studies, may either be an event which occurs very early in disease or an inherent trait causing animals to react to infection with a Th1 or Th2 type response, continued throughout infection. The clinical stage of disease is apparently correlated to the loss of both the cell mediated and the humoral adaptive responses, due to loss of $CD4^+$ T cells (Koets et al., 2002) possibly through induction of apoptosis. None of the animals in this study, either HFS, IS or LFS, developed clinical signs of disease, despite the fact that the experimental inoculum was taken from an animal with active clinical disease and a prolonged high dose scheme was used. Although this phenomenon has been observed in other studies with experimental infection the cause of this finding remains enigmatic.

## Conclusion

In conclusion our study demonstrates that oral experimental infection of calves with a field isolate of *M.a.p.* led to establishment of infection in all animals, although in this study none of the animals developed clinical signs of disease. Discrimination between the group of high frequent shedders and low frequent shedders by antigen specific cell mediated reactions was possible until first parturition only, afterwards the specific cellular immunity was not significantly different between the groups, although maintenance of higher proportions of $CD4^+$ T cells as compared to $\gamma\delta$ T cells at the time of parturition was related to low faecal shedding. Antibody responses however do differentiate between the two groups. High frequent shedders produced anti-PPD-P antibodies as early as one year after infection, the production of which continued during the five years period in ever increasing amounts. Moreover, a boost in antibody production was observed following every parturition. The correlation between high antibody production and high frequent shedding has been made previously, however, the current data argue against this correlation as representative for a Th1 to Th2 shift, together with development of clinical disease, in individual animals. A theory is favoured in which different, likely genetically determined, immune responses are mounted upon infection which are continued throughout. Although early cell mediated immune responses appear to favor the control of mycobacterial disease, the fact that truly protective immune-responses leading to clearance of bacteria were not observed, as well as the fact that prolonged type 2 responses do not lead to clinical disease, further add to the complexity of this mycobacterial disease.

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***Mycobacterium avium ssp paratuberculosis recombinant* heat shock protein 70 interaction with different bovine antigen presenting cells**

## Abstract

Heat shock proteins (Hsp) can deliver antigen into the MHC class I presentation pathway of antigen presenting cells (APC), a process called cross-priming, thus stimulating antigen specific $CD8^+$ T- cell reactions. Hsp were also shown to elicit pro-inflammatory responses in APC. Both processes require interaction of Hsp with APC via specific receptors. In the current study we show receptor mediated interaction of recombinant Hsp70 of *Mycobacterium avium subspecies paratuberculosis* (rHsp70) with RAW 264.7, as has been described previously for Hsp from other sources. Interaction of rHsp70 with bovine peripheral blood mononuclear cells (PBMC) was restricted to $CD14^+$ cells. To investigate interaction of rHsp70 with different bovine APC, we used characterized monocyte derived macrophages (MDM), dendritic cells (DC) and Bomac, an immortalised bovine macrophage cell line. We demonstrated saturation of immature DC with high concentrations of rHsp70 and we showed that interaction of rHsp70 with DC was related to the maturation stage of the DC. Involvement of CD91 as a cellular receptor for rHsp70 was demonstrated however competition studies with immature DC demonstrated that other receptors exist on bovine APC. These data suggest that rHsp70 based vaccines may be useful for the successful immunisation of cattle.

## Introduction

Over the past few years heat shock proteins (Hsp) have been shown to fulfil many different roles in cell physiology and immunity. Intracellularly, Hsp are involved in the folding and unfolding of endogenous proteins (Young, 1990), while extracellularly occurring Hsp were shown to be proteins capable of inducing the maturation of dendritic cells (DC) (Singh-Jasuja et al., 2000), and acting as a cytokine, called chaperokine, thereby linking innate and adaptive immunity (Asea et al., 2000). Hsp can function as antigen carriers, shuttling antigen from outside the cell into the MHC class I-restricted antigen presentation pathway (Castellino et al., 2000; Suzue et al., 1997), a process called 'cross-priming' (Thery and Amigorena, 2001).

To exert their function, specific, receptor mediated interactions between the heat shock proteins and antigen presenting cells (APC) are necessary (Arnold-Schild et al., 1999; Singh-Jasuja et al., 2001; Singh-Jasuja et al., 2000). Different receptors have been identified including CD91 (the low density lipoprotein receptor-related protein/ $\alpha$2macroglobulin receptor (LRP /$\alpha$2M receptor)) (Basu et al., 2001), CD14 (Asea et al., 2000), CD40 (Becker et al., 2002; Wang et al., 2001), lectin-like oxidized LDL receptor (LOX) (Delneste et al., 2002), and Toll-like receptors (TLR) 2 and 4 (Asea et al., 2002; Vabulas et al., 2002), while the existence of additional receptors can not be excluded.

Induction of cytotoxic T-lymphocytes (CTL) against known and unknown epitopes has been proven to be possible with the use of Hsp, either as a carrier of peptide or as a fusion protein (Cho et al., 2000; Huang et al., 2000; MacAry et al., 2004; Moroi et al., 2000; Noessner et al., 2002; Suzue et al., 1997; Tobian et al., 2004). This offers great opportunities for vaccine development (Hoos and Levey, 2003), when immunisation is needed in a population consisting of different MHC types where protection against incompletely characterised antigens is required (Li et al., 2002), as long as this protection can be provided by cytotoxic T-cells.

Studies using mouse models and macrophage cell lines (*e.g.* RAW 264.7 (Binder et al., 2000)) *in vitro* indicated working principles of the Hsp-APC interaction and the induction of a CTL reaction (Cho et al., 2000; Harmala et al., 2002; Kuppner et al., 2001; More et al., 2001; Suzue et al., 1997; Tobian et al., 2004; Udono et al., 2001; Wang et al., 2002). The Hsp in these studies were of either eukaryotic or prokaryotic origin (partially summarized in: (Wallin et al., 2002)). Fusion with antigenic proteins or single peptides loaded to the Hsp are shown to be important for the specificity of the CTL reaction, but not for the adjuvanticity of Hsp (Asea et al., 2000). Extrapolation of the results obtained in these model studies mainly led to applications in the field of tumor immunology (Blachere et al., 1997; Hoos and Levey, 2003; Noessner et al., 2002) but could also apply to infectious diseases.

Based on the idea of a potentially protective role for cytotoxic CD8+ T-cells in mycobacterial infections (Kaufmann, 1991), we hypothesized that CTL would be important in immunity against bovine paratuberculosis, a disease in which the intracellularly residing *Mycobacterium avium ssp. paratuberculosis* (*M.a.p.*) leads to chronic intestinal inflammation (Cocito et al., 1994). As in the battle against bovine paratuberculosis no effective vaccine is currently available, there is a need to develop an immunisation method that induces a CTL reaction in cattle. For this purpose Hsp are a potential candidate.

Preliminary work indicated that recombinant Hsp 70 of *M.a.p.* (rHsp70) interacted with bovine APC (Langelaar et al., 2002). The objective of the current study was to study murine and bovine APC and their interaction with rHsp70 to evaluate whether the basic requirements for initiating cross-priming studies could be fulfilled.

The results from the current study indicate that rHsp70 interacts with the murine macrophage cell line RAW 264.7 similar to other mycobacterial Hsp. Using phenotypically characterized bovine monocytes, monocyte derived macrophages (MDM), monocyte derived

dendritic cells (DC) and Bomac, an immortalised bovine macrophage cell line (Stabel and Stabel, 1995), we showed that rHsp70 interaction with bovine PBMC is restricted to CD14 positive cells. We have found indications that the receptor involved in cross-priming, CD91 plays a role in uptake of rHsp70 by bovine APC, although other cellular receptors are likely involved as well. Together, these data justify follow-up studies on immunisation of cattle with rHsp70 based vaccines.

## Materials and methods

***Animals***

The animals used in this study were Holstein-Friesian cows, conventionally housed at the Department of Farm Animal Health of the Faculty of Veterinary Medicine, Utrecht, the Netherlands. Use of the animals for these experiments was approved by the Ethical Committee.

***Cell culture***

The murine macrophage cell line RAW 264.7 (ATCC TIB-71) and the bovine macrophage cell line Bomac (Stabel and Stabel, 1995) were cultured in culture medium (CM):RPMI 1640, supplemented with 10%FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, 2mM L-glutamine and $5.10^{-5}$ M β-mercaptoethanol. Every 3-4 days, when cells formed a confluent layer, cells were detached with AccutaseR (Sigma) and diluted 1:20 in fresh CM.

Bovine blood was taken from the V.jugularis and collected into heparin containing tubes (BD Vacutainer, Beckton, Dickinson). PBMC were isolated by density gradient centrifugation on Histopaque (1,077 density) (Sigma). Mononuclear cells were collected from the interphase, washed twice in PBS, counted and diluted in CM.

To generate monocyte derived macrophages (MDM) or monocyte derived dendritic cells (DC), $CD14^+$ cells were isolated from the PBMC using the MidiMACS system. PBMC were incubated with αHuCD14 (mouse antibody isotype IgG2a), cross reactive with bovine CD14 (Sopp and Howard, 1997), coupled to super paramagnetic microbeads (Miltenyi Biotec GmBH). After ten minutes of incubation at RT, cells were washed twice in PBS and transferred to a MACS separation column ( Miltenyi). After three washes with cold PBS supplemented with 2% FCS, the magnetically labelled cells were collected. The monocytes obtained by this procedure were 85-98% pure, as analysed by flow cytometry, using FITC-conjugated αHuCD14 (TÜK-4, CLB, Amsterdam, the Netherlands) (data not shown). Monocytes, at a concentration of $1\text{-}2.10^6$ /ml were allowed to adhere to the bottom of culture flasks and cultured in CM for the generation of MDM. DC were cultured in CM supplemented with bovine rIL-4 and bovine rGM-CSF (COS cell supernatant at concentrations predetermined optimal) at a concentration of $0{,}8\text{-}1.10^6$ cells/ml in 6-well culture plates (Costar, Corning, NY). After four days of culture 1 ml CM/ well was replaced by fresh medium. For use in flow cytometry experiments, cells were collected after gently detaching the loosely adherent cells with Cell Dissociation Fluid (Sigma). DC were used after 3 days (DCd3), 5 days (DCd5) and/or 7 days (DCd7) of culture.

***Phenotypes of bovine cells***

Cells were plated in 96-well microtiter plates (Costar, Corning, NY) at a concentration of $1\text{-}2.10^5$/100 µl PBS and incubated at 4 $^0$C with relevant monoclonal antibodies: IL-A42 (immunoglobulin G2a [IgG2a], anti-bovine CD2 [α-bo CD2], ILRAD), GB21A ( IgG1, α-bo γδ TCR, VMRD), MM8 (IgG1, α-bo CD14, VMRD), GB25A (IgG1, α-bo CD21, VMRD), IL-A88 (IgG2a, α-bo MHC I, Compton), CC158 ( IgG2a, α-bo MHC II, Compton), CC149 (IgG2b, α-bo Myd-1, Compton), IL-A159 (IgG1, α-bo CD80, ILRI), IL-A190 (IgG1, α-bo CD86, ILRI), and

IL-A156 (IgG1, α-bo CD40, ILRI) (α-bo CD80, α-bo CD86 and α-bo CD40 are a gift of Dr. N. McHugh, ILRI, Kenya). After 15' of incubation, cells were washed and incubated with a fluorescent labelled secondary antibody (GAM-PE, diluted 1:1000, BD Biosciences). After 15' cells were washed again, transferred to microtubes in a final volume of 200 μl in Facsbuffer (PBS containing 1% of BSA and 0,1% Sodium azide) and fluorescence was measured by flow cytometry (Facs Calibur, Becton Dickinson, San Jose, CA). Gates were set around the population of live cells as determined by propidium iodide (PI) exclusion, and based on the forward and side scatter profiles predetermined for monocytes and monocyte derived cells. Analysis of results was carried out using Cellquest software.

***Protein preparations***

Recombinant *M.a.paratuberculosis* Hsp70 was produced and purified as described previously (Koets et al., 2001). Briefly, *E.coli* Top10 bacteria, transformed with plasmids encoding for *M.a.paratuberculosis* Hsp70, were induced to produce the rHsp70 with a N-terminal histidine tag. The protein was affinity purified using Ni-NTA columns, eluted with 20 mM imidazole in the elutionbuffer (50 mM EDTA, 20 mM Tris-HCL). Purity of the protein was verified using SDS-PAGE. The protein concentration was determined using a standard BCA protocol (Pierce BCA Assay Kit).

For the use in protein binding and uptake experiments, fluorescein isothiocyanate (FITC) (Fluorotag TM FITC Conjugation Kit, Sigma) was conjugated to rHsp70, OVA (Sigma) and α2M (α2macroglobulin; MW 725 kD, Sigma prod.nr.M-7151) according to the manufacturer's instructions.

***Cell surface binding and uptake of rHsp70***

To determine the subset of bovine cells that was responsible for rHsp70 uptake, freshly isolated PBMC were incubated with rHsp70-FITC or OVA-FITC as a control at $37^0$ C. At different time-points (1, 5, 10, 15, 20, 30, 40 and 60 minutes) incubation was abrogated, cells were washed to remove excess protein and stained for phenotypic characterization by flow cytometry.

In binding studies, cells at a concentration of $1\text{-}2.10^5$ cells/well were kept on ice in PBS supplemented with calcium and magnesium (PBS-Ca/Mg). Cells were allowed to cool down for 15' to minimize internalisation of protein. Subsequent incubation with protein for 30' also took place on ice. After the incubation, cells were washed twice at 40C and finally transferred, on ice, to tubes and immediately analysed by flow cytometry. To measure dose dependent binding of rHsp70 to the cells, a 10mg/ml solution of FITC-conjugated rHsp70 in PBS was used. Dilutions of this solution were made by mixing the solution with PBS-Ca/Mg to obtain different molarities. Cells were incubated for 30' on ice with the different molarities. For uptake studies, cells were incubated at $37^0$ C to allow internalisation to occur.
To compare OVA and rHsp70 uptake, RAW264.7 cells were incubated with either of the proteins conjugated to FITC. At different time-points (0, 0.5, 1, 2, 3, 4, 6, 8, 10, 15 and 20 minutes) the incubation was abrogated and cells were fixed in 4% paraformaldehyde and kept on ice until measurement.

In competition of uptake experiments, RAW264.7 cells were incubated with 0, 15.6, 31.3, 62.5, 125, 250 or 500 μg of unlabelled rHsp70 or OVA and incubated at $37^0$ C. After 5 minutes, rHsp70-FITC was added to the cells (2 μg rHsp70-FITC/ $2.10^5$ cells in 150 μl total volume/well). After 10 more minutes of incubation, cells were fixed in 4% paraformaldehyde (PF) and placed on ice. Following the same procedure, BoMac were used to compare competition of uptake with rHsp70 and with α2M. The unlabelled rHsp70 was used in a ratio of 0, 10, 50 and 100 μg to 2 μg rHsp70-FITC (0, 5, 25 and 50 times molar excess), α2M was used in a ratio of 0, 50, 250, 500 μg to 2 μg rHsp70-FITC (0, 10, 50 and 100 times molar excess). The same experiment was carried out using DCd3 with 0, 65, 125 and 250

µg of α2M and 2 µg rHsp70-FITC (0, 13, 25, 50 times molar excess). Molarities are calculated with the MW of the α2M subunits being 179.000 (Sigma, technical data sheet M-7151), this size was confirmed when the α2M solution was brought on a SDS polyacrylamide gel under reducing conditions in our lab.

## Results

### *Interaction of rHsp70 with RAW264.7 cells*

*Binding and uptake*

Incubation of the mouse macrophage cell line RAW264.7 with rHsp70-FITC induced a large increase of fluorescence intensity (fig 1A) as compared to untreated control cells, indicating binding of the recombinant protein to these cells. In a subsequent experiment we investigated the uptake of the FITC conjugated rHsp70 by RAW264.7 cells as compared to the uptake of FITC conjugated OVA. After 1 minute of incubation the fluorescence intensity of the rHsp70-FITC incubated cells tripled (geo mean from 35 to 105). By 20 minutes it had increased by almost 600% (geo mean from 35 to 182), at which time-point the cells were not yet saturated as shown by the continuously rising curve (fig 1b). OVA-FITC was not substantially taken up in this time-span. After 20 minutes only a minor rise in fluorescence intensity (geo mean 54 to 64) was observed in cells exposed to OVA-FITC.

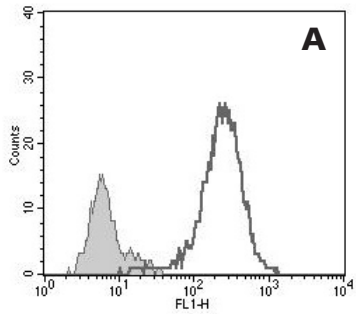
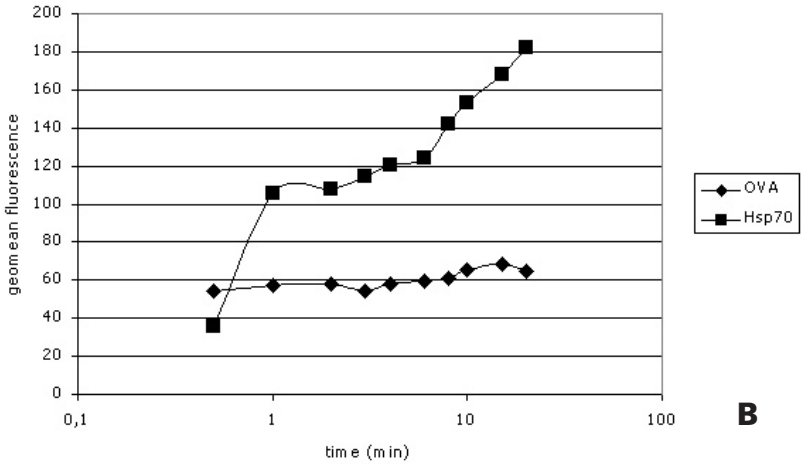



Fig. 1 Binding and uptake of rHsp70-FITC by RAW 264.7 cells.

A. Binding of rHsp70-FITC (fl-1) to RAW 264.7 cells on melting ice. Fluorescence intensity of the cells (on the x-axis) is plotted against cell counts. The gray histogram represents background fluorescence of the cells.

B. Uptake of rHsp70-FITC compared to that of the control protein OVA-FITC by RAW264.7 cells, incubated at 37°C. Geo mean fluorescence intensities were determined by flow cytometry and plotted against the time allowed for uptake of the different proteins.



*Specific uptake of rHsp70*
RAW 264.7 cells incubated with rHsp70-FITC, in the presence of unlabelled protein (rHsp70 or OVA) as a competitor (fig 2), showed a decline in geo mean fluorescence when rHsp70 was used as the competing protein, but not when OVA was used. Only 34% of maximum fluorescence was found when cells were incubated with the maximum concentration of unlabelled rHsp70 (250 times molar excess).

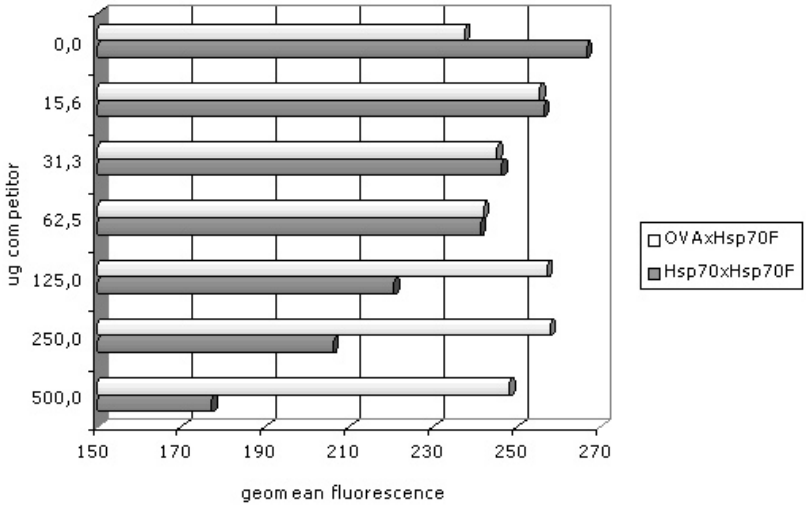



Fig. 2

Competition of rHsp70 uptake by RAW264.7 cells. Cells incubated with 2 µg rHsp70-FITC pre-incubated with increasing amounts of unlabelled protein (rHsp70 or OVA) at 37ºC. Analysis was performed by flow cytometry and geo mean fluorescence plotted against the amount of competitor protein.

### *Interaction of rHsp70 with bovine cells*

*rHsp70 binding and uptake is CD14⁺ cell restricted*
Monocytes within the PBMC population (CD14⁺ cells), but not the remainder of the cells (CD14- cells) clearly showed uptake of the fluorescent labelled rHsp70 (fig 3). The pattern of uptake of rHsp70 in time by CD14⁺ cells was represented by a steep curve, whereas CD14- cells neither took up nor bound rHsp70, as shown by the flat line. OVA-FITC was marginally taken up by the CD14⁺ cells.

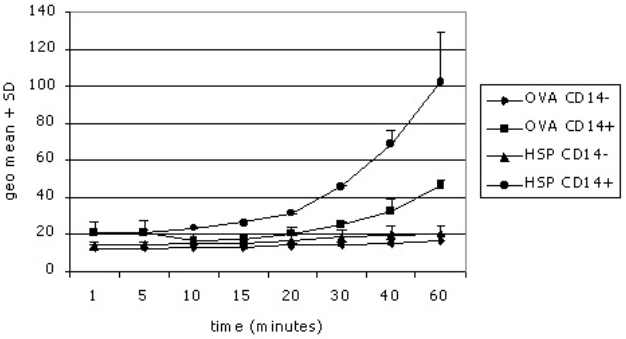



Fig. 3

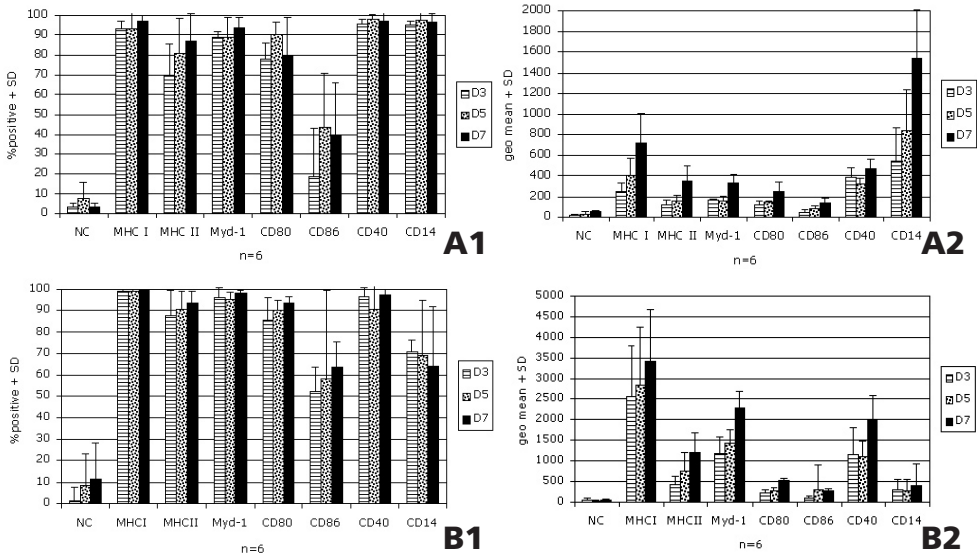
Binding and uptake of rHsp70 by bovine PBMC is CD14⁺ cell restricted and time dependent. Freshly derived PBMC were incubated for 35 minutes at 37ºC with rHsp70-FITC, or OVA-FITC as a control. Rapid endocytosis of rHsp70 compared to a minor quantity of OVA that is slowly taken up. CD14 negative cells (T-cells, B-cells) show no interaction with the proteins.

*Phenotype of bovine cells*

The different bovine APC-like celltypes used in this study (MDM, DC and Bomac) were phenotyped by flow cytometry.

MDM virtually all expressed CD14 (95-100% positive, fig.4A1) and the intensity of staining for this molecule increased with time of culture (fig.4A2).

DC lost surface expression of CD14, at day 3 of culture only 70% of the cells expressed this molecule, declining to 63% at day 7 (fig.4B1), while intensity of expression was low (fig.4B2). DC had a very intense staining with the antibodies against MHC class I, Myd-1 (CD172a) and CD40, and this increased in time. Expression of MHC class II, CD80 and CD86 also increased during the seven days of *in vitro* culture (fig.4B2).




Bomac were positive for MHC class I (95% of the cells), Myd-1 (11%) and CD40 (58%) (fig.4C1). MHC class I was the highest expressed molecule (geo mean 95) and the geo mean of CD40 expression was 27. Some expression of Myd-1 (CD172a) was detected (geo mean 14), however, there was no detectable expression of the other molecules defined by the monoclonal antibodies of our panel (fig.4C2). Morphology of the cells as judged by light microscopy was typical of macrophage-like cells. In addition, Bomac were capable of phagocytosis of fluorescent particles, as measured by flow cytometry and confocal laser scanning microscopy (data not shown).

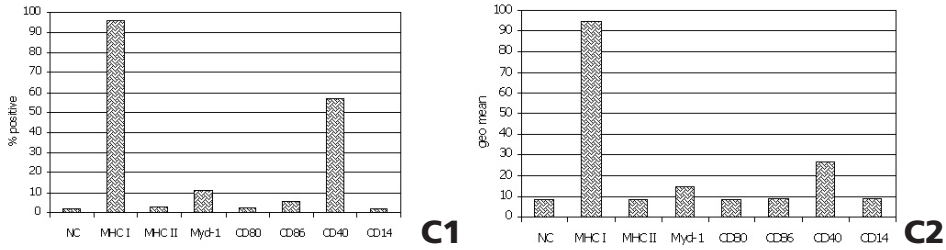



Fig. 4

Expression of surface molecules on MDM (panels A), DC (panels B) and BoMac (panels C), changes during time of culture (day 3, 5 and 7). Left panels represent the percentages of cells positive for a certain molecule, right panels represent the expression level of a certain molecule as expressed by the geo mean fluorescence intensity. Data on MDM and DC are averages calculated from 2 independent experiments, using 3 different animals.

*Binding of rHsp70 to bovine dendritic cells is related to maturity of the cells*
The percentage of cells interacting with rHsp70 decreased in time (approximately 40% decrease from day 3 to day 7 of culture) after an initial increase from day 0 to day 3 (fig.5A). However, intensity of rHsp70 fluorescence but not autofluorescence increased with time, indicating that at day 7 the cells had a higher binding capacity for rHsp70 (fig.5B).

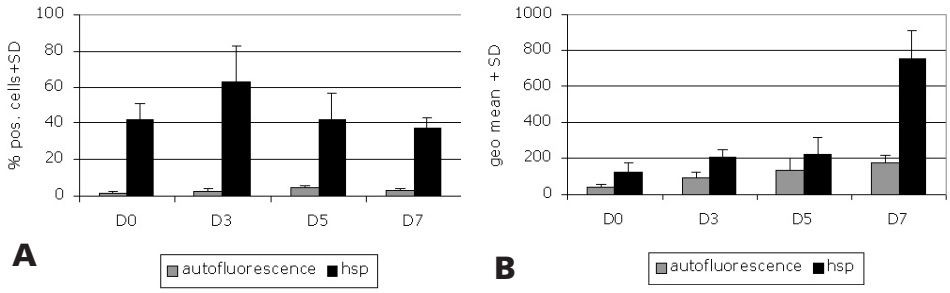



Fig. 5
Interaction with rHsp70-FITC is related to maturity of the DC. The percentage of cells interacting with rHsp70-FITC (A) and the geo mean fluorescence of the positive cells (B) of DC incubated on melting ice, at different stages of maturation (number of days in culture plotted on the x-axis). Data, analyzed by flow cytometry, are represented as mean + SD, of DC cultured from three different animals. The experiment was performed three times with comparable results.

*Dose dependency of rHsp70 interaction with bovine dendritic cells*
Fig.6 demonstrates that day 3 DC incubated with increasing molarities of rHsp70-FITC on ice show dose dependent increases in fluorescence intensity. As much as 70 μM rHsp70 or more had to be added to the cells before the inclination of the curve decreased, nearing plateau values at 150 μM.

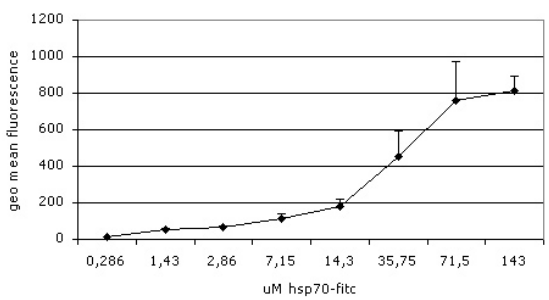



Fig. 6

Interaction of DC with rHsp70 is dose dependent. DC of day3 were incubated with increasing amounts of rHsp70-FITC. Geo mean fluorescence of the cells was plotted against the concentration of rHsp70-FITC (μM). Data represented are the mean of DC of three different animals+SD, of an experiment performed two times with similar results.

*The LRP /α2M -receptor is involved in Hsp70 binding on bovine APC*
Bomac and day 3 DC were used to investigate whether CD91 was involved in uptake of rHsp70 by bovine cells. Bomac rapidly bound and internalised rHsp70-FITC, in a similar manner to bovine monocytes, MDM and DC (data not shown). The labelled protein α2M-FITC was also internalised by bovine cells, this was more pronounced by DCd3 than by Bomac (fig 7A).

BoMac were allowed to take up rHsp70-FITC with either unlabelled rHsp70 or α2M as competitor molecules (fig. 7B). By adding a molar excess (up to 50 times) of unlabelled rHsp70, Bomac uptake of rHsp70-FITC could be inhibited by 8%. A gradual inhibition, up to 60% at a 100 times molar excess, could be observed when α2M was used as a competitor for the rHsp70-FITC uptake.

Uptake of α2M-FITC could be blocked (fig 7C) by α2M itself (maximal inhibition 22%), but when rHsp70-FITC uptake by DCd3 was blocked with α2M, no clear inhibition of uptake could be observed (fig 7D). Blocking of rHsp70-FITC uptake by rHsp70 was not evident either, in contrast increased uptake of rHsp70-FITC in the presence of rising concentrations of unlabelled ligand was observed (fig 7D).

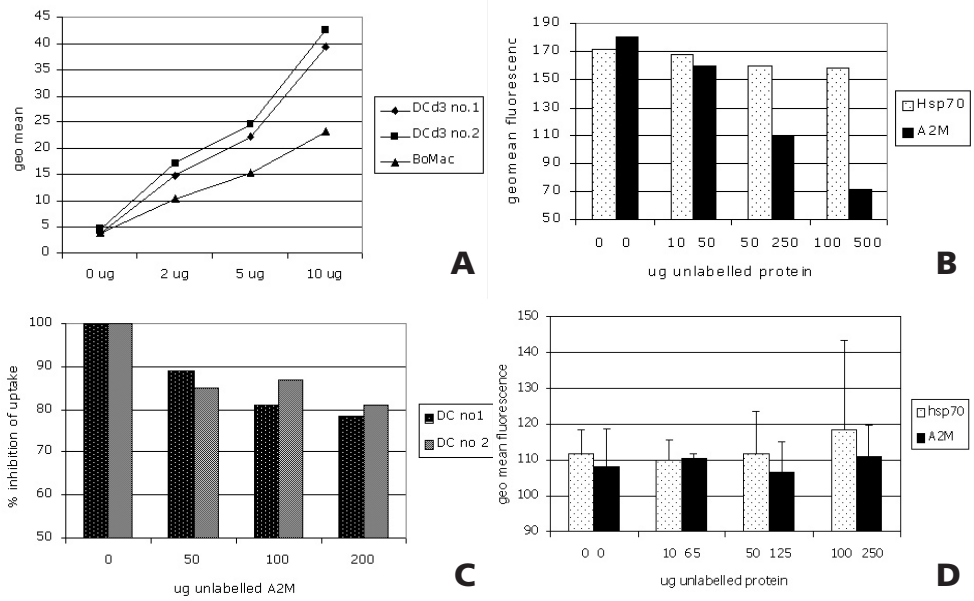



Fig. 7

Uptake and competition of uptake.

A. Uptake of α2M-FITC by BoMac and DCd3 of two different animals. Cells were incubated with increasing concentrations of α2M, for 30 minutes at 370C. Analysis was performed by flow cytometry, geo mean intensity of the cells is plotted against the concentration of protein.

B. BoMac were allowed to take up rHsp70-FITC after pre-incubation with increasing concentrations of unlabelled competing protein; either rHsp70 or α2M. Geo mean fluorescence of the cells is plotted against the concentration of the competing protein .

C. DCd3 of two different animals were incubated with α2M-FITC after pre-incubation with unlabelled α2M as a competitor. Percentage inhibition of uptake of α2M-FITC is plotted against the amount of competing unlabelled ligand.

D. DCd3 of four different animals were incubated with rHsp70-FITC after pre-incubation with increasing concentrations of competing protein; unlabelled rHsp70 or α2M. Geo mean fluorescence of the cells is plotted against the concentration of the competing protein. Data represent the mean of four + SD.

## Discussion

Some APC interact with mammalian Hsp but not with prokaryotic Hsp (Sondermann et al., 2000) and some APC have been reported not to interact with Hsp (Basu et al., 2001; Lipsker et al., 2002; Sondermann et al., 2000). We first demonstrated that recombinant Hsp70 of *M.a.p.* interacted with the murine macrophage cell line RAW 264.7, as has been shown for other, mammalian, Hsp (Binder et al., 2000; Habich et al., 2002). Rapid and preferential uptake of rHsp70 compared to OVA indicated that binding and uptake was a receptor mediated process, a finding substantiated by the possibility to compete this uptake with an

excess of rHsp70 itself, but not with OVA. We then questioned whether the interaction of this rHsp70 with bovine PBMC would be restricted to monocytes, as has been described for Hsp70 interaction with cells of other species (Binder et al., 2000; Fujihara and Nadler, 1999). Uptake of rHsp70 was restricted to monocytes ($CD14^+$ cells) and preferential over uptake of OVA. These results were similar to the results with RAW264.7 cells, indicating receptor mediated endocytosis of rHsp70 by bovine monocytes.

To further study the interaction of rHsp70 with bovine APC, we investigated the phenotypical differences of MDM and DC at different time-points in culture. In our hands, as described earlier (Howard et al., 1999; Werling et al., 1999) culture of monocytes ($CD14^+$ cells) in the presence of the cytokines IL-4 and GM-CSF led to cells with a typical dendritic cell phenotype. When both cell types were followed during seven days, the cells matured, as shown by the upregulation of surface molecules (fig4). However, the level of expression, represented by the intensity of staining of these molecules was 2- to 6-fold higher on DC than on MDM, except for CD14 which was lost on DC while it was present on all MDM. Upregulation of this molecule was most prominent compared to the other surface molecules on MDM. Expression of the costimulatory molecule CD86 was relatively low on both cell types, but more DC than MDM expressed the molecule (64 vs. 40% at day seven of culture). Maturation of cells and divergence of phenotypic features of the bovine cells in our hands was largely similar to that of MDM and DC during culture described in literature (Werling et al., 1999; Woodhead et al., 1998). We thus concluded that bovine monocyte derived dendritic cells after three days of culture could be used as immature DC for studies of the interaction with rHsp70.

Unexpectedly, the immortalised macrophage cell line Bomac expressed only a small number of the molecules expressed by MDM. MHC class I and CD40 expression could be detected on a majority of the cells (95% and 58% respectively) and Myd-1 (CD172a) on a minor population (11%), but intensity of staining was lower for all molecules on Bomac than on MDM. Most strikingly, no detectable level of CD14 expression could be observed. Although functionality of Bomac was disputed before (Sager et al., 1999), and expression of the surface molecules important for antigen presentation differs largely from that in the bovine MDM and DC, the cell line showed phagocytosis and interacted with rHsp70 as good as the other cell populations (monocytes, MDM, DC) used in our studies. For this reason we included Bomac in the investigations of the interaction of bovine APC with rHsp70.

Differences in expression of surface molecules on maturing DC have been explained by functional changes of the cells, from cells with a mainly phagocytic function to cells that are highly specialised in antigen presentation (Reis e Sousa et al., 1999; Woodhead et al., 1998). Hsp70 has been shown to influence monocyte maturation into DC, depending on the maturation stage at which the Hsp70 is administered. Incubation of immature DC with Hsp70 can induce maturation of the cells together with down-regulation of the Hsp70-receptor (Singh-Jasuja et al., 2000), but arrest of maturation from monocytes to DC has also been observed (Kuppner et al., 2001; Sondermann et al., 2000). It has been argued that the maturing effect of Hsp70 on DC is not a property of the Hsp itself, but due to LPS that is present in the recombinant Hsp70 preparations (Bausinger et al., 2002; Beg, 2002). We avoided this influence of rHsp70 on the phenotype of the cells, by studying interaction of rHsp70 and APC at different time-points of cell differentiation. Our data demonstrated that interaction of Hsp70 with bovine APC is related to maturity of the cells per se. Within the population of mature DC (DCd7) 40% of the cells lost the ability to bind rHsp70, compared to immature cells (DCd3). This was comparable to the number of monocytes (day 0) that interacted with rHsp70. However, at day 7 the cells that bound rHsp70 showed increased binding of rHsp70 compared to immature cells. A possible explanation for this could be that immature DC, which have to take up foreign antigen for presentation to naïve T cells, all express sufficient amounts of a Hsp70 receptor on their surface to facilitate uptake of antigen

that is captured in the Hsp70 molecule. Mature DC in turn have switched to the antigen presenting mode in which they activate T-cells. We hypothesized that within the population of mature DC a subpopulation of cells remains ready to take up antigen, even in very low concentrations, therefore highly expressing the Hsp70 receptor.

Features of receptor mediated uptake of protein include rapid endocytosis and saturation of receptor binding (Singh-Jasuja et al., 2000). Immature DC (DCd3) showed dose dependent binding of fluorescent labelled rHsp70 that approached saturation levels only at high molarities (fig.6). It is presumed that presence of the Hsp70 receptor on DCd3 is very abundant and/or that retranslocation of the receptor to the cell surface (Howard et al., 1999) occurs rapidly. Even induced additional expression can not be excluded (fig.7D). Moreover, there might be more than one receptor for Hsp70. The latter idea finds support in a study by Delneste et al.(Delneste et al., 2002) who argued that different receptors for Hsp70 exist on DC, endocytic and signalling receptors, that cooperate to activate APC.

As we observed receptor mediated uptake of rHsp70 by RAW 264.7 cells and we also observed rapid uptake of rHsp70 by bovine monocytes, we focused on the role of CD91 as a possible receptor for rHsp70 on bovine cells. CD91 has been well described as a receptor for Hsp70, Hsp90 and gp96 (Basu et al., 2001), amongst other molecules (Hussain et al., 1995), although literature has also been conflicting on this subject (Berwin et al., 2002). Tobian et al. (Tobian et al., 2004) showed that CD91 acts as an endocytic receptor for bacterial Hsp70 as well as for mammalian Hsp70. Existence of this receptor on bovine cells could be expected as the LRP /$\alpha$2M receptor is a very common molecule on many cell types (APC, hepatocytes, endothelial cells (Moestrup, 1994)), although between APC there are clear differences in CD91 expression. Murine macrophages and bone marrow-derived dendritic cells (Basu et al., 2001), immature human monocyte derived dendritic cells (Lipsker et al., 2002) and human blood monocytes (Hart et al., 2004; Stebbing et al., 2003) but only a subset of human CD11c$^+$ blood dendritic cells (Hart et al., 2004a) and not epidermal dendritic cells (Lipsker et al., 2002) express this receptor. Bomac and immature DC used in this study both internalised $\alpha$2M with similar kinetics as observed for rHsp70. Moreover, uptake of $\alpha$2M-FITC could be inhibited by unlabelled $\alpha$2M, indicative of the presence of a specific receptor on these bovine APC.

After the identification of the LRP/$\alpha$2M receptor on bovine cells, we investigated if it could also be a receptor for rHsp70. Comparison of a part of the sequence of rHsp70 (AA353-386, the C-terminal end of the ATP-ase domain (Flaherty et al., 1990; Zhu et al., 1996)) with a proposed binding motif for $\alpha$2M with the receptor (Nielsen et al., 1996), showed important similarities, including the two Lys residues spaced by three amino acid residues critical for receptor binding. It was unexpected however, when Bomac were investigated, to find that blocking the internalisation of rHsp70-FITC with $\alpha$2M lead to much better inhibition than blocking with unlabelled rHsp70 (60% vs 8%) itself. Presumably, $\alpha$2M would be a better competitor for rHsp70-FITC uptake via CD91 because it was the appropriate molecule for this receptor and would have a higher affinity for the receptor then rHsp70. On the other hand, as a maximum of 100 times molar excess of $\alpha$2M was added it could be argued that it was surprising to still find important uptake (40%) of rHsp70. An explanation for the remaining 40% of uptake would be the existence of another receptor for Hsp70 on the Bomac cell surface. Even total blocking of all LRP/$\alpha$2M receptors would not prevent uptake of rHsp70-FITC, if yet another receptor was involved.

Even less expected was the impossibility to compete for uptake of rHsp70-FITC by d3 DC when either $\alpha$2M or rHsp70 was used, while in contrast incubation with unlabelled rHsp70 seemed to lead to increasing uptake of rHsp70-FITC. Although there was much variation between the DC cultured from different animals (fig.7D), this tendency in kinetics has been observed on several occasions. The same argumentation might apply here as for the difficulty to sature the cells with rHsp70; a logic explanation being the existence of several

different receptors for rHsp70.

## Conclusion

This report provides more insight in the different types of bovine antigen presenting cells and the specificity of the interaction between recombinant Hsp70 of *M.a.p.* and bovine APC. We demonstrated that, based on phenotype characteristics, monocyte derived dendritic cells after three days of culture could be considered as immature DC for further studies on APC-rHsp70 interaction. The bovine macrophage cell line Bomac lost many of the surface molecules typical for macrophages, especially the CD14 molecule, but could still be instrumental in investigations on the specific Hsp70 receptor on bovine cells because interaction of Bomac with rHsp70 was not affected. Endocytosis of rHsp70 within the bovine PBMC population was restricted to bovine monocytes ($CD14^+$ cells). Internalisation of rHsp70 by bovine APC was highly efficient and difficult to saturate. Although we demonstrated involvement of the LRP/$\alpha$2M receptor on Bomac, our results indicate the existence of more receptors for rHsp70 on bovine cells.

Receptor mediated uptake of rHsp70 by bovine APC in this *in vitro* cell culture system supports our idea that in vivo cross-priming of APC using rHsp70 is feasable. Further elucidation of the fate of rHsp70 in bovine cells and the receptors involved, will be relevant when it comes to designing a CTL inducing vaccine.

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APC 4

# Generation of a recombinant heat shock protein 70 fusion construct with enchanced green fluorescent protein

HSP MAP

## Abstract

Paratuberculosis in cattle, caused by *Mycobacterium avium subspecies paratuberculosis* (*M.a.p.*), an intracellularly residing bacterium, is a severe threat to dairy and meat industry. Protective immune reactivity to other intracellular bacteria is shown to at least partly rely on cytotoxic T-lymphocyte (CTL) reactivity. Therefore it is hypothesized that a potential vaccine for paratuberculosis should induce a CTL reaction. Heat shock proteins (Hsp) are molecules that have the capacity to induce such CTL reactions. By receptor mediated endocytosis Hsp introduce carried or fused antigenic proteins into the cytosol of antigen presenting cells (APC), for subsequent processing and CTL induction. In this study we generated a fusion protein, consisting of the receptor binding domain (rbd) of rHsp70 of *M.a.p.* fused to enhanced green fluorescent protein (eGFP) as a model antigen (rbd-H70-GFP). In *in vitro* assays, rbd-H70-GFP interacted with bovine $CD14^+$ cells (monocytes) and with a bovine macrophage line (Bomac), similar to full length rHsp70 of *M.a.p.*. These results enable future study of potential CTL induction in cattle after immunisation with the fusion protein and hopefully will ultimately contribute to protection against *M.a.p.* infection in cattle.

***Keywords***
Mycobacterium paratuberculosis, cattle, fusion protein, heat shock protein, CTL, GFPIntroduction

## Introduction

An ever-growing body of evidence shows that heat shock proteins (Hsp) are useful in strategies for the induction of protective immune responses. Heat shock proteins have been used for their adjuvant activity in DNA vaccines (Reimann and Schirmbeck, 2004), as dendritic cell (DC)-targeting molecules (Hauser et al., 2004), and fused to antigens to elicit cytotoxic T-cell (CTL) activity (Anthony et al., 1999; Cho et al., 2000; Huang et al., 2000; Suzue et al., 1997; Udono et al., 2001), although the mechanisms have not been completely elucidated. Receptor mediated interaction of Hsp with DC is crucial for subsequent priming of antigen specific cells by the DC (Banchereau and Steinman, 1998; Singh-Jasuja et al., 2001; Srivastava, 2002).

The Hsp70 molecule is composed of two functionally different parts: a N-terminal ATPase domain of approximately 44 kD, and a C-terminal substrate binding domain (sbd) (Zhu et al., 1996). The ATPase domain is generally considered to harbour the site that determines receptor binding (Huang et al., 2000; More et al., 1999), thus should be included in fusion proteins.

The Hsp70 of *Mycobacterium avium ssp. paratuberculosis* (*M.a.p.*) (rHsp70) was shown to be an immunodominant antigen in cattle infected with *M.a.p.* (Koets et al., 1999), using the recombinant protein for the detection of cellular immunity. Infection with *M.a.p.* causes a chronic enteritis in ruminants, for which no cure nor effective prophylactic therapy is currently available (Cocito et al., 1994; Harris and Barletta, 2001). Based on recent insights in the immunology of other mycobacterial diseases (Kaufmann, 1991; Silva et al., 2001), we hypothesized that *M.a.p.* specific CTL induction in cattle would contribute to the prevention of paratuberculosis. To investigate the possibilities of CTL induction in cattle by immunisation with rHsp70, the present study aimed at developing a fusion protein of rHsp70 with an antigen.

As only few *M.a.p.* specific antigens are identified to date (Bannantine et al., 2004), that have not been precisely described, we selected enhanced green fluorescent protein (eGFP) as a model antigen to be incorporated in the fusion protein. Enhanced GFP has been shown to contain multiple CTL epitopes for humans (Re et al., 2004) and mice (Stripecke et al., 1999), therefore we assumed that it would also contain bovine CTL epitopes. Our approach was to delete the sequence encoding for the sbd of rHsp70 and to replace this by the eGFP gene. Thus we were able to produce and express a DNA construct resulting in stable expression of a protein, consisting of the receptor binding domain (rbd) of rHsp70 fused to eGFP; rbd-H70-GFP. Incubation of rbd-H70-GFP with bovine PBMC and the immortalised bovine macrophage cell line Bomac (Stabel and Stabel, 1995) showed that reconstruction of the molecule did not compromise the binding capacities of the fusion protein in comparison to the original full length rHsp70.

## Materials and methods

***Expression vectors***

Cloning of the full length gene of heat shock protein 70 of *M.a.p.* (rHsp70) in the pTrcHis bacterial expression plasmid (Invitrogen) and its expression has been described previously (Koets et al., 1999). To construct the fusion protein, the $NH_2$-terminal side of the rHsp70 DNA sequence encoding the substrate binding domain (sbd) was replaced by DNA encoding the eGFP. Following digestion with the restriction endonucleases *AflII* (NE BioLabs, cat.no. R0520S) and *HindIII* (Gibco, cat.no.15207-020) the open vector containing the receptor binding domain (rbd) (Huang et al., 2000) was separated from the cleaved DNA (*i.e.* the

sbd) by gel electrophoresis (1% agarose gel) and isolated using the QIAEXII kit (Qiagen, cat. no.20021). Using T4 DNA polymerase, the *AflII* and *HindIII* ends of the open vector were blunted and subsequently purified with a DNA cleaning kit (Zymo Research, cat.no.D4003). A DNA fragment containing the eGFP sequence was synthesized using the pEGFP-1 plasmid (BD BioSciences) as a template. Forward and reverse primers (table 1.) were designed using the 'Primer2' software (to date replaced by 'Primer3': http://frodo.wi.mit.edu/primer3) and ordered at Invitrogen. PCR was carried out using Pwo polymerase (Roche, cat.no.1644947) that resulted in a blunt ended PCR product of 700 bp. Ligation of the two blunt ended DNA sequences (of the open plasmid containing rbd and of eGFP) was performed using the Quick Ligation Kit (NE Biolabs, cat.no.M2200S), resulting in the pTrcHis expression vector encoding for the fusion protein; rbd-H70-GFP (fig.1).

Expression vectors (the ligation product or the pEGFP-1 plasmid) were transformed into *E.coli* DH5a bacteria (*E.coli* Library Efficiency DH5a Competent Cells, Life Technologies, cat.no.18263-012). Expression of the clones was verified by PCR primer sets (table 1.); for eGFP and for rbd-H70-GFP. Succesful ligation and cloning of the rbd-H70-GFP should give a 1500 bp product. For control on the right orientation of the eGFP gene in the construct, the vector was cleaved with two endonucleases; *AflII* and *NotI* (Gibco, cat.no.15441-025). Correct orientation should give a 600 bp product, while false (reversed) orientation of the eGFP gene would reveal a 1300 bp product.

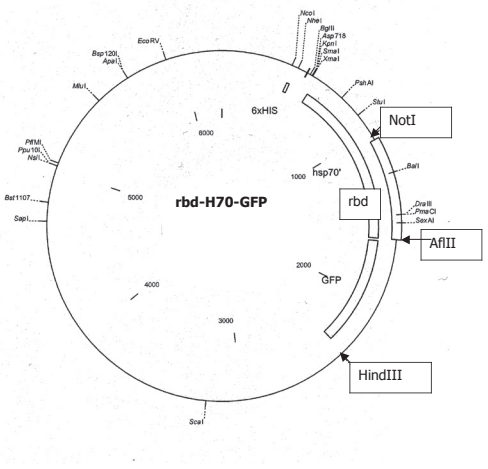



Fig. 1. Expression vector

| PCR product : | | Primer: |
|---|---|---|
| eGFP | Forward-*AflII* | 5'-AAGCCTTAAGATGGTGAGCAAGGGC-3' |
| | Reverse-*HindIII* | 5'-AAGCTTCGAATTGTACAGCTCGTCCAT-3' |
| rbd-H70-GFP | Forward | 5'-AAGCGGATCCTGATGAAGCTCAAGCGCGACG-3' |
| | Reverse-*HindIII* | 5'-AAGCTTCGAATTGTACAGCTCGTCCAT-3' |

Table 1.

***Recombinant protein purification***

*E.coli* DH5a were grown in Luria-Bertani (LB) medium (Biotrading) with 100 μg/ml ampicilline and 50 mM glucose. Protein expression was induced using 1 μM isopropyl thiogalactoside (IPTG) for 5 hours at 37$^0$C and the expression product could be purified as soluble protein. After lysis of the bacteria with 100 μM lysozyme and 3 freeze-thaw cycles, the lysate was centrifugated at 10.000 g and the supernatant was applied to a $Ni^+$-nitrilo-triacetic acid (NTA) column for protein purification, according to the manufacturer's instructions (Qiagen). After extensive washing with Tris-HCl and 60% isopropanol, the protein was eluted and dialyzed against PBS. LPS was removed using the Pierce Endotoxin Removal Kit (Pierce). Protein concentrations were determined using a standard BCA protocol (Pierce BCA Assay Kit). Purity of the protein was verified by 10% sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) and staining of the gel with Coomassie Blue (Bio-Rad), and on Westernblots, using antibodies specific for the His-tag (Amersham, cat.no. 27-4710-01), for eGFP (Living Colors, cat.no. 8362-1), and for an epitope of the N-terminal part of rHsp70 ("7D9")(Koets, 2003), using $\alpha$-mouse-AP (Promega, cat.no.S3721) as a secondary antibody.

***Optimization of protein expression***

*E.coli* DH5a expression clones that were transformed with the rbd-H70-GFP encoding plasmid were analysed by flow cytometry. Twenty colony forming units (cfu) were grown to log-phase ($OD_{600}$ between 0.6 and 1), when IPTG was added. At 0, 2, 4 and 5 hours after induction with IPTG, or without IPTG as a control, 100 µl of bacterial culture was mixed with 100 µl of paraformaldehyde 4% (PF 4%) to kill the bacteria, and directly measured by flow cytometry. Fluorescence of the rbd-H70-GFP expressing bacteria was detected in the Fl-1 channel. Protein expression was determined by fluorescence intensity (MFI) together with the number of bacteria (represented by the number of counts) corresponding to the MFI.

***Cells***

*PBMC*

Bovine blood was taken from the jugular vein and collected into heparinised tubes (BD Vacutainer, Beckton, Dickinson). PBMC were isolated by density-gradient centrifugation (Histopaque (1,077 density) (Sigma). Mononuclear cells were collected from the interphase, washed twice in PBS, counted and diluted to $1\text{-}2.10^6$ cells/ml. (Koets et al., 1999).

*Bomac*

The bovine macrophage cell line Bomac (Stabel and Stabel, 1995) was cultured in culture medium (CM):RPMI 1640, supplemented with 10% FCS, 50 IU/ml penicillin, 50 µg/ml streptomycin, 2mM L-glutamine and $5.10^{-5}$ M $\beta$-mercaptoethanol. Every 3-4 days, when cells formed a confluent layer, cells were detached with AccutaseR (Sigma) and diluted 1:20 in fresh CM. Cells were diluted to $1\text{-}2.10^6$ cells/ml after detachment for use in flow cytometric assays.

***Binding and internalisation of the fusion protein by bovine APC***

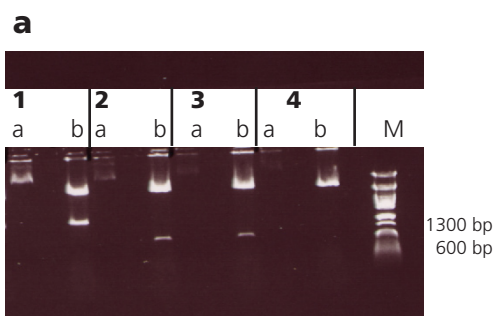
A functional fusion protein requires that binding and internalisation by bovine cells should be similar of that of the full length rHsp70 as described previously (Langelaar et al., 2002). To test this in flow cytometry experiments, PBMC were plated in 96-well microtiter plates (Costar, Corning, NY) at a concentration of $1\text{-}2.10^5$/100 µl PBS-$Ca^{2+}$/$Mg^{2+}$ and incubated on melting ice (for binding) or at $37^0$C (for endocytosis) with rbd-H70-GFP or control proteins (eGFP and rHsp70-GFP (rHsp70 of *M.tuberculosis* (a kind gift of R.A.Young)) for 30'. Cells were washed and incubated with an $\alpha$CD14 antibody (clone MM8, IgG1, VMRD, diluted 1:500), for 15'. Subsequently, cells were washed and incubated with a fluorochrome-labelled secondary antibody (GAM-PE, diluted 1:1000, BD Biosciences). After 15'cells were washed and transferred to microtubes in a volume of 200µl in Facsbuffer (PBS containing 1% of BSA and 0,1% Sodium azide) for assessment by flow cytometry (Facs Calibur, Becton Dickinson, San Jose, CA). The population of $CD14^+$ cells (GAM-PE positive, *i.e.* FL-2 positive) was gated, and the percentage double positive cells ($CD14^+$ and FL-1 positive (*i.e.* eGFP positive)) of the $CD14^+$ cells was determined. Analysis of results was performed using Cellquest software. Percentages of cells that endocytosed the different proteins were compared using a paired student's T-test, $p<0.05$ was considered significant.

Bomac were incubated with rbd-H70-GFP or eGFP as described above, without subsequent surface staining.

## Results

***Production of rbd-H70-GFP***

Successful transformation of *E.coli* DH5a with the correct plasmid for rbd-H70-GFP was verified by PCR. Gel electrophoresis of amplicons of PCR using the the primer pair for the complete rbd-H70-GFP sequence (table 1.) showed a 1500 bp band and with the primer pair for eGFP a 700 bp band (data not shown). Fig.2A shows the result of digestion of the PCR of the rbd-H70-GFP construct with with *NotI/AflII,* that was used to verify the right orientation of the eGFP insertion. One clone showed a 1300 bp band (lane 1) and two clones showed a 600 bp band (lane 2 and 3). As a negative control the open vector containing the rbd sequence, that ligated on itself, was used (lane 4). Westernblot analysis (fig.2B) with antibodies specific for His, rbd (α-7D9) and GFP, showed that when the expressed protein contained the His-tag, the rbd part of rHsp70 and the correctly oriented eGFP respectively, a band was visible at ~70 kD (lane 4 and 5). Three clones, one containing the plasmid for rbd only (lane 1) and two clones containing the plasmid with wrong orientation of eGFP (lanes 2 and 3), were used as a control. A band at ~45 kD was visible when the blots were incubated with anti-His antibodies or α-7D9, but not with antibodies against eGFP. Another control, consistingof eGFP only (lane 6), showed a band at ~30 kD with the α–GFP antibody only.



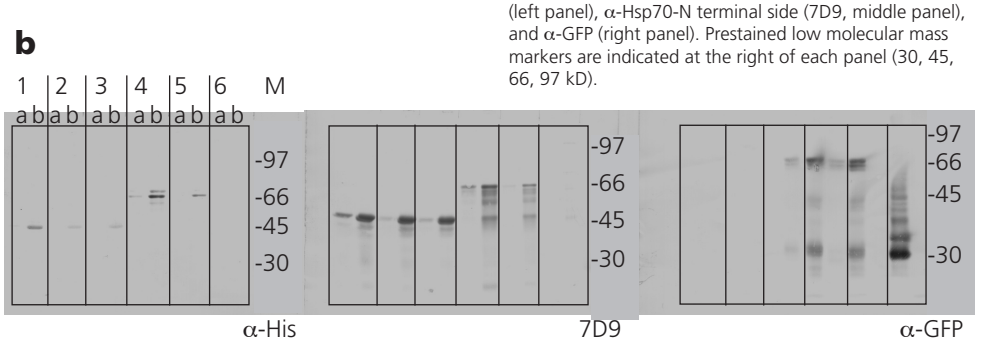




Fig.2
Digestion controls on correct orientation of GFP insertion of clones expressing the fusion protein rbd-H70-GFP (A), and protein expression measured by Western Blot (B).
A: One clone with false orientation (lanes1) shows a 1300 bp band, and two clones with right orientation of GFP (lanes 2 and 3) show a 600 bp band, one negative control (rbd, lanes 4a,b). Lanes a : undigested vector, lanes b: digestion with *NotI/AflII*. M=marker, *PstI* cleaved λ DNA.
B. Western Blot analysisof the negative control (self ligated rbd containing vector, lanes 1a,b), two clones with false orientation of eGFP (lanes 2a,b and 3a,b) and two clones with the right orientation of eGFP (lanes 4a,b and 5a,b) and a positive control for eGFP (lanes 6a,b). Expression clones were induced for 0 (lanes a of each clone) or 4 (lanes b) hours with IPTG. Whole cell preparations were electrophoresed on a 10% SDS-PAGE gel and transferred to nitrocellulose. The membranes were probed with α-His (left panel), α-Hsp70-N terminal side (7D9, middle panel), and α-GFP (right panel). Prestained low molecular mass markers are indicated at the right of each panel (30, 45, 66, 97 kD).

***Optimization of protein expression***

To maximize protein yields from the expression clones, different induction times with IPTG were investigated. Fig.3 shows flow cytometry analysis of bacteria in log phase of growth, after 0, 2, 4, and 5 hours of IPTG induction, compared to uninduced controls at the same time-points. After 5 hours, both IPTG induced and control samples demonstrated the

highest fluorescence, indicative for rbd-H70-GFP expression. Induction of IPTG stimulates more bacteria to express the protein, as demonstrated by higher counts compared to controls at the same time-points.

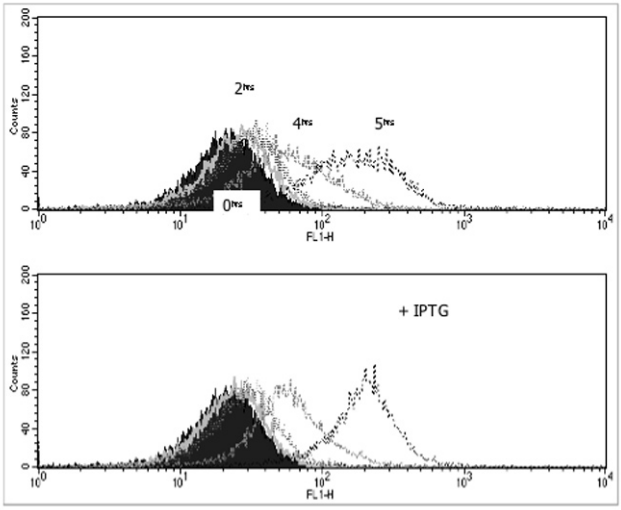



Fig.3.

Influence of incubation time and IPTG coincubation on protein expression. Expression clones in log phase were induced with IPTG (lower panel), or taken as a control (upper panel). Fluorescence of the bacteria expressing rbd-H70-GFP (at the x-axis) against the number of counts, relative to the number of bacteria, on the y-axis. Dark histogram: 0 hours after IPTG induction (*p.i.*). Light, thick, grey line: 2 hours (*p.i.*). Dark, thin, grey line: 4 hours (*p.i.*). Dotted black line: 5 hours (*p.i.*).

***Endocytosis of rbd-H70-GFP by bovine monocytes***

Fluorescent labelling of bovine PBMC due to incubation with the fusion protein rbd-H70-GFP and rHsp70-GFP or eGFP as a control, was measured by flow cytometry (fig.4). In average 11% (+/- 4%) of the monocytes ($CD14^+$ cells) endocytosed the fusion protein, as calculated by subtraction of the percentage of double positive cells ($CD14^+/GFP^+$) observed after incubation on melting ice from that of double positive cells observed after incubation at $37^0$C. During the same incubation period an average of 8% (+/- 5%) of the monocytes endocytosed the full length rHsp70 and 3% (+/- 1%) the control protein, eGFP.

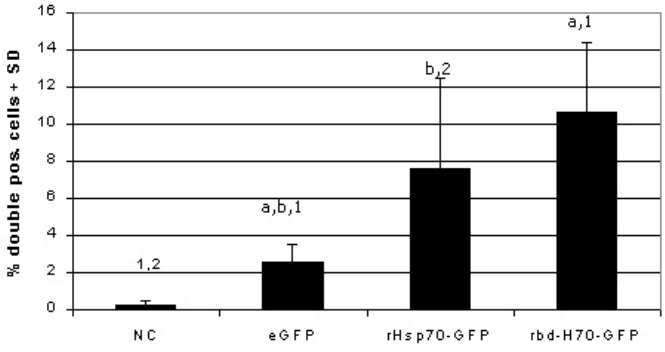



Fig.4

$CD14^+$ cells endocytose rbd-H70-GFP. PBMC were allowed to endocytose rbd-H70-GFP at $37^0$C. 10% of the monocytes endocytosed rbd-H70-GFP, compared to 2% of the monocytes that endocytosed the control protein eGFP ($P<0.01$). Endocytosis was assessed by fluorescence detection of the eGFP by flow cytometry (Values of binding (at $4^0$C) of the protein are subtracted from the values at $37^0$C). Depicted are mean percentage double positive cells ($CD14^+$ /GFP) on the y-axis and different interacting proteins on the x-axis. N=5 animals, a:$p<0.01$, b:$p<0.1$, 1:$p<0.005$, 2:$p<0.05$.

***Endocytosis of rbd-H70-GFP by Bomac***

Figure 5 shows the data obtained by flow cytometry of the Bomac incubated with rbd-H70-GFP or eGFP as the control protein. The fusion protein rbd-H70-GFP was endocytosed by a subpopulation (25%) of the macrophage cell line Bomac, while eGFP only was not internalised during the same incubation period.

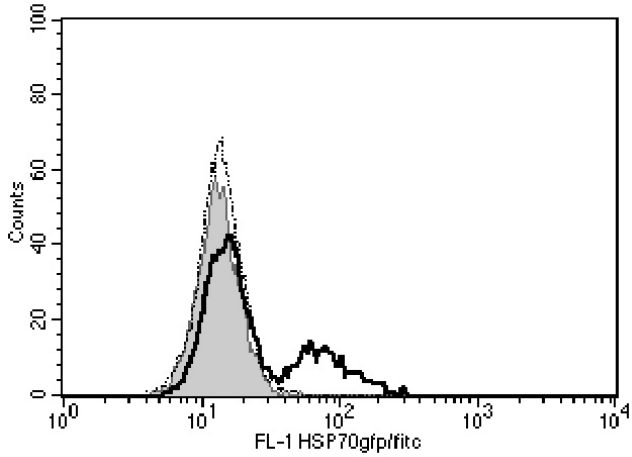



Fig.5

The rbd-H70-GFP fusion protein is endocytosed by Bomac, incubated at 37$^0$C. Endocytosis was assessed by fluorescence detection of the eGFP by flow cytometry. Fluorescence intensity of the cells (on the x-axis) is plotted against cell counts. The light grey histogram represents autofluorescence of the cells, the black dotted line represents Bomac incubated with eGFP only as the control protein, and the thick black line represents incubation with rbd-H70-GFP.

## Discussion

Heat shock protein fusion proteins are promising vaccine candidates (Hoos and Levey, 2003). The current study aimed at construction of a fusion protein of *M.a.p.* rHsp70 with a model antigen, in this case eGFP, likely to induce CTL responsiveness.
We successfully replaced the sbd part of DNA encoding for rHsp70 (Koets et al., 1999), by the eGFP coding sequence, and transformed this expression vector into E.coli, which resulted in expression of the fusion protein rbd-H70-GFP. The eGFP permitted direct insight in the amount of protein expressed, by flow cytometric analysis of the bacteria, that showed to be much higher after 5 hours of IPTG induction than after 4 hours or less. The production protocol was adapted accordingly.

As receptor mediated interaction and endocytosis is important for several of the effector mechanisms of Hsp inlcuding CTL induction(Singh-Jasuja et al., 2001), it was verified whether the rbd-H70-GFP fusion protein was internalised by bovine APC. Flow cytometric analysis indicated that the rbd terminal end of this fusion protein provided the right mode of entry into the cells, as endocytosis could be demonstrated in a number of both monocytes (CD14$^+$ PBMC) (11%) and cells of a bovine macrophage line (Bomac). Endocytosis of rbd-H70-GFP, although by a minor subset of the monocytes, occurred at a similar level as endocytosis of rHsp70-GFP, and was significantly higher than uptake of eGFP as such. A subpopulation (25%) of Bomac also endocytosed the fusion protein avidly. Why only a subpopulation of the cells endocytosed the protein remains to be investigated, but this was also observed when bovine monocyte derived macrophages endocytosed full length rHsp70 (Langelaar et al., 2002).
General consensus exists about the fact that the ATPase domain of Hsp70 contains the

receptor binding side (Huang et al., 2000; More et al., 1999), although interaction of Hsp70 of *M.tuberculosis* with CD40 (Wang et al., 2001) was shown to stimulate chemokine production via the sbd. Our data demonstrate that at least for endocytosis, uptake of the protein devoid of the sbd is not inhibited.

In conclusion these data show the production of a fusion protein consisting of the rbd of heat shock protein and a model antigen (eGFP), and demonstrate that interaction with bovine APC is similar as compared to that of the full length rHsp70. The present results enable future study of the potential induction of CTL in cattle after immunisation with rbd-H70-GFP, and ultimately the contribution of Hsp70 fusion proteins to protection against *M.a.p.* infection in cattle.

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APC

5

# Failure to induce a cytotoxic T cell reaction in cattle by immunisation with a fusion protein consisting of heat shock protein 70 and green fluorescent protein

HSP

MAP

## Abstract

Paratuberculosis is a mycobacterial disease of cattle causing a chronic protein loosing enteropathy. The role of $CD8^+$ cytotoxic T cells (CTL) in this devastating disease is unknown, but it may be hypothesized, based on the knowledge of other diseases caused by intracellular bacteria including mycobacteria, that eliciting a specific CTL reaction in cattle is helpful in preventing paratuberculosis. Heat shock proteins (Hsp) are molecules that are able to induce CTL by shuttling antigen into the MHC class I pathway of antigen presenting cells (APC). As no paratuberculosis specific bovine CTL epitopes are currently known, we aimed at using eGFP as a model antigen. To induce a CTL reaction we immunised cattle with a fusion protein consisting of the N-terminal part of recombinant heat shock protein 70 of *Mycobacterium avium subspecies paratuberculosis* (rHsp70) with enhanced green fluorescent protein (eGFP). After four intracutaneous immunisations, immune responses were assessed with emphasis on cytotoxic responses. Cellular immune responses as determined by lymphocyte stimulation tests (LST) showed recognition of the fusion protein. Humoral responses to the fusion protein were detectable but not significant. Cytotoxic activity, as measured by both a classical $^{51}Cr$ release assay and a flow cytometric assay based on propidium iodide (PI) and annexin-V-FITC staining, could not be detected using reponder cells after restimulation with either the full length fusion protein or peptide pools consisting of nonamer peptides of eGFP during a period of 7 days, despite increased percentages of $CD8^+$ T cells in response to the fusion protein. We concluded that immunisation with this fusion protein is not the appropriate way to induce cytotoxic T cell reactivity in cattle.

***Keywords***

Mycobacterium paratuberculosis, heat shock protein, fusion protein, CTL, chromium release assay, flow cytometry

***Abbreviations***

BCA, bicinchoninic acid; DC, dendritic cell; eGFP, enhanced green fluorescent protein; Hsp, heat shock protein(s); IU, international unit; *L.monocytogenes*, Listeria monocytogens; LAL, limulus amebocyte lysate; LST, lymphocyte stimulation assay; *M.a.p.*, *Mycobacterium avium subspecies paratuberculosis;* M.O.I., multiplicity of infection; MFI,mean fluorescence intensity; Ni-NTA, nickel-nitrilo-triacetic acid; *p.i.*, post immunisation; *p.i.*, post incubation; R:T, ratio of responders to targets; rbd, receptor binding domain; rbd-H70-GFP, fusion protein consisting of the rbd of rHsp70 fused to eGFP; RT, room temperature; sbd, substrate binding domain

## Introduction

Paratuberculosis, a mycobacterial disease affecting cattle, is a considerable threat to dairy and meat industry (Chi et al., 2002) and potentially to public health (Acheson, 2001; El-Zaatari et al., 2001; Greenstein, 2003). Eradication of the disease is hampered by the difficulty of diagnosis in an early stage, the chronicity of the disease and the existence of apparently healthy animals excreting the bacteria (Harris and Barletta, 2001).

Despite intensive research, immune patho-physiology of the disease is far from elucidated (Valentin-Weigand and Goethe, 1999). Humoral responses, *i.e.* antibody production, are found generally in a later stage of disease only, and are known not to protect the animal (Stabel, 1998). Cellular immunity, mediated by $CD4^+$ T-cells with a Th1 phenotype, confers protection to the animal (Bassey and Collins, 1997; Harris and Barletta, 2001). However, when disease progresses from the subclinical to the clinical stage, the host looses control over the pathogen (Stabel, 2000) which coincides with the disappearance of specific $CD4^+$ T-cells (Koets et al., 2002). The contribution of $CD8^+$ T cells (CTL) to protection against paratuberculosis remains unclear so far, however, studies on host defence against intracellular pathogens, including mycobacteria, have revealed the importance of cytotoxic $CD8^+$ T cells (reviewed in:(Serbina and Pamer, 2003; Silva et al., 2001; Wick and Ljunggren, 1999)). We therefore hypothesized that cytotoxic T cells (CTL) contribute to protection to *Mycobacterium avium subspecies paratuberculosis* (*M.a.p.*) infection in ruminants.

To date, an effective vaccine against paratuberculosis is not available. Assuming that CTL are important in combating the disease, a potential vaccine should induce cytotoxic T cell reactions against *M.a.p.* antigen. Heat shock proteins (Hsp), fused or linked to an antigen, have the capacity to induce protective immunity against the antigen they carry (Cho et al., 2000a; Schild et al., 1999), being their own adjuvant in addition(Asea et al., 2000; Todryk et al., 1999). Therefore, Hsp are promising vaccine candidates (Hoos and Levey, 2003; Silva, 1999). In previous studies we demonstrated the interaction of recombinant Hsp70 of *M.a.p.* (rHsp70) with bovine antigen presenting cells (APC) (Langelaar et al., 2002). In the present study a rHsp70 based fusion protein, rbd-H70-GFP, was used to immunise cattle. The fusion protein consisted of the N-terminal side of rHsp70, containing the receptor binding domain (rbd) (Huang et al., 2000), fused to enhanced green fluorescent protein (eGFP) as a model antigen. After repeated intra-cutaneous immunisations, we assessed cytotoxic activity against 9-mer peptides of eGFP in *in vitro* assays, both by a classical $^{51}Cr$ release assay and a flowcytometer based assay.

## Materials and methods

### *Animals*

The animals used in this study were adult Holstein-Friesian cows, conventionally housed at the Department of Farm Animal Health of the Faculty of Veterinary Medicine of the Utrecht University, The Netherlands. The use of the animals for experimental purposes had been approved by the Ethical Committee (DEC). Animals were numbered X01 to X06.

### *Immunisation protocol*

After shaving and skin disinfection, animals were immunised, intra-cutaneously in the neck region, with either 100 μg of eGFP (control; animal nr. X03) or 200 μg rbd-H70-GFP in PBS without adjuvant, three times at three week intervals. A fourth immunisation was given three months later, one dose (*i.e.* 100 μg of eGFP or 200 μg of rbd-H70-GFP) at the

right,- and one dose at the left-hand side of the neck.

***Antigens***

The fusion protein rbd-H70-GFP was produced and purified as described previously for rHsp70 (Koets et al., 2001). Briefly, *E.coli* DH5a bacteria, transformed with plasmids encoding for *M.a.paratuberculosis* Hsp70 in which the substrate binding domain (sbd) had been replaced by the gene encoding for eGFP, were induced to produce the rbd-H70-GFP with an N-terminal histidin tag. The protein was affinity purified using Ni-NTA columns, eluted with 20 mM imidazole in the elution buffer (50 mM EDTA, 20 mM Tris-HCL). Purity of the protein was verified using SDS-PAGE. Protein concentration was determined using a standard bicinchoninic acid (BCA) protocol (Pierce BCA Assay Kit).

A total of 59 nonamer peptides of eGFP with five overlapping amino acids was synthesized. Peptides were prepared by automated simultaneous multiple peptide synthesis and obtained as COOH-terminal amides (Van der Zee, 1995). For the use in cytotoxicity assays and restimulations, eGFP peptides were pooled in pools containing 10 consecutive peptides each (peptides 1-10: pool 1, peptides 11-20: pool 2, peptides 21-30: pool 3, peptides 31-40: pool 4, peptides 41-50: pool 5 and peptides 51-59: pool 6).

***Cell isolation***

Blood was taken from the jugular vein and collected into heparinised tubes (BD Vacutainer, Beckton, Dickinson). PBMC were isolated by density gradient centrifugation on Histopaque (1,077 density) (Sigma). Mononuclear cells were collected from the interphase, washed twice in PBS, counted and diluted in culture medium (CM: RPMI 1640, supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, 2mM L-glutamine and $5.10^{-5}$ M β–mercaptoethanol).

***Cytotoxicity assays***

*Targets*

Target cells were autologuous lymphoblasts; PBMC stimulated for 48 hours with Con A (1.25 μg/ml).

*Restimulation and phenotyping of responder cells*

Responder cells (PBMC) were cultured at $5.10^6$ cells/ml, 10 ml in 25 $cm^2$ culture flasks (Corning) in CM alone (as a negative control) or in CM with antigen. The antigen used was either the whole fusion protein, rbd-H70-GFP, at a concentration of 2 μg/ml or a peptide pool at 1 μg/ml. Three peptide pools per animal were selected based on maximal results in pilot experiments (by the flow cytometric cytotoxicity assay 10 pools were tested per animal, at a responder to target ratio of 50:1; data not shown). After three days of antigen stimulation, rHuIL-2 (Glaxo) 20 IU/ml was added to the cultures. After 7 days of culture the cells were washed in CM and counted. Numbers of viable cells were assessed by trypan blue exclusion, and cells were resuspended accordingly in CM at different concentrations and used for the cytotoxicity assays.

Responder cells were phenotyped using methods described earlier (Koets et al., 2002b). Briefly, cells were plated in 96-wells microtiter plates at a concentration of $1\text{-}2.10^5$/well in 100μl Facsbuffer (PBS containing 1% of BSA and 0,1% Sodium azide). Cells were incubated for 15' at 4 $^0$C with a monoclonal antibody specific for CD8 (IL-A105, ILRAD Nairobi, Kenya), washed and incubated with a fluorescent labelled secondary antibody (GAM-PE, diluted 1:1000, BD Biosciences). After 15' cells were washed, transferred to microtubes in a final volume of 200μl in Facsbuffer and fluorescence was measured by flow cytometry (Facs Calibur, Becton Dickinson, San Jose, CA). Analysis of results was carried out using Cellquest software.

*Chromium release assay*
On the day of the assay, target cells were labelled with $^{51}Cr$ (Amersham Pharmacia Biotech Benelux, Roosendaal, the Netherlands), 50 μCi per $10^6$ cells, incubated for 1 hour and washed three times. Subsequently, targets were plated $5.10^3$ cells/ well in a 96-well plate (Costar, Corning, NY), together with antigen (or medium alone) at a final concentration of 20 μg/ml (rbd-H70-GFP) or 10 μg/ml (peptide pools) and responder cells at different ratios (R:T 12:1, 25:1, 50:1 and 100:1), in triplicate. To determine spontaneous and maximal release, targets were incubated in medium alone and a 2% sodium-dodecyl-sulfate (SDS) solution respectively. After 4-hours of incubation at $37^0C$ and 5% $CO_2$, chromium release in supernatants was assayed in a Wallac Liquid Scintillation Counter 1450 MicroBeta Plus (Perkin Elmer). The percentage specific lysis was calculated according to the following formula: % specific lysis= ((cpm experimental release- cpm spontaneous release)/ (cpm total release/ cpm spontaneous release))x100. Spontaneous release of target cells was less than 28 % in all assays. Specific lysis of more than 10 % at two consecutive R:T ratios (Rosenzweig et al., 2001), represented by a classical dose-response curve, was interpreted as significant.

*Flow cytometric cytotxicity assay*
*Fluorescence labelling of target cells*
On the day of the assay, target cells were labelled with PKH-26 (PKH-26 red fluorescent cell linker kit, Sigma) according to the manufacturer's protocol. Briefly, target cells were washed in Dulbecco's PBS (Bio Whittaker, Cambrex BioSciences, Verviers, Belgium) and resuspended in 1 ml solution C from the kit. The PKH-26 was diluted in 1 ml solution C (10 ml PKH-26/ml). Cells were combined with the dye (1:1) and incubated for 5' at RT, while inverting the tubes several times. The reaction was stopped by adding 1 ml FCS to the mixture, again inverting the tubes several times. Cells were then pelleted by centrifugation, taken up in CM and transferred to new tubes. Cells were washed two more times in CM, counted, resuspended in CM and transferred into 96-well plates (Costar, Corning, NY), $5.10^3$ cells per well.
*Flow cytometric assay*
Antigens, responder cells and target cells were combined as described above for the 51Cr release assay and incubated for 4 hours at $37^0C$ and 5% $CO_2$ . As a positive control for annexin-V-FITC staining of apoptotic cells, targets were incubated for the same period with 330 μM $H_2O_2$ (Laochumroonvorapong et al., 1996). After the incubation period, the reaction was abrogated by putting the cells on ice. Cells were stained with annexin-V-FITC 1:50, 5 μl/well (Nexins research, The Netherlands) and propidium iodide (PI) 0.1 μg (Sigma) according to the protocol (Nexins research, The Netherlands), transferred to Facs tubes and kept on ice in the dark until analysis.

Analysis was carried out using the FACScan (Becton Dickinson, San José, USA). After excitation with an argon laser at 488 nm, the emission of the three fluorochromes was measured through three optical filters: 530 nm=FL-1 for annexin-V-FITC, 585 nm= FL-2 for PKH-26 and >675 nm FL-3 for PI. Spectral overlap was electronically compensated using single colour controls in separate tubes. To distinguish targets from responder cells, the PKH-26 positive cells were gated. Data acquisition was stopped when 2000 events were acquired. Analysis was quantified using the Cellquest software (Becton Dickinson). The percentage of apoptotic cells (early apoptotic: annexin-V-FITC positive and/or late apoptotic: annexin-V-FITC/PI double positive) amongst target cells was calculated. PI single positive cells (necrotic cells) were excluded from the calculations.

***Lymphocyte stimulation assay (LST)***
PBMC were resuspended in CM at a concentration of $2.10^6$ cells/ml and added to triplicate wells of a 96-well microtiter plate (Costar, Corning, NY), using 100 μl of cell suspension

and 100 μl of antigen solution per well. Antigens used were: medium alone as a negative control, concanavalin A (conA) (2,5 μg/ml) as a positive control, and proteins (rbd-H70-GFP in LST 3 weeks after the last immunisation, or rHsp70, rbd, rbd-H70-GFP and eGFP in LST 6 weeks after the last immunisation) at a concentration of 20 μg/ml. Cells were cultured at $37^0$ C and 5% $CO_2$ for four days in a humified incubator. For the final 18 hours 0.4 μCi of [*methyl*-$^3$H]thymidine (Amersham Pharmacia Biotech Benelux, Roosendaal, the Netherlands) was added to each well. After the incubation period, cells were lysed and DNA was harvested onto fibreglass filters and incorporated radioactivity was determined as counts per minute (cpm) by liquid scintillation counting in a Wallac Liquid Scintillation Counter 1450 MicroBeta Plus (Perkin Elmer). Results are expressed as stimulation indices (SI), defined as (mean cpm of stimulated wells/mean cpm of medium cultured wells).

***Multiplex analysis of antigen specific antibodies***

Serum samples were taken three times, starting at the day of the fourth immunisation and subsequently after three and six weeks.

The Luminex Multiplex analysis system was used to test the sera for the presence of antibodies against respectively the fusion protein rbd-H70-GFP, its rbd part only and eGFP. Each antigen was covalently linked to differently coloured beads by a carbodiimide conjugation method as described by the manufacturer (Luminex BV, Oosterhout, The Netherlands). The antibody that interacted with antigen attached to the specific bead was detected by the addition of an anti bovine Ig antibody labelled with PE (GAM-PE, diluted 1:1000, BD Biosciences). The mean fluorescence intensity (MFI) was used as a quantitative measure of the presence of antibody.

## Results

***The CD8 positive subpopulation increases in cultured cells***

When the population of responder cells for the cytotoxicity assays was phenotyped by flow cytometric analysis, a difference was observed in the cells cultured in medium and the cells cultured with rbd-H70-GFP (fig.1). In medium, the population consisted of 23% CD8 positive cells whereas for cells cultured in medium supplemented with rbd-H70-GFP as the antigen, 38% of the population consisted of $CD8^+$ cells (mean of 4 animals). This increase was not observed with the other antigens (data not shown).

***Levels of cytotoxicity measured in $^{51}$Cr release assays***

Cytotoxicity of responder cells against targets pulsed with pooled peptides at different responder to target ratios was evaluated using the $^{51}$Cr release assay. In general, no high percentages of specific lysis were observed when the antigen pulsed targets were compa-

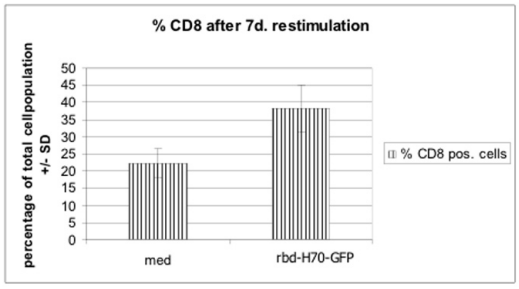


Fig.1

$CD8^+$ T-cell population increases during culture. Percentage of $CD8^+$ T cells within the total population of PBMC after 7 days of culture in CM or CM supplemented with the fusion protein rbd-H70-GFP. Results were compared by a T-test, $P < 0,01$.

red to the medium controls (fig.2). None of the animals expressed a classical dose-response curve with values above the cut-off of 10% specific lysis at consecutive R:T ratios.

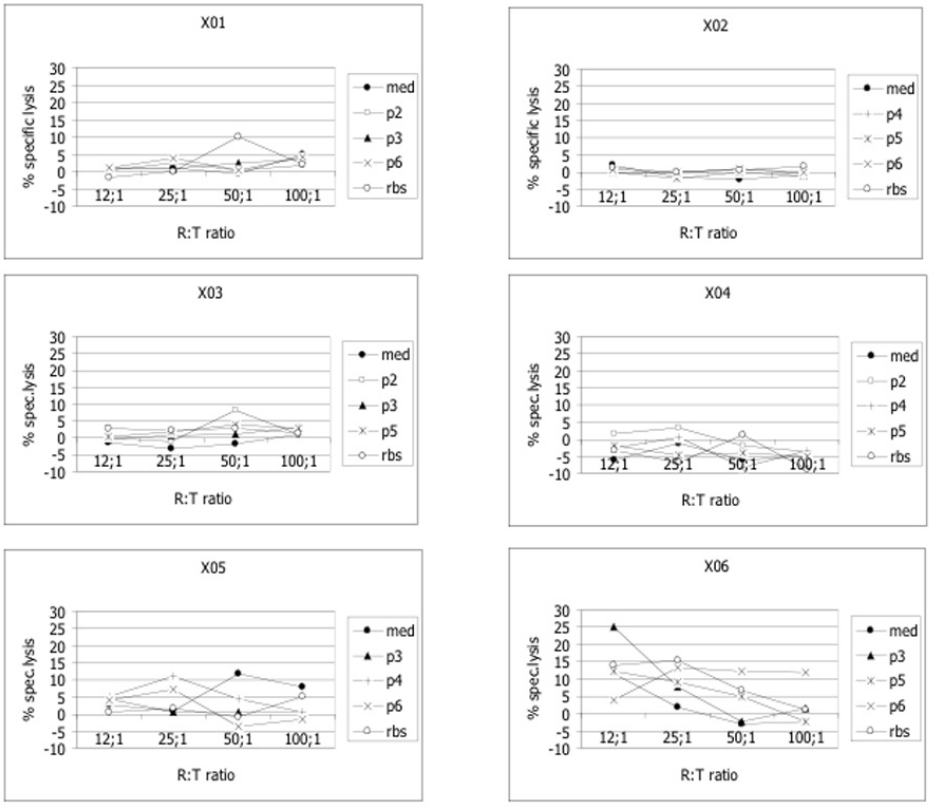


Fig.2

Cytotoxicity as assessed in chromium release assays.
Target cells of six animals incubated with responder cells at different R:T ratios and antigen (or medium as negative control) as indicated in the legends. Mean values of triplicate samples.

***Apoptosis of bovine cells as measured by a tricolour flow cytometric assay***

To measure cytotoxicity in a non-radioactive way, a flow cytometric based assay was set up. When target cells were stained with PKH-26, it was possible to distinguish the target cell population from the responder cells and to determine the percentage annexin-V-FITC positive (early apoptotic) and annexin-V-FITC /propidium iodide double positive (late apoptotic (Fischer et al., 2002)) cells within the targets (fig. 3A).

Apoptosis was induced by $H_2O_2$ (Laochumroonvorapong et al., 1996), leading to positive staining with annexin-V-FITC which makes it a useful positive control for target cell apoptosis (fig 3B).

***Levels of apoptosis measured in flow cytometry* assays**

Determination of cytotoxicity by a flow cytometric assay was carried out in parallel with the standard chromium release assay, with the same targets and responder cells. Cytotoxicity was expressed as the percentage annexin-V positive cells of the PKH26-labelled targets

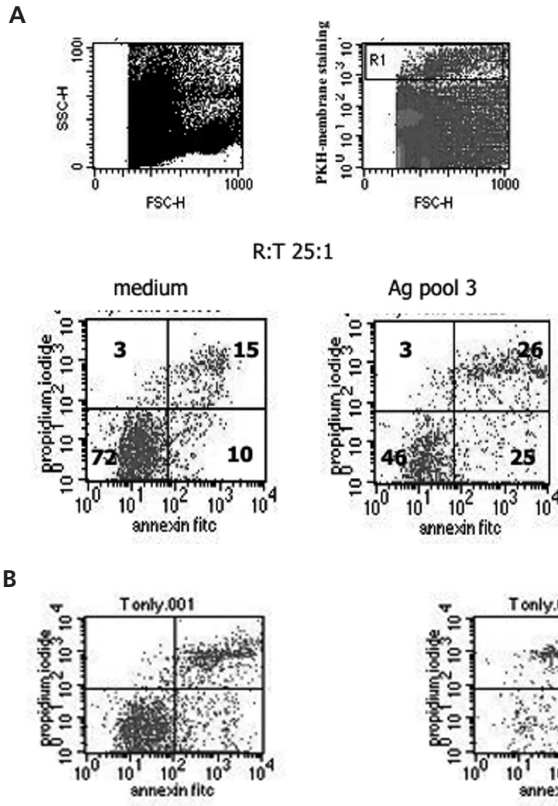


Fig. 3

Typical flow cytometry cytotoxicity assay dot plot (all data derived from the same animal, in one experiment).

A. Targets and responder cells, Forward Scatter (FSC) vs Side Scatter (SSC) and FSC (x-axis)vs PKH26 staining (y-axis). R1= gated target cells. Dot plots of the gated target cells: Horizontal axis, annexin-V-FITC, (FL-1). Vertical axis, PI (FL-3). The percentage annexin-V-FITC positive cells among gated cells is represented by the UR and UL quadrants together. Incubation of cells without antigen and with antigen (peptides of pool 3) during the assay, in a 25:1 R:T ratio.

B. Targets only, incubated in medium or in medium with $H_2O_2$ to induce apoptosis. Annexin-V-FITC staining (x-axis) vs propidium iodide (PI) staining (y-axis)

(fig.4). Triplicate measurements showed results with low standard deviations (SD) for most R:T ratios. In general, high background values were measured of non-pulsed targets incubated with medium cultured responder cells, varying from 15 to 53%. No reactions above the cut-off values were detected.

***Immunised cattle recognise fusion protein in LST***

Proliferative responses to the fusion protein rbd-H70-GFP were detectable 3 and 6 weeks after the last immunisation (*p.i.*). Three weeks *p.i.* proliferation in reponse to the fusion protein was measured in all animals. Six weeks *p.i.*, when in addition proliferative responses to the rbd part of the fusion protein, eGFP and the original full length rHsp70 were tested, all animals reacted to eGFP, 3 animals reacted to the rbd while one animal only reacted against rHsp70 (table 1.).

| Animal nr. | X01 | | X02 | | X03 | | X04 | | X05 | | X06 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Antigen | 3 wks | 6 wks | 3 wks | 6 wks | 3 wks | 6 wks | 3 wks | 6 wks | 3 wks | 6 wks | 3 wks | 6 wks |
| ConA | 431 | 155 | 4411 | 2104 | 4147 | 1257 | 2191 | 3679 | 2676 | 273 | 463 | 3592 |
| Hsp70 | | | | | | | | | | | | 3 |
| rbd-H70-GFP | 4 | | 24 | 18 | 25 | 3 | 4 | 6 | 3 | 3 | 4 | 6 |
| rbd | | | | 8 | | | | 3 | | 4 | | |
| GFP | | 8 | | 11 | | 3 | | 6 | | 3 | | 19 |

Table 1. Proliferative responses of PBMC to rbd-H70-GFP (3 and 6 weeks p.i.), rHsp70, rbd of rHsp70 and eGFP.Indicated in the table are proliferative responses of the six animals, expressed as the stimulation index (SI), that were >2.

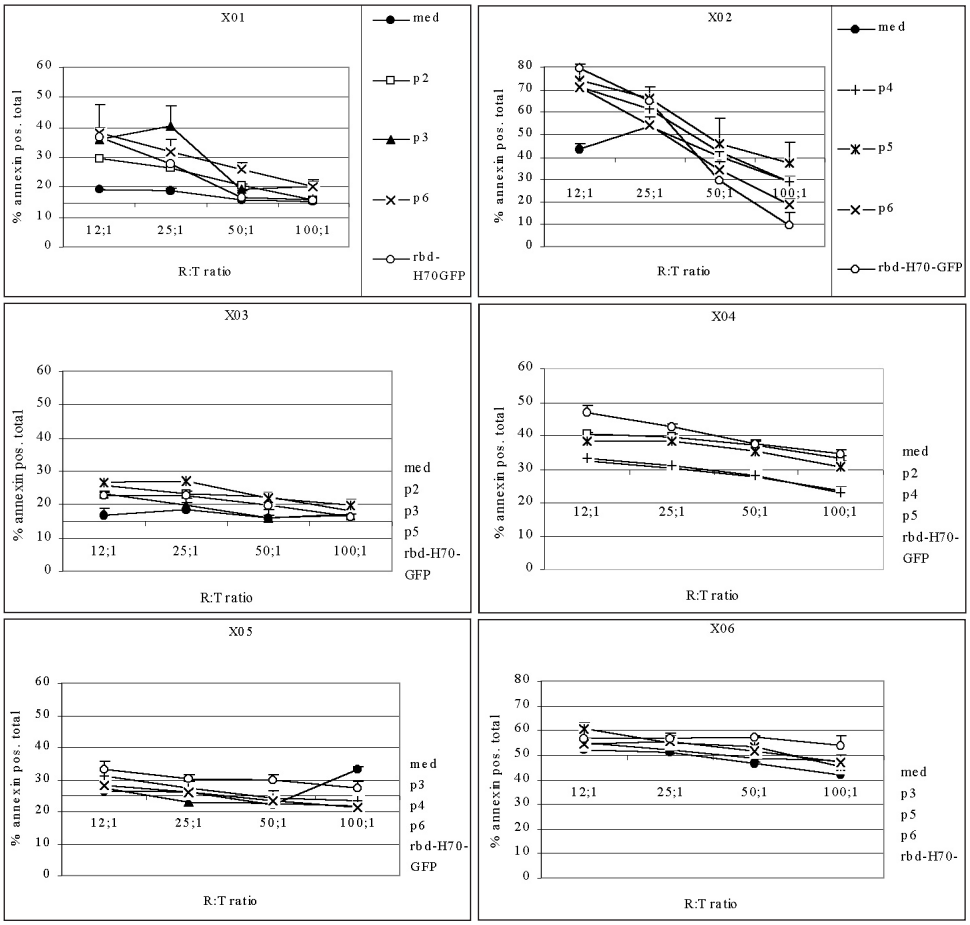


Fig.4

Cytotoxicity as assessed in flowcytometric assay.
Target cells, identified by PKH-26 membrane staining, of six animals incubated with responder cells at different R:T ratios (at the x-axis) and antigen (or medium as negative control) as indicated in the legend. Percentage apoptotic (annexin positive) target cells is depicted at the y-axis. Mean values of triplicate samples + SD.

### *Antigen specific antibody detected in sera of immunised cattle*

Serum samples were taken from the animals at different time-points after immunisation and the presence of antibodies against rbd only, eGFP only or the whole construct, rbd-H70-GFP, was analysed by the Luminex system (fig.5). Animals X04 and X05 had detectable antibody levels at 3 weeks *p.i.*, but the differences with 0 or 6 weeks *p.i.* were not significant.

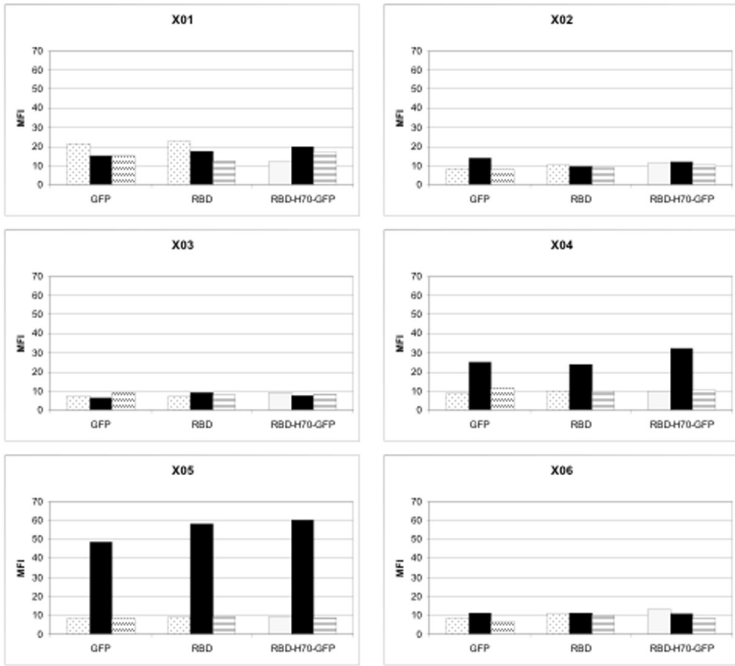


Fig.5

Low antibody responses are detected.
Presence of antibodies specific for eGFP, rbd only or the fusion protein rbd-H70-GFP was analysed using the Luminex Multiplex system. Fluorescence intensity, as a quantitative measure of antibody amounts plotted on the y-axis, antigens coupled to Luminex beads to detect antibodies plotted on the x-axis: eGFP, rbd and rbd-H70-GFP. Low antibody titers were detected but no significant differences were observed between sera at 3 weeks (black bars) or 6 weeks (striped bars) after the last immunisation (dotted bars).

## Discussion

Immunisation with fusion proteins consisting of Hsp fused to antigenic proteins can induce CTL reactions against the antigenic protein (Cho et al., 2000). Therefore, Hsp based fusion proteins are promising vaccine candidates (Hoos and Levey, 2003). In our study, we investigated whether immunisation with the rbd of Hsp70 of *M.a.p.* fused to eGFP, as a model antigen, could induce CTL against eGFP in cattle. Enhanced GFP possesses multiple human (Re et al., 2004), macaque (Rosenzweig et al., 2001) and mouse (Stripecke et al., 1999) CTL epitopes, making it probable that cattle CD8 epitopes can be found as well. Using two different cytotoxicity assays we could not detect the presence of specific CTL, despite the relative abundance of $CD8^+$ T cells in the *in vitro* cultures and although the animals developed cellular immune responses, as we found lymphoproliferation in *in vitro* LSTs in response to the full length fusion protein and to the rbd and eGFP parts of the protein.
We aimed at investigating the CTL population by functional cytotoxicity assays, as in natural infection target cell death by a granule dependent pathway may not only destroy the macrophages harbouring the mycobacteria, but may also have a direct bactericidal effect on *M.a.p.* (Stenger et al., 1997), that can contribute to protection against the disease. A flowcytometer based cytotoxicity assay was set up as has been described earlier (Derby et al., 2001; Fischer et al., 2002), in parallel with the $^{51}Cr$ release assay as a golden standard.

In general, both in the flow cytometric assay and in the $^{51}Cr$ release assay it was difficult to measure antigen specific apoptosis of the lymphoblasts. Aspecific apoptosis however, induced with $H_2O_2$ (Avula and Fernandes, 2002; Laochumroonvorapong et al., 1996) in the flow cytometric assay and with 2% SDS in the $^{51}Cr$ release assay, was inducible using these targets, *i.e.* Con A stimulated lymphoblasts that have also been used in other studies (Beer et al., 1997). The difficulty to identify bovine CTL by measuring target cell death has been mentioned before (De Groot et al., 2003; Gaddum et al., 2003; Woolums et al., 2004).
In responder cell populations cultured with the fusion protein the percentage of CD8 positive cells was 15% higher than in the medium incubated cell populations, but this was not reflected in higher target cell lysis in the cytotoxicity assays. A possible explanation for the increasing percentage of $CD8^+$ T cell proliferation could be a-specific stimulation by the rHsp70 moiety of the fusion protein. As has been described in literature, Hsp70 acts both as a chaperone and as a cytokine, activating APC, which in turn secrete T cell activating cytokines (Asea et al., 2000). To detect functional cytotoxic cells it might be necessary to incubate responder cells for a longer time-period, as has been demonstrated in other studies (Beer et al., 1997).
Literature has been abundant on the possibility to induce cytotoxic T cell reactions with Hsp70, fused or linked to antigen, without the necessity to add an adjuvant, Hsp70 being its own adjuvant (Cho et al., 2000). However, unexpected failure to elicit human melanoma specific CTL with Hsp70 was reported by Fleischer et al. (Fleischer et al., 2004), although a protocol was used that was largely similar to successful CTL induction with Hsp by Noessner et al. (Noessner et al., 2002). Our hypothesis that bovine CTL could be induced with the fusion protein rbd-H70-GFP was based on literature reports that used Hsp70 as a vehicle to induce CTL and on preliminary *in vitro* studies that showed that rHsp70 specifically interacts with bovine APC, while fusion of the rbd of rHsp70 to eGFP did not result in loss of this capacity. However, there is a possibility that the fusion protein uses other, possibly less, receptors on the cell surface of the APC to gain entrance to the cytosol, compared to the full length rHsp70. Especially CD40, that has been shown to be a receptor for Hsp70, was also proven to be a receptor interacting with the substrate binding domain of Hsp70, moreover, this was the case for mycobacterial Hsp70 but not for mammalian Hsp70 (Wang et al., 2001). If the use of this receptor was lost by fusing rHsp70 to eGFP, the final outcome of immunisation with the fusion protein might be different from immunisation with the whole protein.
Because no CD8 epitopes of *M.a.p.* are known, we chose to carry out a proof of principle with eGFP fused to the receptor binding sequence of rHsp70 and we assumed that eGFP contained bovine CTL epitopes. Although a lack of bovine CTL epitopes in eGFP could be one explanation for our results, it is more likely that CTL induction failed due to the incapacity of the rHsp70 moiety to translocate the fusion protein to a bovine APC compartment that would allow for the processing of CTL stimulatory peptides from the eGFP part of the protein and their subsequent presentation in MHC class I.
Other effector roles of CTL could be the excretion of IFN–γ, or an immunoregulatory role by direct cell contact, as has been indicated by Chiodini (Chiodini and Davis, 1993). It has been demonstrated that rHsp70 is a dominant antigen in natural *M.a.p.* infection (Koets et al., 1999), but its influence on the different lymphocyte populations during the disease is not known. Other recombinant Hsp70s in other studies have been shown to enhance disease (Bromuro et al., 1998) or lead to cell apoptosis (Maranon et al., 2000). Spontaneously appearing mycobacterial Hsp70-reactive $CD4^+$ T cells in rat listeriosis were proven to inhibit the protective mechanisms against Listeria infection (Kimura et al., 1998). It could be that the desired induction of cytotoxicity against the antigen cargo of our fusion protein is being overruled by the immunologic reaction elicited by the rHsp70 carrier.

## Conclusion

In this study, we tried to elicit cytotoxic T cell reactions in cattle by immunisations with a rHsp70 based fusion protein. We demonstrated lymphoproliferation in response to the fusion protein and the two different parts, rbd of rHsp70 and eGFP but we were not able to detect CTL reactions *in vitro* using two different cytotoxicity assays, and only very low antibody production. From these data we conclude that immunisation with rbd-H70-GFP is not the appropriate way to induce cytotoxicity in cattle. Future studies should focus more in depth on the fate of rHsp70 of *M.a.p.* after immunisation.

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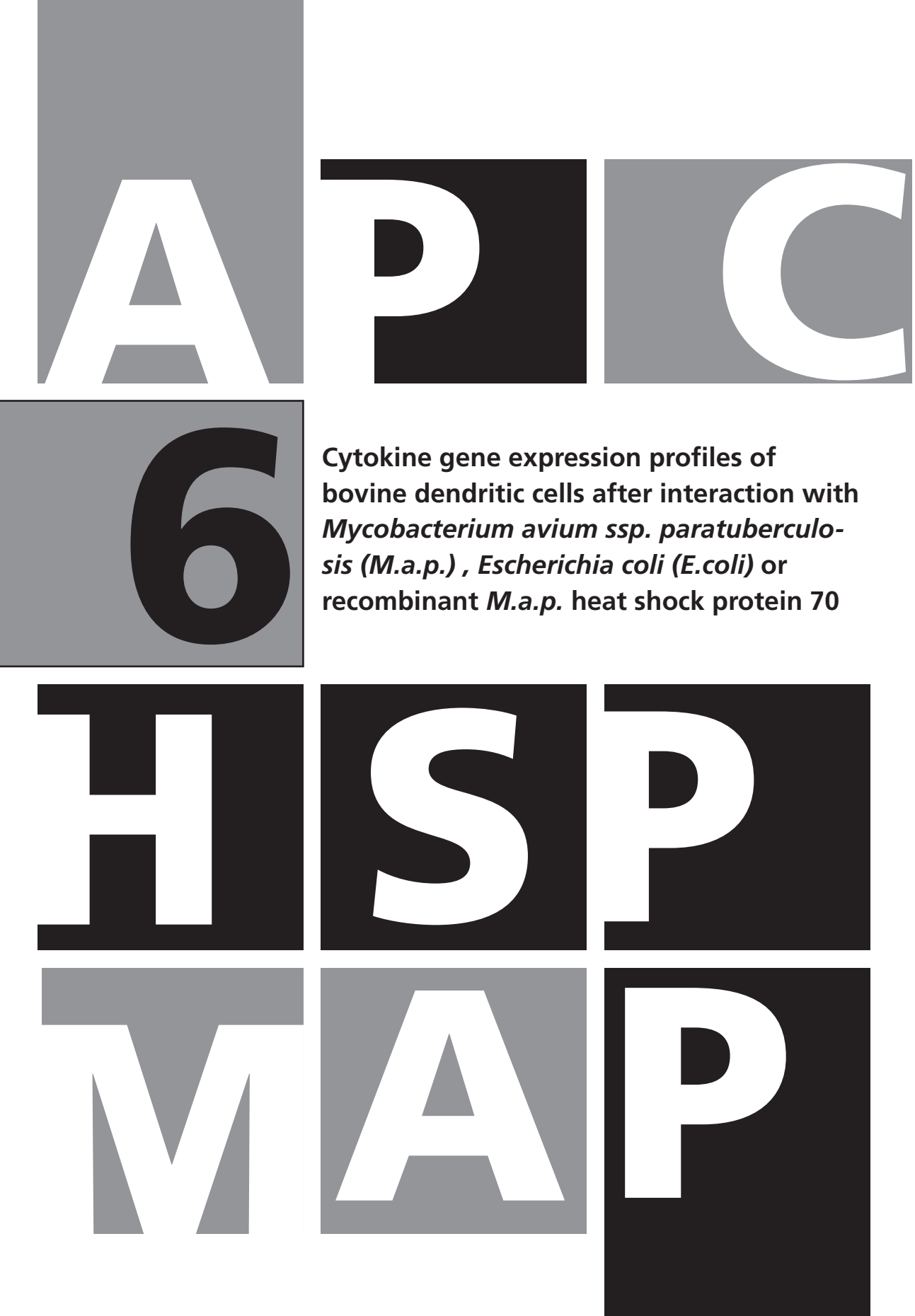


**Cytokine gene expression profiles of bovine dendritic cells after interaction with *Mycobacterium avium ssp. paratuberculosis (M.a.p.) , Escherichia coli (E.coli)* or recombinant *M.a.p.* heat shock protein 70**

## Abstract

*Mycobacterium avium* paratuberculosis (*M.a.p.*) resides and replicates in macrophages. Many mechanisms of immune evasion by *M.a.p.* to survive in the host's cells are known. However, little is known about interactions of *M.a.p.* with dendritic cells (DC). As DC are important cells for induction of protective immunity against infectious diseases, we investigated interference of *M.a.p.* with these cells and used quantitative real-time PCR (RT-PCR) to analyse differential expression of cytokine genes after 6 hours and 24 hours of incubation by immature DC that phagocytosed either *M.a.p.* or *Escherichia coli* (*E.coli*). We hypothesized that phagocytosis of *E.coli* would induce a pro-inflammatory cytokine profile due to abundant presence of LPS and that the cytokine expression profile as induced by phagocytosis of live *M.a.p.* would clearly differ. In addition we hypothesized that incubation of immature DC with rHsp70, an immunodominant antigen of *M.a.p.*, would induce a similar profile of cytokine gene expression as phagocytosis of intact *M.a.p.*. However, phagocytosis of both *E.coli* and *M.a.p.* resulted in a cytokine gene expression pattern representative of a (pro-)inflammatory reaction, dominated by strong induction of IL-12 gene expression, that was higher after 24 hours than after 6 hours of incubation, although the response to *M.a.p.* was less vigorous than to *E.coli*. Incubation with rHsp70 resulted in a more inhibitory type of cytokine gene expression, with delayed IL-12 gene expression. In conclusion, these results indicate that bovine DC are capable of reacting by an immuno-stimulatory, anti-mycobacterial response to infection with *M.a.p.*, while Hsp70 potentially contributes to pathogen virulence by allowing the bacteria to invade and settle in the host cell.



***Keywords***
Mycobacterium paratuberculosis, cytokine, dendritic cell, heat shock protein, RT-PCR


***Abbreviations***
BCA, bicinchoninic acid; DC, dendritic cell; dNTP, nucleotides; *E.coli*, *Escherichia coli*; GM-CSF, granulocyte monocyte-colony stimulating factor; HEYM, Herrold's Egg Yolk Media; Hsp, heat shock protein(s); IU, international unit; LAL, limulus amebocyte lysate; *M.a.p.*, *Mycobacterium avium subspecies paratuberculosis*; M.O.I., multiplicity of infection; mRNA, messenger RNA; Ni-NTA, nickel-nitrilo-triacetic acid; *p.i.*, post incubation; RT, room temperature; RT-PCR, reverse transcriptase-polymerase chain reaction; TGF–β, transforming growth factor-β; TLR, Toll-like receptor;

## Introduction

Dendritic cells (DC) play a key role in the development of antigen-specific immunity. From immature cells, specialized in antigen capture, they differentiate into mature professional antigen presenting cells (APC) under the influence of inflammatory mediators and microbial products, while migrating from the periphery to the secondary lymphoid organs once they have ingested antigen. Mature DC are perfectly prepared to prime naïve T cells to become specific, Th1-, Th2- or regulatory effector cells (Banchereau and Steinman, 1998; Lanzavecchia and Sallusto, 2000).

DC produce IL-12 and pro-inflammatory cytokines upon mycobacterial infection (Demangel and Britton, 2000; Hope et al., 2004), important for orientation towards a Th1 response to control disease. However, it has been demonstrated that different APC; *e.g.* macrophages and DC, react differently to the same stimulus (Hope et al., 2004; Werling et al., 2004). With regard to cytokines, macrophages react primarily by IL-10 production, while DC produce mainly IL-12 upon infection with *M.tuberculosis* (Nau et al., 2002).

Paratuberculosis is a chronic intestinal disease caused by *Mycobacterium avium subspecies paratuberculosis* (*M.a.p.*), affecting cattle throughout the world (Cocito et al., 1994; Valentin-Weigand and Goethe, 1999). *M.a.p.* resides and replicates in macrophages (Harris and Barletta, 2001). Being the host cell for *M.a.p.*, modulation of macrophages by *M.a.p.* has been the subject of many studies (reviewed in:(Sigurethoardottir et al., 2004)). To date, there is no such knowledge on the infection of DC with *M.a.p.*. However, it is known that the induction of an appropriate Th1 type of response is needed to control the disease (Koets et al., 2002b). To induce that response, DC are indispensable.

The aim of our study was to investigate the reaction patterns of DC upon infection with *M.a.p.* compared to phagocytosis of *E.coli*, and uptake of recombinant heat shock protein 70 of *M.a.p.* (rHsp70), as measured by the real time reverse trancriptase- polymerase chain reaction (real time RT-PCR) for relevant cytokines. Bacteria and bacterial components interact with APC via specific receptors, like Toll-like receptors (TLR). Engagement of TLR is an important pathway to induce cytokine gene expression (Janeway and Medzhitov, 2002). Mycobacteria interact via lipoproteins and lipoarabinomannan with TLR2, while Gram-negative bacteria like *E.coli* interact via LPS with TLR4 (Werling and Jungi, 2003). Human Hsp70 has been demonstrated to interact with TLR2 and 4 (Vabulas et al., 2002). In paratuberculosis, heat shock protein 70 (Hsp70) has been described as a dominant antigen (Koets et al., 1999). In addition, Hsp70 has been shown to induce the production of pro-inflammatory cytokines by human DC (Bethke et al., 2002). We hypothesized that phagocytosis of the different bacteria would lead to differences in cytokine mRNA expression as might be expected from studies with infected macrophages (Nau et al., 2002; Weiss et al., 2002). Furthermore we hypothesized that phagocytosis of *M.a.p.* by DC would lead to the production of pro-inflammatory cytokines as has been demonstrated for other mycobacterial infections (Demangel and Britton, 2000; Hope et al., 2004), and that this reaction would also be provoked by one of its antigenic components, Hsp70.

Cytokine expression patterns were largely similar upon phagocytosis of *M.a.p.* and *E.coli* respectively, in general corresponding to a Th1 type cytokine profile. However, incubation of DC with rHsp70 mainly resulted in a cytokine expression profile that would correlate with a delayed and weak Th1 cytokine profile.

## Methods

***Animals***
Young Holstein-Friesian calves, between 10 and 14 days of age, were sampled at a commercial calve trade centre. Blood was taken of six animals, originating from different farms.

***Bovine cells***
Bovine PBMC were prepared from freshly isolated blood, punctured from the jugular vein and collected into heparinised tubes (BD Vacutainer, Beckton, Dickinson). PBMC were isolated by density gradient centrifugation (Histopaque (1,077 density),Sigma). Mononuclear cells were collected from the interphase, washed twice in PBS, counted and diluted in culture medium (CM): RPMI 1640, supplemented with 10% FCS, 50 international units (IU)/ml penicillin, 50 μg/ml streptomycin, 2mM L-glutamine and $5.10^{-5}$ M β-mercaptoethanol. Monocytes were isolated using the Macs separation method: PBMC were incubated with α HuCD14, cross reactive with bovine CD14 (Sopp and Howard, 1997), coupled to super paramagnetic microbeads (Miltenyi Biotec GmBH), 10 μl of bead solution /$10^7$ cells. After ten minutes of incubation at RT, cells were washed twice in PBS and transferred to a Macs separation column ( Miltenyi). After three washes with cold PBS supplemented with 2% FCS, the magnetically labelled cells were collected. The purity of the cells was evaluated by flow cytometry, using PE-conjugated goat anti mouse antibodies (GAM-PE, diluted 1:1000, BD Biosciences) to detect CD14 labelled cells and shown to be 75-96% (data not shown). DC were cultured in CM supplemented with bovine recombinant IL-4 (rIL-4) and bovine recombinant granulocyte monocyte- colony stimulating factor (rGM-CSF) (COS cell supernatant at concentrations predetermined optimal) at a concentration of 0,8-$1.10^6$ cells/ml in 6-well culture plates (Costar, Corning, NY) . Cells were cultured for 3 days to generate immature DC (following the procedure described by Werling et al.(Werling et al., 1999)). Immature DC were incubated with CM (supplemented with rIL-4 and rGM-CSF) only (negative control), or CM combined with bacteria (*M.a.p.*strain 316F at a multiplicity of infection (M.O.I.) of 10:1 or *E.coli* at a ratio of 10 bacteria/cell), or with rHsp70 at a concentration of 10 μg/ml. DC were harvested at 0, 6 and 24 hours of incubation. Cells of the six animals were pooled per stimulus and time-point and washed once in PBS. Cells were then pelleted in eppendorf vials with lid (Eppendorf AG, Hamburg, Germany), $1.10^7$ cells/vial, and stored in 100 μl RNAlater (Ambion, Inc, Austin, USA) at $4^0$ C overnight. Subsequently cells were frozen at $–20^0$ C.

***Protein***
Recombinant *M.a.p.* Hsp70 was produced and purified as described previously (Koets et al., 2001). Briefly, *E.coli* Top10 bacteria, transformed with plasmids encoding for M.a.p Hsp70, were induced to produce the rHsp70 with a N-terminal histidine tag. The protein was affinity purified using nickel-nitrilo-triacetic acid (Ni-NTA) columns, eluted with 20 mM imidazole in the elution buffer (50 mM EDTA, 20 mM Tris-HCL). Purity of the protein was verified using SDS-PAGE. Protein concentration was determined using a standard bicinchoninic acid (BCA) protocol (Pierce BCA Assay Kit). LPS contamination was removed from the solution by using polymyxin B columns (Detoxi gel, Pierce). The final solution contained less than 2 EU endotoxin/mg protein as determined by the limulus amebocyte lysate (LAL) assay (QCL-1000 test Kit, BioWhittaker, Europe).

***Bacteria***
Live *Mycobacterium avium subspecies paratuberculosis* (*M.a.p.*) strain 316F was used to infect DC at a M.O.I. of 10:1 CFU/cell. Bacteria were counted by flow cytometry (Facs Calibur, Becton Dickinson, San Jose, CA ), by comparison with a standard number of fluorescent

beads (FluoSpheres, carboxy-modulate microspheres, size 1 μm, Molecular Probes, Leiden, the Netherlands).As a viability control bacteria were serially diluted and cultured on Herrold's Egg Yolk Media (HEYM) plates (Becton Dickinson) at 37$^0$C for 12 weeks. *E.coli* bacteria were used from a –20$^0$C stock, killed by incubation in ethanol immediately after thawing, and incubated with DC at a ratio of 10:1 bacteria/cell.

***Real Time Reverse Transriptase –Polymerase Chain Reaction (RT-PCR)***

*Extraction of RNA from cultured cells*

Messenger RNA was extracted from $10^6$ cells and purified using commercial kits, the QIAGEN Rneasy Mini Kit and the QIAGEN Rnase-Free Dnase Set respectively (Qiagen Benelux BV), according to the manufacturer's recommendations for cultured cells. The amount of total RNA was spectrophotometrically determined at 260 nm.

*Reverse transcription of mRNA into cDNA*

Using the TaqMan RT kit (Applied Biosystems) 2 μg mRNA was transcribed into cDNA with a final concentration of 5.5 mM $MgCl_2$, 0.5 mM each DNTP, 2.5 μM random hexamers, 0.4 U/μl RNAse inhibitor and 1.25 U/μl Multiscribe Reverse Transcriptase in a reaction volume of 10 ml. The samples were incubated at 25$^0$ C for 10 minutes, followed by transcription at 48$^0$ C (30 minutes) and enzyme inactivation at 95$^0$ C (5 minutes).

*Primers*

Primer sequences were designed to bind specifically to bovine cytokine cDNA using the Primer Express software (Applied Biosystems) according to published bovine cytokine mRNA sequences (GenBank) and purchased from Applied Biosystems. As the housekeeping gene β-2-microglobulin was chosen. The GenBank Accession numbers of the targets as well as the primer sequences are shown in table 1.

*Quantitative real-time RT- PCR*

RT-PCR was performed with the Mx3000P Real-Time PCR Cycler (Stratagene, Amsterdam, the Netherlands) using the Stratagene Brilliant SYBR Green PCR Master Mix (Stratagene) according to the manufacturer's instructions. All PCR reactions were set up in 96-well microtiter plates (Stratagene) using 300 nM of each specific forward and reverse primer and 2.5 μl of cDNA in a reaction volume of 25 μl. Fluorescence was automatically measured during PCR. After a 10 minutes denaturation at 95$^0$ C, the reactions were cycled 40 times at 95$^0$ C for 30 seconds, 60$^0$ C for 1 minute, and 720 C for 30 seconds. To verify that only the specific product was amplified, a melting point analysis was done after the last cycle by cooling samples to 55$^0$ C and then increasing the temperature to 95$^0$ C at 0.2$^0$ C/s. A single product at a specific melting temperature was found for each target. Specificity of the PCR product, based on the predicted size of the product, was also confirmed by gel electrophoresis. All samples were tested in duplicate and the mean was obtained for further calculations. Each run included a no-template control to test for contamination of assay reagents.

*Data analysis*

Real-time PCR efficiencies were calculated, according to $E=10^{[-1/slope]}$. The relative expression ratio (R) of a target gene is calculated based on PCR efficiency and the Ct deviation of an unknown sample versus a control (Pfaffl, 2001), and expressed in comparison to β-2-microglobulin as reference gene using the quantification software REST© (Pfaffl et al., 2002). In the text, T6 and T24 refer to the antigen induced expression of the described cytokine gene relative to the corresponding medium control at 6 hours and 24 hours respectively. The cDNA of untreated control cells normalized to the level of the housekeeping gene (β-2-microglobulin) mRNA have been ascribed a fold induction of 1.

## Results

***Differential expression of cytokine mRNA by immature DC after 6 hours of incubation with antigen***

Cytokine gene expression patterns are depicted in figure 1, where bars representing the n-fold difference are coloured black when this difference is significant as compared to the medium control (p=0.001), and grey when the difference is insignificant (p>0.1). Phagocytosis of dead *E.coli* at T6 (fig.1A) caused increased expression of mRNA for IL-1β (319-fold), IL-6 (16-fold), IL-10 (5-fold), IL-12 (848-fold), TNF-α (25-fold), GM-CSF (44-fold), and down-regulated transcription of the TGF–β gene (2-fold decrease). Phagocytosis of viable *M.a.p.* (fig.1b) resulted in a comparable pattern of upregulation of mRNA expression for the different cytokines. Increased expression of mRNA for IL-1β (48-fold), IL-6 (9-fold), IL-10 (8-fold), IL-12 (369-fold), TNF-α (21-fold) and GM-CSF (51-fold) was observed, while mRNA expression for TGF–β tended to be down-regulated (2-fold decrease). Incubation with rHsp70 (fig.1C) caused increased levels of mRNA for IL-1β (6-fold increase), and decreased levels of IL-12 mRNA expression (3-fold), but only minor increases in IL-6 (2-fold), IL-10 (1.3-fold), TNF-α (1.2-fold), and GM-CSF (1.1-fold) and a decrease in TGF–β (1.5-fold) mRNA expression.

***Differential expression of cytokine mRNA by immature DC after 24 hours of incubation with antigen***

After 24 hours of incubation with *E.coli*, *M.a.p.* or rHsp70, gene expression patterns had changed compared to T6 (fig.1A-C). After incubation of DC with *E.coli* (fig.1D), expression of IL-1β mRNA was increased but less than at T6 (319- to 10-fold increase from T6 to T24), IL-6 mRNA expression lowered from 16-fold increase at T6 to 3-fold increase at T24, and IL-10 mRNA expression that had increased 5-fold at T6, had decreased to 2-fold increase at T24, which was not a significant increase at either time-point. Only IL-12 mRNA expression increased compared to T6 (848-to 3091-fold from T6 to T24 respectively). Expression of TNF-α mRNA decreased compared to T6 (25- to 7-fold increase), as did expression of GM-CSF mRNA (from 44-fold to 42-fold). TGF–β transcription was down-regulated 1.1 fold, which was not a significant decrease.

After 24 hours of infection with *M.a.p.* (fig.1E) the mRNA profiles demonstrated again a similar pattern as described for *E.coli* incubation, except for the expression of IL-10 mRNA, that increased from T6 to T24 (8-fold to 11-fold increase) and GM-CSF mRNA that increased from 51-fold at T6 to 130-fold at T24, the latter being not significant. At T24 the increase of IL-1β mRNA expression was 3-fold (compared to 48-fold at T6), IL-6 increased 1.3-fold at T24 as compared to 9-fold increase at T6, and IL-12 mRNA expression was 4694-fold upregulated (compared to 369- fold at T6). TNF-α mRNA expression changed from 21- to 14-fold increase from T6 to T24 respectively and TGF–β transcription was 1.2-fold down-regulated.

Incubation for 24 hours with rHsp70 (fig.1F) induced down-regulation of IL-1β and IL-6 (36-fold and 7-fold lower expression than control values respectively, while at T6 IL-1β mRNA expression was increased 6 times and IL-6 2 times). After an initial increase in expression, IL-10 mRNA decreased to 1.2 fold down-regulation at T24, neither being significant changes. Expression of IL-12 mRNA increased at T24 to a 4-fold increase, compared to a 3-fold down-regulation at T6. TNF-α gene expression was down-regulated 2-fold (after a 1.2-fold increase at T6) which was significant at this time-point , while TGF–β and GM-CSF mRNA expression remained near control values (1.2 and 1.1-fold upregulation respectively).

***Ratio of TNFα to IL-10 and IL-12 to IL-10***

For a more objective comparison between the different stimuli, we calculated the ratios of

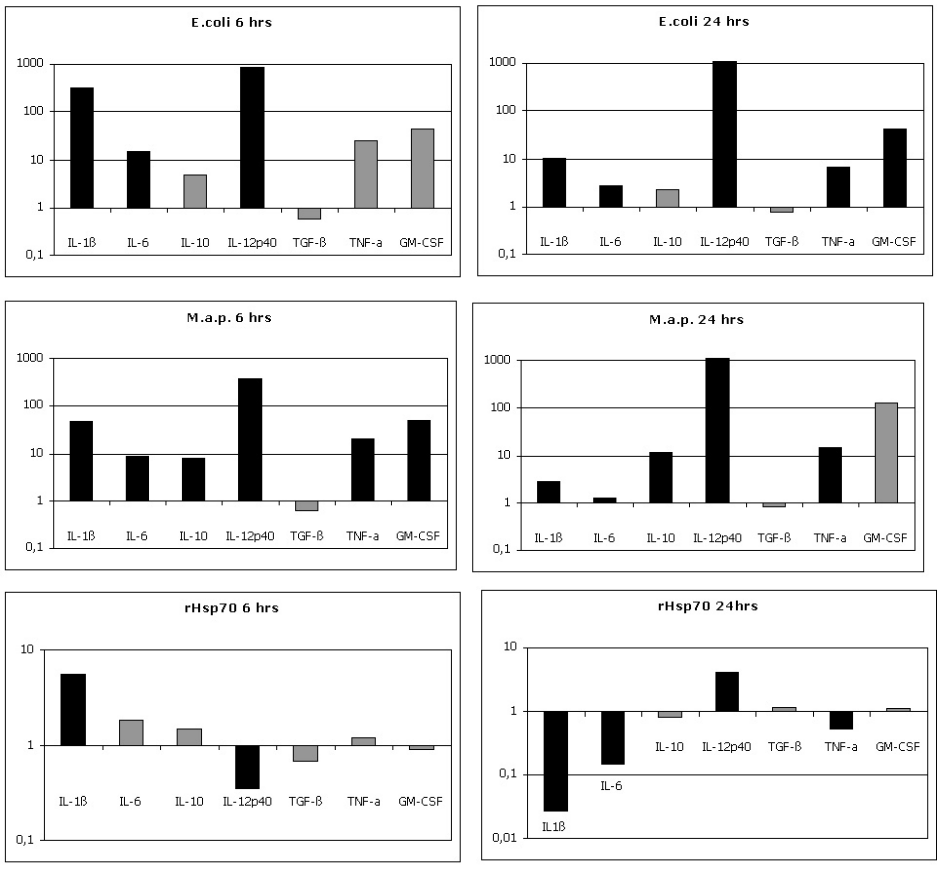


Fig.1.

Cytokine gene expression profiles after 6 and 24 hours of incubation of immature DC with *E.coli* (A,D), *M.a.p.* (B,E) or rHsp70 (C,F). Bars represent the mean fold gene expression (y-axis), expressed as n-fold difference to the untreated controls, which have been ascribed a value of 1. Black bars: significant up/down-regulation of cytokine gene, p=0.001; grey bars: up/down-regulation of cytokine gene not significant (p>0.1).

TNF-α and IL-12 gene expression versus that of IL-10 when DC were incubated with the different antigens (fig.2). The ratio of TNFα versus IL-10 showed more prominent TNFα gene expression by DC incubated with both *E.coli* and *M.a.p.* (ratio of 5 and 3 respectively), at T6. From 6 to 24 hours of incubation these ratios decreased; while the ratio remained higher for *E.coli* (ratio:3), *M.a.p.* induced gene expression of both cytokines was almost equal (ratio ~1) at T24. Incubation with rHsp70 induced equal amounts of both cytokine mRNA at both time-points. Expression of IL-12 versus IL-10 was higher when DC phagocytosed bacteria, a ratio of 171 upon *E.coli* phagocytosis and 46 upon *M.a.p.* phagocytosis at T6. From T6 to T24, the ratios increased 8 times (from 171 to 1343; *E.coli*) and 9 times (from 46 to 419; *M.a.p.*) respectively. Incubation with rHsp70 resulted in overproduction of IL-12 after 24 hours of incubation only, with a low ratio (ratio:5) as compared to the ratios after phagocytosis of bacteria.

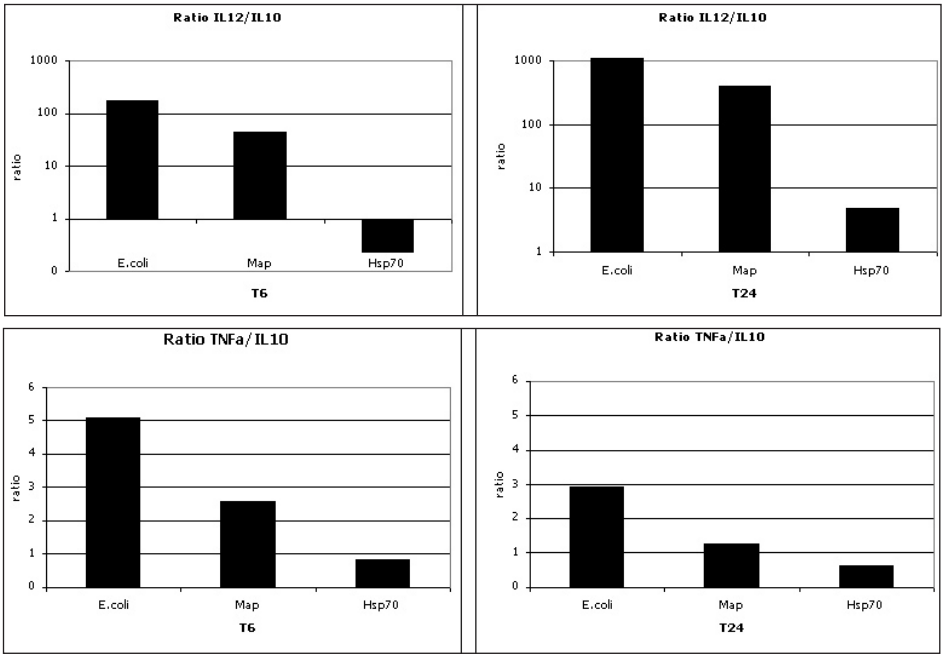


Fig.2.

Relative expression of IL-12 and TNF-α compared to IL-10. Ratio of IL-12/IL-10 (upper panels) and TNF-α/IL-10 (lower panels) at the y-axis, of DC incubated 6 and 24 hours with E.coli, *M.a.p.*, or rHsp70 (x-axis).

## Discussion

The aim of the present study was to compare cytokine gene expression by immature DC, as a result of different activation signals. Focussing on kinetics rather than quantities, cytokine mRNA was measured after 6 and 24 hours of incubation with *E.coli*, *M.a.p.* and rHsp70. Activation signalling of these antigens will presumably occur mainly via TLR (Nau et al., 2002), *E.coli* being a typical ligand for TLR4, *M.a.p.* for TLR2 (Werling and Jungi, 2003) and rHsp70 for both TLR2 and 4 (Nau et al., 2002; Vabulas et al., 2002).
Comparing cytokine expression profiles after phagocytosis of *M.a.p.* or *E.coli* did not reveal striking differences for the cytokine genes chosen in this study. While there were slight differences in the magnitudes of the mRNA expression of the different cytokines, the direction of the change (*i.e.* increase or decrease) was the same for both bacteria from T6 to T24, except for IL-10 and GM-CSF. As mycobacteria have been described as virulent pathogens with many capacities to impede immune reaction by the host cell (Ferrari et al., 1999; Gatfield and Pieters, 2000; Weiss et al., 2001) and were shown to modulate DC function (Geijtenbeek et al., 2003; Mariotti et al., 2002; Nau et al., 2002), it could be expected to find differences in reaction between phagocytosis of *E.coli* and of *M.a.p.*. Possibly, in *in vitro* situations, where DC have been generated in a standardised manner and where the different bacteria are the variable, DC act in a typical manner, triggered by phagocytosis. It could be that the same receptors (*e.g.* scavenger receptors) are engaged upon phagocytosis of both types of bacteria, or that the engagement of different receptors (*e.g.* TLR2 and TLR4) finally leads to the same signalling pathway intracellularly. The latter is suported by a study of Werling et al. (Werling et al., 2004), who demonstrated production of IL-12 by bovine DC upon triggering with different ligands engaging different TLR.

Although the kinetics of cytokine gene expression were largely similar for both bacteria, the ratios of different cytokines might give more accurate information, as they provide more objective data and reflect the subtle balances between the different cytokine genes that were induced. The ratios of TNFa/IL-10 and IL-12/IL-10 expressed the same kinetics for both bacteria, TNF-α/IL-10 decreased from T6 to T24 and IL-12/IL-10 increased from T6 to T24 with a similar magnitude, but the values of the ratios after *E.coli* ingestion were at every time-point higher than after *M.a.p.* infection. This could reflect a hampered capacity of *M.a.p.*-infected DC to mount a vigorous immune reaction, which might be related to the virulence of *M.a.p.*.

Incubation with rHsp70 showed a different pattern of gene expression compared to that observed after phagocytosis of bacteria. Although the genes for the pro-inflammatory cytokines IL-1β, IL-6, GM-CSF and TNF-α were upregulated, IL-12 was down-regulated at T6. Induction of pro-inflammatory cytokines after incubation of DC with Hsp has been described as an immunoregulatory function of Hsp to activate the immune system (Bethke et al., 2002; Wan et al., 2004). Although our experiments demonstrated the transcription of different pro-inflammatory cytokine genes by DC after incubation with rHsp70, though far lower than in case of incubation of the DC with the bacteria, this effect was short lived as peak induction seemed to have passed between 6 and 24 hours *p.i.* or even before 6 hours. At T24 the down-regulation of IL-1β, IL-6 and TNF-α was even more important than the initial upregulation at T6. Upregulation of IL-12 mRNA induced by rHsp70 occurred only at T24, after an initial down regulation at T6, both in absolute values and relative to IL-10 gene expression. A delayed onset of IL-12 production has been described for macrophages infected with *Toxoplasma gondii (T.gondii)* parasites (Denkers, 2003) and the effect has been attributed to the Hsp70 produced by the parasite (Denkers, 2003; Dobbin et al., 2002). This Hsp70 was demonstrated to be secreted by the parasite and inhibited temporarily the nuclear factor- B (NF-κB) signalling pathway, causing reduced transcription of cytokine genes (Dobbin et al., 2002). Virulence of the different *T.gondii* strains could even be directly related to the Hsp70 produced by the strains, although there was only a minor difference (seven residue repeat units) at the amino acid level for the genes encoding for the protein (Dobbin et al., 2002). Blocking the *T.gondii* Hsp70 for a relatively short period (72 hours) before infection of mice with the parasite resulted in reduced numbers of the virulent parasite strain found after 4 days in infected mouse spleens (Dobbin et al., 2002). This indicates that the first interactions of pathogens with their host cell, and the subsequent signalling cascades and cytokines produced, are of importance for the capacity of the pathogen to steadily settle itself and replicate in the host. The delayed onset of IL-12 production we observed could indicate that *M.a.p.* Hsp70 forces the host to allow *M.a.p.* to infect APC safely, as was shown for *T.gondii*.

## Conclusion

Cytokine expression profiles of cattle DC incubated with *M.a.p.*, *E.coli*, and recombinant *M.a.p.* hsp70 provided information on interference of mycobacteria with professional APC, thus on early modulation of specific immunity. It appeared that infection of DC by *E.coli* and *M.a.p.* triggered the production of pro-inflammatory cytokine gene expression, dominated by IL-12, which would lead to the appropriate cell mediated immune reaction to combate infection. However, the response to *M.a.p.* infection was less vigorous than to *E.coli*. In contrast, incubation with rHsp70 led to delayed production of IL-12 gene expression, which indicated that rHsp70 had an inhibitory effect on the induction of a Th1 inflammatory profile, potentially contributing to a situation in which pathogens are able to invade and settle in host cells.

# APC 7

## Summarizing Discussion

HSP MAP

The contribution of $CD8^+$ cytotoxic T lymphocytes (CTL) to protective immune responsiveness and pathofysiology of bovine paratuberculosis is largely unknown, therefore the general aim of this thesis was to gain more insight in the role CTL play in the various phases of the disease process. Contribution to protective immunity against *M.a.p.* infection by CTL was hypothesized, similar to that demonstrated for other infectious diseases caused by intracellular bacteria. Furthermore it was hypothesized that CTL reactivity could be induced in cattle by using heat shock protein 70 (Hsp70) as a cross-priming agent.

A longitudinal study of *M.a.p.* infected calves was conducted to gain insight in the relationship between fecal bacterial shedding patterns, disease developmen, humoral and cellular immune responsiveness, and the presence and activity of $CD8^+$ T cells (chapter 2). The use of rHsp70 as a cross-priming agent and carrier of CTL inducing antigen requires receptor mediated interaction with bovine antigen presenting cells (APC), as described in chapter 3. A fusion protein, consisting of the receptor binding part of rHsp70 fused to a model antigen, was constructed and its interaction with bovine APC as compared to the native *M.a.p.* Hsp70 was investigated (chapter 4). Subsequently, it was used to immunise cattle and two different cytotoxicity assays were used to detect CTL activity *in vitro* (chapter 5). As dendritic cells (DC) prime antigen specific T cells and the phenotype of the T cells is determined by the cytokines they produce, the cytokine gene expression profiles of DC incubated with *M.a.p.* and with rHsp70 were studied to determine potential differential T cell priming by DC depending on the antigens used (chapter 6). Finally, the results of the different studies and the potential implications for the role of immune responses to Hsp70 occurring during natural *M.a.p.* infection are discussed (this chapter).

## The versatility of Hsp70

In this study, rHsp70 was shown to be a dominant antigen in bovine paratuberculosis, eliciting strong cellular immune responses during experimental infection. At the same time, rHsp70 was used as a carrier for a model antigen, *i.c.* eGFP, with the ultimate intention to immunise cattle with rHsp70 fused to a *M.a.p.* specific antigen to protect against *M.a.p.* infection. However, literature reports on the favourable effects of the use of mycobacterial Hsp70 in prevention or modulation of auto-immune diseases seem in contradiction with the use of Hsp70 to combat infectious diseases.

Opinions that Hsp70 can be exploited both to fight tumor diseases and infectious diseases, and also auto-immune diseases are presented by two 'schools'. One school, the line of thoughts of which was followed when we developed our hypotheses, is the one that argues that Hsp70 can be used as an immunological tool because Hsp70, taken up into the cytoplasm via specific receptors, activates APC and in the end gives rise to cross-presentation of antigen carried by the Hsp70 (Srivastava, 2002; Tobian et al., 2004b). In summary, this means that the properties of Hsp70 that are being exploited are helpful in eliciting specific CTL and Th1 type cellular immune responses. The second school, in contrast, studies Hsp70 for its virtues in combating auto-immune diseases by the induction of IL-10 and Th2 type or regulatory T cells (Prakken et al., 2001; van Eden et al., 2003). Both schools emphasize the much conserved nature of Hsp between the species and even between prokaryotic and eukaryotic organisms, which explains the cross-reactivity between different Hsp.

To explain the apparent opposite properties of the same molecule one should consider three 'faces' of the Hsp70 molecule (figure). First, it is a 'neutral' molecule that, via a specific endocytic receptor, enters an APC and shuttles its cargo antigen into the cytoplasm of the cell. Second, it is a signalling molecule, that can interact with different types of receptors and thus activate different types of signalling cascades. These receptors can exist solitarily or in combination on the surface of the APC. The type and maturation stage of the APC determines which receptors are expressed. The combination of receptors used determines the

signalling cascade and the final production of cytokines and/or accessory surface molecule expression. Thirdly, it is an antigen. This antigen will be taken up by APC, by phagocytosis and/or macropinocytosis, and antigenic epitopes will be presented, leading to development of specific T cells.

The first and second aspects of Hsp are independent of the MHC background of the host. Despite the high homology of the Hsp, differences between prokaryotic and eukaryotic Hsp have been reported for receptor interaction with mammalian APC. Bearing in mind the three DC signals needed to induce specific T cells (antigen presented to the TCR, accessory molecule expression and cytokine environment (Kapsenberg, 2003)), the mutual influence between cytokines, APC phenotype, receptors expressed and Hsp determines the outcome of Hsp and APC interaction and subsequent T cell priming which may be very diverse, and more complicated than can be predicted on the basis of *in vitro* experiments with one type of APC and one type of Hsp.

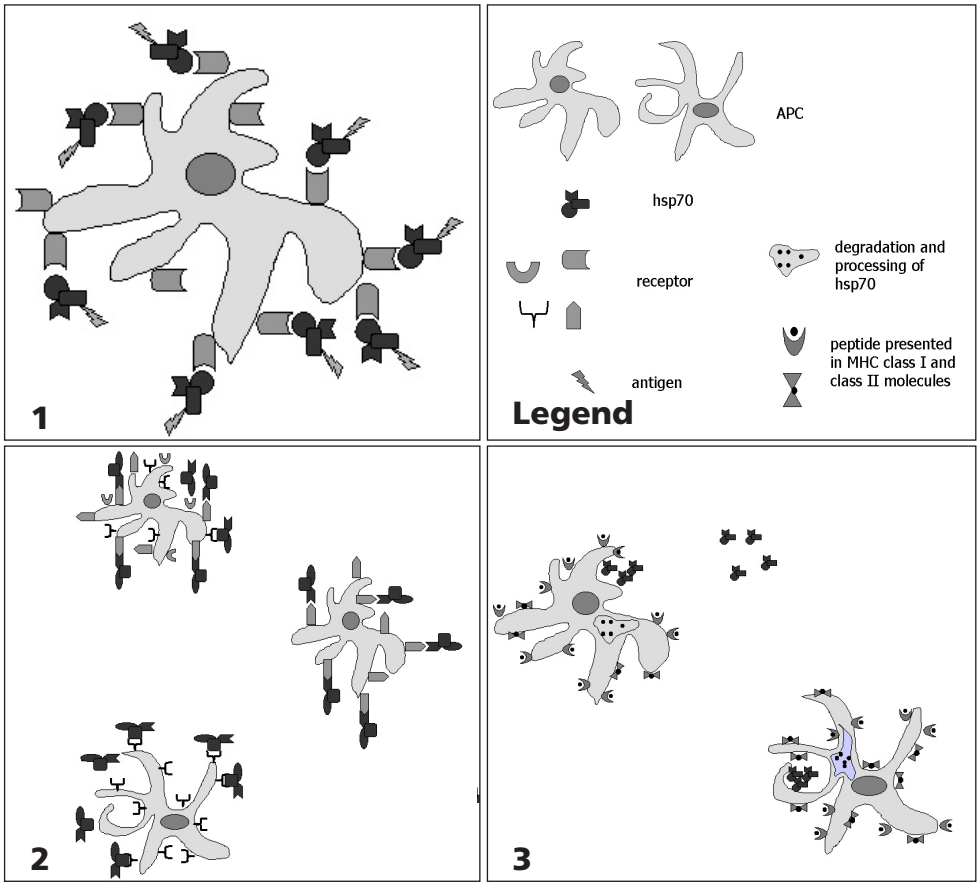


Figure of the three faces of Hsp70. 1: carrier of antigen, 2: signalling molecule, 3: antigen

The third aspect, Hsp70 as an antigen itself, is dependent on MHC. Common epitopes between Hsp exist, due to their conserved nature. Prokaryotic Hsp contain human (Adams et al., 1997) and bovine (Norimine et al., 2004) CD4 epitopes, as well as human CD8 epitopes (Charo et al., 2001). The Hsp70 epitopes that were described to induce regulatory T cells,

such as in rheumatoid arthritis (Prakken et al., 2001), in leprosy (Mutis et al., 1994) and in *L.monocytogenes* infection (Kimura et al., 1998) are of benefit in autoimmune diseases but detrimental in the case of intracellular infections.

## CTL, Hsp and *M.a.p.*

The longitudinal study described in chapter 2 demonstrated that the third aspect of rHsp70 as described above, its antigenic properties, plays a dominant role in *M.a.p.* infection. In comparison to the *M.a.p.* recombinant Hsp65, proliferative responses to rHsp70 were much more vigorous, a finding that is in agreement with previous work (Koets et al., 1999). Although the subset determination of lymphocyte proliferation in response to rHsp70 and *M.a.p.* (chapter 2, figure 7, CFSE assay) revealed that a major contribution to rHsp70 specific responses could be attributed to γδ T cells, more data are needed to assess the involvement of the different lymphocyte subsets in response to rHsp70 in time.

In this study, we could not find evidence for $CD8^+$ T cells as a major population of lymphocytes, contributing to protective immune-reactivity against *M.a.p.* infection. Neither could lymphocyte subpopulations or other functional parameters of cellular immunity be correlated to protective immunity, or discriminate between the populations of high- and low frequent shedders, that was clearly distinguishable by PPD-P specific antibody production, from first parturition onwards. Moreover, despite the absence of clinical signs in both groups and long delay of the onset of fecal shedding in some animals, it was obvious that none of the animals was able to clear the infection. Early events in infection might be decisive for the outcome of disease, which makes it even more plausible that CTL, provided they can be induced, could be helpful in prevention of paratuberculosis.

We showed that the interaction of rHsp70 with bovine APC was receptor mediated and dependent on the type and the differentiation stage of the APC (chapter 3). The well described receptor CD91 (Basu et al., 2001) was involved, but the difficulty to saturate receptor binding places together with the impossibility to compete for labelled rHsp70 uptake by immature DC with either rHsp70 or α2M (the CD91 ligand), provided evidence for the existence of more receptors. Enhanced GFP was used as a model antigen since no *M.a.p.* specific epitopes are known, while eGFP was shown to contain several CTL epitopes for different species, making it probable that bovine epitopes could also be found. When fusing the presumed receptor binding domain of rHsp70 to eGFP, the interaction with bovine APC was not lost (chapter 4). The findings on rbd-H70-GFP interacting with bovine APC, in agreement with the literature on receptor dependent interaction of Hsp with APC, strengthened the idea that CTL induction in cattle with the fusion protein, exploiting the carrier function of rHsp70 to bring eGFP antigen into the cytoplasm of the bovine APC for subsequent degradation and presentation in the context of MHC class I to CTL (figure, panel 1), would be feasable. Immunisation with the fusion protein (chapter 5) led to proliferative responses in LST in response to the full length fusion protein as well as its constituents, rbd of rHsp70 and eGFP. However, no cytotoxic T cell reaction could be detected *in vitro*, by using both a classical chromium release assay and a flow cytometric assay.

Several explanations for the absence of eGFP specific CTL, which are not mutually exclusive, might exist and are discussed hereafter. Cells may be present that produce IFN–γ during the *in vitro* assay, but that are not cytotoxic in response to the peptide loaded targets. Enhanced GFP might not contain bovine CTL epitopes, despite the CTL epitopes that exist within this protein for several other species. Alternatively, the rHsp70 moiety of the fusion protein might be the main cause of the failure to induce CTL, in several ways. First, although endocytosis of the fusion protein by bovine monocytes and Bomac was shown, comparable to endocytosis of the full length rHsp70, the use of different and possible less cell surface

receptors for the uptake of the fusion protein in comparison to the full length rHsp70 can not be excluded. Second, although rHsp70 was supposed to be a 'neutral' carrier of antigen (figure, panel 1), rHsp70 of *M.a.p.* might be less neutral than expected, and, via specific receptors, induce bovine DC to produce cytokines (figure, panel 2) that polarize the DC to prime T cells with a regulatory or suppressive phenotype. Finally, epitopes that induce regulatory or suppressor T cells (Mutis et al., 1994; Prakken et al., 2001) might be present in rHsp70 (figure, panel 3). Taking into consideration the results of the study and the possible explanations, it was concluded that with this immunisation protocol, rbd-H70-GFP was not the right construct to induce CTL in cattle.

Dendritic cells are pivotal in priming of naive T cells. Therefore, cytokine gene expression patterns of immature DC that were incubated with *M.a.p.*, rHsp70 or *E.coli* were determined (chapter 6). Two interesting observations were made. First, incubation with either *M.a.p.* or *E.coli* induced largely similar cytokine gene expression patterns, dominated by IL-12 mRNA expression. In literature, *in vitro* infection of bovine DC with different bacteria was shown to lead to IL-12 production (Hope et al., 2004; Werling et al., 2004), while infection of macrophages with the same bacteria (Werling et al., 2004) led to IL-10 production, but also to more than ten times more NO produced than in DC. This demonstrated the differential outcome of stimulation of different APC with the same stimulus, as we already observed (chapter 3) with rHsp70 interacting with APC. Moreover, although this was an *in vitro* situation, which may not account for differences in cytokine environment that exist in the in vivo situation, it seemed like a 'default' reaction of bovine APC upon stimulation with these different bacteria. This would imply that DC are perfectly capable of mounting a Th1 type reaction upon infection with mycobacteria (chapter 6), while in the literature macrophages were demonstrated to produce sufficient microbicidal substances to overcome mycobacterial infection (Werling et al., 2004). Where does immune reactivity go wrong for the host that develops paratuberculosis? Although there is no proof as yet, the results of the longitudinal study (chapter 2) point towards important involvement of genetic factors in answer to this question. High frequent fecal shedding was shown to be related to the presence of PPD-P specific antibodies detectable from one year *p.i.* onwards, while in the group of low frequent fecal shedders no antibodies could be detected during the five years investigation period. Therefore, the relationship between containment of infection and the production of antibodies in response to infection, which is a heritable trait (Mortensen et al., 2004), indicated that the response to *M.a.p.* infection is at least partially genetically determined.

While phagocytosis of *E.coli* and *M.a.p.* led to similar cytokine gene expression profiles, dominated by IL-12 mRNA expression, the second observation was that incubation of immature DC with rHsp70 resulted in delayed IL-12 gene transcription. Although it is speculative to extrapolate this finding to the failure to induce CTL (chapter 5), the possibility should be investigated. The cytokine profile needed to induce a strong CTL response is predicted an immediate IL-12 response together with the production of pro-inflammatory cytokines (Demangel and Britton, 2000; Trinchieri, 2003). If delayed IL-12 gene expression, together with relatively early down-regulation of pro-inflammatory cytokine genes, as was demonstrated (chapter 6), would be suggestive of the priming of T cells with a more regulatory or suppressive phenotype (Chieppa et al., 2003), this would call for a more cautious approach when rHsp70 is to be used in a fusion protein.

## Possible roles of Hsp70 in natural infection

The receptor mediated uptake of rHsp70 by bovine APC demonstrated was a prerequisite for our immunisation study, but at the same time the pathophysiological consequences of this interaction in the natural situation are not clear. Mycobacterial Hsp70 will certainly accu-

mulate at the site of infection (Galdiero et al., 1997) and influence the APC that are present locally. The question then arises as to whom can use this Hsp70 to its benefit, the pathogen or the host.

According to literature, *M.a.p.* Hsp70 could induce maturation of DC, release of pro-inflammatory cytokines and enhance uptake of antigen, captured in the Hsp70 molecule. This would be advantageous for the host, as it activates host defence and thereby aids *M.a.p.* destruction. Although we did not study all of these aspects, our data of cytokine gene expression by immature DC (chapter 6) do not plead in favour of this theory, but rather indicate that rHsp70 delays the development of anti-mycobacterial immunity.
The Hsp70 could also be used in the pathogen's advantage, depending on the APC that encounter the Hsp70 first. Monocyte development into DC was demonstrated to be inhibited by Hsp70 (Kuppner et al., 2001). By that means, Hsp70 would hamper the development of appropriate anti-mycobacterial immunity in a very early stage.

Possibly, an apoptotic signaling pathway can be activated via TLR2 (Aliprantis et al., 2000), a receptor for Hsp70 (Asea et al., 2002; Vabulas et al., 2002). Although killing of macrophages renders mycobacteria devoid of their home, selective killing of activated macrophages expressing TLR2 would on the contrary protect mycobacteria from macrophages with enhanced phagocytic capacity and leave unactivated macrophages intact.

Yet another effect of mycobacterial Hsp70, competing for receptor places with host molecules, is a more passive effect. A receptor like CD91 is involved in the immune response in several ways. The natural ligand for CD91, $\alpha$2-macroglobulin ($\alpha$2M), is a protease inhibitor that is present in relatively high concentrations in serum and leaks into sites of inflammation (Chu and Pizzo, 1993). It is involved in uptake and cross-presentation of antigen by APC (Chu and Pizzo, 1993; Hart et al., 2004), but is also decribed as a carrier of cytokines (Borth and Luger, 1989; Gourine et al., 2002; James, 1990). Occupation of CD91 by Hsp70 may hinder antigen presentation and development of appropriate specific anti-mycobacterial responses. Furthermore, occupation of CD91 by Hsp70 may interfere with the clearance of cytokines by $\alpha$2M, which as a consequence may lead to a cytokine microenvironment that is advantageous to the pathogen.

There is no hard evidence supporting these assumptions, but the knowledge that *M.a.p.* Hsp70 interacts with bovine APC merits more investigation that will provide better insight in the subtle balance between pathogen and host.

## Concluding remarks

The study described in this thesis aimed at gaining more insight in the role of CTL in bovine paratuberculosis and at inducing CTL by immunisation with a rHsp70 based fusion protein. The decisive events on the outcome of *M.a.p.* infection seem to occur very early after infection when cellular immune responses of low frequent shedders are still a differentation mark with high frequent shedders. This enforces the idea that the presence of inducible *M.a.p.* specific CTL early in infection might indeed very well help in preventing disease establishment, at the time when the intestinal macrophages are not yet loaded with the pathogen. Induction of CTL with a fusion protein consisting of the rbd of rHsp70 fused to a specific *M.a.p.* antigen should not be encouraged, as the use of rHsp70 fused to eGFP did not elicit eGFP specific CTL, and moreover the rHsp70 moiety of such a fusion protein cannot be considered as a neutral carrier of antigen but should be regarded as a dominant antigen that will influence the ensuing immune reaction.

Further research on the involvement of rHsp70 in both specific cell mediated immune reactions and the interaction with bovine APC will certainly provide deeper insight in immune-pathology of paratuberculosis which might provide us with clues to prevent this detrimental disease.

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NL


**Nederlandse samenvatting**

| | |
|---|---|
| **antigeen** | deeltje, vaak een eiwit, dat een immuunreactie opwekt |
| **APC** | Antigeen Presenterende Cel |
| **CD** | Cluster of Differentiation, karakteristiek molecuul op het oppervlak van een witte bloedcel. De CTL hebben bijv. CD8, de T-helper cellen hebben CD4 |
| **CTL** | Cytotoxic T Lymphocyte, ofwel cytotoxische T-cel |
| **cytokine** | cytokines zijn signaalstoffen. Cellen communiceren met elkaar via rechtstreeks contact (cel-cel contact), of indirect via signaalstoffen, bijv. cytokines. Cellen hebben grote hoeveelheden receptoren op hun oppervlak, als een soort antennes, om de boodschappen door te geven naar het binnenste van de cel. Receptoren en de bijbehorende signalen, direct of indirect, passen bij elkaar als een sleutel en een slot. |
| **DC** | Dendritische Cel, cel die behoort tot de APC, belangrijkste cel die een specifieke immuunreactie initiëert doordat hij de T-cellen 'instrueert' |
| **fagocytose** | 'opeten' , APC kunnen lichaamsvreemde deeltjes opeten en delen daarvan aan de buitenkant van hun celoppervlak presenteren, waardoor specifieke cellen, die dit herkennen, een reactie in gang zetten tegen dit deeltje |
| **GFP** | Green Fluorescent Protein, fluorescent eiwit van een kwal, fluoresceert groen zoals ook bekend is van vuurvliegjes en gloeiwormen. De DNA-volgorde van dit eiwit is bekend en het kan daarom nagemaakt worden. In deze studie is het gebruikt als een model-antigeen, en gekoppeld aan een stukje van het Hsp70. Op die manier is groen fluorescerend Hsp70 verkregen |
| **HFS** | high frequent shedder |
| **Hsp70** | Heat shock protein 70, eiwit dat in cellen wordt gemaakt onder invloed van stress (warmte, verzuring etc.) |
| **leuk** | www.dnacode.nl; www.johnes.org; www.cellsalive.com |
| **LFS** | low frequent shedder |
| ***M.a.p.*** | *Mycobacterium avium subspecies paratuberculosis*, de bacterie die paratuberculose veroorzaakt |
| **macrofaag** | macrofagen behoren net als DC tot de APC. Macrofagen zijn 'veelvraten', ze zijn bekend om hun grote fagocytose capaciteit, ze kunnen gefagocyteerd materiaal verwerken en aan hun oppervlak presenteren en daarmee een immuunreactie van specifieke cellen opwekken. Die specifieke cellen moeten dan wel eerst door een DC geinstrueerd zijn. Macrofagen zijn ook de cellen waar de *M.a.p.* bacterie in overleeft en zich vermenigvuldigt, terwijl het eigenlijk de taak van de macrofaag is om de bacterie zo snel mogelijk af te breken |
| **MHC** | Major Histocompatibility Complex, molecuul dat stukje antigeen presenteert aan de buitenkant van een cel. Dit geheel, het MHC met antigeen erin, wordt herkend door specifieke T-cellen. CTL herkennen MHC klasse I met daarin antigeen, T-helper cellen herkennen MHC klasse II met daarin antigeen. Als de T-cel 'zijn' specifieke combinatie van MHC met antigeen tegenkomt, zal hij een interactie met de presenterende cel aangaan en in actie komen. De actie kan zijn: maken van cytokines (T-helper cellen), of dood maken van de cel die het antigeen presenteert (CTL). Het MHC klasse I komt op alle cellen voor, het MHC klasse II op APC |
| **monocyte** | voorstadium van macrofagen en DC, die in het bloed circuleert |
| **peptide** | stukje eiwit |
| **recombinant** | recombinant eiwit: eiwit waarvan de genetische code bekend is, waardoor het mogelijk is om dit eiwit te laten produceren door bijv. bacteriën |

## Hoofdstuk 1

Evolutionair gezien hebben zowel microben als hun gastheer maar één doel: het in stand houden van de soort waartoe ze behoren. De microben (in dit proefschrift een bacterie: *Mycobacterium avium subspecies paratuberculosis*, kortweg: *M.a.p.*) en hun gastheer (in dit proefschrift: koeien), hebben ieder strategieën 'verzonnen' om dat doel te bereiken. De *M.a.p.*-bacterie heeft de gastheer koe nodig om zich te kunnen vermenigvuldigen. Aangezien de koe deze ziekteverwekkende bacteriën niet bij zich wil dragen, probeert hij er weer vanaf te komen. Daartoe moeten de cellen van het afweersysteem, de witte bloedcellen, in actie komen. Dit gaat via een soort tweetraps raketmechanisme: allereerst wordt de algemene afweer (innate immunity) in werking gezet, die actief is tegen allerlei soorten microben. Zo snel mogelijk worden er ook specifieke cellen (van de 'adaptive immunity') geactiveerd, om juist deze *M.a.p.* te lijf te gaan. Ondertussen heeft *M.a.p.* allerlei mechanismen ontwikkeld om aan deze aanvallen te ontkomen, en rustig de tijd te nemen om zich te vermenigvuldigen in z'n gastheer.

***De ziekte***
Het bijzondere aan de ziekte die door *M.a.p.* veroorzaakt wordt, paratuberculose, is dat de klinische verschijnselen (ernstige, niet te behandelen diarree, waardoor het dier sterk vermagert en uiteindelijk dood gaat) pas optreden als het dier enkele jaren oud is. De besmetting vindt echter plaats in kalveren. In de tussenliggende periode is aan het dier uiterlijk niets te zien, terwijl ondertussen de bacterie zich vermenigvuldigt en bovendien ook met de mest uitgescheiden wordt. Dit betekent dat het dier alweer andere kalveren kan besmetten, zonder dat dat opgemerkt wordt.

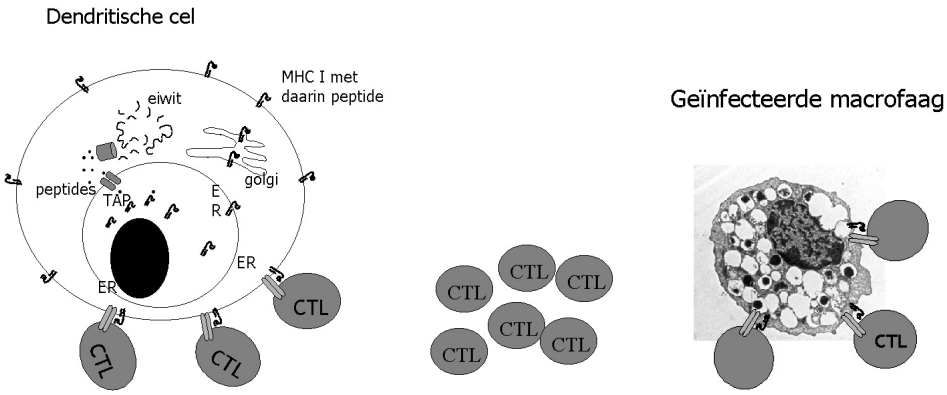
***De afweer***
Een andere bijzonderheid van de *M.a.p.* bacterie is dat hij zich ophoudt en vermenigvuldigt in de macrofagen van de darm. Macrofagen zijn cellen van het afweersysteem, die eigenlijk als voornaamste functie hebben om lichaamsvreemd materiaal (bijvoorbeeld bacteriën) te fagocyteren (op te eten) en af te breken. Dit afbraakproces vindt plaats in vacuoles (blaasjes) in de macrofaag, die sterk verzuren en die daardoor de bacterie dood maken. Stukjes bacterie worden dan gekoppeld aan MHC (Major Histocompatibility Complex) moleculen van klasse II, naar de oppervlakte van de cel getransporteerd en aan de buitenkant van de cel gepresenteerd. Het stukje vreemd eiwit (antigeen) in MHC klasse II kan dan herkend worden door T-helper cellen, andere cellen van het afweer systeem. Deze T-helper cellen zullen sterk vermenigvuldigen, en iedere keer als ze weer macrofagen tegen komen met dit specifieke stukje eiwit in MHC klasse II aan het oppervlak, zullen ze cytokines, signaalstoffen, gaan produceren. Deze cytokines activeren dan nieuwe macrofagen om sneller en actiever bacteriën dood te maken. Op de één of andere manier weet de *M.a.p.*-bacterie dit hele mechanisme te omzeilen. De verzuring van de vacuoles is veel minder, waardoor de bacterie gewoon in de macrofaag kan blijven leven. De macrofaag kan bovendien de MHC klasse II moleculen niet goed aan het oppervlak celoppervlak presenteren. Het is niet precies bekend, maar het wordt wel verondersteld, dat de door *M.a.p.* geïnfecteerde macrofagen zelfs een negatief effect hebben op de T-helper cellen die contact maken met het MHC klasse II dat eventueel toch met een stukje bacterie aan het oppervlak van de macrofaag verschijnt. Het negatieve effect resulteert in dood (door 'apoptose') van de T-helper cellen.
Het is bekend bij paratuberculose dat de T-helper cellen hun werk goed uit moeten voeren om de infectie te weerstaan. Het is ook bekend dat die T-helper cellen in eerste instantie, als de infectie nog niet zo lang aanwezig is, inderdaad aantoonbaar zijn en blijkbaar kunnen helpen om de infectie in toom te houden. In de loop van de tijd verdwijnen die T-helper cel-

len echter, en wordt het dier ziek terwijl het steeds meer bacteriën uit gaat scheiden.

***CTL en Hsp70, de hypothese***

Bij de studie die in dit proefschrift wordt beschreven wilden we gebruik maken van een andere manier van afweer, zoals in gang gezet wordt bij infecties met virussen. Virussen die een cel infecteren dringen als het ware hun genetische materiaal op aan de gastheercel, die de viruseiwitten en vervolgens virusdeeltjes gaat maken. De virusdeeltjes komen dan uit de cel en gaan op weg om nieuwe cellen te infecteren. Bij het maken van viruseiwitten zal de cel ook alweer eiwitten afbreken, en presenteren in het MHC klasse I molecuul. Dit wordt herkend door een ander soort T-cellen, de cytotoxische T-cel of CTL (cytotoxic T lymphocyte), meestal een zogenaamde 'CD8 positieve T-cel'. Deze CTL zal ook cytokines maken, net als de T-helper cel, maar vooral maakt hij stoffen die de geïnfecteerde cel dood maken. Een virus heeft de gastheer cel nodig om zich te vermenigvuldigen; als nu voortdurend de cel waar hij in zit wordt dood gemaakt, zal hij uiteindelijk geen plaats meer vinden om zich te vermenigvuldigen en dooft de infectie uit. In het geval van paratuberculose is het idee dat als er CTL geactiveerd worden die de geïnfecteerde macrofaag dood maken, de *M.a.p.*-bacterie al snel geen huisje meer heeft, en de infectie, op soortgelijke manier als bij virussen, uit zal doven.

Om effectieve CTL te maken die door *M.a.p.* geïnfecteerde macrofagen zullen doden, zullen de CTL in eerste instantie door dendritische cellen 'geïnstrueerd' (geprimed) moeten worden. Daarna zullen ze op zoek gaan tot ze (op de geïnfecteerde macrofaag) opnieuw het stukje specifieke eiwit tegen komen in MHC klasse I, waarna ze die macrofaag zullen doden (zie figuur 1)

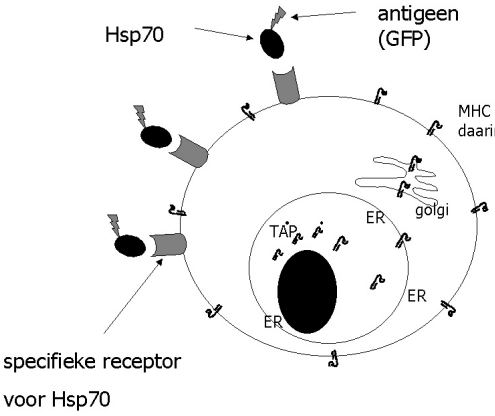



Figuur 1.

Een dendritische cel verwerkt eiwit (van *M.a.p.*) in de cel tot peptides, waarmee het MHC klasse I geladen wordt. Dit wordt gepresenteerd aan CTL. De CTL die specifieke interactie met dit MHC klasse I-peptide complex aangaan, vermenigvuldigen zich. De specifieke CTL zullen interactie aangaan met geinfecteerde macrofagen, die hetzelfde peptide samen met MHC klasse I aan hun oppervlak vertonen. De interactie leidt tot dood van de geinfecteerde macrofaag.

Een probleem is echter om een stukje bacterie in het MHC klasse I te krijgen. Eigenlijk komt alleen eiwit in de MHC klasse I terecht dat in de cel zelf gemaakt is. Er zijn echter 'trucs' beschreven, die ook op natuurlijke wijze optreden, om een eiwit 'van buitenaf' toch de cel in te krijgen en dan in het MHC klasse I. Eén van die 'trucs' is het gebruik van zogenaamde 'heat shock proteins'. Deze eiwitten worden normaal gesproken gevormd in cellen onder stresscondities, ze vervullen in de cel dan een rol in het vouwen en transporteren van eiwit-

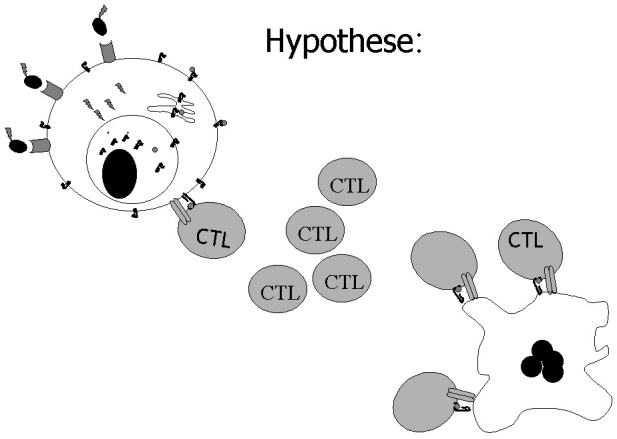
ten door de cel heen. Ze kunnen, als de cel waarin ze gevormd zijn kapot gaat, ook vrij komen, en dan het eiwit dat ze aan het transporteren waren mee nemen naar 'buiten'. Als ze dan een antigeen presenterende cel (APC) tegen komen, dat zijn macrofagen, maar ook zogenaamde dendritische cellen, dan zullen ze door die cel, via speciale receptoren, naar binnen gehaald worden en ook het eiwit dat ze bij zich hebben mee naar binnen nemen. Op die manier komt dat getransporteerde eiwit binnen in de macrofaag of dendritische cel terecht, waar het kan worden afgebroken en gepresenteerd in MHC klasse I. Deze omweg, die 'cross-priming' wordt genoemd, resulteert erin dat eiwitten die van buiten de cel naar binnen komen, toch in de MHC klasse I terecht komen (figuur 2).




Figuur 2.

Hsp70 gaat via specifieke receptoren de cel binnen, en neemt het antigeen mee. Het antigeen wordt dan in de cel afgebroken tot peptides en in de MHC klasse I gepresenteerd.

De hypothese die ten grondslag lag aan het in dit proefschrift beschreven onderzoek was in twee delen opgesplitst. Eén: voor het bestrijden van de infectie met *M.a.p.* zijn CTL belangrijke cellen. Twee: die CTL kunnen opgewekt worden door een dier in te spuiten met heat shock eiwit (in dit geval: Hsp70), waaraan een stukje eiwit zit (in dit geval: GFP) waartegen die CTL gemaakt zullen worden (figuur 3).




Figuur 3.

CTL worden geprimed tegen peptides van GFP, dat door het Hsp70 mee de DC in genomen is. De CTL zullen macrofagen doden die het GFP ook opgenomen hebben en peptides ervan presenteren in MHC klasse I.

## Hoofdstuk 2

In dit hoofdstuk wordt de infectie beschreven van een twintigtal kalveren met de *M.a.p.*-bacterie. De dieren zijn op jonge leeftijd geïnfecteerd waarna gedurende vijf jaar gemeten is of er bacteriën in de mest aantoonbaar waren (door middel van een kweekmethode), of er verschuivingen waren in de populaties witte bloedcellen (T-cellen, B-cellen en monocyten) en of er specifieke antilichamen aantoonbaar waren. De witte bloedcellen werden ook getest in reactie op verschillende antigenen van *M.a.p.* (PPD-P, Hsp65, Hsp70) en op de hele bacterie. Gedurende de studieperiode zijn de dieren niet zichtbaar ziek geworden. Wel splitste de groep vrijwel direct op in een groep van dieren die bijna bij elke mestkweek positief waren (hoog frequente uitscheiders) en een groep met dieren die niet of pas veel later in de periode bacteriën uit gingen scheiden (laag frequente uitscheiders). Van laag frequente uitscheiders (LFS) kan men zeggen dat ze dus een goede beheersing van de ziekte hebben, terwijl de ziekte bij hoog frequente uitscheiders (HFS) zich sneller uitbreidt. Bij de HFS groep waren ook al na een jaar antilichamen in het bloed aantoonbaar, terwijl die bij de LFS nooit aangetoond werden. Op basis van de kennis over paratuberculose werd verwacht dat de LFS een duidelijk verschillende reactie zouden vertonen voor wat betreft de cellulaire activiteit (de T-helper cellen die van belang zijn om de infectie in toom te houden), maar dit bleek niet het geval te zijn. Alleen de eerste twee jaar van de infectie waren er duidelijke verschillen meetbaar met de HFS, maar deze verschillen verdwenen na de periode rondom het afkalven, en kwamen daarna niet meer terug. Ook in de percentages van de verschillende T-cellen en de B cellen en monocyten in het bloed waren weinig verschillen tussen de groepen (HFS en LFS). Wel gold voor beide groepen dat het percentage T-helper cellen enorm afnam rondom de partus (het afkalven). Dit fenomeen is vaker beschreven bij koeien, het heeft onder andere te maken met hormoonschommelingen rondom het kalven en de energiestatus van het dier. Het is mede de oorzaak van de algemene weerstandsdaling rondom het afkalven. We concludeerden dat de cellulaire afweer alleen heel vroeg in de infectie meebepalend is voor de uiteindelijke uitkomst van de ziekte en dat het waarschijnlijk voornamelijk genetisch bepaald is of een dier terecht komt in de groep van HFS of LFS. De belangrijkste trigger voor het op gang komen van een antilichaamreactie en het gaan uitscheiden van bacteriën in de mest ligt in de afkalfperiode.

## Hoofdstuk 3

In het derde hoofdstuk wordt onderzocht of het recombinante Hsp70 van *M.a.p.* interactie aangaat met antigeen presenterende cellen (APC; macrofagen en dendritische cellen) van koeien. Antigeen presenterende cellen worden onder andere gekarakteriseerd door verschillende oppervlakte structuren, zoals het MHC klasse I en II, maar ook zogenaamde co-stimulatoire moleculen als CD80 en CD86 en verschillende soorten receptoren. Deze oppervlakte structuren kunnen in meer of mindere mate aan het oppervlak van de cel gepresenteerd worden, afhankelijk van het stadium waarin de cel zich bevindt. Voor dendritische cellen (DC) wordt de verandering van oppervlaktestructuren maturatie (rijping) genoemd. Immature (onrijpe) DC hebben een hoge fagocytose capaciteit, doordat er veel fagocytosereceptoren aan het oppervlak van de cel beschikbaar zijn, maar wanneer de DC rijpt verdwijnen die receptoren en komen er meer structuren aan het oppervlak die te maken hebben met het presenteren van antigeen aan T-cellen. De DC van koeien noemen we immatuur na drie dagen kweek en matuur na zeven dagen kweek. Door het Hsp70 te labelen met een fluorescente 'tag' kunnen de reagerende cellen gemeten worden. Het blijkt dat de APC interactie aangaan met het Hsp70, en dat het Hsp70 ook via (meerdere, verschillende)

specifieke receptoren de cel binnengaat. Binnen een populatie immature DC hebben een groot aantal cellen (ongeveer 60%) receptoren voor het Hsp70 aan het oppervlak, terwijl binnen de populatie mature DC veel minder cellen de receptor aan het oppervlak hebben, maar de cellen die reageren met het Hsp70 hebben wel veel méér receptoren aan het oppervlak. Om gebruikt te kunnen worden in een fusie-eiwit was het van belang te weten dat het Hsp70 interactie aangaat met APC, omdat dat de eerste cellen van de specifieke afweer zijn, die de CTL moeten primen.

## Hoofdstuk 4

In het vierde hoofdstuk wordt beschreven hoe er een fusie-eiwit gemaakt is. Dit fusie-eiwit bestaat uit het 'voorste' deel van het Hsp70 (volgens de literatuur is dat het stuk dat aan de celreceptor bindt) met daaraan een ander eiwit, het Green Fluorescent Protein (GFP). Dit GFP is gebruikt als model-antigeen, om eerst te kijken of het systeem van CTL opwekken met een Hsp70 zou lukken. Het fusie-eiwit ging ook interactie aan met de APC. Er werd besloten dat dit het juiste eiwit was om koeien mee in te spuiten teneinde na te gaan of er CTL gegenereerd konden worden.

## Hoofdstuk 5

Hoofdstuk vijf beschrijft de immunisatie van zes koeien met het fusie-eiwit. Na herhaalde immunisaties in de huid werd bloed genomen van de koeien. De witte bloedcellen uit dit bloed werden nog een week gerestimuleerd met het fusie-eiwit, of delen daarvan, om de door immunisatie opgewekte specifieke T-cellen de tijd te geven zich te vermenigvuldigen. Daarna werd met twee verschillende proefmethodes (een zg. klassiek chroom release assay en een assay gebaseerd op de flowcytometer) gekeken of specifieke CTL te vinden waren. Bovendien werd er gekeken naar antilichamen tegen het fusie-eiwit en naar proliferatieve responsen van T-cellen in reactie op het fusie-eiwit. De T-cellen bleken te reageren op het fusie-eiwit, hetgeen wil zeggen dat de immunisatie gelukt is en dat er geheugencellen zijn aangemaakt die het antigeen herkennen. Bij restimulatie van de cellen in het laboratorium, in reactie op het fusie-eiwit, kwamen er meer CD8 T-cellen dan zonder toevoeging van het fusie-eiwit. Echter, cytotoxische activiteit tegen peptides (stukjes antigeen) van het GFP of het hele fusie-eiwit was niet aantoonbaar, met geen van beide tests. Hieruit blijkt dat deze methode, het gebruik van dit stukje Hsp70 met daaraan GFP, zonder toevoeging van een adjuvans, een helperstof, niet werkt om in het rund CTL tegen het GFP op te wekken. Ook al zijn er wel andere T-cellen die het fusie-eiwit herkennen.

## Hoofdstuk 6

In hoofdstuk zes werden DC van het rund opnieuw bestudeerd. Immature DC werden geïncubeerd met *E.coli* bacteriën, *M.a.p.* of met Hsp70, en er werd gekeken naar het afschrijven van verschillende genen voor cytokines (signaalstoffen). Ook al is het afschrijven van het gen niet direct hetzelfde als de daadwerkelijke eiwitproductie (cytokineproductie), men is er doorgaans van overtuigd dat de mate waarin een bepaald gen afgeschreven wordt toch direct gekoppeld is aan de mate waarin een eiwit ook echt geproduceerd wordt. Hier bleek dat de fagocytose van *E.coli* en van *M.a.p.* leidde tot de afschrijving van cytokinegenen

die vrijwel hetzelfde waren. Omdat bekend is dat *M.a.p.* allerlei manieren heeft om de gastheercel om de tuin te leiden, terwijl dat voor *E.coli* niet zo is, was ditzelfde cytokine patroon niet verwacht. Het patroon van DC die geïncubeerd waren met Hsp70 zag er echter heel anders uit. Met name de afschrijving van het gen voor IL-12, een cytokine dat van groot belang is bij het initiëren van de juiste immuunreactie om bacteriën te elimineren, vond pas na 24 uur en slechts in beperkte mate plaats. Dit betekent dat een 'los onderdeel' van *M.a.p.*, het Hsp70, een andere reactie opwekt dan de hele bacterie, voor wat de cytokine productie betreft. Het betekent bovendien dat Hsp70 niet in eerste instantie een immuunreactie opwekt die de juiste T-cellen aanzwengelt die infectie moeten couperen, een eigenschap die in de literatuur wel aan het Hsp70 wordt toegedicht.

## Hoofdstuk 7

In het laatste hoofdstuk worden alle voorgaande hoofdstukken nogmaals besproken. Het is niet mogelijk gebleken om met Hsp70 een CTL-reactie op te wekken, maar Hsp70 is wel een belangrijk antigeen in de infectie. Ook wordt bediscussieerd wat de rol van het Hsp70 zou kunnen zijn gedurende de natuurlijke infectie. Is het een eiwit dat snel herkend wordt door de gastheer die vervolgens een reactie ontwikkelt die bescherming kan bieden tegen de infectie, of is het juist een eiwit dat door de bacterie gebruikt wordt om aan de afweer van de gastheer te ontsnappen? Voor beide mogelijkheden zijn argumenten aan te dragen en derhalve verdient de rol van het Hsp70 in natuurlijke infectie nader onderzoek. Verder zal gezocht moeten worden naar andere methodes om CTL op te wekken.

Gedurende de promotieperiode stond het gewone leven ook niet stil. Dood, geboorte, verhuizing, grote en kleine gebeurtenissen, in vijf jaar tijd kan er veel gebeuren. Ook al is het niet direct aan het schrijven van dit proefschrift gelieerd, zonder de steun en vriendschap van heel veel mensen had ik vast minder stevig in mijn schoenen gestaan. Ik ben enorm dankbaar voor het bestaan in mijn nabijheid van: mijn familie, Langelaar en Bakker Schut, mijn schoonfamilie, mijn extended 'Vreelandse familie', Bert, de Vosjes, lagere- en middelbare school vrienden en vriendinnen, studiemaatjes, Z13 huisgenoten, bestuursvriendjes, mijn so-not-called jaarclub, 'de vriendjes', en de nieuwe vrienden sinds de geboorte van de kinderen.

Natuurlijk zijn er ook mensen die ik speciaal wil bedanken voor het tot stand komen van dit proefschrift.

*Dames eerst.*
Elena Pinelli, het is alweer lang geleden maar de stage die ik bij je liep sterkte me in het idee dat ik het onderzoek in zou willen. Ik vond het een ontzettend leuke tijd en daarmee is het ook bepalend geworden voor het gaan doen van promotie-onderzoek bij de afdeling immunologie de afgelopen jaren. Het salsadansen is helaas niet blijven hangen op de afdeling.
Kerstin Müller, jouw kennis van het rund is al veel geroemd, en terecht. Ik verkeerde altijd graag in je buurt om er zoveel mogelijk van op te doen. Tot mijn grote geluk regelde je ook nog dat ik in het paratuberculose-onderzoek bij de afdeling Immunologie terecht kon. Enorm veel dank daarvoor.

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Willem van Eden, nou heb ik nog steeds je buiten niet gezien, terwijl we toch bijna buren zijn geworden. Het zal er vast nog eens van komen. Om eerlijk te zijn had ik in het begin niet zoveel op met het Hsp70, dat ik toch een beetje als jouw 'pet-molecule' (die kreet is gejat maar ik weet niet meer van wie) beschouw. In de loop van de tijd echter ben ik er vreselijk door geïntrigeerd geraakt, en inmiddels houdt het me nog net niet uit m'n slaap. Dat laatste geldt eigenlijk voor 'de immunologie' en ik ben daarom erg blij dat ik de afgelopen jaren op de afdeling heb mogen werken.
Victor Rutten, hoeveel promovendi je al begeleid hebt weet ik niet, maar ik wens toekomstige aio's allemaal toe dat ze bij jou terecht komen. Je rust en relativerende kijk op de zaken op momenten dat het me niet zo goed ging hebben me enorm gesteund. Aan werkbesprekingen heb ik vooral goeie herinneringen en het idee dat we ook veel gelachen hebben, terwijl er toch echt hard gewerkt is. Je bent waarschijnlijk de enige die me een vel vól correcties in handen kan geven met de mededeling 'alleen een paar kleine opmerkinkjes', waarvan ik dan inderdaad aan kan nemen dat het wel mee valt. Ik ben je ook erg dankbaar voor de digitale begeleiding de afgelopen maanden, alhoewel ik je meer vakantie gegund had.
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dingen des levens te hebben. Alhoewel, dat kan ook best bij een glas wijn.

Aad, Marije, Bart, Ruth, Corinne, Kirsten, Daphne, Ildiko, Lisanne, Marieke, Hanneke, Wiebren, Mariska, kortom: para-troepers van het eerste en latere uren, dank voor alle gezelligheid en inspiratie, maar zeker ook de hulp; bij chromium release assays, het maken van (LPS-vrij!) Hsp70 en ontwikkelen van een fusie-eiwit, om maar enkele zaken te noemen.
Collega's van de afdeling Immunologie, jullie allen hebben veel bijgedragen aan het werkplezier. Door inhoudelijke of juist informele gesprekken, het organiseren van allerhande sociale aangelegenheden, maar ook (dank!) door het helpen zoeken in het magazijn, het uitrekenen van eiwitconcentraties, het redden van mijn computer en het maken van buffers, om maar enkele dingen te noemen die in je eentje tot gróte moedeloosheid kunnen leiden. Overigens lijkt het een eigenschap van met name de analisten van de afdeling te zijn dat ze naast drukke werkzaamheden toch altijd tijd weten vrij te maken voor andermans (mijn) praktische rompslomp. Nogmaals: dank.
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Dear collegues from Compton, especially Jayne and Chris, I would like to thank you here for the very pleasant stay I had at the IAH, and the good contacts and support for my research ever since.

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Mooier is er niet.

*Wilnis, december 2004*
Merel

Merel Frederiek Moira Langelaar werd geboren op 9 januari 1968 te Loenen aan de Vecht. De middelbare school opleiding werd gestart op het Gemeentelijk Gymnasium te Hilversum maar na een verhuizing werd de vijfde klas en eindexamen gedaan aan het Vossius Gymnasium te Amsterdam. Het eindexamen werd gedaan in acht vakken en in mei 1985 cum laude afgerond.

Nadat zij voor de studie Diergeneeskunde uitgeloot was vertrok Merel naar Parijs, om daar een jaar als jeune fille au-pair te werken en Frans te leren. In september 1986 kon zij alsnog starten met de studie Diergeneeskunde aan de Faculteit Diergeneeskunde te Utrecht. Daar behaalde zij in 1987 de propedeuse cum laude en werd in 1991 het doctoraal examen behaald. Gedurende de doctoraalperiode werden de studiejaren ten volle benut voor zowel studie als nevenactiviteiten en was zij lid van achtereenvolgens de Veterinaire Almanakcommissie (1987-1988), assessor van het bestuur van de Diergeneeskundige Studenten Kring (1988-1989) en voorzitter van het dagelijks bestuur van de stichting Diergeneeskunde In Ontwikkelingssamenwerking (1989-1991).

Na het behalen van het doctoraaldiploma vertrok zij opnieuw naar Parijs, ditmaal naar het Institut d'Elevage et de Médecine Vétérinaire Tropicale, waar een postdoctorale 'Tropenopleiding' werd gevolgd, inclusief een stage van vier maanden in Benin, West-Afrika. Deze opleiding besloeg de periode eind 1991 tot eind 1992, waarna in januari 1993 begonnen kon worden aan de tweede fase van de studie Diergeneeskunde. Na het theoretische deel hiervan bleek er een wachttijd te ontstaan voor de start van de co-schappen, die tijd werd nog wat verder opgerekt en besteed aan ruim zes maanden stage bij de afdeling Immunologie van de Faculteit Diergeneeskunde (februari-augustus 1994). Gedurende deze stage werd de eerste onderzoekservaring opgedaan in het onderzoek naar *Leishmania infantum*, onder leiding van Elena Pinelli. Een *in vitro* infectiemodel voor macrofagen met deze parasiet werd ontwikkeld.

De studie Diergeneeskunde werd uiteindelijk afgerond, na wederom een stage in Afrika (in begin 1996 in Maputo, Mozambique), op 4 april 1996. Het dierenartsexamen, in de richting Landbouwhuisdieren, werd met genoegen gehoord. Ook met genoegen werd het ja-woord gehoord, op diezelfde 4 april 1996, des ochtends in het gemeentehuis te Loenen aan de Vecht, waar Merel in het huwelijk trad met Henk Antonis. Kort daarop werd vertrokken naar Sarras, Frankrijk, waar op 1 juni de deuren open gingen van de veterinaire praktijk die daar samen met Henk gesticht werd.

Na twee jaar intensieve samenwerking met de veehouders in de noordelijke Ardeche en Drôme bleken er echter te weinig koeien en te hoge bergen in dat gebied te zijn en werd besloten weer naar Nederland terug te keren. Daar vond zij een baan vanaf juli 1998 tot oktober 1999 bij de Buitenpraktijk van de Faculteit Diergeneeskunde, bij de afdeling Herkauwers. Zij werkte daar als dierenarts/docent en reed dagelijks uit met de co-assistenten. Het onderzoek bleef echter zijn grote aantrekkingskracht op haar uitoefenen en in november 1999 kon zij beginnen, als aio, aan het onderzoek bij de afdeling Immunologie, Hoofdafdeling Infectieziekten en Immunologie van de Faculteit Diergeeskunde, zoals dat in dit proefschrift beschreven is. Vanuit de afdeling Landbouwhuisdieren behield zij de begeleiding door prof. Jos Noordhuizen en na de laatste re-organisatie is zij lid van de afdeling Klinische Pathofysiologie, terwijl het onderzoek bij de afdeling Immunologie begeleid is door dr. Ad Koets en dr.Victor Rutten, en prof. Willem van Eden daar haar promotor is.

Het onderzoek dat beschreven wordt in dit proefschrift werd mogelijk gemaakt door en is uitgevoerd bij de afdeling Immunologie, Hoofdafdeling Infectieziekten en Immunologie en de Hoofdafdeling Gezondheidszorg Landbouwhuisdieren van de Faculteit Diergeneeskunde te Utrecht

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