Heat shock protein 70 vaccination induced immune responses in bovine paratuberculosis

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Stress eiwit Hsp70 vaccinatie geïnduceerde immuun responsen in bovine paratuberculose
(met een samenvatting in het Nederlands)

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Voor mijn familie
Voor mijn moeder en schoonmoeder
Voor Denise, Max en David
In herinnering aan allen die mij lief waren

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Contents

Abbreviations

aa	aminoacid
Ab	Antibody
Ag	Antigen
APC	Antigen Presenting Cell / AlloPhycoCyanin
BoLA	Bovine Leucocyte Antigen
BSA	Bovine Serum Albumine
CD	Crohn's Disease
CD#	Cluster of Differentiation # (e.g. CD25)
cfu	colony forming units
CPM	Counts Per Minute
Con A	Concanavalin A
CTL	Cytotoxic T lymphocyte
DC	Dendritic Cell
DDA	Dimethyl-Dioctadecyl-Ammoniumbromide
DTH	Delayed Type Hypersensitivity test
ELISA	Enzyme-Linked ImmunoSorbent Assay
Elispot	Enzyme-linked immunosorbent spot assay
FACS	Fluorescence Activated Cell Scanner/Sorter
FcR	Fc Receptor
FCS	Fetal Calf Serum
FITC	Fluorescein IsoThioCyanate
FSC	Forward SCatter
Foxp3	Forkhead box protein 3
HE	Hematoxilin-Eosin
Hsp	Heatshock protein
IEL	Intra Epithelial Lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IS	Insertion Sequence
kD	kilo Dalton
LAM	LipoArabinoMannan
LPL	Lamina Propria Lymphocyte
LST	Lymphocyte Stimulation Test
mAb	Monoclonal antibody

MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MHC	Major Histocompatibility Complex
OD	Optical Density
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	PhycoErythrin
PP	Peyers Patches
PPD	Purified Protein Derivative
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
SFC	Spot Forming Cells
SI	Stimulation Index
SSC	Side SCatter
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th	T helper lymphocyte
TNF	Tumor Necrosis Factor
Treg	T regulator lymphocyte
WC	Workshop Cluster
ZN	Ziehl-Neelsen

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Chapter 1

General introduction

INTRODUCTION

Paratuberculosis (Johne's disease) was first described in 1895 by H.A. Johne and L. Frothingham in Germany [81] and called pseudotuberculosis or Johne's disease in 1906 [9]. It was not until 1910 that Koch's postulates were fulfilled and the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) was grown in the laboratory. The disease was reproduced in experimentally infected cattle [185, 184] and has since been known to be a threat to ruminants worldwide [29]. Paratuberculosis is considered to be one of the most serious chronic bacterial diseases of ruminants in agriculturally developed countries [88]. The bacterium, causing a severe gastroenteritis in ruminants worldwide, is common in domesticated and wild ruminants like deer, elk or bison. Non ruminants like horses [103], rabbits [33, 189], non human primates [113], fox, stoat, weasel, crow, rook, jackdaw, rat, wood mouse, hare, and badger are under debate in the epidemiology of this disease, after identifying infections by culture and histopathological analysis [11], as wildlife reservoirs or after experimental infection like those performed in pigs and chickens [152]. In wildlife species *MAP* can be isolated from different tissues. Histopathological lesions are observed however proved to be subtle in comparison to lesions found in advanced paratuberculosis in domesticated ruminants. Clinical signs have not been described for these naturally infected species. The host range is much wider than previously thought and the positive fecal cultures from these species suggest that environmental contamination with *MAP* can occur and can thereby pose a risk to grazing livestock and farms adjoining paratuberculosis infected properties.

In 2000 55% of the herds in The Netherlands showed serologically positive cows as tested by an absorbed ELISA and high economic losses were observed [14, 125]. In most western countries the prevalence and economic losses are similar to the situation in the USA where Dairy studies 2002 and 2007 (National Animal Health Monitoring System, U.S. Department of Agriculture's) showed the percentage of positive herds to be 20-40% resp 68% (approximately 90% of herds will be infected by 2020) with at least one sample cultured positive for *MAP* and an estimated annual economic loss of $ 1.5 billion in the United States only [171].

Although a potential role of *MAP* in the induction of Crohn's disease (CD) [47] is still debated [60, 162] there is sufficient reason to minimize exposure of man via milk, meat [39, 170] or water [194] by controlling the disease in ruminants. Based on similarities between CD and paratuberclosis, the presence of *MAP* in CD lesions [114] and serology, *MAP* is considered to be involved in the pathogenesis of CD in humans and by some considered to be a potential zoonosis however many studies did

not confirm these results [58, 115].
Control of paratuberculosis may be achieved by improved management as well as by a strategy of diagnosis and culling of shedders and finally by vaccination [38, 62, 82, 154].
This thesis reports use of *MAP* heat shock protein (Hsp) 70 vaccination as a novel measure to control bovine paratuberculosis, and analysis of T and B cell epitopes in *MAP* Hsp70 in cattle. Since regulatory cells seem to play a role in chronic gradual progressive mycobacterial disease, and accompanying changes in immuneresponsivess, the presence and function of regulatory cell types in cattle was investigated.

PARATUBERCULOSIS

Mycobacterium avium subspecies *paratuberculosis*

Mycobacteria (genus *Mycobacterium (M.)*), comprising more than 100 gram positive species, are mostly saprofytic but also important human and animal pathogens have been identified. These pathogenic members are usually characterized by their slow growth in culture, furthermore mycobacteria are characterized by their acid-fastness and lipid-rich cell wall composed of a thick waxy mixture of lipids and polysaccharides. The main pathogenic mycobacteria include *Mycobacterium avium (MAV)*, *M. bovis (MBO)*, *M. leprae (MLE)*, and *M. tuberculosis (MTU)*.
Genotypic and phenotypic tests and correlations with DNA-DNA hybridization tests led to the subdivision of the *M. avium* group in three subspecies [182]. The *M. avium* complex (MAC), consisting of *M. avium* subsp. *avium* (*MAA*) and *M. avium* subsp. *intracellulare (MAI)* has been associated as the most prevalent opportunist pathogens causing infection among immunosuppressed individuals like advanced HIV-1 infection [77]. Less commonly, pulmonary disease in nonimmunocompromised persons is a result of infection with MAC. In children, the most common syndrome is cervical lymphadenitis. AIDS patients with *M. avium* infections suffer considerable morbidity and shortened life span [17, 79].
The other two subspecies in the *M. avium* group are *MAP* and *M. avium* subsp. *silvaticum* (*MAS*) are rarely classified as members of the MAC, although *MAP* infections in AIDS patients have been reported [151].
MAP is a small (0.5 x 1.5 micron) gram-positive, acid fast, and rod-shaped bacterium and it is the causative agent of paratuberculosis in cattle [184], sheeps, goats and other

ruminants. It is thought not able to grow and multiply in the environment because of its inability to produce mycobactin (a chemical needed to transport iron), which is unique among members of the mycobacterial family. Thus *MAP* can only multiply inside animal cells where it uses iron from its host and lives as an obligate parasitic pathogen. If found in soil or water samples, it can be assumed that *MAP* is simply persisting in those places, sometimes for years, after being deposited there through fecal contamination from an infected animal. In vitro growth of the bacterium is established on egg based media supplemented with mycobactin [181] [182]. In culture *MAP* is characterized by slow growth, with generation times of 12-24h [100], and typical colonies depending in part on the type of culture medium.

Infection, clinical course and pathogenesis

Infection

The primary source of infection for neonatal [177] and young calves [140] is feces of infected cows [32, 36]. Most infections are probably aquired by ingestion of the organism through drinking contaminated milk, food products or licking contaminated surfaces. After ingestion, *MAP* travels through the gastro-intestinal tract and targets the mucosa-associated lymphoid tissues of the host [108] where they are endocytosed by M cells [129] of ileal Peyer's patches (PP). The highest concentration of PP in calves is found in the terminal ileum, a predilection site of infection. The ileal PP (in contrast to jejunal PP) start regressing after birth and disappear in the first 6 to 15 months of life [66]. Especially in young calves the ileal PP may represent up to 90% of the total PP of the gut associated lymphoid tissue (GALT) contributing to the sensitivity of the calves during the first year of life. Inside the PP, after passing the M-cell *MAP* is phagocytosed by subepithelial and intraepithelial macrophages [108, 121], and in the phagosomes the bacterium hides, multiplies and evades the immune system [86, 121]. Similar to other species of pathogenic mycobacteria, *MAP* is an intracellular pathogen of macrophages and monocytes [199] and found in almost any tissue in which macrophages are present like in spleen [141], fetus [157], semen and testis [104] even including milk [178].

Clinical course

Following infection, disease progression is classified in four stages, silent infection, subclinical (start low shedding *MAP* in faeces), clinical and advanced clinical disease [193]. Some animals develop clinical disease (≤ 10% of infected animals), preceded by shedding bacteria without clinical signs of disease [36], these shedders contribute

to the infection of other animals by contamination of the environment. In some of the chronically infected cows the long asymptomatic stage (2 to 4 years) is followed by rapid progression to a clinical stage due to protein-losing enteropathy. Animals suffer from chronic diarrhoea, dehydration, weightloss, decreased milk production, edema, anemia and infection related symptoms as signs of paratuberculosis [33, 36, 171] and ultimately death of the host. Gross pathology of paratuberculosis is characterised by chronic enteritis, chronic lymphangitis, or mesenteric lymphadenopathy [25].

Pathogenesis

The pathogenesis of *MAP* infection at the macrophage level is incompletely understood, with much of our current understanding derived from the *MAV* and *MTU* literature and from *in vitro* studies on *MAP* infection in bovine and murine macrophages. Collectively, these data suggest that if macrophages can be appropriately activated, they will kill intracellular *MAP* [76, 137, 200]. Migration of *MAP* infected macrophages or free bacteria transported via the lymphatic or venous system and taken up by macrophages on site is thought to be responsible for the wide dissemination in tissues [25, 186]. Intestinal granuloma's develop after macrophage activation, cytokine production (interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α)) and initiation of cellular immune responses in the draining lymph nodes aiming at clearance of the infection [32, 36]. In Johne's disease the relationship between granuloma morphology and mycobactericidal activity is not well defined as it is for human granulomas. In human granulomas control of mycobacterial infection correlates with granuloma morphology, with tuberculoid granulomas imparting a more favorable outcome than lepromatous types [59, 75]. Intestinal lesions present as granulomatous enteritis for paratuberculosis, showed features close to the lepromatous type of human leprosy i.e. granuloma morphology (showing poorly delineated granuloma morphology, high bacterial burden, and polygonal and histiocytic macrophages) however variations in the morphology and infiltrates in granuloma's may be encountered [33, 75, 98]. Sometimes they are patchy, mild, or servere depending of the clinical stage of the animal and usually localized in the distal ileum, lymphnodes, tonsils and the cecal valve [8, 25], they may show infiltrations of lymphocytes, macrophages, and plasma cells. Functioning of these cells are associated with strong humoral and weak cell-mediated immune responses typical of clinical stages of bovine Johne's disease [75]. The inflammation leads to the major component of the pathogenesis being manifestations of affected swollen and club shaped villi of the mucosal epithelium of the ileum and the associated malnutrition syndrome.

Diagnosis of MAP infection

In vitro cultivation of fecal *MAP* bacterium is considered to be the most reliable method [117] to diagnose *MAP* infection but is a time consuming procedure (6 weeks to 6 months depending on the culture system and media used), requires mycobactin, has a moderate sensitivity (apprx. 50%) (due to decontamination procedures) and high level of test failure due to contamination [38, 192]. Acid-fast (Ziehl Neelsen) stainings of fecal smears is still used for diagnosis, it has very low sensitivity and *MAP* is only detectable in subclinical or intermittent shedder animals decreasing reliability of the assay. In addition, the specificity is also poor as all acid fast organisms are detected.
Detection of the bacterium can also be based on amplification of the *MAP* specific genetic element IS 900 [37, 65] by the highly specific polymerase chain reaction (PCR), via this insertion sequence specific for *MAP* and/or additional use of IS 901 specific for some *M. avium* strains (not present in *MAP* strains), but often sensitivity is low in direct testing of fecal or other samples due to sample preparation (purifying *MAP*) in order to perform PCR.
Despite the fact that *MAP* specific antigens (Ag) [10, 69] are being discovered at an increasing rate due to elucidation of the *MAP* genome [106], data regarding recombinant *MAP* antigen specific cell mediated immune responses during various stages of paratuberculosis are still limited [92, 94, 161].
In contrast to these methods detecting the presence of *MAP* directly, cellular or humoral host immune responses to *MAP* are indirect indicators of past encounters with the organism, however often the sensitivity of these assays is low. Detection of humoral immune response i.e. antibodies specific for *MAP* Ag like PPDP, heatshock proteins [95, 94] or *MAP* cell wall components like lipoarabinomannan (LAM) is performed by enzyme-linked immunosorbent assays (ELISA) with [148] or without [179] pre-absorption to *M. phlei* to reduce false positive [176]. Complement fixation tests (CFT) [116] and agar gel immunodiffusion (AIGD) [159] are also described to measure the humoral immunity. Cellular immune responses assessed i*n vivo* by a delayed type hypersensitivity test (DTH) with intradermal johnin shows positive results early in infection and high cross-reactivity between probably other mycobacteria [53]. *In vitro* lymphoproliferative assays (lymphocyte stimulation tests (LST)) [74, 91, 93, 94, 118], IFN-γ assays [169] performed with white blood cells of animals in different stages of *MAP* infection have also been used to measure cell mediated immunity. Sensitivity of these assays is often hampered by aspecific reactions against antigens shared with apathogenic mycobacterial species especially when using crude extracts (e.g. PPD) or cell wall components.

Vaccination and disease prevention

Although in The Netherlands paratuberculosis control programs have been carried out since 1942 [13], the inability to accurately detect subclinically infected animals has hampered these programs. The more recent control strategies such as the Paratuberculosis Program Netherlands (PPN) [67] are based on management and hygienic measures and removal of shedder and suspect animals, as identified by annual fecal culture or ELISA to decrease the infection risk for young susceptible animals at the farm level. At present a new program based on milk ELISA aims at control, <1000 bacteria per liter milk, of paratuberculosis, rather than eradication. This strategy is considerably cheaper than the earlier subsidized test-and-cull program. In other countries like France and the USA [166] test and cull programs are established based on fecal culture or serological testing but success rate is limited due to lack of reliable and rapid detection of subclinically infected animals [84]. Use of vaccination by experimental vaccines, based on heat killed or live attenuated whole bacterium preparations [62] performed in mice, goats and sheep and incidental experimental vaccination in cattle under field conditions [57, 126, 168] with inactivated *MAP* bacterium and/or improved calf management [82] proved successful in reducing clinical paratuberculosis. However, even long-term use of a vaccine does not prevent fecal shedding of the bacteria [97], does not lead to elimination of the infection from herds [84], and interferes with tuberculosis diagnostics in cattle [96] [126]. Besides in its current formulation, including oil adjuvant, it often causes severe local tissue reaction [139] [150]. Often the vaccinated animals are found positive in immune response based diagnostic tests and [57] *MAP* carrier animals have to be detected by antigen detection tests. In order to eradicate the disease completely, a potent vaccine without many side effects and disadvantages is needed [15] in combination with hygienic measures and improved calf management. Development of an efficient subunit vaccine, would offer possibilities to overcome some of the disadvantages of whole cell vaccines. In this thesis, and previous studies, we focuses on vaccination with *MAP* Hsp70, searching for protection associated immune responses, for usage as an potent subunit against paratuberculosis.

Immune responses: Overview, dogma and model

Overview

Immunological interactions between *MAP* and its hosts are complex and incompletely understood for several reasons. Mycobacterial infections are difficult to study because of the slow progression, chronic nature of the disease, and the intracellular macrophage/phagosome environment protecting the bacterium from immune surveillance. The complex structure of mycobacteria, in particular the cell wall of mycobacteria composed of sugars and lipids of exotic structure, many of which contribute to its pathogenicity [64]. In addition the emphasis of Johne's disease research has been directed towards the development of better tests for diagnosis of subclinical infection in cattle and not towards understanding the complex biological relationship between the natural host (ruminants) and the pathogen. After mycobacterial infection, in many cases, a cytotoxic and early proinflammatory response occurs to control this intracellular bacterium within macrophages. A $CD4^+$ Th1 (cell mediated immunity biased) T-cell response develops characterized by the release of IL-2 and the proinflammatory cytokines such as gamma interferon (IFN-γ) [169], interleukin-1α (IL-1α), and IL-6 [43]. During the late subclinical phase the proinflammatory Th1 response is often decreased or lost and in the late stage of disease a Th2 (humoral response biased activity) seems to predominate [43, 172] as can be observed after mycobacterial infections in different species [6, 56]. Controlling mycobacterial infections in transgenic knockout mice is presumably due to the immunomodulating characteristics of IL-10 [124]. Furthermore IL-4 [175] (a classic Th2 cytokine) and TNF-α [85] (activates macrophages and essential for granuloma formation) were shown to give rise to enhanced pathology in IL-4 or TNF-α deficient mice. Cytokines known to regulate IFN-γ such as IL12 (especially the p40 subunit) [42] and IL-18 [174] have been shown to be more susceptible to mycobacterium-induced diseases as was shown in IL-12 or IL-18 deficient mice. In outbred species, such as cattle, the T helper cell dichotomy is not as strict as compared to inbred mice. Additional $CD4^+$ T cells have been described, referred to as Th0, which express all combinations of cytokines, like immunomodulary subsets of Th cells like Th3 producing TGF-β and Tr1 producing IL-10 described to be preferentially present in the gut [68, 109]. In the lamina propria lymphocytes (LPL) the Th population appears to be skewed to a Th2-like phenotype [66], the preferential site of pathology of *MAP* to the Th2 biased LPL compartment may be more dan coincidental. Regulation of Th responses may be different in the intestinal wall compared to secondary lymphoid organs (IFN-γ and IL-4 regulated), whereas in the intestine IL-4 is less pronounced. Control of

Th1 seems to be regulated, unique for the intestinal mucosa, by TGF-β, and IL-10 [68, 128, 144]. Migration of phagocytic cells (professional antigen presenting cells (APC)) is suggested from clinical cases by which *MAP* disseminates through the body and the bacterium can be recovered from all sites sharing adhesion markers and adressins with the GALT system. The role of cytokines in cattle is less clear as most studies are performed on mycobacteria other than *MAP* in deficient mice, thanks to functional genomic tools and quantitative real time PCR (qRT-PCR) during natural and experimental infection of cattle with *MAP* recently the role of cytokines involved in the host immune response is becoming more clear [2, 45, 191]. Expression profiles of at least 42 genes are inherently different in freshly isolated PBMCs from *MAP* infected cattle when compared to similar cells from uninfected controls. Gene-expression differences observed following microarray analysis were verified and expanded upon by qRT-PCR [46].

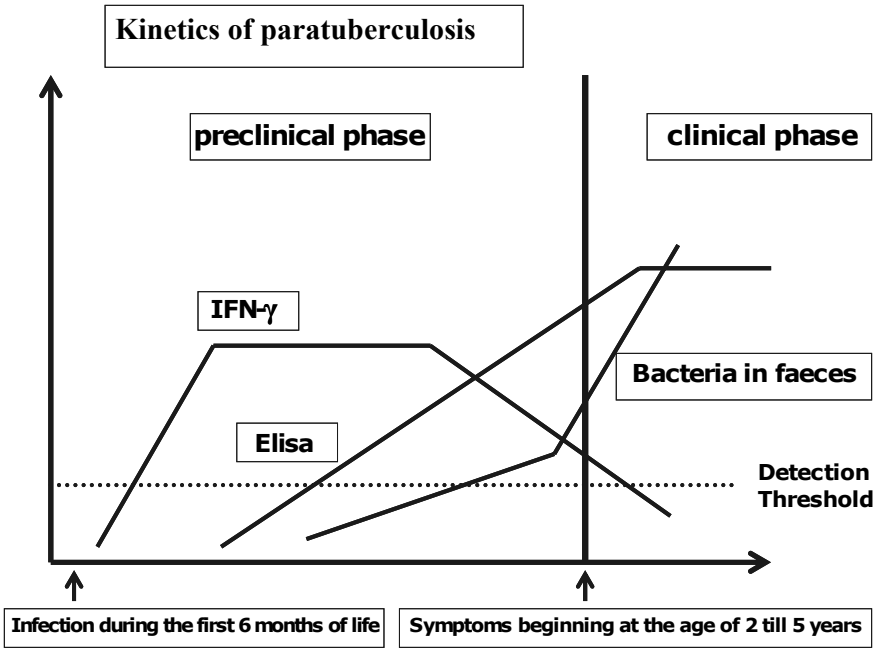

Fig. 1. Kinetics of paratuberculosis; schematic representation of the immunological dogma described in bovine paratuberculosis divided in a preclinical and clinical phase. Cellular responses represented by IFN-γ (Th1) measurements and humoral responses as tested by Elisa techniques and bacteria in feces according to fecal culture techniques in time. Detection threshold is included for the measured parameters.

Dogma

The current dogma is that in early stages of paratuberculosis (mycobacterial infections) protective Th1 type cell-mediated immune responses, driven by IFN-γ responses [24, 122] predominate, whereas in the clinical stage of the disease the humoral responses prevail possibly signaling a switch in immune reactivity related to disease progression (Figure 1). It appears that intracellular multiplication of *MAP* in macrophages and killing (via NO release) occurs simultaneously, reflecting an initial Th1 cellular immune response of the host that switches to Th2 in animals with clinical disease. Isotype specific ELISA, especially IgG1 (type 2 response-Th2)/IgG2 (type 1 response-Th1) to measure Th1/Th2 balance changes, measuring *MAP* antigen specific responses by LAM, PPDP and recombinant Hsp60 and Hsp70 [95] or *MAP* protoplasmic Ag [197] have been described and showed contadictory results comparable to studies performed in tuberculosis and leprae [54, 167]. The results performed in cattle indicated that there is no uniform association with increased antibody responses during the progression from the asymptomatic stage to the clinical stage of bovine paratuberculosis, but rather a loss of Th1 reactivity [95]. Buergelt, Hall, McEntee and Duncan (1978) also noted marked thymic involution in animals with clinical signs of disease, compared to age matched healthy controls. Whether this involution contributes to the immunological unresponsiveness in clinical animals, by means of altered T cell-generation, is unknown [25]. Studies with experimentally infected calves have challenged the dogma, demonstrating that humoral immune responses may occur very early after inoculation [190], but dosage, timing of exposure, and routes of entry in these studies were likely different than those in natural exposure, complicating interpretation. Other studies, using experimental infection through oral dosing and natural infection, suggest that the humoral response does not dominate until later in the infection cycle [120].

Model

A proposed model [44] for interaction of *MAP* with the immune system of cattle, starting after initial *MAP* infection and colonization of the intestine by IFN-γ activated macrophages [127] under influence of adhesion molecules and other factors upregulated at sites of infection [2], is based on data from numerous laboratories. Initially an early development of a proinflammatory T-cell population within lymph nodes draining sites of infection followed by expansion of non-cytotoxic suppressor cell populations capable of producing IL-10, which may require close contact with Ag, proinflammatory cells or products produced for activation was shown. Increased levels (expression and/or production) of proinflammatory cytokines /transcripts like

IL-1 produced by activated macrophages, IL-6 and IFN-γ at sites of infection and IL-8 as a consequence of IL-1α overexpression and stimulation of macrophages, probably without requirement of antigen, were measured in cattle [45]. Signaling of TNF-α doesn't appear to be involved in macrophage activation at sites of *MAP* infection in cattle and probably limited by the *MAP* infection [45, 105]. However, TNF receptor-associated factor 1 (TRAF1) [28] is found to be upregulated by enhanced IL-1α production, and limits programmed cell death (apoptosis). In combination with failure to express sufficient TNF-α increased macrophage recruitment occurs and prolonged survival ensures survival of intracellular *MAP*. Tissue damage is probably due to the chronic inflammatory immune response, IL-1α production, and release of reactive oxygen produced by activated macrophages or neutrophils. *In vivo* development of a suppressor population restricting local proinflammatory and cytotoxic immune reactivity would be helpful in limiting tissue damage. Yet this allows expansion of *MAP* infection and PBMC responses to *MAP* will be quite variable depending upon the balance between proinflammatory and suppressor cell populations. These suppressor T cells may be of the γδ T-cell subtype but it is considered to be more likely that the initial noncytotoxic suppressor cells are Th2 like $CD4^+$ cells or other T regulatory cells producing IL-10 (major early regulator cytokine) [18]. These cells are suggested to be rather gentle in circulation to proinflammatory cells and upon activation produce IL-10 to quiet the proinflammatory response at the site of infection. After proinflammatory and suppressor cell populations a potential cytotoxic regulatory cell type, probably a Th2 like $CD8^+$ T cell or a γδ TCR^+ T cell is suggested [30, 31] to function. The mechanism of T cell anergy, often observed in very-late-stage clininical animals, could be explained by cytotoxic immunoregulatory cells. In this model no active switch between Th1 and Th2 is proposed rather an IgG1 response can develop at any time, depending on the balance established by the suppressor cells that deplete proinflammatory cells, infection progresses and a predominant IgG1 immune response is left and animals are classified as clinical and terminal.

Immune regulation

How the immune system discriminates between self and non-self is probably one of the most important questions in immunology. When harmful microorganisms invade the organism they are a threat to immune homeostasis which depends on a balance between responses that control the infection and counteracting responses that prevent chronic inflammation. Disbalance can lead to insufficient clearance of the pathogens i.e. chronic infection and/or severe reciprocal responses like chronic inflammation and

tissue damage. Besides elimination of self-reactive cells (clonal deletion) and their functional inactivation (clonal anergy) substantial evidence for a third mechanism of self-tolerance called T-cell-mediated suppression of self reactive T cells has been presented [155, 160]. The immunological responses in paratuberculosis involved are depending of a complex interplay between T cells, B cells and antigen presenting macrophages.

Gershon and Kondo showed that T cells not only enhance (Th1 and Th2) but also suppress immune responses, these T cells called suppressor or regulatory T cells (Treg) down regulated immune responses and proved to be different from helper T cells [61]. Nowadays immunoregulation comprises complex mechanisms involving the activities of various immune cell subtypes ($CD4^+$, $CD8^+$, NKT, DC, monocytes, B cells, γδ T cells) among which natural and induced Treg cells were shown to play an important role in humans and rodents [119] as essential part of the activity and functionality of the immune system. Natural Treg are produced in a functionally mature state direct from the thymus as $CD4^+CD25^+$ (IL-2 $receptor^+$ $CD4^+$ T cells) and are "naturally" ready to mediate immunosuppression. Subsequently, $CD4^+/CD25^+$ natural Treg were shown to function in controlling and regulating the immune system in infectious diseases [99], self-tolerance and autoimmune diseases [7].

Intracellular Foxp3 transcription factor is regarded as one of the more constant hallmarks of natural Treg cells and described in many human and rodent related articles as being critical important for the development and function of natural Tregs ($CD4^+/CD25^{high}/Foxp3^+$) [198] and is considered necessary and sufficient for Treg development and functioning in maintaining peripheral tolerance. The two defining factors of $CD4^+/CD25^{high}$ natural Treg are their functional regulatory activity and their anergic state (non-proliferative) upon stimulation. Other cell types are also involved in immune regulation, apart from $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells different cell types have been suggested as potential regulatory cells (i.e. $CD8^+$ T cells, $CD14^+$ cells and ($WC1.1^+$, $WC1.2^+$ in cattle) γδ T cells in humans, rodents [16, 101, 135, 145, 163] and cattle [23, 89, 133, 153, 158] but has until now not been proven functionally *ex vivo* in cattle.

Furthermore no information is known concerning the presence and functioning of bovine natural Treg. Naturel Treg limit the magnitude of effector responses which may lead to a failure to adequate control an infection, in addition it also limits tissue damage by limiting antimicobial immune responses. To establish this equilibrium can be a major target for an eventual therapeutical usage [12, 19]. In the intestine natural Treg are considered to be one of the most important regulating subsets involved in controlling immune responses and intestinal homeostasis and a deficiency

of regulatory cells ($CD4^+CD45RO^+CD25^+$) is suggested in intestinal diseases like ulcerative colitis (UC) [180]. Leukocytapheresis followed by purifying natural Treg and thereafter being returned to the patients or purifying and expanding natural Treg before injecting back in patients could well prove to be a potent therapy for certain intestinal disorders to restore intestinal cellular homeostasis as is shown in colitis mice [123, 147].

γδ T cells

The principles governing bovine γδ T cell specificity and diversity in function remains an enigma. Commonly hypothesized is their innate functioning by immunoregulation and immunosurveillance (as reviewed by Hayday (2000) [70] and Carding et al., (2002) [27]). They are capable of generating more unique antigen receptors than αβ T cells and B cells combined, however their repertoir is limited in recognizing numbers of antigens dominated by specific subsets. Many similarities can be defined for γδ T cells compared to αβ T cells, for example cell-surface proteins and effector capabilities like cytokine/lymphokine/chemokine (IFN-γ, IL-10, IL-4, RANTES, MIP1β and lymphotaxin) production and cytotoxicity (by cytolytic effectors as granzyme, FAS-ligand and lymphotactin) are described. In most adult animals, unlike αβ T cells, γδ T cells constitute only a small percentage of the lymphocytes that circulate in most adult animals. In ruminants γδ T cells comprise a major proportion of the PBMC population. Especially in young calves γδ T cells represent up to 75% of all T cells [72] which distinguish cattle from most other animals, a role for the abundantly present γδ T cells in young ruminants is suspected however the biology behind these observations remains unclear as is the function of these cells [89, 91, 149]. Ruminant γδ T cells express a very diverse repertoire of T cell receptors in contrast to mice and humans and may have a better developed capacity to recognize diverse ligands. However in most animals γδ T cells are more widespread within epithelium-rich tissues, such as skin, intestine and reproductive tract comprising up to 50% of T cells present, and are known for their functional interaction with many cell types. In the bovine skin a massive sustained γδ T cell migration takes place *in vivo* suggesting constant replenishment and in the tissues fulfillment of more than exclusively local functions by these γδ T cells (predominantly Vγ1, Vγ3 and Vγ7 T cells) [188]. Their absence (or rare usage) of MHC restriction or non-classical MHC I molecules and ability to recognize soluble proteins and non-protein antigens [3] like lipids possibly by presentation in CD1 molecules [143] of endogenous origin also distinquishes them from αβ T cells. Fresh purified γδ T cells respond to pathogen associated molecular

patterns (PAMPs) and encode a variety of PAMP receptors suggesting that rapid response to PAMP receptors may be an innate role of γδ T cells [71], also Toll-like and scavenger receptors were detected. The antigen recognition by γδ TCRs [51, 130, 149, 165] is fundamentally different compared to αβ T cells, the lack (minority) of processing and MHC restriction allows the γδ T cells to recognize a wide array of antigens suggesting a more innate functioning in which the CD3δ region of human γδ T cells has been described to be involved in recognizing tumor cells [196]. Heat shock proteins (Hsp) produced by 'stressed' cells as a stress signal has been suggested to be recognized by γδ T cells [21] but has never been demonstrated convincingly [87]. Besides antigenic recognition, antigenic presentation by γδ T cells has also been described for cattle [40] and humans [22] (Vγ2Vδ2 T cells) which would be a very effective way of presentation in combination with PAMP recognition. Considering γδ T cells as a single group of cells, instead of a family of many subpopulations with high diversity, which functioning is dependent of local microenvironment, tissue distribution, structure of their antigen receptors, and probably how and to what stage they become activated, is probably one of the biggest mistakes made. Bovine γδ T cells can be divided in distinct phenotypical subsets by unique high molecular weight molecules (WC1 and GD3.5), WC1 [52, 138] molecules are encoded by a family of genes belonging to the scavenger receptor cysteine rich (SCRC) domain family [34] which are not expressed on human or mouse γδ T cells [195].

An immunoregulatory role is suggested for human Vδ2 T cells which are heterogeneous and comprise distinct populations defined by surface marker expression and effector functions induced in the presence of BCG infected dendritic cells (DC) [111]. An effective immune response against the intracellular bacterium *MTU* [55] and increased γδ T cell cytotoxicity leading to advanced tuberculosis [135] raises the question if γδ T cells in infections are beneficial or deleterious. This proofs the importance in influencing the timing, magnitude and composition of the γδ T cell response and consideration that different subsets of γδ T cells contribute to the overall response [5]. In progressive bovine paratuberculosis a local loss of $CD4^+$ T cells and increased frequency of γδ T cells is observed in ileum lamina propria lymphocytes and related decreased cell-mediated responses also raises the question if these cells are beneficial [91]? The regulatory role of γδ T cells is supported by alterations in resistance to a variety of pathogens in the absence of these cells [49, 131]. Human tumor-infiltrating Vδ1 T cells suppressing T and DC function [142] and murine Vδ1 T cells interacting with activated macrophages have been described [50]. Literature suggests immunomodulation by γδ T cells [23, 149] and in addition potential age related differences in immune regulatory roles of $WC1^+$ γδ T cell subsets in ruminants

[89, 153]. A functional regulatory/suppressive role of $WC1^+$ γδ T cells, comprising $WC1.1^+$, $WC1.2^+$ [73, 110] and $WC1.3^+$ subsets, and $WC1^-$ γδ T cells has not been shown thus far *ex vivo*.

T cell effector mechanisms of γδ T cells include tissue repair as is shown in protection of the intestinal mucosa by intraepithelial γδ T cells in a murine colitis model [80] and lysis of malignant cells [63]. Furthermore a role in autoimmunity via regulation of experimental autoimmune encephalomyelitis (EAE) [90] or via a pathogenic role in EAE [134], allergy [48] and infection (reviewed in [27]) has been implicated suggesting an overall function in maintaining or regaining homeostasis in the tissues in which they reside.

The rapid production of IFN-γ and other pro-inflammatory cytokines after infection by the intracellular bacteria *Listeria monocytogenes* suggests an initial effector response by murine Vγ1 T cells [112]. The increased IFN-γ production in the absence of γδ T cells as response to *Listeria monocytogenes* and an exaggerated Ag specific adaptive immune response [163] suggests an essential function in downmodulating the immune response to *Listeria monocytogenes* and is consistent with a role for γδ T cells in regulating inflammation.

In cattle $WC1^-$ [83], $WC1^+$ [20] and $WC1.1^+$ [153] cells have been shown to be IFN-γ secretors, after specific defined stimulation, that can act as innate immune cells potentially by directing adaptive immune responsiveness. However, in addition expression of IL-10 (known to be antagonistic for IFN-γ functioning) could also be detected in $WC1^+$ γδ T cells [40, 153].

Antigen selection for a subunit vaccine

In this study we tested *MAP* Hsp70 as a subunit vaccine, previously shown to be an immunodominant antigen in bovine paratuberculosis [95, 94], similar to other mycobacterial diseases such as tuberculosis and leprosy. A number of studies on mycobacterial diseases, mainly in murine model systems, have indicated a potential use of Hsp as vaccine components. Our previous studies indicated that immune responses to recombinant *MAP* Hsp70 proteins were predominantly cell mediated contrary to recombinant *MAP* Hsp65, which apparently induced mainly antibody responses and minor cell-mediated responses [91, 95]. Heat shock proteins (Hsp) are a family of proteins expressed in all cells constitutively or in response to cold, heat and other environmental stresses. They increase heat tolerance and perform functions essential to cell survival under these conditions. Some serve to stabilize proteins in abnormal configurations, and play a role in folding and unfolding of proteins, acting

as molecular chaperones. There are four major subclasses: Hsp90, Hsp70 (*MAP*) [173] , Hsp60 (*MAP*) [41] and small Hsp (*MAP*) [35] that are known to be highly conserved but still very immunogenic/immunodominant in many infections and other diseases. Heat shock proteins have shown to be recognized in mycobacterial diseases such as tuberculosis, leprosy [1, 78, 136], bovine paratuberculosis [91, 94, 102] and bovine tuberculosis [146, 164]. Mycobacterial Hsp70 is known to specifically bind to innate receptors present on macrophages [26] and dendritic cells [102], leading to production of pro-inflammatory signals, which may in turn lead to IFN-γ production by T and NK cells. In bovine paratuberculosis *MAP* Hsp70, being a well defined antigen in contrast to the widely diagnostically studied purified protein derivative of paratuberculosis (PPD-P) [4, 62] and cell wall components like lipoarabinomannan (LAM) [64, 176], induces *in vivo* and *in vitro* cell mediated as well as humoral immune responses [95, 94] as was also found in humans [132]. A multitude of functions both pro- and anti-inflammatory [78, 107, 156, 187] has been proposed for the Hsp70 stress protein, suggesting a possible regulatory function directed away from chronic inflammatory functioning. *MAP* Hsp70 has a length of 623 amino acids (aa) [95, 173] and is composed of two functional domains: an ATPase domain (NH_2 terminal) and a substrate binding domain (sbd) (COOH terminal part) [183].
For application of subunit vaccines using antigenic preparations with potentially less epitopes available, we furthermore studied to which extend outbred animals are able to respond to such a subunit vaccine, aiming for fine tuning/identification of immunogenic *MAP* Hsp70 regions by measuring fine specificity (epitopes) of T and B cell responses.

AIM AND OUTLINE OF THE THESIS

The whole bacterin vaccines induce substantial reduction of the number of clinically affected animals in herds but do not protect against infection and shedding of *MAP*, interfere with tuberculosis diagnostics in cattle and often cause severe local tissue reaction [150]. Availability of an effective subunit vaccine, would overcome some of the disadvantages of whole cell vaccines.
Protective immunity to the intracellular mycobacterial pathogens is thought to be cell mediated in origin, in *MAP* infected animals it has been shown that *MAP* Hsp70 is an immunogenic/ immuno-dominant antigen that predominantly induced cell mediated immune responses [93]. For that reason the main aim of this thesis was to study the potential of recombinant *MAP* Hsp70 as a subunit vaccine in cattle that were

experimentally infected with *MAP* (chapter 2) [92].
As the use of subunit vaccines implicates using antigenic preparations with potentially less epitopes available it is important to study to which extend outbred animals are able to respond to such a subunit vaccine and hence fine specificity of responses in the T cell compartment (chapter 3) and the B cell compartment (chapter 4) was studied.
Finally since regulatory T cells (Treg) are regarded essential components for maintenance of immune homeostasis, it has been suggested that increasing immunosuppressive activity of regulatory T cells is responsible for the anergy observed in clinically diseased cattle. In addition Hsp70 has been implicated as a molecule which can regulate immune responses away from chronic inflammatory pathways. Since knowledge on bovine regulatory T cells is limited, experiments were conducted to functionally identify bovine regulatory cell populations *ex vivo*, with special emphasis on $CD4^{+}CD25^{high}$ and $CD4^{+}CD25^{low}$ T cells as potential natural Treg in comparison to $WC1^{+}$ γδ T cells, $CD8^{+}$ T cells, NK^{+} and $CD14^{+}$ subpopulations as described in chapter 5.
Concluding this thesis, the findings of the studies are summarized and discussed in chapter 6.

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2

Chapter 2

Mycobacterial 70 kD heat-shock protein is an effective subunit vaccine against bovine paratuberculosis

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SUMMARY

Paratuberculosis is a chronic granulomatous inflammation of the small intestine of cattle and other ruminants, caused by infection with *Mycobacterium avium ssp. Paratuberculosis* (*MAP*). The disease can be found in ruminant herds worldwide, causing substantial economic losses at farm level due to premature culling and production losses.

In previous studies it has been shown that immune responses to recombinant *MAP* Hsp70 proteins were predominantly cell mediated. As protective immunity to the intracellular mycobacterial pathogens is thought to be cell mediated in origin, we have studied the use of a recombinant *MAP* Hsp70 as a subunit vaccine in cattle experimentally infected with *MAP*.

The results of the current study demonstrate that recombinant *MAP* Hsp70 can be successfully used as a subunit vaccine against bovine paratuberculosis, significantly reducing shedding of bacteria in feces during the first 2 years following experimental infection.

INTRODUCTION

Paratuberculosis is a chronic granulomatous inflammation of the small intestine of cattle and other ruminants, caused by infection with *Mycobacterium avium ssp. paratuberculosis*. The disease can be found in ruminant herds worldwide, causing substantial economic losses at farm level due to premature culling and production losses. In addition it may represent a potential food safety issue by transmission of *MAP*, the causative organism, to humans via dairy and other products [37, 42].

Transmission of paratuberculosis involves the presence of the bacterium in several substrates. From infected animals, bacteria are mainly excreted in feces and when the disease progresses towards the clinical stage of infection also milk. Following excretion the organism is able to survive in the environment for prolonged periods of time in substrates such as surface water, and soil [37, 43].

Eradication of paratuberculosis is hampered by a lack of reliable diagnostic tools with high specificity and sensitivity and could be aided substantially with a vaccine [27]. The currently available vaccine consists of various variations of whole bacterins with adjuvants. These vaccines have been shown to have a variable efficacy in field studies [21, 28]. From these studies it has become apparent that the current vaccine prevents the occurrence of the clinical stage of the disease to a high degree, thereby limiting a substantial amount of the direct economical damage. In cattle however, the vaccine

does not prevent infection and limits the frequency of subclinically infected animals, which shed bacteria in their feces intermittently, only marginally at best. Other major drawbacks of the whole bacterin vaccines are the interference tuberculosis and paratuberculosis diagnostics and eradication due to the fact that vaccinated animals become false positive in regular tuberculosis and paratuberculosis diagnostic assays [15, 19, 21, 28, 38]. A third drawback is that the vaccine in its current formulation causes substantial local tissue reaction, in terms of prolonged swelling and granuloma formation at the site of injection [30].

Relative little effort has been put in studying recombinant protein antigens of *MAP* in general and in vaccine studies in particular. With previous work we have documented immune responses to mycobacterial heat shock proteins (Hsp) in various stages of bovine paratuberculosis. The Hsp are a family of evolutionary conserved proteins, expressed in both prokaryotic and eukaryotic organisms. The expression of Hsp is upregulated during the cellular stress that occurs in both host and pathogen in response to infection and inflammation. We have previously shown that the Hsp are immunodominant antigens in bovine paratuberculosis [20, 18], similar to other mycobacterial diseases such as tuberculosis and leprosy, eliciting strong cell mediated and antibody responses. A number of studies on mycobacterial diseases, mainly in murine model systems, have indicated a potential use of Hsp as vaccine components. Our studies indicated that immune responses to recombinant *MAP* Hsp70 proteins were predominantly cell mediated contrary to recombinant *MAP* Hsp65 which apparently induced mainly antibody responses and minor cell mediated responses [17, 20].

As protective immunity to the intracellular mycobacterial pathogens is thought to be cell mediated in origin, we have studied the use of a recombinant *MAP* Hsp70 as a subunit vaccine in cattle experimentally infected with *MAP*. New candidate vaccines against bovine paratuberculosis should have the potential to limit transmission of infection between susceptible animals to aid in eradication strategies. In the current study we therefore studied immune parameters as well as fecal excretion in the first two years of experimental paratuberculosis to determine the potential of the recombinant *MAP* Hsp70 as a subunit vaccine in a relatively short timeframe considering the natural course bovine paratuberculosis.

MATERIALS AND METHODS

Animals and experimental design

A total of 40 female calves (aged 29 ± 9 days at the start of the experiment) were used in the current study. The calves were raised using conventional procedures and feeds,

and were checked daily for general health. The calves were randomly assigned to one of the 4 experimental groups, according to a 2x2 factorial design. (Table 1).

Table 1.
Experimental design

Group	*n*	Infection	Immunization
G1	10	No	No
G2	10	No	Yes
G3	10	Yes	No
G4	10	Yes	Yes

Calves in groups 1 and 2 were physically separated from calves in groups 3 and 4, and rigorous hygienic measures were taken to prevent infection of the control groups. Calves allotted to groups 1 and 2 were randomly co-mingled in group housing pens for 10 calves each, to prevent possible influence of a treatment effect. Similarly calves from group 3 and 4 were housed mixed. Blood samples were taken every 2 weeks for the first 12 months of the experiment and monthly for the remainder. Heparinized samples were used for isolation of lymphocytes, and serum samples were taken for serological analysis. Body weight was recorded on the same time points as blood samples were taken. Fecal samples were taken 7 times during the experiment, at days 0, 14, 126, 280, 406, 532 and 644.

Ethics

The use of animals in the experiments described in these studies was approved by the Ethical Committee of the Utrecht University and performed according to their regulations.

Infection of calves

Calves assigned to groups 3 and 4 were infected orally using feces from a *MAP* infected cow which was characterized as a consistent shedder by fecal culture of the mycobactin-J dependant and IS900 PCR positive *MAP*. The calves received 9 dosis of 20 grams of feces, mixed with 100 ml milk replacer per dose by gavage feeding, during the first 21 days of the experiment at regular intervals. Semi-quantitative fecal culture indicated that > 100 cfu / gr of feces were present in the inoculum. Hence, calves received a minimum total dose of 1.8×10^4 cfu each.

Immunization of calves

Calves assigned to groups 2 and 4 were immunized once at the start of the experiment (day 0). The immunization consisted of the administration of 200 µg of recombinant *MAP* Hsp70 in 1 ml phosphate buffered saline (PBS) containing 20 mg/ml dimethyl dioctadecyl ammonium bromide (DDA) adjuvant (Sigma Aldrich, USA), subcutaneously in the dewlap. The same animals received a second immunization at day 308 of the experiment. Recombinant *MAP* Hsp70 was produced as published previously [20].

Fecal culture of *MAP*

Diagnosis of paratuberculosis infection was performed using the routine fecal culture system, based on published methods [14], at Veterinary Health Service, Deventer, The Netherlands. Samples were checked for bacterial growth by colony count every 4 weeks, the first observation at 8 weeks post inoculation, and considered negative if after a culture period of 16 weeks no bacterial growth was observed. Bacterial growth was confirmed to be *M. avium* ssp. *paratuberculosis* based on mycobactin dependence of the culture and the confirmation of the presence of the specific IS900 insertion sequence by PCR [40]. Results of fecal culture were scored semi-quantitatively between 0 and 9 based on the combination of time to positive (TTP), respectively 8, 12 or 16 weeks and the number of colonies counted (CFU) per gram of feces, as outlined in Table 2.

Table 2.
Fecal culture score

	Time to positive culture (weeks)		
	16	12	8
Negative	0	0	0
1–10 cfu/gr	1	2	3
10–100 cfu/gr	4	5	6
>100 cfu/gr	7	8	9

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from aseptically taken, heparinized blood samples using density gradient centrifugation, and cultured as published previously [18].

Flowcytometric analysis of lymphocyte subsets

Three colour flowcytometric analysis was performed with monoclonal antibodies against bovine lymphocyte markers that have been described earlier (see Table 3). Three color flowcytometry was performed as follows. Approximately 200.000 cells per well, suspended in 200 µL of facs buffer (FB; PBS, 1% BSA, 0.01% Sodium-Azide), were put in 96 well plates in 7-fold, and centrifuged for 2 min at 1200 rpm in a cooled centrifuge (4ºC). Subsequently the FB was discarded and the pellet resuspended. This wash step was repeated once more before the unlabeled antibodies (see Table 3) were added to the cells, in predetermined optimal concentration diluted in FB.

Table 3.
Monoclonal antibodies (mAb) used in flow cytometric analysis

mAb	Isotype	Antigen identified	Refs.	Modification
ILA-42	IgG2a	Bovine CD2	[38]	biotinylated
ILA-12	IgG2a	Bovine CD4	[39]	-
CACT138A	IgG1	Bovine CD4	[40]	-
ILA-51	IgG1	Bovine CD8	[41]	-
ILA-29	IgG1	Bovine WC1 (γδ T cells)	[42]	-
CACT61A	IgM	Bovine TCR1-N12 (γδ T cells)	[43]	-
GB25A	IgG1	Bovine CD21-like (B-cell)	[44]	-
ILA-59	IgG1	Bovine Immunoglobulin light chain	[44,45]	FITC labeled

In one well no primary antibody was added as negative control, in another well an optimized mixture of the unlabeled antibodies against CD4, CD8, N12, CD14 and CD21 was added to enable an estimation of the total percentage of cells that could be identified per sample. Cells were incubated for 15 minutes in the dark at 4ºC. Next cells were washed twice as described above. Subsequently a phyco-erythrin (PE) conjugated secondary goat-anti-mouse antibody (Southern Biotech, USA) was added in predetermined optimal concentration diluted in FB and again cells were incubated for 15 minutes in the dark at 4ºC. Following two more washes the biotinylated anti-CD2 and the FITC labeled ILA-59 were added and incubated for 15 minutes in the dark at 4ºC. Again cells were washed twice and streptavidin-ALEXA633 (Molecular Probes, USA) was added and incubated as described above. Finally, cells were washed twice and resuspended in 100 µL FB prior to measurement on a Facs Calibur flowcytometer (Becton-Dickinson, USA). Data of at least 10.000 events was analyzed using Cellquest software (Becton-Dickinson, USA).

Antigens

Recombinant *M. a. paratuberculosis* Hsp 65kD and Hsp70 kD were produced according to methods described in detail earlier [6, 20]. Purity of the recombinant Hsp65 and Hsp70 was checked using SDS-PAGE and preparations were tested for LPS contamination by Limulus assay (Sigma, St. Louis, USA).

Purified protein deviate was prepared from *M. a. paratuberculosis* strain 3+5/C culture supernatant (PPD-P) and *M. a. avium* strain D4 culture supernatant (PPD-A) according to the OIE manual [11] at the Institute for Animal Health and Science (Lelystad, The Netherlands).

M. a. paratuberculosis strain 316F and *M. a. avium* strain D4 were grown at the Institute for Animal Health and Science (Lelystad, The Netherlands). *E. coli* strain DH5α was grown overnight in Luria Bertani (LB) medium at 37ºC.

Concanavalin A was used as a positive control (2.5 mmg/ml) and medium alone as a negative control.

Elispot assay for bovine IFNγ secreting cells

The sterile 96-wells PVDF filter plates (Millipore, USA) were coated overnight at 4 ^{o}C with 100 µl/well anti-bovine IFN-γ capture monoclonal antibody 5D10 (1 µg/ml, Biosource, USA) in filter sterilized bicarbonate coating buffer (pH9.6). Subsequently, the plate was blocked with 200 µL RPMI1640 supplemented with 10% fetal calf serum (FCS) for 2 hours at 37°C. After 2 hours the blocking medium was replaced with culture medium (CM) (RPMI1640 supplemented with 10% FCS, 50 IU/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine) with or without antigen according to the description below. Subsequently we added 2.10^5 cells per well in 100 µL CM to the Elispot plate and cultured the plate for 20 - 24 h in humidified incubator at 37 ^{o}C and 5% CO_2. Following incubation, spots were developed as follows. The wells were washed twice with H_2O_d and then trice with PBS-0.05% Tween 20 (PBS-T). Subsequenlty, 100 µl/well rabbit anti-bovine IFN-γ (1:200 in PBS-0.05% Tween 20-2% BSA, (PBS-TB)), a generous gift from Martin Vordermeier, VLA Weybridge, UK, and incubated for 30 min at room temperature. Then plates were washed 3 times with PBS-T, and wash fluid was removed thoroughly. Next, 100 µl/well of monoclonal anti-rabbit IgG-alkaline phosphatase conjugate (Sigma, USA, diluted 1:2000 in PBS-TB) was added and incubated for 30 min at room temperature. Subsequently, plates were washed 6 times with PBS-T. Then 100 µl/well of BCIP/NBT (Sigma, USA) dissolved in 10 ml H_2O_d was added. Once spots developed substrate was removed and plates were washed with copious amounts of tap water. Plates were air dried and stored in the dark before counting spots. Spots were counted, and total spot area was calculated using an automated Elispot reader according to instructions provided by

the manufacturer (A.EL.VIS GmbH, Hanover, Germany). Spot counting results were expressed as delta-spot forming cells (dSFC), calculated by subtracting the number of spots in medium control wells from the number of spots in antigen stimulated wells, unless stated otherwise. Medium alone was used as a negative control, concanavalin A (2.5 µg/ml) was used as positive control. PPD-P, PPD-A, Hsp70 and Hsp65 were used at a predetermined optimal concentration of 10 µg/ml. *M. a. paratuberculosis* strain 316F, *M. a. avium* strain D4, and *E. coli* DH5α were used at MOI 1:1 with the PBMC. All tests were performed in triplicate. To perform a relative estimation of the amount of IFNγ produced, the percentage of area that the spots covered per well was calculated and expressed as percentage of the total area of the well. Subsequently the antigen specific spot area was calculated by subtraction of medium control values from values in antigen stimulated wells.

Serology of calves

Serological responses to recombinant *M. a. paratuberculosis* Hsp70 protein were measured using a previously described ELISA technique, with modifications [20]. All sera were diluted 10 times in blocking buffer and 100 µl was measured in duplicate. In addition, in each plate a positive and a negative control sample were added in duplicate. The modifications consisted of the use of a biotinylated anti-bovine IgG secondary antibody (Sigma-Aldrich, USA) and an avidin-peroxidase conjugate (Sigma-Aldrich, USA), both according to instructions provided by the manufacturer, before adding substrate. Results are expressed as S/P (sample to positive) ratio.

For a general serological screening of the samples, a commercially available absorbed ELISA assay for the serodiagnosis of paratuberculosis was used according to instructions provided by the manufacturer (Institute Pourquier, Montpellier, France). The antigens used in this ELISA are derived from a protoplasmic extract of *MAP*. Absorbence values were expressed as S/N ratio (sample to negative), and values higher than S/N ratio 59 were considered positive [7].

Statistical analysis

The data from the fecal culture test were analyzed using the R program version 2.0.1 [31] for statistical analysis using a logistic regression for non-normal repeated measurements models [25]. Cow was taken as the random effects term. Fecal culture, scored semi-quantitative (table 2), was used as the outcome in the model. Based on the lowest AIC for best fit, the final model contained the continuous variable time (month and month2), the factor treatment (treated and untreated) as well as the interaction month x treatment as explanatory variables. A linear mixed effects model has been

performed to analyze Elispot data as the response variable and group, time and the interaction between group and time as explanatory variables. For the Elispot medium control data the logarithm was taken to meet the normality assumption. To model the correlation between consecutive time points an autoregressive order 1 (AR1) correlation structure was introduced. The variance was not constant in time, for this a variance structure was used which allows a different variance at each time point. Cow was added to the model as the random effects term to take into account the dependence of the data. For the analysis of the data the program R version 2.1.1 was used with the nlme library using maximum likelihood estimation of the effects. The likelihood ratio test was used for best model fit using a significance level of 0.05. Multiple comparisons were made, following a Bonferroni correction, between groups per time point. The relationship between the number of spots and the area covered by the spots was calculated using the least squares fit for a line represented by y=mx+b in which m is the slope and b is the intercept. R squared was used to judge goodness of fit. A linear mixed effects model was performed to analyze flow cytometry data as the response variable and group, time and the interaction between group and time (age) as explanatory variables. To additionally analyze the age related differences in subset frequencies, the flow cytometric data was grouped in 5 age intervals namely animals aged from 20-43 days, 44-120 days, 121-240 days, 241-360 days and 360-644 days. Analysis of variance was performed on the flow cytometric data using the Kruskal-Wallis test, followed by Dunn's multiple comparisons test to test for differences between the age intervals.

RESULTS

Observations on general health status

The growth of the animals was monitored throughout the experiment and the results indicated that there were no differences in growth between the different treatment groups. (data not shown) Around day 56 an outbreak of ringworm skin disease, caused by *Trichophyton verrucosum*, affected all animals in groups 3 and 4, this infection resolved spontaneously. Around day 70, 16 out of 20 animals in groups 1 and 2 showed signs of a respiratory infection, while 10 cases resolved spontaneous, 6 were successfully treated with antibiotics and anti-inflammatory drugs. During the experiment 3 animals had to be culled for reasons unrelated to paratuberculosis. These animals belonged to 3 different treatment groups. Two animals were culled based on severe respiratory infections that were non-responsive to treatment; one animal had to be culled due to paresis posterior following a fall.

Side effects of vaccination

The effect of primary vaccination with Hsp70 with DDA adjuvant in the dewlap was a palpable swelling with a maximum diameter between 2 and 5 cm at a week post immunization in animals of both the vaccinated groups G2 and G4. The swelling was in the majority of cases not painful and resolved to a small, apparently inert, nodule of approximately 1 cm diameter in the course of 3 weeks. The effect of the second immunization at 308 days was similar in animals of group G2 (vaccination, no infection), however less swelling (maximum diameter 3 cm, and 4 cases with no palpable swelling, one week post immunization) was observed in animals of group G4 (vaccination and infection).

Fecal culture results

The results from the fecal culture tests are summarized in Fig. 1. The animals on the control groups 1 and 2 remained culture negative throughout the experiment. From the calves in group 3, infection with *MAP* only, in total 8 out of 10 animals were tested positive for *MAP* at least once of 7 time points tested, and 2 animals tested positive 5 out of 7 time points. In group 4 calves, which were both infected and immunized, 5 out of 10 animals tested positive once at day 126 of the experiment. Only 1 animal tested positive for *MAP* on 2 subsequent occasions. Based on the logistic regression model, the vaccinated animals in G4 shed bacteria in their feces significantly less frequent compared to unvaccinated calves in G3 ($p<0.01$) (Fig. 1).

Phenotyping of PBMC

With the panel of monoclonal antibodies we defined 7 subtypes of lymphocytes in the PBMC as is outlined in Table 4. The use of the mixed set of antibodies as defined in the material and method section also led to the identification of a subset of lymphocytes that stained positive for CD2, however did not stain with any of the other markers. Based on staining similarities we designated this population as NK-like cells. Data analysis indicated that there were no significant differences between the 4 treatment groups during the study (data not shown). On the other hand, age was a major factor regarding observed frequency differences in certain lymphocyte subsets. For analysis of the age effect, the data was subsequently grouped for all animals in different age groups (20-43, 44-120, 121-240, 241-360, 360-644 days of age), as shown in Table 4. The $CD4^+$ cells were present at relatively stable frequencies, 20.1 – 21.4%, when comparing the age intervals with the exception of the interval between 44-120 days when a significant elevation to 26.2% was observed ($p<0.001$ for all comparisons). In the $CD8^+$ T cells there were more frequency fluctuations during the first 2 years of life,

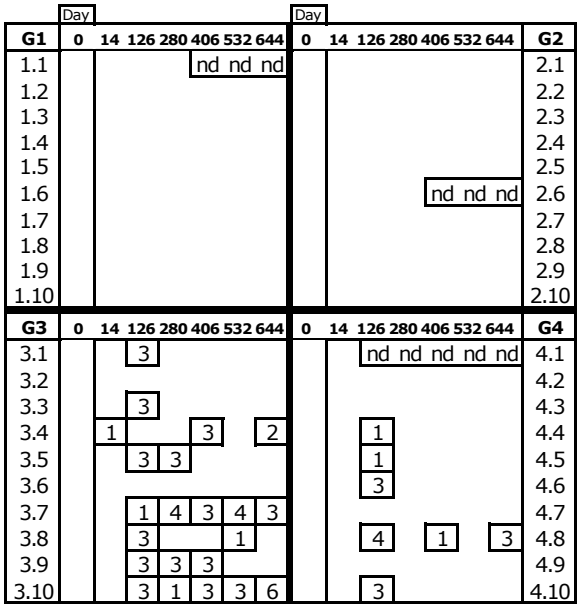

Fig. 1. In the 4 panels all the results from the fecal culture tests are shown per treatment group. G1 represents the animals that were neither immunized nor infected, G2 animals only received the immunization, G3 animals only received the infection; and G4 contains the animals that were both immunized and infected. Squares with scores >0 indicate a positive fecal culture for MAP for a given animal and a given time point scored according to table 2, (nd) indicates time points were respective animals were not tested because they were culled from the experiment.

while an average frequency of 8.6% was measured overall, most notably the period 20-43 days and 241-360 days showed a similar and significantly lower frequency of 8.0% and 7.2% respectively. The $N12^{+}CD2^{+}WC1^{-}$ subset of γδ T cells was low (2.5%) in youngest animals then rose in frequency in age intervals 44-120 and 121-240, up to 6%, and subsequently declined again to 3.3% in age interval 360-644. The $N12^{+}CD2^{-}WC1^{+}$ subset of γδ T cells was highest in the lowest age interval (26.0%), fluctuated between 16.2% and 20.6% in the intermediate intervals to decline to 15.1% in highest age interval. The B cell frequency was lowest in the 20-43 day (10.2%, $p<0.001$ for all comparisons) and 44-120 day (18.9%, $p<0.001$ for all comparisons) interval to stabilize between 32.3-35.8% in the remaining three age intervals. In addition we observed a high frequency of $CD14^{+}$ monocytes in young calves (29.8%, $p<0.001$ for all comparisons), which subsequently declined to an average frequency of 8.3% in the highest age group.

Table 4.
Age related differences in lymphocyte subset frequencies in blood mononuclear cells
The average frequencies (95% confidence interval) of the 7 different lymphocyte subsets detected in the blood mononuclear lymphocytes are shown for the 5 different age (days) groups. Lymphocyte frequencies between age groups that do not share letters are significantly different ($p<0.05$). As no differences between treatment groups were detected the results of the animals from the different treatment groups have been combined.

Lymphocyte subset	Age(days)									
	20–43		44–120		121–240		241–360		360–644	
$CD4^+$	20.8^a	(19.5–22.0)	26.2^b	(24.7–27.7)	21.4^a	(20.4–22.4)	20.1^a	(19.0–21.1)	21.1^a	(20.2–22.2)
$CD8^+$	8.0^a	(7.2–8.8)	9.7^{ab}	(8.5–10.8)	9.9^b	(9.0–10.8)	7.2^a	(6.4–8.1)	9.0^{bc}	(8.2–9.8)
$N12^+CD2^+WC1^-$	2.5^a	(2.2–2.8)	5.7^{bc}	(4.8–6.5)	6.0^b	(5.3–6.6)	4.9^{bc}	(4.4–5.5)	3.3^d	(3.0–3.6)
$N12^+CD2^-WC1^+$	26.0^a	(23.9–28.2)	16.2^b	(15.0–17.5)	17.5^{bcd}	(16.2–18.8)	20.6^d	(18.8–22.4)	15.1^{bc}	(14.1–16.1)
B cell	10.2^a	(9.1–11.4)	18.9^b	(17.4–20.4)	35.8^c	(34.4–37.3)	33.1^c	(31.1–34.9)	32.3^c	(30.7–33.9)
$CD14^+$	29.8^a	(27.4–32.2)	15.6^b	(14.1–17.0)	12.1^{bd}	(10.9–13.3)	9.7^{cd}	(8.7–10.6)	8.3^c	(7.2–9.4)
$CD2^+NK$ like	11.2^a	(10.0–12.5)	8.9^{ab}	(8.0–9.8)	10.2^a	(9.3–11.1)	8.2^{bc}	(7.1–9.2)	6.7^c	(5.7–7.7)

IFN-γ Elispot

In the young calves there was a high number of cells which spontaneously produced IFN-γ following density gradient isolation and overnight culture as is shown in Fig. 2A (medium control values). This background was most prominent in the first 3 months of life however, to a certain extend it remained present during the first 2 years of life. In general, most differences in IFN-γ production were observed in the period between 224 and 336 days post infection for both PPDP (Fig. 2D) and *MAP* (Fig. 2E) antigen. Statistical significant differences in IFN-γ responses to PPDP between the infected group G3 and control groups G1 and G2 were observed at 308 days post infection. For the animals in G4 significant differences were observed at the 3 consecutive time points between 280 and 336 days post infection. Significant differences in IFN-γ responses to *MAP* between the infected group G3 and control groups G1 and G2 were observed at the 2 time points between 308 and 336 days post infection. For the animals in G4 significant differences were observed at the 4 time points between 252 and 336 days post infection. To a lesser extend a similar distinction between exposed/infected and uninfected controls could be made during the final part of the second year. Both at 588 and 644 days post infection animals in G3 had statistical significant elevated IFN-γ production in response to PPDP and *MAP* antigens. For animals in G4 these differences were not statistically significant. With respect to IFN-γ response to Hsp70 only at days 280 and 308 post infection a statistical significant difference between G4 and control groups G1 and G2 was observed. No other statistically significant differences between groups were identified for stimulation with Hsp70. Apart from the number of IFN-γ producing cells in response to various stimulations and controls we also calculated the total area of the well covered by the spots as an

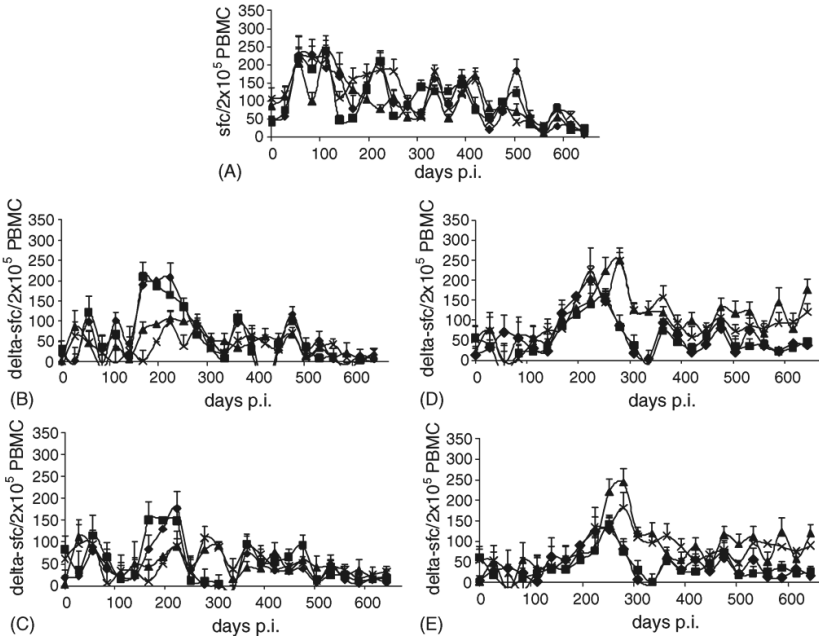

Fig. 2. The production of IFN-γ in the IFN-γ Elispot assay is expressed as the number of spot forming cells (sfc) / 2.10^5 PBMC for the medium control (panel A) and as the delta-sfc, that is the number of spots in the antigen stimulated wells minus the number of spots in medium control wells, for antigens E. coli (panel B), MAP Hsp70 (panel C), PPDP (panel D) and MAP (panel E). The 4 treatment groups are indicated by (♦) for G1 (no infection, no immunization), (■) for G2 (no infection, Hsp70 immunization), (▲) for G3 (infection with MAP, no immunization) and (x) for G4 (infection with MAP and Hsp70 immunization).

estimation of the total amount of IFN-γ produced. The results indicated that there was a significant linear correlation between the number of spots per well and the total area the spots covered in medium control wells (Fig. 3A) and Hsp70 stimulated wells (Fig. 3B). In case of the medium control wells the relation was characterized by y=0.0203x + 0.0754, R^2=0.93, and for the Hsp70 stimulated wells this was y=0.0213x + 0.0937, R^2=0.93. For the other antigens tested, similar linear correlations were observed (not shown).

Serological responses

The animals in group 2 and 4 which were vaccinated with Hsp70 at day 0 and day 308 showed clear and prolonged antibody responses to the immunogen as is shown in Fig. 4. The second immunization showed a more prolonged antibody response compared to the primary immunization. Where levels were close to baseline a year after primary

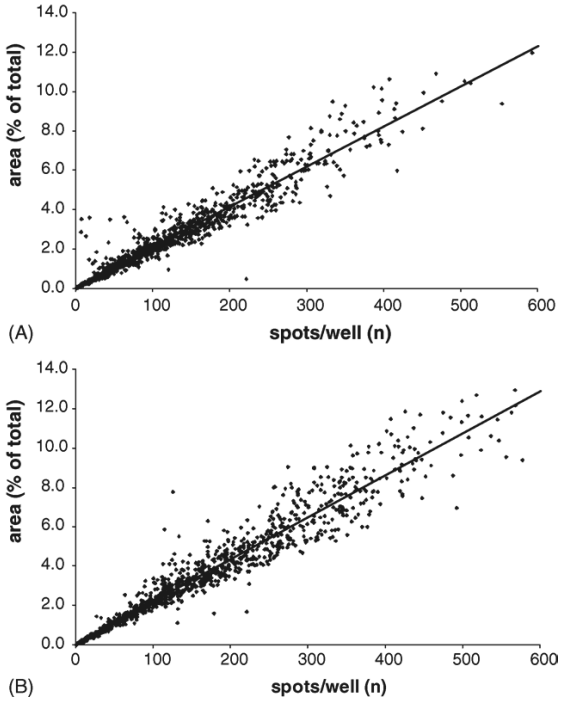

Fig. 3. The correlation between the number of spots per well and the percentage of area covered by the spots per well is shown in panel A for the medium controls and in panel B for well stimulated with recombinant MAP Hsp70 protein. For the medium control wells the relation between total spot number and percentage area covered was characterized by $y=0.0203x + 0.0754$ ($R^2=0.93$), and for the Hsp70 stimulated wells this was $y=0.0213x + 0.0937$ ($R^2=0.93$).

immunization, they remained high until the end of year 2. No antibody responses were observed in the uninfected control group G1 and the infected non immunized group G3 except for minor peaks at day 56 and day 70 of the experiment.

The results from the commercially available absorbed ELISA are depicted in Fig. 5. Serum antibody responses were determined in samples from the same time-points at which fecal samples were tested. The first serological responses can be seen at 406 days post infection, 98 days past the second immunization with Hsp70 in G2 and G4. Apart from responses in the *MAP* infected groups G3 (4 of 10) and G4 (1 of 9), responses were also detected in 4 of 9 G2 animals, while none of the G1 animals showed responses. At day 532 1 animal in G3 and 3 animals in G4 were positive while animals in G1 and G2 were all negative. At day 644 1 animal in G2, 2 animals in G3 and 1 animal in G4 tested positive.

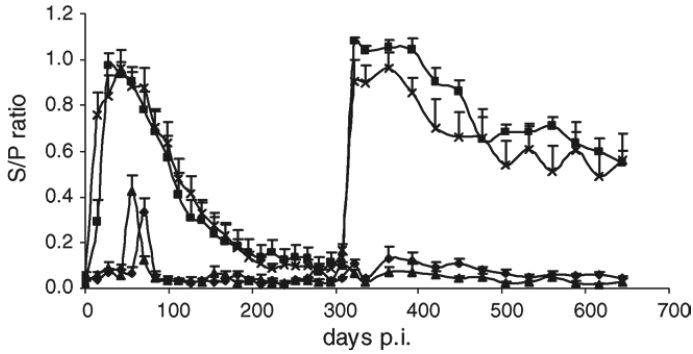

Fig. 4. Total IgG responses to Hsp70 protein measured by ELISA and expressed as the S/P ratio + SEM. Antibody responses of immunized animals (G2, ■, non infected; and G4, X, infected) and non-immunized (G1, ♦, non infected and G3, ▲, infected) are shown during the course of the experiment. Animals of G2 and G4 were immunized at day 0 of the experiment. A second immunization was performed at day 308 of the experiment.

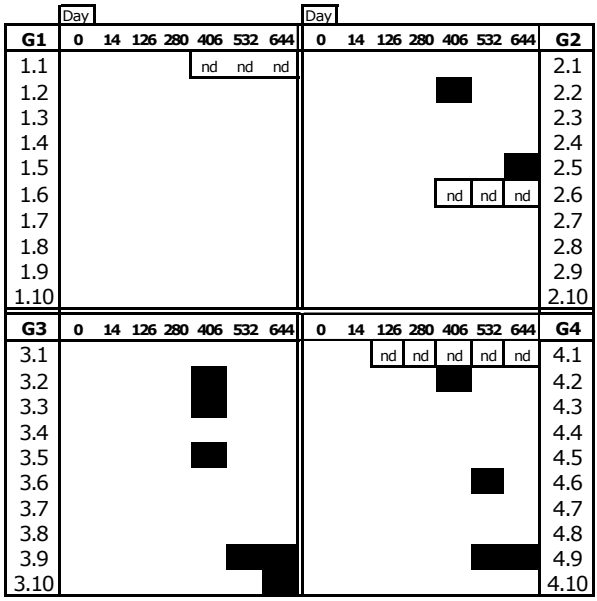

Fig. 5. In the 4 panels all the results from the absorbed ELISA is shown per treatment group. G1 represents the animals that were neither immunized nor infected, G2 animals only received the immunization, G3 animals only received the infection; and G4 contains the animals that were both immunized and infected. Black squares (■) indicate a positive ELISA S/N ratio for a given animal and a given time point, (nd) indicates time points were respective animals were not tested because they were culled from the experiment.

DISCUSSION

To our knowledge this is the first report describing a, heat shock protein based, subunit vaccine approach to bovine paratuberculosis which leads to significantly decreased shedding of bacteria into the feces. Heat shock proteins have been shown to be dominant antigens for the immune system in many infectious diseases (reviewed in [45]). The Hsp70 of *M. tuberculosis* and *M. bovis* has been used previously in DNA vaccination studies, both in calves and in murine model systems, with variable success as therapeutic [26] or a preventive [34] vaccine against tuberculosis. Our previous studies provided evidence that the mycobacterial Hsp70 protein was a dominant T cell antigen in bovine paratuberculosis [17, 18]. In addition we also showed that in naturally infected animals the antibody responses to Hsp70 were generally low and did not allow for discrimination between infected and uninfected animals. This was in contrast with the immune responses to the Hsp60 antigen which induced little T cell activation and high levels of antibody in *MAP* infected animals shedding the bacteria in the feces [20]. As protective immunity against mycobacterial infection is considered to be cell mediated in origin (reviewed in [32, 35]), we decided to use the recombinant Hsp70 protein in a subunit vaccine using DDA as a Th1 skewing adjuvant [24]. Based on the current insights in transmission of bovine paratuberculosis, characterized by the fact that the most susceptible individuals, neonates, are born into an infectious environment [37], we did not to use a classical prime-boost vaccination before experimental infection as in our view this would not represent the natural sequel of events. Therefore vaccination and challenge were synchronized, and in addition a single vaccination was used to enable monitoring of a potential differential development of immune responses due to the concomitant infection in comparison to vaccination in uninfected control animals. The results of the current study demonstrate that recombinant *MAP* Hsp70 can be successfully used a subunit vaccine against bovine paratuberculosis in terms of significantly reducing shedding of bacteria in feces during the first 2 years following experimental infection. Our study is too short to evaluate the protective effect of the vaccination in terms of preventing the progression to the clinical stage of the disease, which is a hallmark of the whole bacterin vaccine [39]. However the fact that the subunit vaccine significantly reduces shedding, which is something the whole bacterin fails to do [19, 41], is a promising aspect of the Hsp70 immunization indicating a higher level of control on bacterial growth.

Although we selected the Hsp70 protein based on its cell mediated immunity stimulating properties in our own and other studies, the lack of clearly different IFN-γ production in the vaccinated animals was unexpected. Antigen specific IFN-γ production were

present from day 280 onwards in the study, however while significant differences could be measured they were related to challenge, not to treatment. The effect was similar when comparing PPD-P and whole *MAP* bacteria as antigen. At certain time points significant differences in Hsp70 induced IFN-γ could be measured, the difference was also related to challenge rather than treatment. These results indicated that the Elispot assay could detect *MAP* infected individuals in different stages post challenge based on IFN-γ. There was IFN-γ production in response to Hsp70 protein during the two years of the study only to reach baseline at the end of the second year. Possibly, as Hsp70 is an evolutionary conserved antigen with high homology with other bacterial and even eukaryotic Hsp70, cross reactive T cell responses obscured vaccine specific T cell responses. In addition, it has also been shown that mycobacterial Hsp70 specifically binds to innate receptors present on macrophages [4] and dendritic cells [23], leading to production of pro-inflammatory signals which may in turn lead to IFN-γ production by T and NK cells. The observation that all animals in this study have high frequencies of monocytes, gradually decreasing with age, adds to this hypothesis.

The whole blood IFN-γ assay performs better in populations of young cattle as compared to serum antibody ELISA [13]. However, the fact that young ruminants have high non-specific production of IFN-γ puts constrains on the use of IFN-γ based diagnostics of bovine paratuberculosis in animals less than 2 and especially less than 1 year of age [2]. A role for the abundantly present γδ T cells in young ruminants is suspected however the biology behind these observations remains unclear as is the function of these cells [16, 17, 33]. Recently, NK cells have been implicated in the production of IFN-γ, especially in young calves, and in response to certain mycobacterial antigens [29]. The NKp46 antibody [36] was not available during our study for confirmation and our flow cytometry data indicate we overestimated the number of NK cells in the population we called NK-like, although we observe similar frequency kinetics [22]. The proportions of γδ T cells, CD4+ and CD8+ T cells were comparable to those reported previously for various age groups [22].

The fact that little antibody can be detected during the first two years of bovine paratuberculosis infection is a common characteristic of the natural infection, hampering the use of serum antibody ELISA diagnostic assays at these early stages of infection [13]. In the current study, when using a commercially available serum antibody ELISA for paratuberculosis we found the first animals in both the treated and untreated infected group in the second year for the first time. In addition we had two responders in treated but uninfected control group, which may indicate that some Hsp70 may be present in the antigen used in the commercial ELISA leading to a positive response. The data also supports previous observations that few antibodies to

Hsp70 are formed during the infection with *MAP* [20]. The extensive and prolonged production of serum antibodies to Hsp70 was prominent, and restricted to the treatment groups. No significant difference between challenged and uninfected animals was observed considering the total anti-Hsp70 IgG produced. Also, the high levels of anti-Hsp70 antibodies observed in this study subsequent to vaccination at least suggested that antibody responses to this protein in the context of paratuberculosis infection are not detrimental per se, as they were correlated with decreased shedding of bacteria. The priming of animals with Hsp70 using DDA adjuvant induced an immune response which is clearly different from encountering the protein during infection. This difference does enable a differentiation between vaccinated and infected animals which may be clearly an advantage in the application of the vaccine. As the Hsp70 is an evolutionary conserved antigen it remains to be investigated whether Hsp70 vaccinated cows will become false positive in diagnostic assays for bovine tuberculosis, such as the intra-dermal skin test.

In conclusion we have demonstrated that vaccination of cattle with a Hsp70/DDA vaccine significantly reduces shedding of *MAP* in the feces which in turn may reduce transmission of infection, has little direct and long term side-effects and enables differentiation between vaccinated and infected animals and as such may contribute to the paratuberculosis eradication strategies.

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3

Chapter 3

Epitopes of mycobacterium paratuberculosis 70 kD heat shock protein activating bovine helper T cells

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SUMMARY

The recombinant 70 kD heat shock protein of *Mycobacterium avium subspecies paratuberculosis* (*MAP* Hsp70) has proven to be an immunodominant antigen and a subunit vaccine candidate for paratuberculosis. The aim of the present study was to define *MAP* Hsp70 specific T cell epitopes in cows immunized with *MAP* Hsp70 and cows experimentally infected with *MAP*. Nine peptides were shown to induce proliferation of lymphocytes from a *MAP* Hsp70 immunized high responder cow. From 28 cows experimentally infected with *MAP* 82% responded to at least one of the 5 most immunodominant peptides, indicating relevance of the epitopes during infection. Since 15 different BoLA class II types were present in these 28 animals it was concluded that the peptides were presented by multiple BoLA class II alleles thus to be promiscuous in nature, which may add to the potential of the *MAP* Hsp70 subunit vaccine.

INTRODUCTION

Bovine paratuberculosis [16] is caused by infection of young calves with the intracellular pathogen *Mycobacterium avium paratuberculosis* [35]. The infection may ultimately result in chronic disease characterized by granulomatous lesions in the ileum with some infected animals developing a lethal progressive protein loosing enteropathy. Paratuberulosis is a worldwide problem with a prevalence of 20-40% in US dairy herds and an estimated annual economic loss of $ 1.5 billion in the United States [43], and similar prevalence and economic losses in the Netherlands [4, 28]. Although a potential role of *MAP* in the induction of Crohn's disease (CD) is still debated [40] there is sufficient reason to minimize exposure of humans by controlling the disease in cattle, for example through vaccination.

The current vaccines against *MAP*, experimental as well as commercially available, are based on killed or live attenuated whole bacterium preparations. These vaccines induce substantial reduction of the number of clinically affected animals in herds but do not protect against infection and shedding of *MAP*, interfere with tuberculosis diagnostics in cattle [22] and often cause severe local tissue reaction [34]. Development of an effective subunit vaccine, would offer possibilities to overcome some of the disadvantages of whole cell vaccines.

Despite the fact that due to elucidation of the *MAP* genome [23], *MAP* specific antigens (Ag) [3] are being discovered at an increasing rate, data regarding recombinant *MAP* antigen specific cell mediated immune responses during various

stages of paratuberculosis are still limited [39]. Heat shock proteins (Hsp) are very immunogenic/immunodominant antigens and have been shown to be recognized in mycobacterial diseases such as tuberculosis, leprosy [1, 15], bovine paratuberculosis [20] and bovine tuberculosis [42].

Apart from their chaperone function both pro- and anti-inflammatory functions [15, 24, 50] have been proposed for the Hsp70 (DnaK) stress protein. In bovine paratuberculosis *MAP* Hsp70 induces *in vivo* and *in vitro* cell mediated as well as humoral immune responses [19, 21, 31].

In a recent vaccination/challenge experiment in which calves were vaccinated with a subunit vaccine, containing recombinant *MAP* Hsp70 in dimethyl dioctadecyl ammonium bromide (DDA), reduced shedding of bacteria in feces persisted for up to 2 years following experimental infection [19]. In the present study our aim was to characterize *MAP* Hsp70 specific immune responsiveness at the epitope/peptide level. Responsiveness as defined by cell proliferation and IFN-γ secretion in these animals was analysed in the context of their BoLA class II types [25, 53], known to be highly polymorphic [12], to asses potential promiscuity of the Hsp70 peptides (epitopes) which is relevant for subunit vaccine efficacy in outbred populations.

MATERIALS AND METHODS

Animals

A total of 44 cows and calves from conventional dairy farms in the Dutch paratuberculosis program with no history of paratuberculosis were used in the current study. The calves were raised using conventional procedures and feeds, and all animals were checked daily for general health.

Ethics

The use of animals in the experiments described was approved by the Ethical Committee of the Utrecht University and performed according to their regulations.

Experimental infection of calves

A group of calves (n=28) (aged 16 ± 11 days at the start of the experiment) were experimentally infected orally as previously described [19]. In short; feces from a *MAP* infected cow was orally administered in 9 doses of 20 grams mixed with 100 ml milk replacer per dose by gavage feeding, during the first 21 days of the experiment at regular intervals. Proliferative responses of PBMC to *MAP* Hsp70 and selected immunodominant *MAP* Hsp70 peptides were studied on d210.

Paratuberculosis infection status

All animals used in these studies were monitored for paratuberculosis infection status by performing routine fecal culture of *MAP* 4 to 12 times during the experiments described according to methods published previously [19].

***MAP* Hsp70 immunization of calves**

In the first immunization experiment 10 cows (2 years of age) were divided in two groups. One group of animals received two immunizations with 200 µg *MAP* Hsp70/DDA the other 500 µg *MAP* Hsp70/DDA at days 0 and 14. Immunization was performed by injection of 1 ml volumes subcutaneously in the dewlap.

In the second immunization experiment calves (n=3) were immunized with 200 µg *MAP* Hsp70 / DDA at day 0 (age 29 ± 9 days of age at the start of the experiment) and boosted at d308, proliferation was tested on d893, while control calves were sham immunized with PBS only (n=3).

Isolation of peripheral blood mononuclear cells

Heparinized blood samples were taken aseptically from the jugular vein of the animals. Peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation, and cultured as published previously [20].

Lymphocyte Stimulation Test

Prior to and at indicated time points following infection or immunizations, PBMC were tested in lymphocyte stimulation tests (LST) as described previously [18].

In short LST were performed in 96 well microtiter plates (Corning-Costar, Cambridge, MA, USA) using 100 µl of PBMC suspensions ($2x10^6$/ml) or T cell lines ($4x10^5$/ml) and 100 µl of antigen per well, all tests were performed in triplicate. Cells were cultured in RPMI 1640 tissue culture medium (Gibco®-Invitrogen, Breda, The Netherlands) supplemented with 10% FCS (Bodinco BV, Alkmaar, The Netherlands), 50 IU/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, $5x10^{-5}$ M β-mercaptoethanol (Flow Laboratories, Ivume, UK) and 0.5 µg/ml Amphotericin B (Sigma, St. Louis, MO, USA) (designated complete medium (CM)). The following antigens were used in the LST at predetermined optimal concentrations of 10 µg/ml; *MAP* Hsp70 peptides and PPD-P, recombinant *MAP* Hsp70 was tested at 5-10-20 and 40 µg/ml. Bacterial strains of *MAP* and E. coli were used in a concentration of $1.4x10^6$ CFU/ml. Concanavalin A (Con A, Sigma) was used as a positive control stimulant (2.5 µg/ml) and CM 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 Health, Little

Chalfont, Buckinghamshire, UK) was added to each well and cells were cultured for an additional 18 hrs. Subsequently, cells were harvested onto glass fiber filters and incorporation of ^{3}H thymidine was measured by liquid scintillation counting (Perkin Elmer / Wallac micro-Betaplate, Waltham, Massachusetts, USA) and expressed as counts per minute (cpm).

Magnetic cell sorting (MACS®) of bovine T cells

T cells were isolated from PBMC using positive selection by MACS®-beads according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). Briefly, cells were labeled with a mouse anti-bovine CD3 mAb (MM1A, VMRD, Pullman, WA, USA; Table 1) at a predetermined dilution. After washing the cells with 10 ml PBS, goat-anti-mouse IgG coated MACS®-beads were added and after 10 min of incubation the $CD3^{+}$ cells were isolated on a MACS® LS column mounted in a VarioMACS™ magnet. Positively sorted cells were eluted by flushing the column with PBS, washed once in PBS, and resuspended in complete medium for further use.

Generation of T cell lines / T cell clones

T cell lines specific for peptides of *MAP* Hsp70 were derived from PBMC of the high responder animal 2636 selected from immunization experiment 1. Briefly, cells were grown in bulk in 25 cm^2 tissue culture flasks (Corning Costar) at an initial concentration of $2x10^6$ cells/ml and 10 µg/ml *MAP* HSP70 in complete medium. After 10-14 days vital cells were recovered using density gradient isolation. T cells were restimulated with 10 µg/ml *MAP* HSP70 using irradiated (3000 cG) autologous PBMC as antigen presenting cells (APC) in a 2:1 APC to T cell ratio, at a total number of $3x10^6$ cells in 1ml CM in wells of a 24 well plate (Corning Costar) for 4 days, after which vital T cells were recovered using density gradient isolation. T cells were then subsequently cultured for another 10 days in CM supplemented with 40 µg/ml gentamycin (Gibco®-Invitrogen) and 100 u/ml recombinant human IL-2 (a generous gift from Wim den Otter, Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht, The Netherlands) at a final concentration of $1x10^6$ cells/ml in wells of a 24 well plate. This is one 14-day restimulation cycle. After the first restimulation cycle with *MAP* Hsp70 two additional restimulation cycles were performed using *MAP* Hsp70 peptides P105, P111 or P113 (defined as dominant immunogenic peptides by LST) as antigens. The resulting T cell lines were phenotyped by flow cytometric analysis and tested in LST as described below.

In addition bovine T cell clones were grown from MACS® isolated T cells (of the high

responder animal 2636) in CM + 10 % bovine factor (BF = supernatant of bovine PBMC stimulated with 5 µg/ml Con A + 5 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma)) + irradiated (3000 cG) allogenic PBMC + irradiated (6000 cG) human EBV (Epstein Barr virus) transformed B cell lines (CP + DG). In short, cloning was performed by limiting dilution in a 96 wells roundbottom plate (Corning Costar); 1-2x10^5 irradiated allogenic cow PBMC + 1x10^5 irradiated human EBV CP + DG + MACS purified T cells of animal 2636 (0.5 cell/well, 5 cell/well and 8 cell/well) were cloned in CM containing 20 U human IL-2/ml and 20 ng/ml human IL-15/ml (Peprotech EC Ltd, London,UK), clones were grown for 2 to 3 weeks after which the 14-day restimulation protocol was followed. After the first restimulation with *MAP* Hsp70 or *MAP* bacterium and two extra restimulations with *MAP* Hsp70 peptide P113 the T cell clones were phenotyped by flow cytometric analysis and tested for Ag specificity in a LST, performed as described above.

Flow cytometric analysis of lymphocyte subsets

Cell lines and clones were characterized by flow cytometry using murine monoclonal antibodies (mAb) specific for bovine cell markers (ILRI, Nairobi, Kenia and VMRD, Pullman, WA, USA; Table 1) as described previously [19]. Briefly, approximately 200,000 cells were washed twice in cold FACS buffer (FB: PBS supplemented with 1% BSA (Sigma), and 0.01% Na-azide (Sigma). The cells were incubated with an unlabeled monoclonal antibody for 15 minutes in the dark at 4ºC, washed twice and subsequently a phycoerythrin (PE) conjugated (secondary) goat-anti-mouse antibody (Becton-Dickinson biosciences Pharmingen, San diego, CA, USA) or an Alexa fluor 633 conjugated (secondary) goat-anti mouse IgG antibody (Alexa 633) (Molecular Probes, Oregon, USA) was added and again cells were incubated for 15 minutes in the dark at 4ºC. After two more washes, cells were resuspended in 100 µL FB prior to measurement in a FacsCalibur flowcytometer (Becton-Dickinson). Data for at least 5000 events were analyzed using Cellquest software (Becton-Dickinson).

Ex vivo IFN-γ Elispot assay

Elispot assays were performed as described previously [19] on generated cell lines and clones. In short, to anti-bovine IFN-γ capture mAb 5D10 (1 µg/ml, Biosource, Wheatley, United Kingdom)(Table 1) coated PVDF filter plates (Millipore, MA, USA) CM was added with or without antigen and subsequently 1x10^5 cells per well were added. Concanavalin A (Con A 2.5 µg/ml) (Sigma) was used as positive control stimulant. PPD-P, *MAP* Hsp70 and *MAP* Hsp70 peptides were used at a predetermined optimal concentration of 10 µg/ml. *MAP* strain 316F [35], MAA strain D4 (MAA),

and E. coli strain DH5α (E. coli) were used at MOI 1:1 with generated *MAP* Hsp70 specific T cell lines/clones. CM alone was used as a negative control. All tests were performed in triplicate. Following incubation, spots were developed by adding rabbit anti-bovine IFN-γ (1:200, a generous gift from Martin Vordermeier, VLA Weybridge, UK). Plates were washed and monoclonal anti-rabbit IgG-alkaline phosphatase conjugate (Sigma, diluted 1:2000) was added. After incubation plates were washed and BCIP/NBT (Sigma) was added. Once spots developed substrate was removed and plates were washed. Plates were air dried and stored in the dark before counting spots. Spots were counted using an automated Elispot reader according to instructions provided by the manufacturer (A.EL.VIS GmbH, Hannover, Germany). Results were expressed as delta-spot forming cells (dSFC), calculated by subtracting the number of spots in CM wells from the number of spots in antigen stimulated wells.

Antigens

Recombinant *MAP* Hsp70 kD was produced according to methods described in detail earlier [21]. Purity of the recombinant Hsp70 was checked using SDS-PAGE and preparations were tested for LPS contamination by Limulus lysate assays (Sigma). Purified protein derivative was prepared from *MAP* strain- 3+5/C culture supernatant (PPD-P) according to the OIE manual [14] at the Institute for Animal Health and Science (Lelystad, The Netherlands). *MAP* strain 316F [35] and Mycobacterium avium avium strain D4 (MAA) [21, 20] were grown at the Institute for Animal Health and Science (Lelystad, The Netherlands). Escherichia coli strain DH5α (E. coli) was grown overnight in Luria Bertani (LB) medium at 37ºC. Concanavalin A (Con A - Sigma) stimulated PBMC were used as positive controls for proliferative responses. Peptides were synthesized according to the *MAP* Hsp70 sequences [21, 45] by automated simultaneous multiple peptide synthesis (SMPS) as described previously [49]. Peptides were analyzed by reversed phase HPLC and electrospray mass-spectrometry on an ion-trap mass spectrometer (LCQ; Thermoquest, Breda, The Netherlands). Purity of the peptides ranged between 50 and 90%. A set of 124 synthetic 14-mer peptides, with a 5 amino acid (aa) shift and an overlap of 9 aa, covering the complete sequence of *MAP* Hsp70 was produced and tested initially in 11 pools of 10 peptides + 1 pool of 14 peptides for proliferative responses of PBMC. Single immunodominant peptides were identified from pools which induced high proliferation by individually testing the peptides from these pools and for confirmation of the total amount of detected positive peptides all 124 peptides were individually tested. To define fine specificity of responses the epitopes of two immunodominant *MAP* Hsp70 peptides, P111 and P113, were tested using series of 14-mer peptides which were shifted 1 aa per peptide

in both sequence directions to perform epitope pepscan analysis [48]. Furthermore peptides containing alanine substitutions at potential anchor positions of P111 and P113 were synthesized to confirm the anchor and core region. To identify the core region of one *MAP* Hsp70 peptide (P111) peptides ranging in length from 8-16 aa were synthesized to test the minimal peptide length still capable of inducing T cell proliferation in vitro.

MHC class I and II typing

Bovine Leucocyte Antigen [27] typing was performed by hybridization of biotinylated genomic PCR products to short, 15-22 base, oligonucleotide microarrays as previously described [32]. The only significant modification of the published procedure was that biotinylated PCR products for each exon of interest – class I exon 2, class I exon 3 and DRB3 exon 2 – were generated by direct amplification rather than by heminested PCR. The BoLA class I primers amplify exon 2 or exon 3 from most classical and non-classical class I loci. The microarray typing system, therefore, defines class I haplotypes rather than alleles at individual class I loci. Consequently, class I, A-region haplotype (AH) nomenclature is used for class I haplotypes identified by microarray typing. AH with established serotypes, which includes all AH carried by cattle in this study, have the same number as the narrowest serological BoLA class I specificity associated with these haplotypes [7]. DRB3 allele names are those assigned by the BoLA Nomenclature Committee of the International Society for Animal Genetics [8, 37]. Information on BoLA sequence based nomenclature is available on the IPD – MHC Sequence Database (http://www.ebi.ac.uk/ipd/mhc/bola/index.html) [13]. Class IIa, D-region haplotypes (DH) carried by the cattle were inferred on the basis of the class I and DRB3 typing [44].

***MAP* Hsp70 peptide presentation and T cell recognition in context of MHC class II**

T cel interaction with different MHC class II/*MAP* Hsp70 peptide 105 interactions were studied by combining peptide specific $CD4^+$ T cells and peptide presenting BoLA class II matched/mismatched $CD14^+$ (MM61A, IgG1, VMRD) (Table 1) APC. FACS sorted $CD4^+$ T cells (IL-A11, IgG2a, generous gift of Jan Naessens, ILRI) (Table 1) shown to respond to *MAP* Hsp70 peptide P105 (0, 2.5, 5 and 10 μg/ml) and concanavalin A (Con A / 0, 2.5, 5 and 10 μg/ml as a positive control) (Sigma), were used as a readout system for P105 presentation. The $CD4^+$ T cells of cow 2636 (DRB3*0101/*1101)(50,000 / 96 round-bottom well (RB) Corning Costar) were co-cultured with FACS sorted $CD14^+$ APC (150,000 / well) of two animals 100% BoLA MHC class II matched (100% M / 1375 and 0076 / DRB3*0101/*1101), two animals

100% BoLA MHC class II mismatched (100% MM / 0461 / DRB3*1201/*14011 and 0464 / DRB3*1201/*2703) and one animal 50% matched (50% M / 0469 / DRB3 *1101/*1201) in the presence of 10 ug/ml P105 in triplicates. Co-cultures were also performed adding anti-bovine MHCII (anti-BoLA DR) mAb IL-A21 (10 µg/ml, IgG2a, generous gift of Jan Naessens, ILRI) (Table 1) and isotype control murine IgG2a mAb. After 72h at 37°C ^{3}H-thymidine was added and 18h later analyzed for ^{3}H-thymidine incorporation as a measure for proliferation. Proliferation was calculated as stimulation indices (S.I.) by dividing the cpm from the co-cultured wells by the cpm of the CM control wells.

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

mAb	Isotype	Antigen identified	References
MM1A	IgG1	Bovine CD3	[9]
ILA-11	IgG2a	Bovine CD4	[2]
ILA-51	IgG1	Bovine CD8	[11]
ILA-29	IgG1	Bovine WC1	[6]
GB21A	IgG2b	Bovine δ chain TCR1-N24	[10]
GB25A	IgG1	Bovine CD21-like	[5]
MM61A	IgG1	Bovine CD14	[38]
IL-A21	IgG2a	Bovine MHC II (DR)	[47]
5D10	IgG1	Bovine IFN-γ	[54]

Bioinformatics: Epitope prediction and homology comparison of Hsp70 500-589 sequences

Data on the protein sequences of *MAP* Hsp70 (DNAK_MYCPA), *MAA* Hsp70 (A0QLZ6_MYCA1), M. bovis (*MBO*) Hsp70 (DNAK_MYCBO), M. leprae (*MLE*) Hsp70 (DNAK_ MYCLE), M. tuberculosis (*MTU*) Hsp70 (DNAK_MYCTU) and E. coli Hsp70 (DNAK_ECOLI) were taken from the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) under the accession numbers; Q00488, A0QLZ6, P0A5C0, P19993, P0A5B9 and P0A6Y8 respectively.

Prediction of human HLA-DR-restricted determinants within the 9 identified *MAP* Hsp70 peptides were performed using ProPred (the MHC class II binding peptide server) (http://www.imtech.res.in/raghava/propred/) [41, 53].
Homology percentages between the complete sequences of *MAP* Hsp70, those of the other 4 mycobacteria spp. listed above and E. coli Hsp70, as well as between the aa region 500-589 of *MAP* Hsp70 (containing the 5 most immunodominant peptides) and the other Hsp70, were determined after multiple sequence alignment by CLUSTALW (http://align.genome.jp/).

RESULTS

Paratuberculosis infection status

The animals used in immunization experiment 1 and 2 remained fecal culture negative throughout the experiment. For the calves experimentally infected with *MAP*, 12 out of 28 animals were fecal culture positive for *MAP* on 1 to 3 of 6 time points tested prior to evaluation of *MAP* Hsp70 specific immune responses.

MAP Hsp70 specific responses in cows after immunization experiment 1 and selection of *MAP* Hsp70 high responder

The first and second immunization of animals in immunization experiment 1 at the age of 2 years induced substantial proliferative responses to the *MAP* Hsp70 protein. In Fig. 1 the proliferative responses to various doses of Hsp70 at day 28, 2 weeks after the second immunization, are shown for the two Hsp70 treatment groups. Whereas the first immunization induced substantial proliferation a clear increased secondary response was not observed following the second immunization. Furthermore *MAP* Hsp70 responses also showed a short maximum response 14-21 days after primary immunization and a rapid decrease to lower memory levels from d30 (data not shown).
The low dose group of adult animals (200 μg) showed a higher average response but also a higher variation in S.I. as compared to the high dose group (500 μg). High proliferative responses were measured in cow 2636 (low dose group) and this cow was selected for further studies. Responses to *MAP* Hsp70 remained high in this animal throughout the follow up period.

Identification of 9 immunogenic *MAP* Hsp70 peptides by proliferative PBMC responses of cow 2636

The high responding animal 2636 was used to test responses against pools of *MAP* Hsp70 peptides. Out of 12 peptide pools, pool 61-70, pool 101-110 and pool 111-

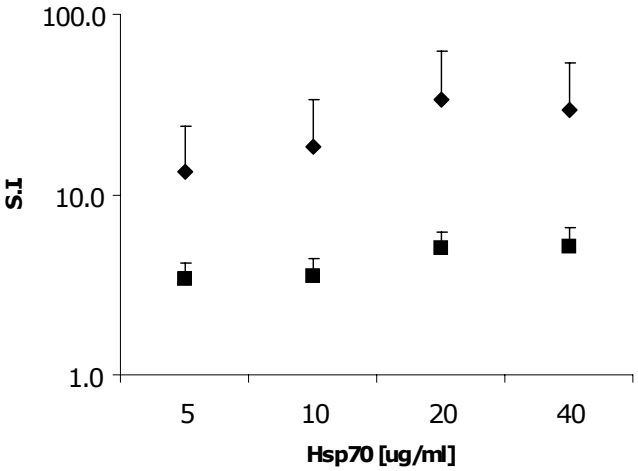


Fig. 1. Selection of the MAP Hsp70 high responder cow 2636. Proliferative responses of PBMC from 2-year-old cows immunized with MAP Hsp70, data for d28 after MAP Hsp70 immunization are shown. Five cows were immunized with 200 µg/ml MAP Hsp70/DDA (♦), including high responder cow 2636, and 5 cows were immunized with 500 µg/ml MAP Hsp70/DDA (■). PBMC with (5, 10, 20 or 40 µg/ml) or without MAP Hsp70 were incubated for 72h at 37°C, then ^{3}H-thymidine was added and 18h later cells were analyzed for ^{3}H-thymidine incorporation as a measure of proliferation. Proliferation is expressed as stimulation index (S.I.) + 1SEM.

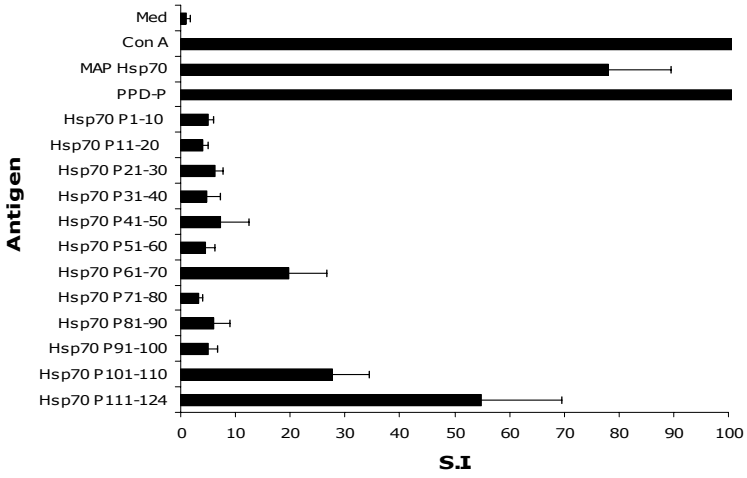
124 were shown to induce strong proliferative responses (Fig. 2A). All 124 peptides were also tested individually and a total of 9 individual peptides (P67, P105 and P108-114 (Table 2), all from pools previously defined as stimulatory, induced proliferative responses (S.I.≥2) (Fig. 2B). From these 9 immunogenic peptides the 5 most dominant peptides (P105, P111, P112, P113 and P114) were selected based on repeated induction of strong proliferative responses.

Analysis of *MAP* Hsp70 peptide specificity and phenotype of T cell lines/clones from animal 2636

Four T cell lines, named 2636-P105.1, 2636-P111.1, 2636-P113.1 and 2636-P113.2 where generated after initial restimulation of PBMC with *MAP* Hsp70, followed by two rounds of restimulation with either peptide P105, P111 or P113 (according to the names of the clones). Phenotyping by flow cytometric analysis showed that they were all $CD4^{+}$ T cell lines. Proliferation experiments revealed a high peptide specific response (64≤S.I.≤1531) of each T cell line to its specific peptide, but not (S.I.=1) to control *MAP* Hsp70 peptides.

From 17 phenotyped T cell clones, stimulated with *MAP* Hsp70 or *MAP and* restimulated with peptide P113, specificity for *MAP* Hsp70 and *MAP* was determined. All 17 clones were $CD4^{+}$ T cells and all proved to be specific for *MAP* Hsp70 and

A



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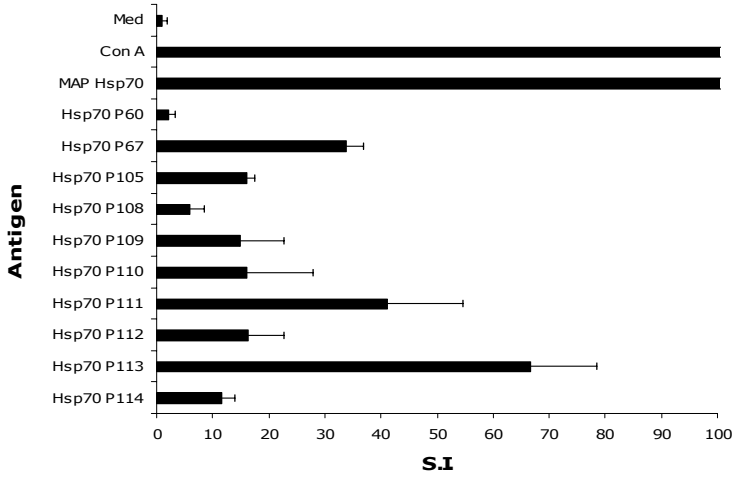
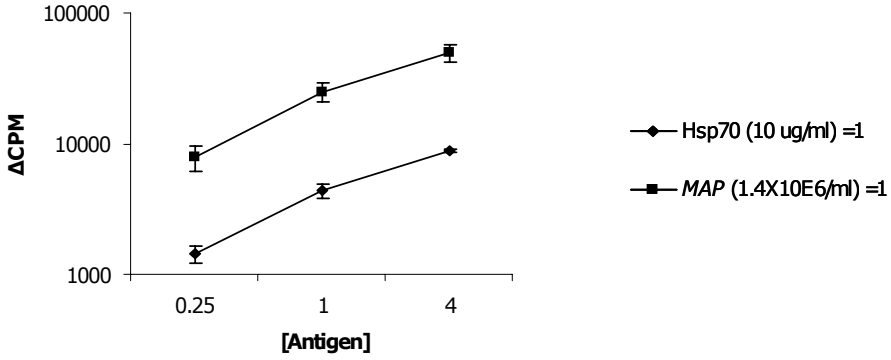


Fig. 2. Selection of immunodominant MAP Hsp70 peptides. (A) Proliferative responses of PBMC from MAP Hsp70 high responder cow 2636 cultured with MAP Hsp70 peptide (P) pools spanning MAP Hsp70, three positive controls: MAP Hsp70, Concanavalin A (Con A) and PPD-P, and medium (med) as a negative control. (B) Proliferative responses (S.I.>2) of PBMC from cow 2636 cultured with individual peptides P1-124 spanning MAP Hsp70, two positive controls: Con A and MAP Hsp70, and two negative controls: med and MAP Hsp70 peptide 60 (Hsp70 P60). PBMC were incubated for 72h at 37°C, ^{3}H-thymidine was added and 18h later cells were analyzed for ^{3}H-thymidine incorporation as a measure of proliferation. Proliferation is expressed as stimulation index (S.I.) + 1SD.

MAP in a dose dependent manner and specific for peptide P113. A typical example is depicted in Fig. 3 showing the specificity of CD4+ T cell clone 2636-P113.3J9 for both *MAP* Hsp70 and *MAP* bacterium in a dose dependent manner (Fig. 3A) and for *MAP* Hsp70 peptide P113, in this case the overlapping sequence P113+1 (aa 559-568) and P114 (Fig. 3B).

A



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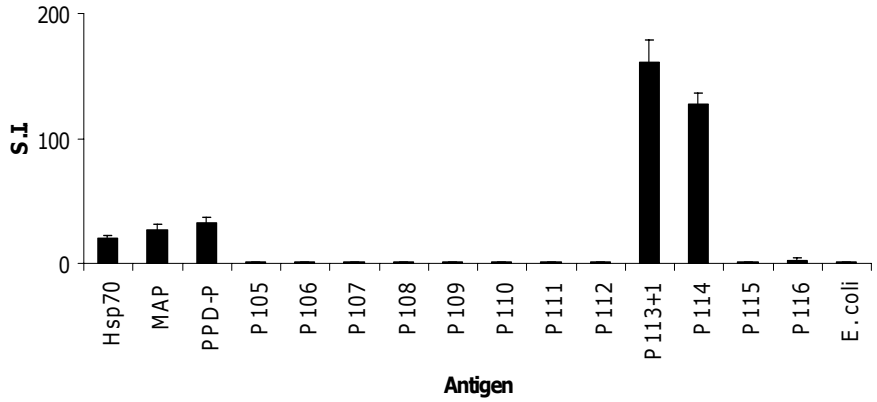

Fig. 3. Characterization of CD4+ T cell clone 2636-P113.3J9. (A) Proliferative dose dependent responses of CD4+ T cell clone 2636-P113.3J9 to MAP bacterium [35] and MAP Hsp70 (Hsp70) expressed as Δ-CPM (cpm stimulated - cpm medium control) ± 1SD and (B) responses for pepscan analysis of P113 compared to MAP Hsp70, MAP bacterium, PPD-P, E. coli and MAP Hsp70 peptides P105-116, expressed as stimulation index (S.I.) + 1SD.

Epitope mapping of 2 *MAP* Hsp70 immunodominant peptides via T cell lines/ clones

Fine tuning of peptides P111 and P113 by pepscan epitope mapping and alanine substitutions using T cell line 2636-P111.1 revealed that the core sequence of the peptide consisted of "**D**AKTA**L**" and the amino acids **D** (Aspartic acid) and **L** (Leucine) as important anchor amino acids for major histocompatibility class II (MHC-II) binding of *MAP* Hsp70 peptide P111. Experiments in which peptides of different lengths were used revealed that the peptide should be larger than 12 aa to induce a proliferative response (Fig. 4). Using T cell lines 2636-P113.1, 2636-P113.2 (Table 2) and T cell clone 2636-P113.3J9 (Fig. 3, Table 2) a "**DI**TAIKSA**ME**" amino acid peptide core sequence was found in which the amino acids **D** (aspartic acid), **I** (iso-Leucine), **M** (methionine) and **E** (glutamic acid) were found to be important anchor amino acids for major histocompatibility class II (MHC-II) binding of *MAP* Hsp70 peptide P113 (data not shown).

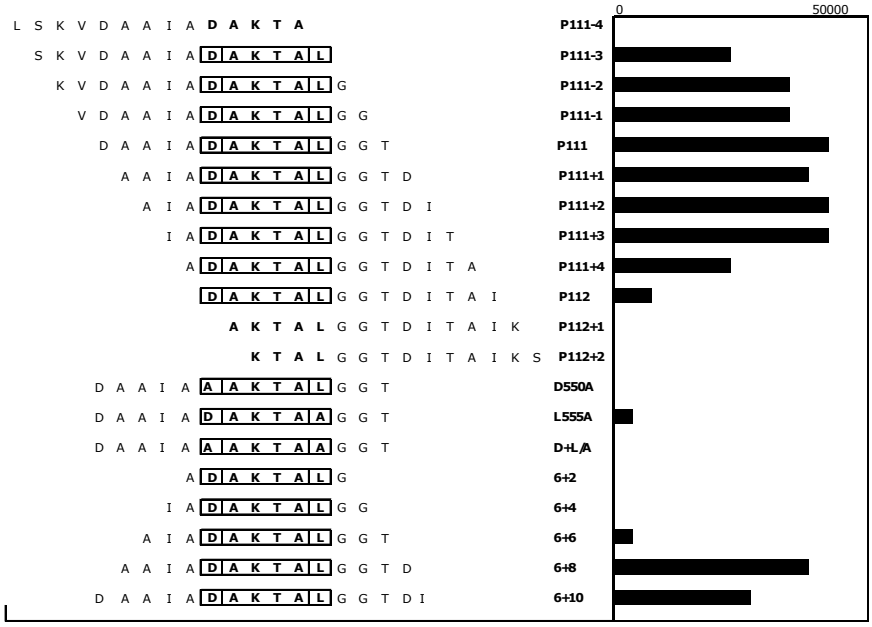

Fig. 4. Pepscan analysis of the $CD4^+$ T cell line 2636-P111.1. Proliferative responses expressed as counts per minute (CPM) for: sequential analogs of MAP Hsp70 peptide P111 (shifted incrementally 1 amino acid (aa) to the right (+1) or 1 aa to the left (-1); P111 alanine (A) substitution analogs (substituting aa 550 (D), 555(L) and 550(D)+555(L) for (A)); and P111 length analogs (peptide core of 6 aa supplemented by 2, 4, 6, 8 or 10 adjacent aa.

Table 2.
Immunogenic pools/peptides of MAP Hsp70 recognized by high responder cow 2636. Immunogenic MAP Hsp70 peptide pools and peptides and amino acid numbering / sequence are shown. The peptide cores are **bold** and the peptide anchors are **bold** + underscored.

Pool	Peptide	Number of amino acids	Amino acids
61-70	P67	326-339	VKELTGGKEPNKGV
101-110	P105	515-528	SLVYQTEKFVKDQR
101-110	P108	530-543	AEGGSKVPEETLSK
101-110	P109	535-548	KVPEETLSKVDAAI
101-110	P110	540-553	TLSKVDAAIADAKT
111-120	P111	545-558	DAAIA**D**AKTA**L**GGT
111-120	P112	550-563	DAKTALGGTDITAI
111-120	P113	555-568	LGGT**DI**TAIKSA**ME**
111-120	P114	560-573	**I**TAIKSA**ME**KLGQE

IFN-γ production by *MAP* Hsp70 peptide specific T cell lines/clones

To determine functional characteristics of the 4 T cell lines and the 17 T cell clones generated, IFN-γ Elispot assays were performed to assess IFN-γ production after Ag specific activation. Concurrently the same T cell lines/clones were also tested in a LST to confirm their peptide specificity (P105, P111, P113).

All cell lines and clones showed a significant increase in amounts of IFN-γ spot forming cells (SFC) and proliferative responses after being stimulated by their specific peptide compared to another *MAP* Hsp70 peptide/Ag or medium control. As a representative example for all peptide specific T cell lines/clones the *IFN-γ* Elispot results of T cell line P113.2 are shown (Fig. 5A). Numerous spots were present after *MAP* Hsp70 peptide P113 (dSFC = 585) and Con A (dSFC = 428) stimulation whereas very few spots were observed after stimulation with another Ag (3-5 SFC) or medium control (3 SFC). In a proliferation experiment performed in parallel (Fig. 5B) *MAP* Hsp70 peptide P113 (82000 cpm), Con A (35000 cpm) and *MAP* Hsp70 (21000 cpm) induced robust proliferation but cpm for the negative controls P112 (26 cpm) or medium (55 cpm) were low.

Responses of PBMC from calves immunized with *MAP* Hsp 70 to 5 immunodominant *MAP* Hsp70 peptides (immunization exp 2)

PBMC from a group of 3 *MAP* Hsp70 immunized (day 0 and 308), *MAP* culture negative, calves showed a tendency for higher proliferative responses to the five

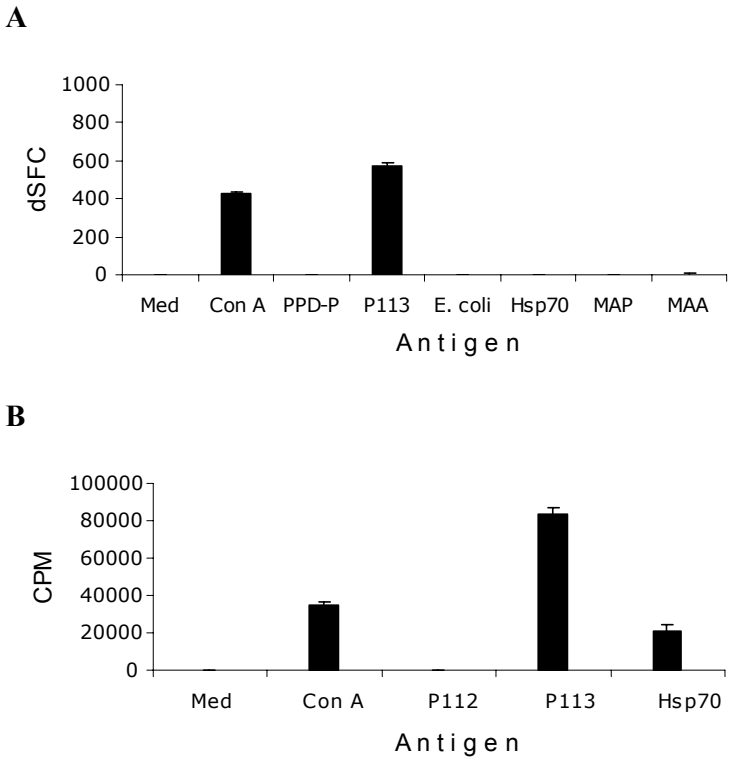

Fig. 5. IFN-γ production and Ag specificity of a MAP Hsp70 peptide P113 antigen specific, $CD4^+$ T cell line 2636-P113.2. (A) IFN-γ production is shown as delta spot forming cells (dSFC) + 1SD following stimulation with concanavalin A (Con A), PPD-P, MAP Hsp70 peptide 113 (P113), E. coli, MAP Hsp70 (Hsp70), MAP and MAA. Medium (Med) was included as a negative control for spontaneous SFC production. (B) Proliferative responses following stimulation with MAP Hsp70 peptide P113 (P113). As positive Ag controls MAP Hsp70 (Hsp70) and concanavalin A (Con A) were included, medium (Med) and MAP Hsp70 peptide P112 (P112) were included as negative controls. Proliferation is expressed as counts per minute (CPM) + 1SD.

most immunodominant *MAP* Hsp70 peptides identified with stimulation indices 1≤S.I.≤7 as compared to CM (S.I.=1) on day 893 (Fig. 6, black bars)**.** The PBMC also exhibited strong proliferative responses to PPD-P, *E. coli* and *MAP* (68≤S.I.≤207) (data not shown). PBMC from 3 untreated (not *MAP* Hsp70 immunized) cows, raised under conventional housing conditions showed low responses to one of the five *MAP* Hsp70 peptides or *MAP* Hsp70 (1≤S.I.≤3) as compared to CM (S.I.=1) (Fig. 6, open bars). They did, however, show proliferative responses to PPD-P, *E. coli* and *MAP* (14≤S.I.≤45) (data not shown).

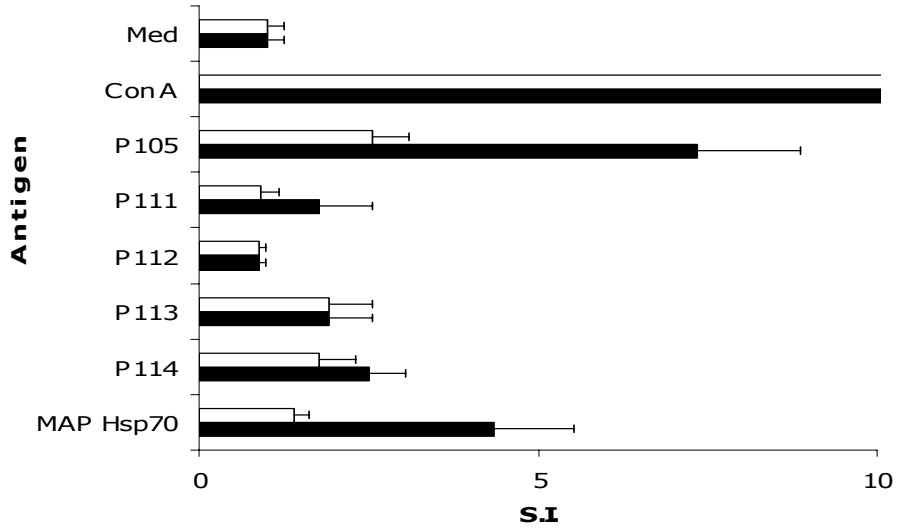

Fig. 6. Ag specific proliferative responses in animals without and after MAP Hsp70 immunization. Mean proliferative responses of PBMC, tested on day 893, from 3 conventionally housed untreated cows (open bars) and 3 MAP Hsp70 immunized cows (immunized on days 0 and 308; black bars) cultured with MAP Hsp70 and the 5 most dominant peptides (P) of MAP Hsp70: P105, 111, 112, 113 and 114. Concanavalin A (Con A) was used as a positive control and medium (Med) was used as negative control. After 72h at 37°C ^{3}H-thymidine was added and 18hrs later ^{3}H-thymidine incorporation was measured. Proliferation is expressed as mean stimulation index (S.I.) + 1SEM.

BoLA MHC class I + II typing and proliferative responses of PBMC of animals experimentally infected with *MAP*

Of 28 experimentally *MAP* infected animals 26 were typed for BoLA and animals with 15 different MHC genotypes, combinations of two MHC class I + II haplotypes, were identified (Table 3). From these 26 typed animals, 17 (65%) had one of six common MHC I/II genotypes; AH11-DRB3*0101/AH13-DRB3*2703, AH11-DRB3*0101/AH15-DRB3*1101, AH13-DRB3*2703/AH20-DRB3*1201, AH14-DRB3*0902/AH20-DRB3*1201, AH15-DRB3*1101/AH20-DRB3*1201 or AH20-DRB3*1201/AH20-DRB3*1201. The immunized high *MAP* Hsp70 responder animal 2636 was identified to be AH11-DRB3*0101/AH15-DRB3*1101 and showed high proliferative responses to all five peptides and *MAP* Hsp70 (Table 3).

Peptide 105, peptide 113 and complete *MAP* Hsp70 protein tended to stimulate the strongest proliferative responses in infected animals. Proliferative responses (S.I.≥2) to peptide 105 (17≤S.I.≤31) were observed in 82% of the infected animals and to peptide 113 (7≤S.I.≤12) in 68% of the infected animals. Proliferative responses

(S.I.≥2) to peptides P111, 112, and P114 (2≤S.I.≤13) were observed in 14-36% of the infected animals. The frequency of proliferative responses (S.I.≥2) to *MAP* Hsp70 (6≤S.I.≤11) in these animals was 43% (Fig. 7A and Fig. 7B). After coupling infection status and proliferative responses to *MAP* Hsp70 we found that 25% of all animals remained fecal culture negative and these animals also showed no proliferative response to *MAP* Hsp70 (data not shown).

Table 3.

BoLA MHC Class I and II types and MAP Hsp70 and MAP Hsp70 peptide specific proliferative responses after experimental MAP infection. Proliferative responses by PBMC of 28 MAP challenged cows and MAP Hsp70 high responder animal 2636 (K2636) to five dominant MAP Hsp70 peptides (P105, P111-P114) and MAP Hsp70 (Hsp70). Proliferative responses are expressed as stimulation index (S.I.) and combined with BoLA class I and II types determined in microarray analysis.

Cow	Class I	DRB3*	Haplotype #1	Haplotype #2	P105	P111	P112	P113	P114	Hsp70
K1406	AH11/AH12	*0101/*1701	AH11-DRB3*0101	AH12-DRB3*1701	3,0	1,1	0,7	2,1	3,3	0,8
K0467	AH11/AH13	*0101/*2703	AH11-DRB3*0101	AH13-DRB3*2703	3,9	1,5	0,9	2,5	2,6	1,1
K1372	AH11/AH13	*0101/*2703	AH11-DRB3*0101	AH13-DRB3*2703	30,0	0,6	1,0	4,3	18,1	11,9
K0463	AH11/AH15	*0101/*1101	AH11-DRB3*0101	AH15-DRB3*1101	0,7	0,7	0,3	0,9	0,3	0,7
K1375	AH11/AH15	*0101/*1101	AH11-DRB3*0101	AH15-DRB3*1101	6,7	1,3	1,4	3,6	1,0	2,8
K2636	*AH11/AH15*	**0101/*1101*	*AH11-DRB3*0101*	*AH15-DRB3*1101*	*14,0*	*41,1*	*16,2*	*66,6*	*11,5*	*149,0*
K0460	AH11/AH20	*0101/*1201	AH11-DRB3*0101	AH20-DRB3*1201	4,9	1,7	2,9	3,2	1,1	1,7
K1407	AH12/AH13	*1701/*2703	AH12-DRB3*1701	AH13-DRB3*2703	13,0	0,8	1,0	1,9	2,5	4,5
K1409	AH12/AH19	*0901/*0701	AH12-DRB3*0901	AH19-DRB3*0701	3,1	0,9	2,2	2,2	1,0	1,4
K0470	AH12/AH20	*1701/*1201	AH12-DRB3*1701	AH20-DRB3*1201	3,8	0,6	0,9	3,3	0,9	1,5
K1376	AH13/AH14	*2703/*0902	AH13-DRB3*2703	AH14-DRB3*0902	0,7	0,3	0,4	0,7	0,4	0,5
K0465	AH13/AH20	*2703/*1201	AH13-DRB3*2703	AH20-DRB3*1201	29,0	17,1	1,5	7,8	28,4	44,4
K1371	AH13/AH20	*2703/*1201	AH13-DRB3*2703	AH20-DRB3*1201	14,1	1,3	1,2	3,4	2,9	2,1
K1381	AH14/AH19	*14011/*0701	AH14-DRB3*14011	AH19-DRB3*0701	1,8	1,0	0,9	1,2	0,7	0,8
K0466	AH14/AH20	*0902/*1201	AH14-DRB3*0902	AH20-DRB3*1201	1,4	1,0	1,4	1,2	0,9	1,3
K1374	AH14/AH20	*0902/*1201	AH14-DRB3*0902	AH20-DRB3*1201	37,1	1,1	1,0	30,0	1,2	1,4
K1379	AH14/AH20	*0902/*1201	AH14-DRB3*0902	AH20-DRB3*1201	2,4	0,8	9,5	1,1	1,0	0,8
K1382	AH14/AH20	*0902/*1201	AH14-DRB3*0902	AH20-DRB3*1201	17,9	1,4	1,2	6,7	1,1	3,3
K0462	AH15/AH20	*1101/*1201	AH15-DRB3*1101	AH20-DRB3*1201	18,5	0,9	0,9	15,9	2,2	2,5
K0468	AH15/AH20	*1101/*1201	AH15-DRB3*1101	AH20-DRB3*1201	73,6	18,0	1,1	36,0	2,0	2,0
K0469	AH15/AH20	*1101/*1201	AH15-DRB3*1101	AH20-DRB3*1201	45,9	1,0	1,1	27,0	1,1	4,8
K1380	AH15/AH20	*1101/*1201	AH15-DRB3*1101	AH20-DRB3*1201	9,2	0,9	1,0	1,6	0,7	1,4
K0459	AH20/	*1201/	AH20-DRB3*1201	AH20-DRB3*1201	0,7	0,7	1,1	1,5	0,9	0,8
K1377	AH20/	*1201/	AH20-DRB3*1201	AH20-DRB3*1201	27,6	1,2	1,1	10,4	1,5	27,5
K1378	AH20/	*1201/	AH20-DRB3*1201	AH20-DRB3*1201	11,0	0,8	0,8	2,1	0,6	1,3
K0461	AH20/Blank	*1201/*14011	AH20-DRB3*1201	Blank-DRB3*14011	147,7	11,6	28,1	36,1	113,0	49,4
K0464	AH20/Blank	*1201/*2703	AH20-DRB3*1201	Blank-DRB3*2703	28,7	13,1	26,5	17,4	44,6	37,5
K1373	ND	ND	ND	ND	111,1	0,9	0,8	33,7	1,0	18,2
K1408	ND	ND	ND	ND	2,1	0,6	0,8	1,6	0,9	1,0

MHC class II restricted *MAP* Hsp70 peptide presentation and T cell recognition

FACS sorted $CD4^+$ T cells, of the high responder cow (2636), showed dose dependent proliferative responses to Con A (Con A / 0, 2.5, 5 and 10 µg/ml) and, in the absence of APC, no responses to the *MAP* Hsp70 P105 peptide (0, 2.5, 5 and 10 µg/ml) alone (Fig. 8A). The T cells were furthermore co-cultured with FACS sorted $CD14^+$ APC from two animals that were 100% BoLA MHC class II matched, two animals that were 100% BoLA MHC class II mismatched and one animal that was 50% matched in the presence of 0 - 10 ug/ml P105. Positive S.I. were measured after presentation by 100 or 50% matched animals (dose dependent S.I. as high as S.I. = 29) and

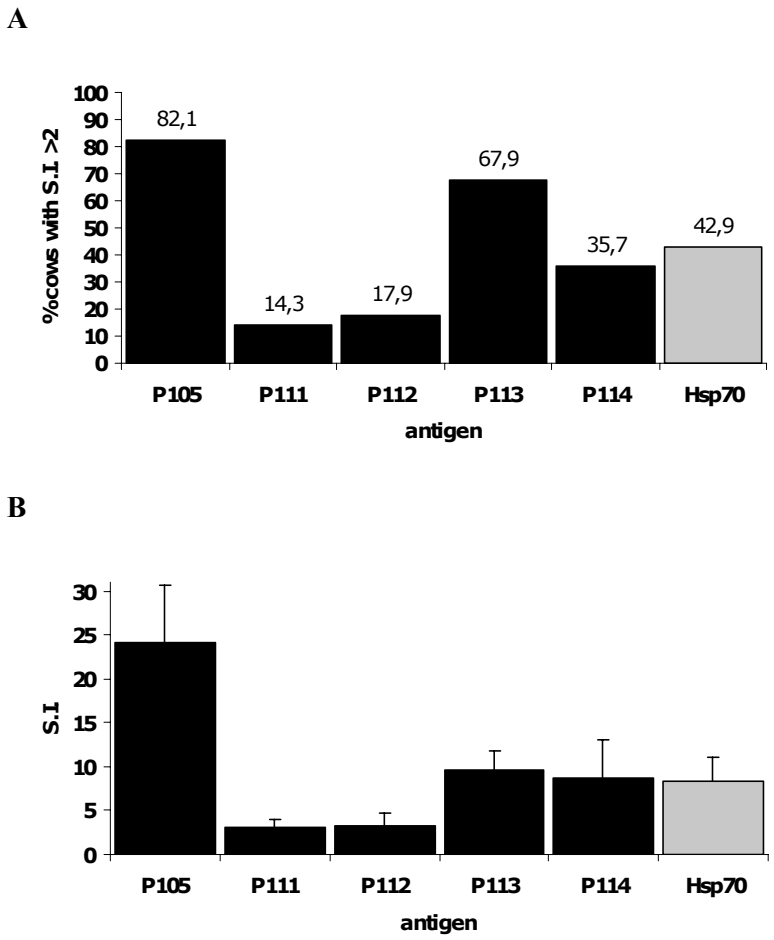

Fig. 7. MAP Hsp70 and MAP Hsp70 peptide specific proliferative responses after experimental MAP infection. (A) Proliferative responses of PBMC from 28 conventionally housed MAP challenged cows cultured with different Ag, peptides (P) 105, 111, 112, 113 and 114 of MAP Hsp70 expressed as percentages and (B) expressed as stimulation index (S.I.) + SEM of cows with S.I.≥2. PBMC were stimulated for 72 hours at 37°C thereafter ^{3}H-thymidine was added and 18h later ^{3}H-thymidine incorporation was measured.

negative or low S.I. by 100% mismatched animals (Fig. 8B). Co-cultures incubated with anti-bovine MHCII mAb IL-A21 (10 μg/ml – IgG2a) showed complete blocking of P105 stimulation (all S.I. < 2) (Fig. 8C). Incubation of co-cultures with isotype matched murine IgG2a control mAb (10 μg/ml) resulted in dose dependent responses for the 100 or 50% MHC matched animals (as high as S.I. = 11) but no response for the 100% mismatched animals (≤ S.I. = 3 / Fig. 8D).

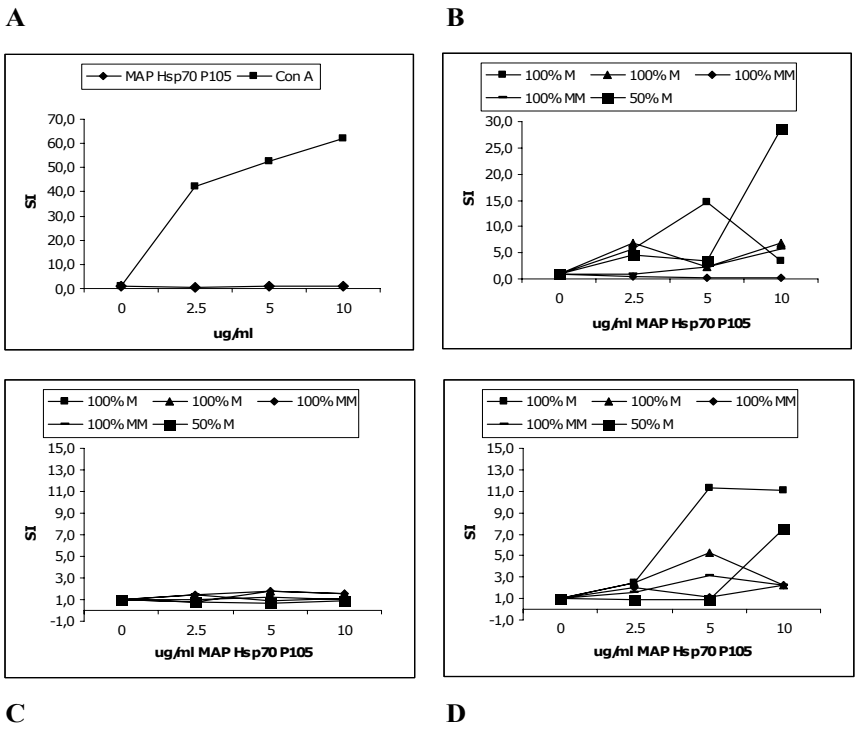

Fig. 8. MHC class II restricted MAP Hsp70 peptide presentation and T cell recognition.
(A) Responses of FACS sorted $CD4^+$ T cells from a high responder cow (2636 / DRB3*0101/*1101), stimulated by MAP Hsp70 peptide P105 (0, 2.5, 5 and 10 µg/ml) and concanavalin A (Con A / 0, 2.5, 5 and 10 µg/ml) in the absence of APC. (B) These same T cells co-cultured with FACS sorted $CD14^+$ APC from two 100% BoLA MHC class II matched animals (100% M; 1375 and 0076 DRB3*0101/*1101), two 100% BoLA MHC class II mismatched animals (100% MM; 0461 DRB3*1201/*14011 and 0464 DRB3*1201/*2703) and one 50% BoLA MHC class II matched animal (50% M; 0469 DRB3*1101/*1201). Co-cultures as in B were in the presence of (C) anti-bovine MHCII mAb IL-A21 (10 µg/ml) and (D) isotype matched murine IgG2a control mAb (10 µg/ml). After 72h of culture at 37°C ^{3}H-thymidine was added and 18h later ^{3}H-thymidine incorporation was measured. Proliferation is expressed as stimulation index (S.I.).

Bioinformatics: Epitope prediction and homology comparison of *MAP* Hsp70 and *MAP* Hsp70 500-589 sequences

Bioinformatics tools for in silico analysis and prediction of bovine class II restricted *MAP* Hsp70 epitopes are not yet available. Consequently, we choose to start with in vitro screening to identify peptides that are immunogenic after *MAP* Hsp70 immunization and after infection by *MAP*. Subsequently, we compared these empirically defined peptides with predictions made by the ProPred program, predicting human HLA-DR-binding determinants which are known to be structurally related to

BoLA-DR. Protein sequences of *MAP* Hsp70, 4 mycobacterial spp. Hsp70 and *E. coli* Hsp70 were used to predict HLA-DR-restricted determinants and for homology studies. The ProPred software predicted 4 out of 9 (44%) experimentally defined peptides to be HLA-DR binding motifs as can be seen in Table 4**.** No human HLA-DR binding motifs were predicted for the *MAP* Hsp70 P67, P108, P109, P110, and P111 peptides. After submitting the sequences of *MAP* Hsp70 P105, P112, P113 and P114 peptides presentation by 3 DR1 alleles (DRB5*0101, *0105 and DRB1*0102), 2 DR4 alleles (DRB1*0405 and *0410) and 2 DR7 alleles (DRB1*0701 and *0703) was predicted (Table 4)

Table 4.

MAP Hsp70 peptide numbers selected as proliferation inducing peptides and ProPred HLA-DR binding predictions. Peptide numbers and sequences for experimentally identified immunogenic peptides and ProPred HLA-DR binding predictions (ProPred threshold: 3% threshold = percentage of best scoring natural peptides) that correspond to immunogenic MAP Hsp70 peptides identified by pepscan analysis are shown. The peptide cores are in **bold** and the peptide anchors are in **bold** + underscored in the experimental identified as well for the ProPred predicted peptides.

Peptide number	**Peptide sequences**	**HLA-DR**
MAP Hsp70 P67	VKELTGGKEPNKGV	None predicted
MAP Hsp70 P105	SLVYQTEKFVKDQR	DRB1_0102: S**L**<u>**L**</u>**VYQTEKFVK**DQR DRB5_0101: SL<u>**V**</u>**YQTEKFVK**DQR DRB5_0105: SL<u>**V**</u>**YQTEKFVK**DQR
MAP Hsp70 P108	AEGGSKVPEETLSK	None predicted
MAP Hsp70 P109	KVPEETLSKVDAAI	None predicted
MAP Hsp70 P110	TLSKVDAAIADAKT	None predicted
MAP Hsp70 P111	DAAIA<u>**D**</u>**AKTA**<u>**L**</u>GGT	None predicted
MAP Hsp70 P112	DAKTALGGTDITAI	DRB1_0701: DAKTA<u>**L**</u>**GGTDITAI** DRB1_0703: DAKTA<u>**L**</u>**GGTDITAI**
MAP Hsp70 P113	LGGT<u>**DI**</u>**TAIKSA**<u>**ME**</u>	DRB1_0405: LGGTD<u>**I**</u>**TAIKSAME** DRB1_0410: LGGTD<u>**I**</u>**TAIKSAME** DRB1_0701: <u>**L**</u>**GGTDITAI**KSAME DRB1_0703: <u>**L**</u>**GGTDITAI**KSAME
MAP Hsp70 P114	<u>**I**</u>**TAIKSA**<u>**ME**</u>KLGQE	DRB1_0405: <u>**I**</u>**TAIKSAME**KLGQE DRB1_0410: <u>**I**</u>**TAIKSAME**KLGQE

Homology identification by sequence alignment between 5 mycobacterial and *E. coli* Hsp70 sequences, and especially the 500-589 aa region (containing 5 out of 9 immunodominant peptides in *MAP* Hsp70), was performed to determine potential cross reactivity with the corresponding regions of the bacterial Hsp70 sequences. Percentage homology, after alignments between the complete sequences of *MAP* Hsp70 (DNAK_MYCPA) and that of the other 4 *Mycobacterium spp.* and *E. coli* Hsp70, proved to be for *MAA* 99.8% (A0QLZ6_MYCA1), *MLE* 93.5% (DNAK_ MYCLE), *MTU* 92.5% (DNAK_MYCTU), *MBO* 92.4% (DNAK_MYCBO) and *E. coli* 53.7% (DNAK_ECOLI).

A dendrogram depicting evolutionary changes from ancestral to descendant forms, based on shared homology was designed for all the used Hsp70/DnaK (aa 500-589) sequences (Fig. 9).

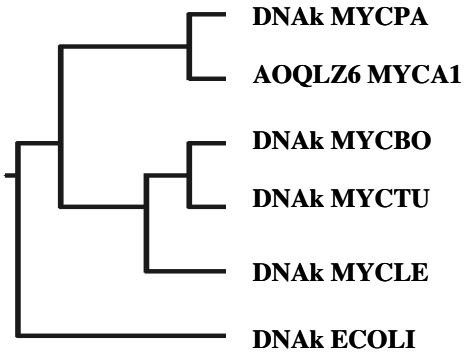

Fig. 9. Dendrogram designed after CLUSTALW analysis of aa 500-589 Hsp70 (DNAk) sequences. Comparison of Hsp70 aa sequences from M. avium paratuberculosis (DNAk MYCPA), M. avium avium (A0QLZ6 MYCA1), M. bovis (DNAk MYCBO), M. tuberculosis (DNAk MYCTU), M. leprae (DNAk MYCLE) and E. coli (DNAk ECOLI). For this region of Hsp70, two pairs of sequences had 100% homology: M. avium paratuberculosis and M. avium avium, as well as M. bovis and M. tuberculosis.

Homology between the 500-589 regions of HSP 70 of *MAP* (DNAK_MYCPA, containing the 5 most immunodominant peptides), the other 4 *Mycobacterium spp.* and *E. coli* Hsp70 proved to be for *MAA* 100% (A0QLZ6_MYCA1), *MTU* 87.8% (DNAK_MYCTU), *MBO* 87.8% (DNAK_MYCBO), *MLE* 85.5% (DNAK_MYCLE) and *E. coli 30*% (DNAK_ECOLI) (Fig. 10).

Within the 500-589 regions of *MAP*/*MAA* Hsp70 the sequence homology of the 5 identified dominant *MAP* Hsp70 peptides were compared to determine the epitopes sequence homology between the bacterial Hsp70. We found 11 aa of the *MTU* and

MBO sequence to be different (12%), 7 of these 11 different aa (64%) were part of 1 of the 5 dominant *MAP* Hsp70 peptide sequences. In *MLE* 13 aa were different (14%) and 7 of these 13 aa (54%) were part of 1 of the 5 dominant *MAP* Hsp70 peptide sequences. Furthermore in all three mycobacterial Hsp70 sequences a significant difference was detected at *MAP*/*MAA* Hsp70, aa 550 (D=aspartic acid), with the anchor aa for binding peptide P111 being exchanged for E=glutamic acid. Finally in the *E. coli* Hsp70 90 aa sequence, 63 aa were different from *MAP*/MAA (70%) and of these 63 aa, 28 aa (44%) were part of one of the 5 dominant *MAP* Hsp70 peptide sequences including exchanges of the anchor aa 550, 560 and 568 from the core of P111 and 113 (Fig. 10).

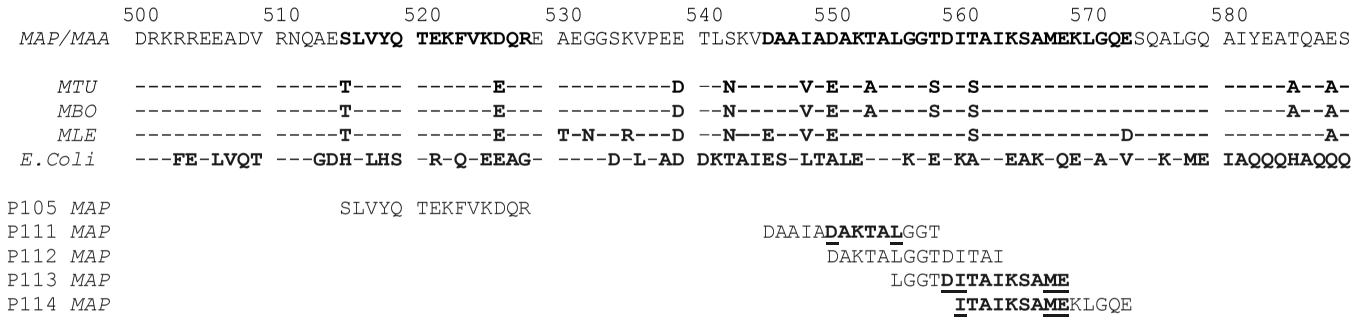

Fig. 10. CLUSTALW analysis of aligned amino acid 500-589 Hsp70=DNAk sequence. Alignment of M. tuberculosis (MTUB), M. bovis (MBOV), M. leprae (MLEP) and E. coli compared to M. avium paratuberculosis [35] / M. avium avium (MAA) (100 % homology) Hsp70 (DNAk) sequences (amino acids 500-589). In the MAP/MAA sequences the 5 most immunodominant peptides are in **bold.** In the aligned MTUB, MBOV, MLEP and E. coli DNAk amino acid differences are in **bold**, and in the sequences of the 5 most dominant peptides (P105, 111, 112, 113 and 114) of MAP mapped epitope cores are in **bold** and anchors **bold** + underscored.

DISCUSSION

To our knowledge this is the first report describing *MAP* Hsp70 specific peptide epitopes recognized by $CD4^+$ T cells from outbred cattle experimentally infected with *MAP* or immunized with *MAP* Hsp70. Recently we found that Hsp70 could be successfully used as a subunit vaccine against bovine paratuberculosis, significantly reducing shedding of bacteria in feces during the first 2 years following experimental infection [19]. Hsp are known to be very immunogenic/immunodominant antigens and have been shown to be recognized in many infectious diseases (reviewed by Zugel in 1999 [55]) including mycobacterial diseases such as tuberculosis, leprosy [1, 15], bovine paratuberculosis [20] and bovine tuberculosis [42]. Definition of kinetics/mechanism and fine tuning the specificity of the priming of cellular immunity, which is thought to be most effective towards paratuberculosis infection [18, 20], are instrumental to further develop *MAP* Hsp70 subunit vaccination [19]. In order to study the fine specificity of *MAP* Hsp70 specific cellular responsiveness, a *MAP* Hsp70 immunized subunit vaccine using DDA as a Th1 skewing adjuvant [26] high responder cow was selected for primary identification of T cell activating peptide epitopes. Overlapping synthetic peptides, covering the complete *MAP* Hsp70 sequence, revealed 9 peptides that reproducibly induced proliferation of PBMC. Recognition of P105 and *MAP* Hsp70 was confirmed in Hsp70 immunized animals. The immunized animals exhibited antigen specific T cell responses at d893, 585 days after being boosted on d308, that were absent in sham treated animals. In immunized animals (immunization 1), maximum *MAP* Hsp70 responses were observed 14-21 days after primary immunization/boost with a rapid decrease to lower memory levels (data not shown).

Using 5 immunodominant peptides 21 *MAP* Hsp70 specific T cell lines/clones were generated. All of the lines/clones produced IFN-γ and were comprised of peptide specific $CD4^+$ Th1 cells. The importance of such Th1 cells in the immune response to mycobacterial infections has been well established in small-animal models as well as in natural host species, like cattle infected with *M. bovis* (*MBO*) [51] or *MAP* [17].

In order to asses potential promiscuity, defined as the recognition of a peptide in the context of more than one BoLA class II molecule, experiments were performed to confirm BoLA class II binding and epitope mapping. Robust proliferative responses of CD4+ T cell lines to 14 aa and 16 aa peptides and weak responses to 12 aa peptides indicated binding to and presentation by MHC class II molecules [36]. Furthermore, testing activation of *MAP* Hsp70 specific T cell lines/clones via pepscan techniques and alanine substitutions revealed that the amino acid peptide core sequences of the immunodominant *MAP* hsp70 peptides P111 (**DAKTAL**) and P113 (**DITAIKSAME**)

contain important anchor (binding pocket) amino acids (underscored) involved in major histocompatibility class II (MHC-II) binding..

BoLA-DR and HLA-DR are known to be structurally related orthologous loci and show high sequence similarities (DRA and DRB) justifying the usage of human HLA-DR targeting computer programs to predict bovine epitopes, as no prediction tools are available to predict BoLA class II-restricted peptides [53]. To identify peptide epitopes that might bind to BoLA class II motifs, sequence analysis was performed using the virtual-matrix-based HLA-DR prediction program ProPred [41] which identifies promiscuous binding regions in proteins useful in selecting peptide vaccine candidates like those identified in *MBO* [53] and *MTU* [29]. For a few HLA-DR molecules similar detailed motifs are known for HLA-DR1, DR3 and DR4 MHC subtypes [33] often associated with autoimmune and infectious diseases. T cell recognition of *MLE* and *MTU* Hsp70 is often restricted in the context of DR3 MHC subtypes known to bind mycobacterial Hsp70 peptides [1, 30]. However, other HLA-DR (DR1, 2, 5,7, DRw53 and DRw52) phenotypes are described, which enable the discrimination between *MTU* and *MLE* [31].

The *MAP* Hsp70 peptide P111 was not predicted to be a MHC class II (HLA-DR3) binding peptide in the ProPred analysis, although the binding motif of peptide *MAP* Hsp70 P111 is identical to the binding motif known for HLA-DR3 (HLA-DRB1*0301 alias DR3w17) [33]. This is probably due to the obligatory P1 anchor residues allowed for HLA-DR3 in the ProPred motif according to Sturniolo et al. [46]. Peptide P113 was predicted to bind 2 DR4 and 2 DR7 MHC class II alleles. No human HLA-DR binding was predicted for the *MAP* Hsp70 peptides P67, P108, P109 and P110. After submitting the sequences of *MAP* Hsp70 P105, P112, and P114 peptides we found 3 DR1 alleles, 2 DR4 alleles and 2 DR7 alleles (Table 4). No DR3 MHC types were predicted to bind the 9 identified *MAP* Hsp70 peptides.

The range of BoLA subtypes able to present the 5 most immunodominant peptides was assessed using 28 *MAP* infected, MHC typed cows. A short oligonucleotide microarray was used for DRB3 typing; DRB3 is known to be the most polymorphic bovine MHC class II gene [32]. Promiscuity was examined in this outbred cohort of 28 *MAP* infected cows; we confirmed that the 5 most immunodominant peptide epitopes were recognized by T cells from cows with a broad range of BoLA class II MHC subtypes. Of the 28 animals 26 were typed for BoLA, 15 different combinations of MHC class I/II were identified, proving that despite controlled breeding cattle still exhibit high MHC diversity [12]. Responses to the 5 immunodominant peptides ranged from 14 – 82%, with P105 being recognized by 82% of the animals. Due to the nature of our screening (5 immunodominant peptides from 9 identified peptides determined

in a *MAP* Hsp70 high responder animal) additional epitopes may be present in the Hsp70 molecule, possibly resulting in an underestimation of the percentage of animals responding to Hsp70. Furthermore, this may be an underestimate because many of the animals that failed to respond to P105 (S.I. < 2) also failed to respond to *MAP* Hsp70 (S.I. < 2) and were fecal culture negative. The fecal culture negative animals that failed to respond to *MAP* Hsp70 had probably cleared the *MAP* infection or failed to become experimentally infected. When animals not responding to *MAP* Hsp70 are ignored, 100% of the remaining animals responded (S.I. ≥ 2) to one or more of the 5 immunodominant peptides. These results clearly indicate that when outbred cattle are infected with *MAP*, the immunodominant peptides recognized by the *MAP* Hsp70 immunized animal, stimulate T cell proliferation in a high percentage of animals.

Finally the binding and presentation of peptide by BoLA class II was tested using proliferation assays in which T cell recognition of peptide P105, the most promiscuous BoLA class II binding peptide, was assessed in the context of matched and mismatched $CD14^+$ APC. This experiment showed that peptide P105 was recognized by the TCR of sorted $CD4^+$ helper T cells from high responder animal 2636 only when peptide was presented in the context of matched BoLA class II isoforms. Blocking the MHC class II binding by a mAb confirmed the class II binding of this *MAP* Hsp70 peptide. The apparent promiscuity of 5 immunodominant epitopes from *MAP* Hsp70 and confirmation of binding/ presentation by BoLA class II, confirms the suitability of *MAP* Hsp70 as a subunit vaccine for outbred cattle.

In order to investigate the biological specificity of the 5 immunodominant *MAP* Hsp70 peptides, sequence homology was studied by alignment between 5 mycobacterial Hsp70 sequences and an *E. coli* Hsp70 sequence. Special attention was given to the 500-589 aa region containing the 5 most immunodominant peptides out of the 9 immunogenic peptides in *MAP* Hsp7. Both sequence similarity and potential cross reactivity between the corresponding regions of the bacterial Hsp70 sequences were examined. As proteins or genes are derived from the same ancestral gene or protein, homologous sequences of closely related organisms are usually very similar. Alignment of the Hsp70 sequence of *MAP/MAA* (99.8% homology), with *MBO*, *MTU* and *MLE* showed homology of 92 to 94%, while the similarity between *MPA* and *E. coli* was 53%. This demonstrates the conserved nature of these immunodominant proteins. Nevertheless, for the *MAP* Hsp70 500-589 aa region containing the 5 most dominant peptides there was 100% homology between *MAP and MAA*, while homology with *MBO*, *MTU* and *MLE* was 85 to 89% and homology with *E. coli* was only 30%. Most strikingly twice as many aa substitutions were observed in the 500-589 aa sequence compared to the mean number of substitutions per 90 aa in

MAP Hsp70, and of the variable aa in this region 54-64% were part of one of the 5 identified immunodominant *MAP* Hsp70 peptides. These results indicate that this is a rapidly evolving region of the Hsp70 gene of these mycobacterial strains. This rapid evolution may be due to the influence of immune responses of infected individuals to these immunodominant peptides giving rise to mycobacterial strain Hsp70 specific peptides. Nonetheless, we acknowledge that prediction of species specificity by comparing sequence identity/homology alone is not sufficient and that individuals with diverse major histocompatibility complex constellations need to be tested to characterize the cross-reactivity or species specificity of peptide-based reagents [52].

In conclusion, the results of this study indicate that immunization with r*MAP* Hsp70/DDA leads to activation of Th1 type T cells with specificity for a limited number of epitopes. These peptide epitopes were also recognized by a large proportion of outbred *MAP* infected animals, indicating that the epitopes are relevant targets during infection. Combined these results support previous data indicating the potential of the *MAP* Hsp70 subunit vaccine in outbred cattle as a way to control bovine paratuberculosis.

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4

Chapter 4

Identification of B cell epitopes of *Mycobacterium avium ssp. paratuberculosis* 70 kD heat shock protein

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SUMMARY

Paratuberculosis is a chronic mycobacterial infection of the small intestine of ruminants that causes substantial economic losses at farm level worldwide, particularly in cattle. A previous study with a novel Hsp70/DDA subunit vaccine showed a significant reduction in fecal shedding. The main post-immunization immune response to *MAP* Hsp70 was a pronounced antibody production against *MAP* Hsp70. The aim of the current study was to determine which immunogenic regions of *MAP* Hsp70 protein are recognized using different 70kD heat shock proteins and to map the B cell epitopes using synthetic peptides. In conclusion this study demonstrated that at least two dominant linear B cell epitopes are present in the Hsp70 molecule as determined by monoclonal antibodies. These epitopes are also immunogenic in vaccinated calves and goats, and are accessible to antibodies under a number of denaturing, non-denaturing, and native conditions. This study also provided indications that the Hsp70 protein is present in the bacterial cell wall of *MAP* and it may be argued that vaccination induced antibodies can bind intact bacteria and play a direct role in the protective effect shown after Hsp70/DDA subunit vaccination against bovine paratuberculosis.

INTRODUCTION

Paratuberculosis is a chronic mycobacterial infection of the small intestine of ruminants that causes substantial economic losses at farm level worldwide, particularly in cattle. In addition it may represent a food safety issue due to transmission of the causative organism *Mycobacterium avium* ssp. *paratuberculosis (MAP)*, to humans via dairy products.

Transmission of *MAP* occurs by excretion in feces into the environment where it may survive for prolonged periods of time. When the disease progresses towards the clinical stage of infection, *MAP* may also be present in milk.

Currently a vaccine which can be used to control paratuberculosis in cattle is not available since the whole cell vaccine registered for use in sheep interferes with the control programs against bovine tuberculosis. Individual *MAP* proteins as subunit vaccine candidates may overcome this interference. Previously we documented immune responses to mycobacterial heat shock proteins (Hsp) in the various stages of disease after natural infection of cattle with *MAP* [16].

The expression of the Hsp, a family of evolutionary conserved proteins, in both prokaryotic and eukaryotic organisms is upregulated during the cellular stress that occurs in both host and pathogen in response to infection and inflammation.

In bovine paratuberculosis [17, 16], similar to other mycobacterial diseases such as tuberculosis and leprosy, Hsp elicit strong cell mediated and antibody responses. Our studies indicated that immune responsiveness to recombinant *MAP* Hsp70 proteins in naturally infected animals was predominantly cell mediated. In contrast, the predominant immune reactivity observed against recombinant *MAP* Hsp65 was antibody production and to a far lesser extend cell mediated responsiveness [14, 17]. Since protective immunity to the intracellular mycobacterial pathogens is thought to be cell mediated, recombinant *MAP* Hsp70 protein was used as a subunit vaccine in cattle prior to experimental infection with *MAP*. It induced protection as indicated by significantly reduced bacterial shedding [15], and prevented occurrence of clinical disease (unpublished observations). Surprisingly and in strong contrast with our previous observations in field cases of bovine paratuberculosis the hall marks of the post-immunization immune response to *MAP* Hsp70 in this immunization-challenge study were the lack of a cell mediated response against *MAP* Hsp70 and pronounced antibody production specific for the protein [15].

The contribution of antibodies to protection against mycobacterial infections is considered minimal by some (reviewed in [2, 13]), but not all (reviewed in [11]). Most of the recent studies on serum therapy of *M. tuberculosis (MTb)* infection report protective effects of antibodies specific for polysaccharide cell wall antigens such as lipoarabinomannan (reviewed in [12]). A monoclonal IgA antibody directed against a small surface-expressed mycobacterial heat shock protein (the 16kD α-crystallin homologue) also confers passive protection against early infection of murine lungs with tuberculosis [23]. Thus we hypothesize that Hsp70 specific antibodies may be relevant in the protection observed in cattle vaccinated with the recombinant *MAP* Hsp70 subunit vaccine.

Therefore the aim of the current study was to study which parts of the *MAP* Hsp70 protein are recognized using different 70kD heat shock proteins and *MAP* B cell epitopes using synthetic peptides. We generated mouse monoclonal antibodies to determine the (dominant) B cell epitopes of the recombinant protein and studied whether these antibodies recognized native *MAP* Hsp70 protein and interact with intact bacteria. Subsequently the specificity of antibody responses induced by vaccination of cattle and goats with recombinant *MAP* Hsp70 was determined to assess whether these animals recognize the same protein regions and/or epitopes identified with the monoclonal antibodies, and could potentially contribute to protection against paratuberculosis.

MATERIALS AND METHODS

Animals and experimental design

Mice

Two Balb/c mice were used for Hsp70 immunizations and the generation of monoclonal antibodies.

Goats

A total of 30 female goat kids (Saanen breed dairy goats, age 14 ± 3 days at the start of the experiment) were used. The kids were raised using conventional procedures and feeds, and were checked daily for general health. The kids were randomly assigned to one of the four experimental groups, according to a 2×2 factorial design. Kids in groups 1 (*n=7)* and 2 (*n=8)* (uninfected controls) were physically separated from kids in groups 3 (*n=7)* and 4 (*n=8)* (*MAP* infected). Kids assigned to groups 2 and 4 were immunized once at the start of the experiment (day 0). The immunization consisted of the administration of 200 μg of recombinant *MAP* Hsp70 in 1 ml phosphate buffered saline (PBS) containing 10 mg/ml dimethyl dioctadecyl ammonium bromide (DDA) adjuvant (Sigma Aldrich, St.Louis, MO, USA) in the final preparation, subcutaneously in the lower neck region.

Blood samples were taken from the V. jugularis on a weekly basis for a period of 3 months. Serum was stored for analysis upon completion of the experiment. Goats were necropsied at the end of the experiment and tissue samples from ileum, jejunum, the ileocecal and a jejunal mesenteric lymph node were analyzed using *MAP* specific IS900 PCR [22], bacterial culture on mycobactin J supplemented HEY medium (BD Biosciences Europe, Erembodegem, Belgium) and histopathology.

Infection of goat kids

Goat kids assigned to groups 3 and 4 were infected orally with 3 oral doses, one day apart, of $2x10^9$ cfu of *MAP* strain G195, originally isolated from a goat with clinical signs of paratuberculosis, grown on Middlebrook 7H10 supplemented with OADC and Mycobactin J (a generous gift from D. Bakker, CIDC, Lelystad, The Netherlands).

Cattle

Sera from cattle were analyzed to characterize *MAP* Hsp70 specific antibody responses. The sera were taken from cattle that participated in a Hsp70 vaccination – challenge experiment details of which have been published previously [15]. In short a total of 40

female calves (4 groups of 10 calves, randomly assigned to one of the 4 experimental groups, aged 29 ± 9 days at the start of the experiment) were used in that study. Serum samples were taken every 2 weeks for the first 12 months of the experiment and monthly for the remainder. The immunization was identical as described in the goat kids above, administered subcutaneously in the dewlap. The same animals received a second immunization at day 308 of the experiment.

Ethics

Animal experiments were approved by the Ethical committee of the Utrecht University, and performed according to their regulations.

Antigens

The following antigens were used for vaccination and definition of fine specificity of monoclonal antibodies (mAb) specific for *MAP* Hsp70: recombinant *MAP* Hsp 65kD (r*MAP* Hsp60) and Hsp 70kD (r*MAP* Hsp70) were produced according to methods described in detail earlier [5, 17]. Purity of the recombinant Hsp60 and Hsp70 was checked using SDS-PAGE, and Western blot. SDS-PAGE and Western blot were performed according to methods described in detail elsewhere [8, 9].

A recombinant C-terminal deletion mutant protein was constructed, comprising the receptor binding part of the Hsp70 molecule (containing the N-terminal amino acids 1-359 of wildtype Hsp70), with a molecular weight of approximately 45kD and named RBS70. This recombinant deletion mutant *MAP* Hsp70 molecule was constructed by restriction endonuclease digestion of the original recombinant *MAP* Hsp70 pTrcHis expression vector with AflII (NE Biolabs, Ipswich, MA, USA) and HindIII (Gibco®-Invitrogen, Breda, The Netherlands) using 5 units of each enzyme per μg DNA. The digested fragment was separated from the vector DNA by agarose gel (1%) electroforesis and isolated from the gel using a QIAEXII kit (Promega Benelux bv, Leiden, The Netherlands) according to instructions provided by the manufacturer. The vector DNA was blunted by using T4 DNA polymerase (Fermentas Gmbh, St. Leon-Rot, Germany) subsequently purified using a DNA cleaning kit (Zymo Research Corp., Orange, CA, USA) , religated using T4 DNA ligase (Quick Ligation kit, NE Biolabs, USA) and purified using the DNA cleaning kit (Zymo Research Corp., USA). Finally chemically competent Top10 bacteria (Invitrogen) were transformed with the vector DNA using a heatshock protocol provided by the manufacturer. Transformed bacteria were selected and protein expression and purification was performed similar to the procedure described for recombinant *MAP* Hsp70 [17].

In addition, the following antigens were used: recombinant *M. tuberculosis* Hsp70

(*MTb*), recombinant *Escherichia coli* (*E. coli)* Hsp70 and bovine Hsc70 purified from bovine brain (generous gifts from Stressgen, Vancouver, Canada). The purified protein derivatives (PPDs) were produced at CIDC (Lelystad, The Netherlands) as previously described [10], from *MAP* 3+5/C (10 µg/ml), PPD *MAP* (PPD-P), *M. bovis (MB)* strain AN5 (10µg/ml), PPD *MB* (PPD-B), and *M. avium ssp. avium (MAA)* strain D4 (10 µg/ml), PPD *MAA* (PPD-A). *MAP* strain 316F was grown at the CIDC (generous gifts from D. Bakker).

The following GenBank derived sequences were used to define peptides for the screening of monoclonal antibodies and sera from cattle and goats: Q00488 (*MAP* Hsp70); A0QLZ6 (*MAA*); P0A5C0 (*MB*); P0A5B9 (*MTb*); P04475 (E.coli); NP776975 (*Bos taurus* Hsp70-1A).

A first set of one hundred twenty-four synthetic 14-mer peptides, with an aminoterminal cysteine, a 5 amino acid (aa) shift and an overlap of 9 aa, covering the *MAP* Hsp70 molecule, was synthesized using the simultaneous multiple peptide synthesis (SMPS) technique described previously [21]. To enable di-sulphate binding of peptides to the solid phase ELISA plate, an amino-terminal cysteine residue was coupled to each peptide during synthesis. For primary screening peptides were pooled in 11 groups of sequential peptides. Positive pools were retested on the single peptide level.

To enable coupling of peptides to streptavidin coated carrierbeads for the Luminex system (see below) a separate set of immunogenic 14-mer *MAP* Hsp70 peptides, selected based on the first screening with the 14-mer peptides, was synthesized using SMPS and modified using amino-terminal biotinylation. A third set of 15-mer peptides consisting of mycobacterial, *Bos taurus* and *E.coli* homologues to identified *MAP* Hsp70 linear epitopes was also synthesized using SMPS and modified using amino-terminal biotinylation.

Generation and screening of monoclonal antibodies

The generation of monoclonal antibodies has been described previously [4]. Briefly, 100 µg of recombinant *MAP* Hsp70 protein in 80 µl PBS was mixed with 100 µl Specol [3] (ASG, Lelystad, The Netherlands) to obtain a water in oil emulsion used for i.p. immunization of Balb/c mice. This immunization was repeated 3 weeks later. Another 3 weeks later, four days prior to hybridoma production the mice were boosted i.v. with 50 µg of the antigen in 50 µl PBS. After 4 days spleen cells were fused with mouse myeloma cells (Sp2/0) using polyethyleenglycol (PEG, Merck, Darmstad, Germany). Antigen specific antibody producing hybridoma's were selected by ELISA [20] and subcloned in limiting dilution.

Isotyping monoclonal antibodies

The isotype of the monoclonal antibodies was determined using the Mouse Hybridoma Subtyping Kit (Roche, The Netherlands) according to instructions provided by the manufacturer. In short, the monoclonal antibodies were tested in three concentrations (1.0, 0.1 and 0.01 µg/ml) diluted in blocking buffer. Following an incubation of 30 min at room temperature, the plate was washed and subsequently incubated with peroxidase conjugated goat-anti-mouse (GAM)-IgA-, IgG-, IgG1-, IgG2a-, IgG2b-, IgG3-, IgM, λ-light chain and κ-light chain diluted 1:2000 in blocking buffer. Following an incubation of 30 min at room temperature the plate was washed extensively, and 100 µl ABTS (2,2'-azinobis (3 ethyl) benzthiazolinsulfonic acid) substrate buffer was added to each well. The optical density (OD) was measured after 10 minutes at 405 nm on a spectrophotometric Elisa reader (Bio-Rad laboratories, Hercules, CA, USA). Absorbence values of hybridoma supernatants were subsequently analysed.

ELISA

The *MAP* Hsp70 protein ELISA

The *MAP* Hsp70 protein ELISA to measure antibody responses in cattle sera has been described previously [17]. Briefly, 96 well EIA plates (Corning Costar Corp., Acton, MA, USA) were coated with 100 µl of antigen (1 µg/ml Hsp70, or 0.5 µg/ml RBS70) diluted in sodium bicarbonate buffer (pH 9.6), for 20 min at 37°C. All subsequent incubations were performed for 20 min at 37°C, and after each incubation step plates were washed 3 times with PBS containing 0.05% Tween 20. Wells were blocked with 200 µl blocking solution (Roche, Almere, The Netherlands). Sera of immunized/infected goats or hybridoma supernatants were used in a predetermined optimal dilution or were serially diluted in blocking buffer as indicated. Plates were washed and subsequently incubated with polyclonal goat anti-mouse peroxidase (PO) conjugated antibodies (Sigma Aldrich, USA) to detect murine monoclonal antibodies, and rabbit anti-goat IgG-PO (Sigma Aldrich, USA) to detect goat antibodies. Finally plates were washed extensively, and 100 µl ABTS (2,2'-azinobis (3 ethyl) benzthiazolinsulfonic acid (Roche, Almere, The Netherlands) substrate buffer was added to each well. The optical density (OD) was measured after 10 minutes at 405 nm on a spectrophotometric Elisa reader (Bio-Rad laboratories, USA). Absorbance values of sera or hybridoma supernatants were subsequently analysed.

The whole mycobacterial cell ELISA

The whole mycobacterial cell ELISA was a modification to the protein ELISA. In brief, 96-well plates (Corning Costar Corp., USA) were coated with 100 µl of washed bacteria (both *MAP* and *MAA*; $1x10^8$ cfu/ml), diluted in sodium bicarbo-nate buffer pH 9.6 for 60 min at room temperature, while shaking at 300 rpm on a electronic MTS shaker (IKA® Werke, Staufen, Germany). All subsequent incubations were performed for 30 min shaking at room temperature. After each incubation step plates were washed three times with PBS containing 0.01% Tween 20. Wells were blocked with 200 µl of Post Coating Buffer (PCB) (Roche Diagnostics GmbH, Mannheim, Germany), containing 50 mM Tris-HCl, 150 mM sodium chloride and a 1% (w/v) protein mixture that was obtained by proteolytic degradation of purified gelatin, at pH 7.4. Sera or pure antibody-solutions were diluted in PCB; followed by incubation Goat anti Mouse (GAM)-PO (ELISA subtyping kit, Roche, The Netherlands) 1:2000 diluted in PCB. Finally plates were washed extensively, and 100 µl ABTS (2,2'-azinobis (3 ethyl) benzthiazolinsulfonic acid; Roche, Almere, The Netherlands) substrate buffer was added to each well. The optical density (OD) was measured after 10 minutes at 405 nm on a spectrophotometric Elisa reader (Bio-rad laboratories, USA). Absorbance values were subsequently analysed.

Peptide ELISA

Peptide ELISA was used for the initial epitope mapping of the monoclonal antibodies generated against *MAP* Hsp70. To obtain di-sulphate binding of cys-linked peptides to the solid phase ELISA plates, 96-well CovaLink NH F8 plates (Nunc, Roskilde, Denmark) were pretreated with 100 µl of 0,5 mM SPDP (N-succinimidyl-3-(2-pyridyldithio) propionate, a bifunctional cleavable crosslinker containing a N-hydroxysuccinimide esther and a 2-pyridyl disulfide group (Pierce, Rockford, IL, USA) in propanol, diluted in PBS for 30 min at 37 °C. After incubation, the plates were washed two times with double distilled water. The different cys-linked peptides were diluted in 0.1 M Tris-HCl, pH 8.0 at a concentration of 15 µg/ml, directly before transfer of 100 µl per well to the plate, and incubated for 60 min at 37 °C. The plates were washed two times with double distilled water, and subsequently three times with tap water. Wells were blocked with 200 µl of post coating buffer (Roche, Almere, The Netherlands) for 15 min. Antibody-solutions were diluted to a concentration of 1 µg/ml in post coating buffer (Roche, Almere, The Netherlands); this was followed by incubation with peroxidase conjugated goat-anti-mouse antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands) 1:2000 diluted in ELISA blocking buffer (Roche, Almere, The Netherlands) for 30 minutes. Next, the plate was washed extensively, and 100 µl ABTS (2,2'-azinobis (3 ethyl)

benzthiazolinsulfonic acid; Roche, Almere, The Netherlands) substrate buffer was added to each well. The optical density (OD) was measured after 10 minutes at 405 nm on a spectrophotometric Elisa reader (Bio-rad laboratories, USA). Absorbence values of hybridoma supernatants were subsequently analysed.

Flowcytometric analysis of monoclonal antibodies binding to mycobacteria.
To study whether monoclonal antibodies bind to intact bacteria, indicative of the presence of *MAP* Hsp70 in the bacterial cell wall, suspensions of *MAA* strain D4 and *MAP* strain 316F (generous gifts from D. Bakker, CIDC) were prepared from log phase liquid cultures. Suspensions of *MAA* and *MAP* (both 10^{10} bacteria/ml in PBS) were diluted 1:100, washed three times by centrifugation (1 min at 14000 RPM in an Eppendorf centrifuge (5417 R, Eppendorf)) and resuspended in PBS. These suspensions were diluted 1:100 in PBS supplemented with 1% BSA and 0.01% sodium azide (both from Sigma Aldrich, USA) and divided in volumes of 100 µl. Hsp70 specific monoclonal antibodies were added in a concentration of 5 µg/ml. After incubation for 25 min at room temperature (RT) and three washes with PBS supplemented with 1% BSA and 0.01% sodium azide (FACS buffer), FITC-labelled Goat anti-mouse antibodies (Becton-Dickinson, San Jose, CA, USA) were added and incubated for 25 min at RT. After three more washes, 10000 bacterial cells were used for analysis by FACScan (Becton-Dickinson, San Jose, CA, USA).

Negative contrast electron microscopy of antibodies binding to mycobacteria
Bacterial suspensions (*MAP* and *MAA*, both 10^8 bacteria/ml in PBS) were divided in volumes of 150 µl, washed three times in ELISA blocking buffer (Roche, The Netherlands) and resuspended in 100 µl ELISA blocking buffer Ten µl of a 100 µg/ml Hsp70 specific monoclonal antibody solution and respective isotype control antibodies were added and incubated on a tube rotator for half an hour at room temperature. Bacteria were washed 3 times with 1 ml PBS supplemented with 0,1% Tween 20 to remove unbound antibodies, and resuspended in ELISA blocking buffer (Roche, The Netherlands). Subsequently, protein A–10 nm colloidal gold (Aurion, Wageningen, Netherlands, 1:400 in blocking buffer) was added and again incubated on a tube rotator for half an hour at room temperature. Bacteria were washed 3 times, resuspended in 100 µl blocking buffer and stored at 4˚C for a maximum of two days. Five µl drops of stained *MAA* and *MAP* suspensions were put onto a clean plastic support and a negative-stain immuno electron microscopy Ni or Cu-grid was placed on top of the drop for 15 min at room temperature. The grid was rinsed three times 5 min on phosphate buffered saline drops containing 50 mM glycine. Next, the grid was placed on incubation buffer (0.1% acetylated bovine serum albumin in PBS) for 3 times 10

min and then washed 4 times 5 min on double distilled water. Grids were blotted dry very short on filter paper, tipped on 5 µl potassium phosphotungstate solution (2%) and blotted completely dry on filter paper. Finally, stained grids were viewed using a transmission electron microscope (Philips, Eindhoven, The Netherlands).

Luminex multiplex immunoassay

To ensure that binding between Hsp70 peptides and antibodies was not influenced by the addition of the amino-terminal cysteine residue and to enable screening of large numbers of peptide antigens with limited volume serum samples the Luminex xMAP technology platform was used. A set of 14-mer peptides containing a selection of *MAP* Hsp70 peptides, based on the peptide ELISA results, as well as (myco)bacterial homologues present in *MB*, *MTb*, *E. coli* and the bovine Hsc70 homologue were used. Multiplex peptide specific antibody measurements were performed using biotinylated peptides linked to avidin coated fluorescent microspheres (LumAv, Luminex corp., Austin, TX, USA) on a Luminex 100 platform according to instructions provided by the manufacturer (Luminex corp, Austin, TX, USA). Individual peptides were coupled to beads with a unique dual fluorescent label so that they can be identified in the multiplex assay. A total of $2.5x10^5$ beads (100 µl) per uniquely labeled beadset were washed twice with PBS, and subsequently incubated with 10 µmol biotinylated peptide for 10 minutes at 20˚C. Following the incubation unbound peptide was removed by washing the beads twice with PBS. Finally the beads were resuspended in their original volume (100 µl) using PBS supplemented with 1% bovine serum albumine (Sigma Aldrich, USA) and 0.01% sodium azide, and stored in the dark at 4˚C until further use. For multiplex analysis 20 µl of resuspended coated beads of each of up to 20 unique beadsets were pooled in an eppendorf container.

To the total volume of 400 µl and additional 400 µl of PBS was added and mixed. In a round bottom 96 well microtiter plate, 10 µl of the mixed beads was added per well. Subsequently 100 µl of goat or calve serum samples were added per well. This mixture was incubated for 30 min at 4˚C and subsequently washed once with PBS supplemented with 1% bovine serum albumine (Sigma Aldrich, USA) and 0.01% sodium azide. Next, bead-bound antibodies were labeled with 50 µl 1:5000 diluted protein-A-RPE (Prozyme, USA). This mixture was incubated for 30 min at 4˚C at which point 100 µl PBS supplemented with 1% bovine serum albumine (Sigma Aldrich, USA) and 0.01% sodium azide was added. The 96 well plate was placed in the Luminex 100 analyzer and per sample the amount of PE derived fluorescence was measured for each of the 20 unique beadsets by acquisition of data of 100 beads per beadset and expressed as mean fluorescence intensity (MFI) as a measure for antibody bound to the peptide coupled to the designated beads.

Immunohistochemistry

Selected Hsp70 specific monoclonal antibodies recognizing linear epitopes were tested in immunohistology to study whether the identified epitopes are detectable in wildtype *MAP* present in infected lesional tissue, as opposed to recombinant Hsp70. For immunohistological evaluation of selected monoclonal antibodies formalin fixed, paraffin embedded tissues samples were used from the goat kid infected in the present study (groups 3+4) , and from archived formalin fixed, paraffin embedded tissues samples from cattle diagnosed with paratuberculosis and uninfected control animals. Microbiological and immunological characterization of these cattle samples has been published previously [14].

Tissue specimens were fixed in 4% buffered formaldehyde, embedded in paraffin, and processed by routine methods for microscopic examination using a Hematoxylin and Eosin (H&E) and Ziehl Neelsen (ZN) stains. Mycobacterial Hsp70 antigen was detected by an avidin-biotin complex (ABC) method. Tissue sections were dewaxed in xylene and rehydrated through graded alcohols for 2 min each step till distilled water. They were then pre-treated with Citrate buffer pH 6.0 in microwave 700 Watt for 10 min. Endogenous peroxidase activity was suppressed by 1% H_2O_2 in methanol for 30 min. This was followed by treatment with 10% normal horse serum (NHS) 1:10 in PBS for 15 min for removal of non-specific reactivity and by incubation with primary antibody (4°C overnight). The secondary antibody (biotin labelled horse anti-mouse 1:125, Dako, Glostrup, Denmark) was applied for 30 min at room temperature. The two solutions A and B of the ABC kit were diluted 25 times in PBS, mixed and the ABC reagent was stored for 30 min. until further use. Then the slides were incubated for 30 min with ABC-complex at room temperature. Conjugate binding was detected by adding the substrate chromogen (3.3-diaminobenzidine, DAB) and color was allowed to develop for 10 minutes. Finally, tissue sections were washed with distilled water, counter-stained with haematoxylin, rinsed, dehydrated and mounted.

RESULTS

Characterization of recombinant *MAP* Hsp70 specific hybridoma's

From spleen cells of the mice immunized with recombinant *MAP* Hsp70 that recognized r*MAP* Hsp70 in ELISA a total of 21 hybridoma's were generated.

The *MAP* Hsp70 protein ELISA

From the 21 supernatants tested 8 reacted with *MAP* rHsp70, in the remaining 13 supernatants no reactivity with *rMAP* Hsp70 was observed and these were not

considered for further analysis. None of these 8 supernatants reacted with r*MAP* Hsp60 or PPD-A control antigens, 3 supernatants recognized their epitope in PPDP (KoKo.B03, KoKo.B05, KoKo.B06) (Fig. 1A).

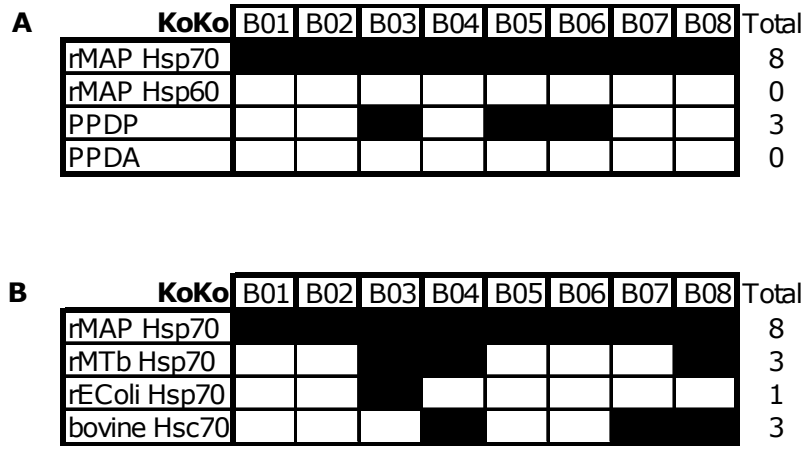

Fig. 1. Screening of specificity of hybridoma supernatants generated following immunization of mice with recombinant *MAP* Hsp70. Depicted in panel A is the reactivity of hybridoma supernatants to the recombinant M. avium spp paratuberculosis (*MAP*) Hsp70 protein, a recombinant control protein (recombinant *MAP* Hsp60 protein), and Johnin (PPD-P) and avian tuberculin (PPD-A), representing complex protein mixtures from *MAP* and *MAA* culture supernatants. Panel B depicts the reactivity of hybridoma supernatants to several Hsp70 proteins, recombinant mycobacterial Hsp70 from *MAP* and M. tuberculosis (*MTb*), recombinant Hsp70 from E. coli and purified bovine Hsc70 (constitutive bovine Hsp70 from brain). Top rows of representative table indicate the clone name (KoKo.B01 – B08). Cells that have been filled (■) indicate ELISA responses in which OD405nm > (average OD405nm of background control samples + 3 x SD) in two separate experiments.

Furthermore culture supernatants of the 8 *MAP* Hsp70 recognizing hybridoma's were screened for reactivity with recombinant Hsp70 from *MAP, MTb, E.coli and purified* bovine Hsc70 to identify cross reactivity. Four out of these 8 supernatants reacted only with *MAP* Hsp70 (KoKo.B01, KoKo.B02, KoKo.B05, KoKo.B06), three supernatants also recognized 2recombinant Hsp70 from *MTb* (KoKo.B03, KoKo.B04, KoKo.B08), 3 supernatants recognized bovine Hsc70 (KoKo.B04, KoKo.B07, KoKo.B08) and only one supernatant recognized recombinant Hsp70 from *E. coli* (KoKo.B03) (Fig. 1B).

Analysis of antibody binding to intact mycobacteria

Reactivity of three antibodies, KoKo.B01, KoKo.B02 (results not shown) and KoKo. B03 with whole *MAP* and *MAA* bacteria was tested. Antibodies KoKo.B01 and KoKo. B02 were selected as antibodies recognizing a less conserved epitope, KoKo.B03 was selected as a cross-reactive antibody recognizing a more conserved epitope. Despite high sequence similarities of *MAP* and *MAA* Hsp70 protein (99.8% similarity, the only difference being Q198H), reactions with intact *MAA* were significantly lower in ELISA ($p<0.001$) compared to reactions with intact *MAP* (Fig. 2A-I+II), a low reaction was observed with *MB*.

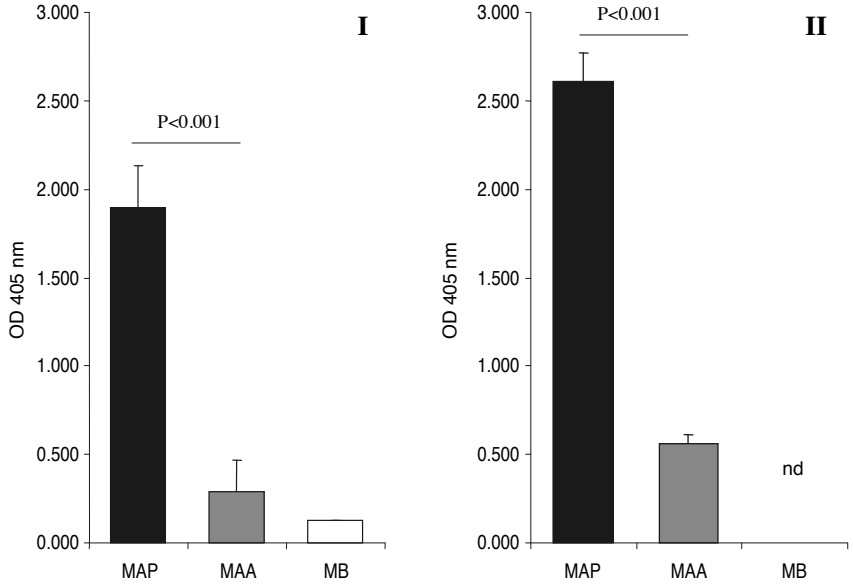

Fig. 2A. Screening of specificity of 2 hybridoma supernatants generated following immunization of mice with recombinant *MAP* Hsp70. Panel I) whole mycobacterial cell ELISA testing binding of antibody KoKo. B03 to M. avium spp. paratuberculosis (*MAP*), M. avium spp. avium (*MAA*), and M. bovis (*MB*) and Panel II) testing of antibody KoKo.B01 (*MB* not determined). Results are presented as the average optical density at 405 nm (OD405) + SD, p values reflect comparison of *MAP* and *MAA*, $p<0.001$.

Similar data were obtained for KoKo.B01 and KoKo.B03 using a flowcytometric approach to address the binding of antibodies to intact living mycobacteria, an example of which is shown in Fig. 2B. In addition, studies using negative contrast electron microscopy also indicated more extensive binding of Hsp70 antibodies (KoKo.B03) to *MAP* bacteria in comparison to *MAA* bacteria (Fig. 2C).

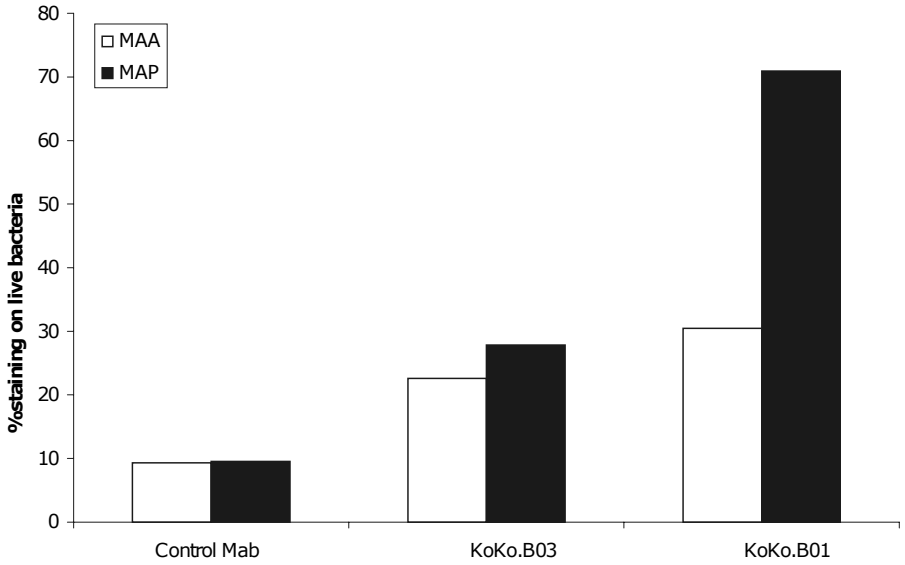

Fig. 2B. Flow cytometric analysis of the selected *MAP* Hsp70 specific antibodies KoKo.B01 and B03 after binding to live bacteria. White bars are results obtained using M. avium ssp. avium (*MAA* strain D4), solid black bars are results using M. avium ssp. paratuberculosis (*MAP* strain 316F). Results are expressed as the percentage of propidium iodine negative bacteria which display positive staining with the FITC conjugated Goat-anti-Mouse second step antibody. Isotype control mAb for KoKo.B03 (IgG2a) and KoKo.B01 (IgG1) were included to investigate background staining. For each condition staining of 10.000 live bacteria was analyzed.

Peptide ELISA

All 8 antibodies reacting with r*MAP* Hsp70 were subsequently tested for recognition of synthetic *MAP* Hsp70 peptides. In a primary screening 3 antibodies displayed reactivity to specific pools of *MAP* Hsp 70 peptides (Fig. 3: KoKo.B01 (3A), KoKo.B02 (3B), KoKo.B03 (3C)). The remaining 5 monoclonal antibodies did not recognize linear peptide epitopes, an example of which is shown for KoKo.B04 (Fig. 3D). Subsequent fine mapping of the epitopes using the single peptides of the pools in a solid phase ELISA confirmed that the 3 peptide reactive monoclonal antibodies (KoKo.B01, KoKo.B02, KoKo.B03) recognized linear epitopes in *MAP* Hsp70. The antibodies KoKo.B01 (IgG1 isotype) and KoKo.B02 (IgG2b isotype) recognized the aminoacid sequence P595-603 (PDGAAAGGG)(Fig. 4A+B), located in the C-terminal part of *MAA* and *MAP* Hsp70 but not present in *MB* and *MTb* Hsp70 according to published sequences. The third antibody, KoKo.B03 (IgG2a isotype), recognized a conserved epitope in the N-terminus of the *MAP* Hsp70 protein with the apparent core region sequence P111-124 (ITDAVITVPAYFND) (Fig. 4C) which is also present in *MAA*, *MB*), and *MTb* Hsp70.

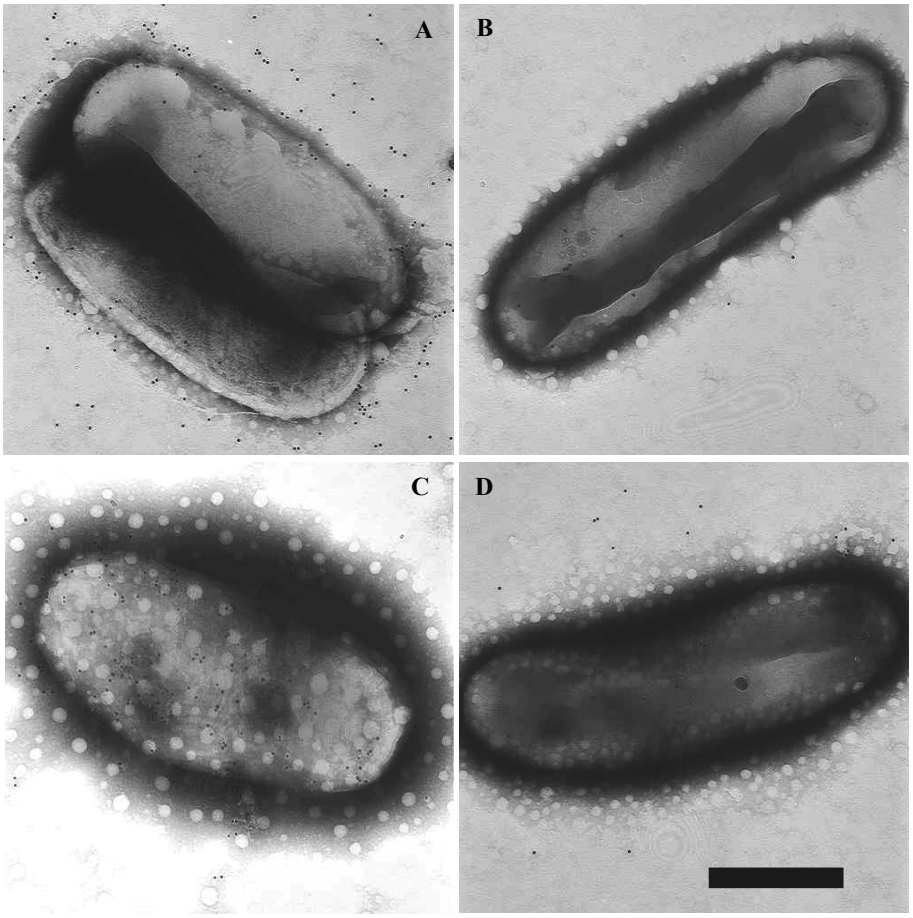

Fig. 2C. Negative contrast EM analysis of the selected MAP Hsp70 specific antibody KoKo.B03 after binding to live bacteria. (panel A) M. avium ssp. avium (*MAA*) stained with KoKo.B03 or (panel B) isotype control (IgG2a). (panel C) M. avium ssp. paratuberculosis (*MAP*) stained with KoKo.B03 or (panel D) isotype control. Bar indicates 500 nm, magnification 28k, Philips transmission electron microscope.

Luminex multiplex immunoassay

A set of peptides, containing relevant *MAP* Hsp70 peptides as well as (myco)bacterial homologues present in *MB*, *MTb*, and the Bos Taurus Hsc70 homologue of the two identified linear epitopes, was tested to confirm specificity of the antibodies. The data indicate that the same epitopes are recognized in this system and binding is not influenced by amino-terminal modifications. Furthermore the data indicate that KoKo.B01 (not shown) and KoKo.B02 recognize an epitope which is present and identical in *MAP* and *MAA*, but absent in *MB*, *MTb*, and *E. coli* (Fig. 5A). Finally

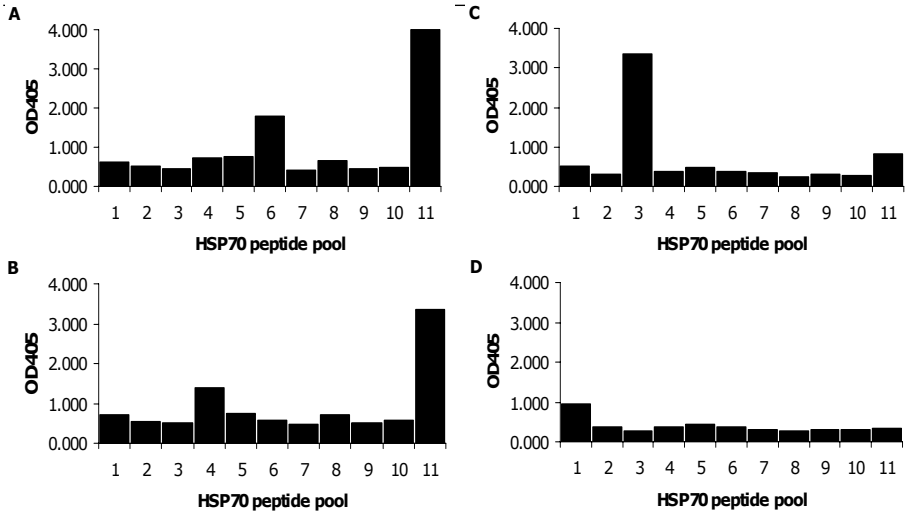

Fig. 3. ELISA screening of Hsp70 specific monoclonal antibodies by *MAP* Hsp70 peptide pools to detect B cell epitopes. Antibodies showing reactivity with recombinant M. avium ssp. paratuberculosis (*MAP*) Hsp70 were tested for reactivity with synthetic *MAP* Hsp70 peptides. 14-mer peptides, with a 5 amino acid (aa) shift and an overlap of 9 aa, covering the *MAP* Hsp70 molecule, were pooled in 11 groups of 11 or 12 sequential peptides. In this primary screening antibody reactivity was tested and 3 antibodies displayed reactivity to specific pools of peptides as depicted in (panel A) KoKo.B01, (panel B) KoKo.B02, and (panel C) KoKo.B03. In contrast (panel D) KoKo.B04 did not react to any pool of peptides. Results are presented as the average optical density at 405 nm (OD405) + SD.

the data regarding KoKo.B03 indicate that conserved mycobacterial homologues (*MB*, *MTb*) are equally well recognized, while recognition of the *E.coli* homologue is at approximately 50% of that of the parent epitope and detection of the bovine homologue is near background levels (Fig. 5B).

Recombinant C-terminal deletion mutant protein ELISA

Comparison of binding of the 8 *MAP* Hsp70 specific monoclonal antibodies in ELISA to the recombinant deletion mutant protein RBS70 (containing the N-terminal amino acids 1-359 of wildtype *MAP* Hsp70) to the recombinant *MAP* Hsp70 protein confirmed that KoKo.B01 and KoKo.B02 recognize an epitope at the C-term of Hsp70, which is not present in RBS70. KoKo.B03 recognizes a N-term linear epitope which is present in RBS70 and Hsp70.

The remaining 5 antibodies (KoKo.B04-B08) recognized conformational epitopes. The antibodies KoKo.B04, KoKo.B05, KoKo.B07 and KoKo.B08 recognize epitopes in the N-term of Hsp70 comprised by the RBS70 mutant molecule. The antibody KoKo.B06 recognizes a conformational epitope in the less conserved C-term of Hsp70 (Fig. 6)

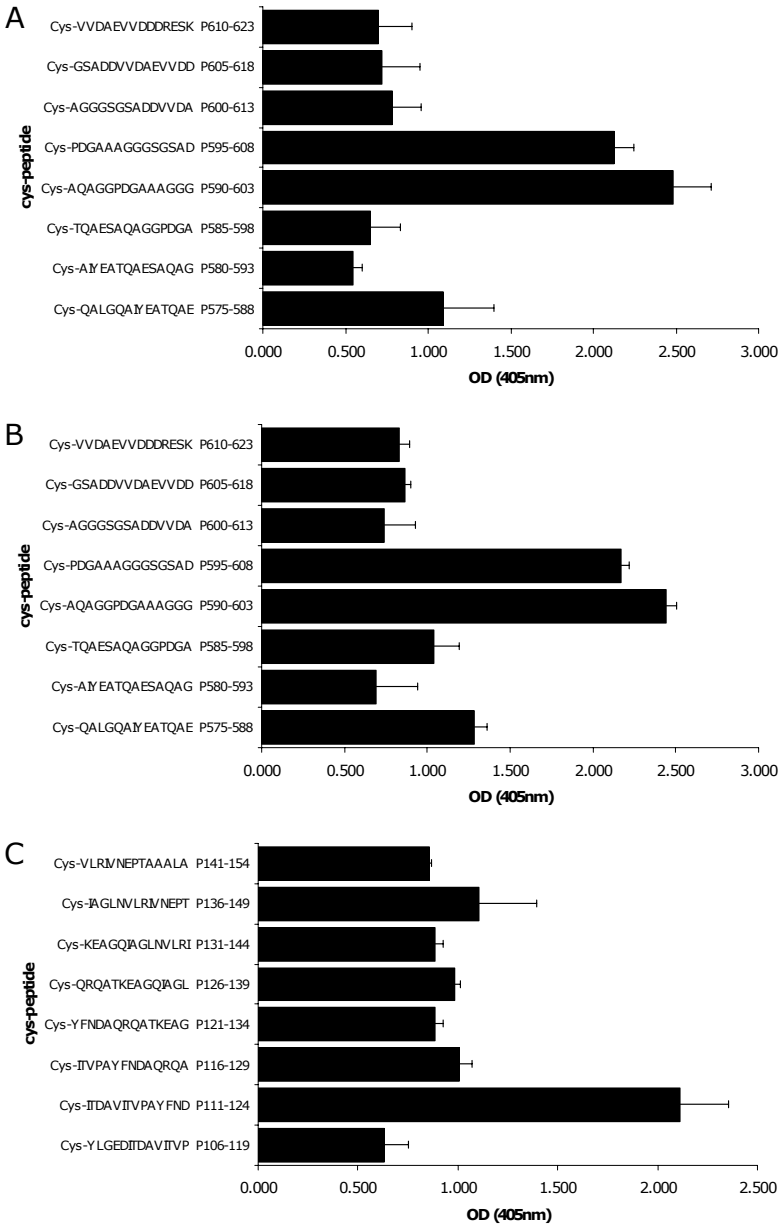

Fig. 4. ELISA screening of Hsp70 reactive monoclonal antibodies by *MAP* Hsp70 peptides to detect B cell epitopes. Antibodies showing reactivity with recombinant M. avium ssp. paratuberculosis (*MAP*) Hsp70 were tested for reactivity with synthetic *MAP* Hsp70 peptides. Antibody reactivity was tested on individual peptides of a positive 14-mer peptide pool, with a 5 amino acid (aa) shift and an overlap of 9 aa, found positive after primary screening of the peptide pools. (Panel A) KoKo.B01, (panel B) KoKo.B02, and (panel C) KoKo.B03 test results are presented as the average optical density at 405 nm (OD405) + SD.

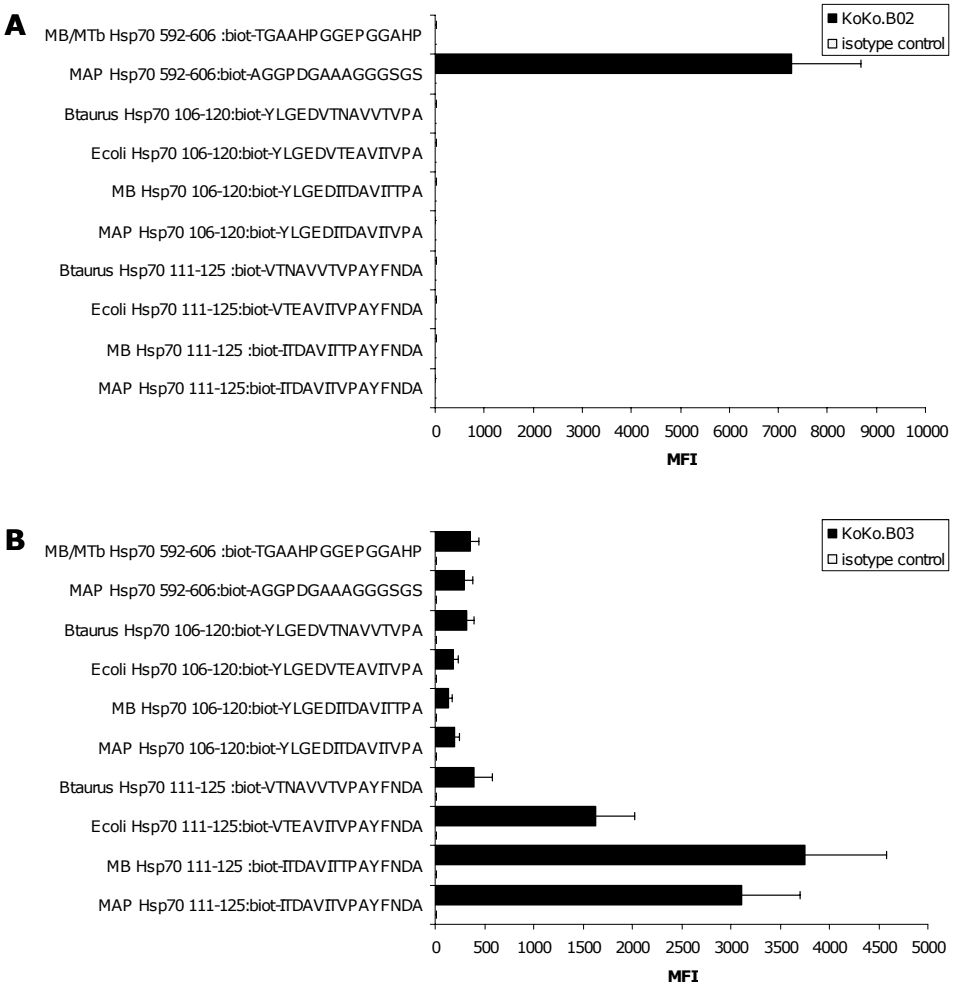

Fig. 5. Multiplex Luminex analysis of *MAP* Hsp70 specific monoclonal antibodies testing specific binding to Hsp70 peptide epitopes from different sources. (Panel A) KoKo.B02 (IgG2b) mAb and isotype control mAb was tested on a set of 15-mer biotinylated peptide epitopes bound to avidin coated fluorescent beads (LumAv), containing relevant M. avium ssp. paratuberculosis (*MAP*) Hsp70 peptides as well as (myco) bacterial homologues present in M. bovis (*MB*), M. tuberculosis (*MTb*), E. coli and the Bos taurus Hsc70 homologue of the two identified linear epitopes to confirm specificity of the antibodies was tested. (Panel B) KoKo.B03 (IgG2a) mAb and isotype control mAb. Test results are presented as the mean fluorescent intensity (MFI) + SD.

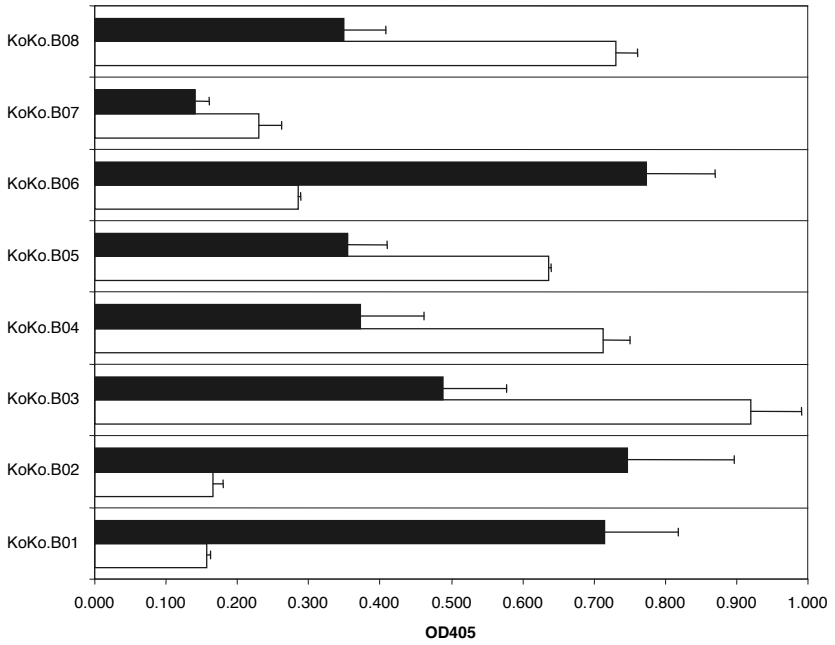

Fig. 6. Comparison of binding of 8 *MAP* Hsp70 specific monoclonal antibodies to *MAP* Hsp70 and a recombinant deletion mutant protein of *MAP* Hsp70. Antibodies (KoKo.B01-B08) were tested for binding the recombinant *MAP* Hsp70 protein (black bars) and the recombinant deletion mutant protein RBS70 (white bars) (containing the N-terminal amino acids 1-359 of wildtype Hsp70). KoKo.B01 and KoKo.B02 recognize a linear epitope at the C-term of Hsp70, which is not present in RBS70. KoKo.B03 recognizes a N-term linear epitope which is present in RBS70 and Hsp70. Results are presented as the average OD405 + SD of 3 independent experiments.

Recognition of linear B cell epitopes of rHsp70 by cattle and goats

In cattle, the highest Hsp70 specific antibody responses in Hsp70 Elisa were detected from 3 weeks post vaccination [15] (data not shown). In goats, the highest peak in Hsp70 specific antibody responses measured by Hsp70 Elisa was detected 4 weeks post vaccination (Fig. 7A).

In the goats, *MAP* Hsp70 specific serum antibody concentrations remain stable between 4 and 12 weeks post vaccination and were not influenced by the exposure of goats to *MAP*. The *MAP* Hsp70 antibody responses in unvaccinated goats remained at background levels during 12 weeks irrespective of exposure to *MAP*. Similar kinetics were observed using the ELISA with the RBS70 molecule (data not shown). Sera obtained at 3 (cattle) and 4 (goats) weeks post vaccination were analyzed for antibodies binding to 20 different Hsp70 peptides using the Luminex xMAP platform.

Results indicated that in vaccinated goats the dominant responses are directed against the peptides P111-124, P357-370, P484-497, P505-518, P605-618, and P610-623. Results for P111-124, and 6 comprising the less conserved C-term of Hsp70 is shown in Fig. 7B.

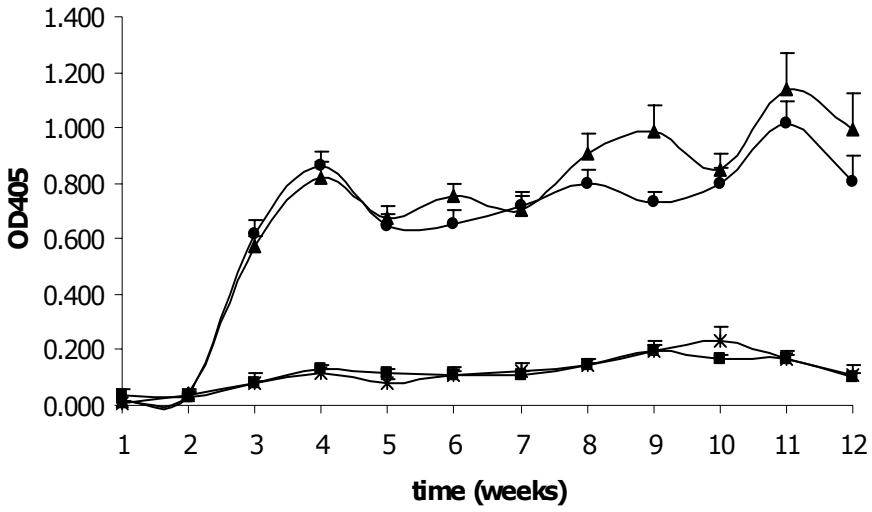

Fig. 7A. Measuring serum antibody (total Ig) response to recombinant *MAP* Hsp70 protein in goat kids by ELISA. Goats were immunized with 200 µg of recombinant M. a. paratuberculosis (*MAP*) Hsp70 in 1 ml phosphate buffered saline (PBS) containing 10 mg/ml dimethyl dioctadecyl ammonium bromide (DDA) adjuvant (group 2 (▲) n=7 and group 4 (●) n=8) at time point week 1. Animal in group 3 (X) and group 4 (♦) were experimentally infected with MAP during the first week with 3 dosis of *MAP* one day apart. Animals in group 1 (■) n=7 were neither immunized nor infected. Sera were prediluted 1:20, results are expressed as the average optical density at 405nm (OD405) of animals in the group + SEM.

Vaccination with simultaneous exposure to *MAP* does not alter responses to P111-124, P484-497, and P605-618. Lower responses are detected for the remaining peptides P357-370, P505-518 and P610-623, in MAP exposed groups as compared to those after vaccination alone. Similar differences were observed at later time points (data not shown).

In calves (Fig. 7C) the dominant responses in vaccinates are directed against the peptides P111-124, P590-603, P600-613, and P610-623. Simultaneous exposure to *MAP* does not alter responses to P111-124; lower responses are detected to P590-603; and P600-613 is recognized preferentially by vaccinated and *MAP* exposed calves. Finally, P610-623 is recognized by rHsp70 vaccinated calves only. Similar data were obtained with sera from calves at later time points post vaccination (data not shown).

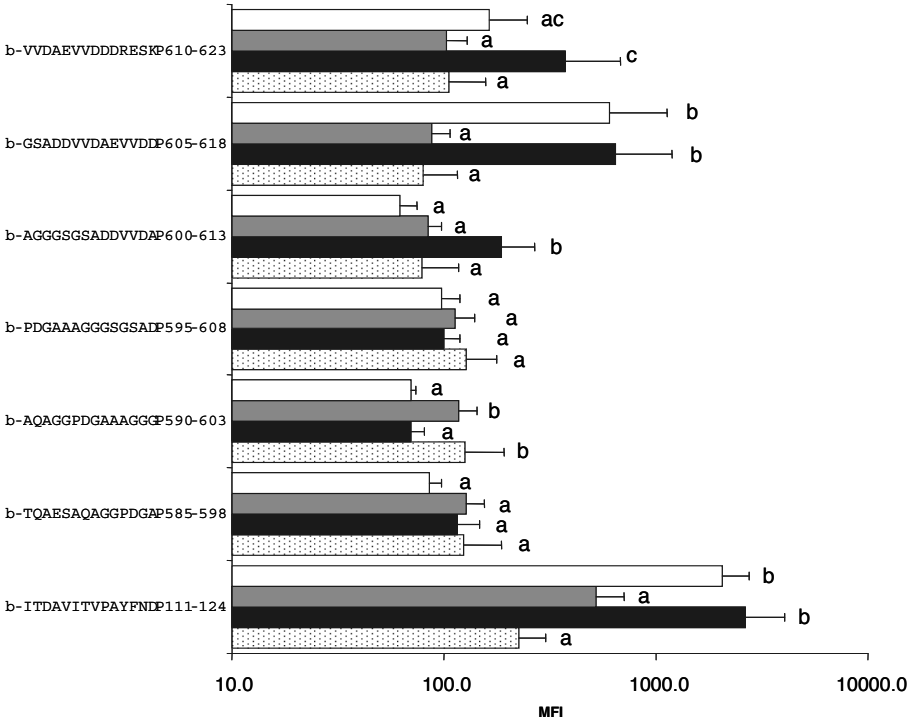

Fig. 7B. Multiplex Luminex analysis of goat serum antibody response to *MAP* Hsp70 peptides. Peptide epitope recognition by goats vaccinated with recombinant *MAP* Hsp70 protein using DDA adjuvant measured by Multiplex analysis of serum antibody binding tested on a set of 14-mer biotinylated immunogenic M. avium ssp. paratuberculosis (*MAP)* Hsp70 peptides bound to avidin coated fluorescent beads (LumAv). Goats were immunized with 200 µg of recombinant *MAP* Hsp70 in 1 ml phosphate buffered saline (PBS) containing 10 mg/ml dimethyl dioctadecyl ammonium bromide (DDA) adjuvant (group 2 (black bars) n=7 and group 4 (white bars) n=8) at time point 0. Animal in group 3 (grey bars) and group 4 were experimentally infected with MAP. Animals in group 1 (dotted) n=7 were neither immunized nor infected. Sera were prediluted 1:2, results are expressed as the mean fluorescence intensity (MFI) of animals in the group + SEM. Bars related to responses to a peptide that do not share letters are significantly different at $p<0.05$.

MAP Hsp70 is present in lesional tissue

The KoKo.B02 and KoKo.B03 antibodies recognizing linear epitopes of *MAP* Hsp70 were tested for suitability in immunohistochemical analysis of formalin fixed, paraffin embedded bovine tissue. Both antibodies could be used to study in situ localization of the bacteria in tissue sections, also indicating that the epitope and therefore the Hsp70 protein is expressed by *MAP* in intestinal lesions. Fig. 8 shows immunohistochemical staining of *MAP* infected intestinal tissue with KoKo.B02; isotype control antibodies were used at equal concentrations and showed no staining (data not shown).

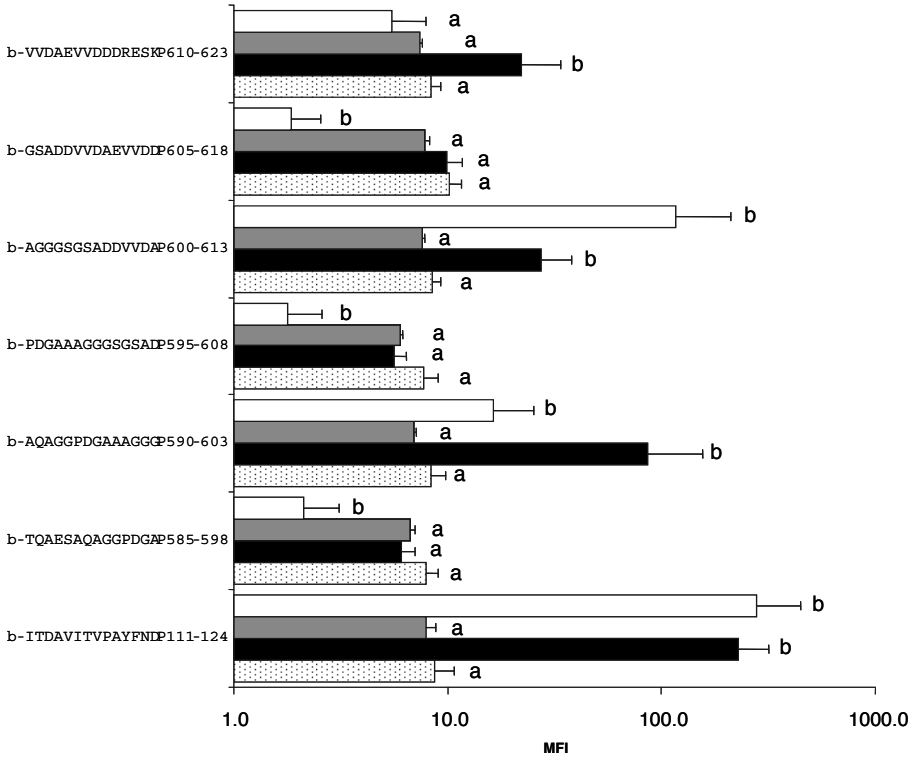

Fig. 7C. Multiplex Luminex analysis of calf serum antibody response to *MAP* Hsp70 peptides. Peptide epitope recognition by calves vaccinated with recombinant *MAP* Hsp70 protein using DDA adjuvant measured by Multiplex analysis of serum antibody binding tested on a set of 14-mer biotinylated immunogenic M. avium ssp. paratuberculosis (*MAP*) Hsp70 peptides bound to avidin coated fluorescent beads (LumAv). Calves were immunized with 200 μg of recombinant *MAP* Hsp70 in 1 ml phosphate buffered saline (PBS) containing 10 mg/ml dimethyl dioctadecyl ammonium bromide (DDA) adjuvant (group 2 (black bars) n=10 and group 4 (white bars) n=10 at time point 0. Animal in group 3 (grey bars) and group 4 were experimentally infected with MAP. Animals in group 1 (dotted) n=10 were neither immunized nor infected. Sera were prediluted 1:2, results are expressed as the mean fluorescence intensity (MFI) of animals in the group + SEM. Bars related to responses to a peptide that do not share letters are significantly different at $p<0.05$

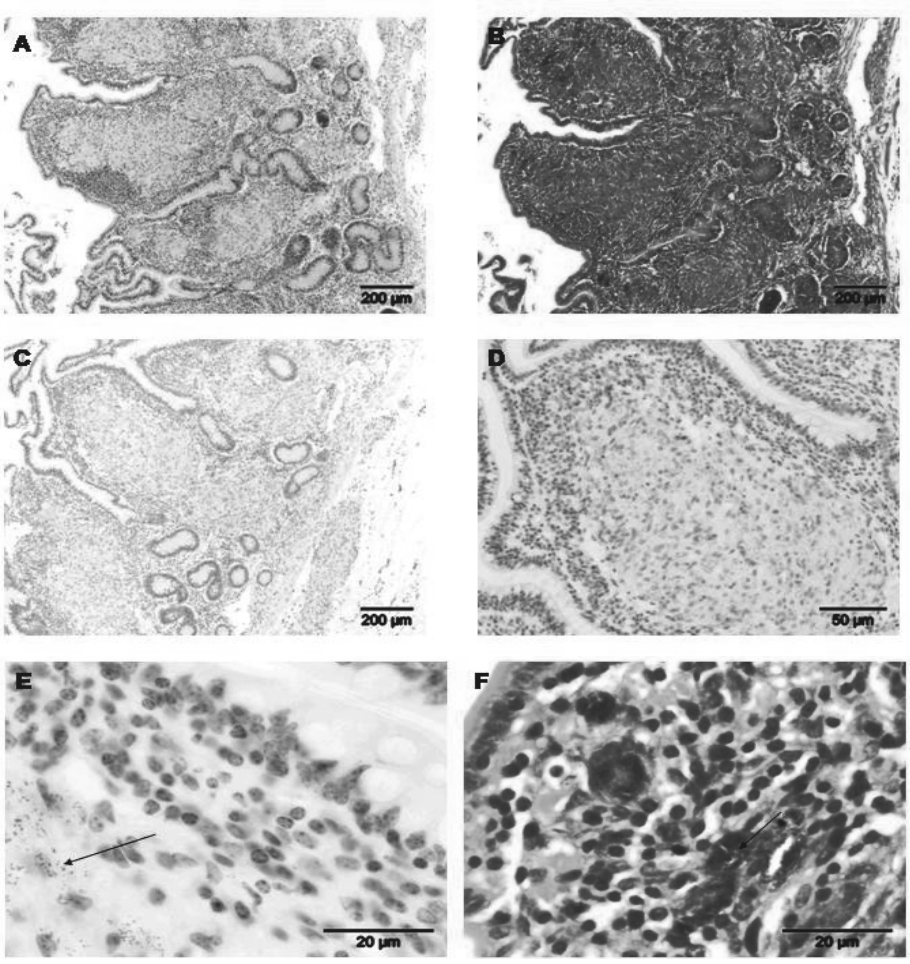

Fig. 8. Staining of lesional tissue sections from the ileum of a cow diagnosed with paratuberculosis by *MAP* Hsp70 specific monoclonal antibody KoKo.B03
Sections of this paratuberculosis diagnosed cow, showing clinical signs, were processed using (panel A) routine HE staining, (panel B,F) Ziehl-Neelsen staining for acid fast bacteria and M. avium ssp. paratuberculosis (*MAP*) stained with KoKo.B02 (Panel C, D and E).

DISCUSSION

In a previous publication we showed that vaccination of cattle with recombinant *MAP* Hsp70 exerted a protective effect as measured by a significantly reduced bacterial shedding [15]. An unexpected finding in this study was the pronounced and prolonged Hsp70 antibody response post vaccination, while Th1 T cell activity measured as IFN-γ production to specific antigens showed no correlation to vaccination or protection.
Hence the role of antibodies requires attention. As a first step the current study describes the characterization of *MAP* Hsp70 epitopes recognized by murine monoclonal antibodies as well as goat and cattle sera.
The observation that immunization of Balb/c mice with either recombinant *MAP* Hsp70 (this study) or whole-cell extract of *MAP* [1] readily induces dominant B cell responses against Hsp70 points out that route and method of exposure through immunization gives an immune response different from natural exposure to the pathogen which does not result in high Hsp70 antibody titers for a prolonged period after infection. In addition immunization of mice with whole-cell extracts of *MAP* led to the generation of monoclonal antibodies against isocitrate lyase (*MAP*1643) and Hsp70 (*MAP*3840) predominantly, indicating that these are proteins which are immunogenic and abundantly present in *MAP* [1].
In the current study, using murine monoclonal antibodies recognizing the Hsp70 protein, a synthetic peptide approach resulted in definition of two linear epitopes. One of those epitopes (recognized by KoKo.B03) is located in the conserved N-terminus of the native protein, the other (recognized by KoKo.B01 and KoKo.B02) is located in the less evolutionary conserved C-terminal region of the protein. Clearly these linear epitopes are accessible in the native recombinant protein, and as evidenced by the immunohistochemistry on formalin fixed tissue also in intestinal lesions caused by *MAP* infection. Thus indicating that potential posttranslational modifications of the protein expressed by *MAP*, in contrast to the *E.coli* recombinant protein, are not likely to interfere with the binding of the antibodies in this region obviously of relevance for species specific responsiveness [18]. Additionally it indicates that the Hsp70 protein is expressed by mycobacteria in vivo and is present in intestinal lesions found in field cases of bovine paratuberculosis.
In addition, the combined use of the C-term and N-term epitopes, of Hsp70 also offers possibilities to obtain a certain degree of species specificity enabling distinction between *MAA* and the pathogenic *MTb*-complex species as well as *M. leprae (MLE)*. With the known specificity of these antibodies, they may serve as an alternative to Ziehl-Neelsen staining, increasing specificity and potentially also sensitivity for detection of mycobacteria in diagnostic specimen. In addition, outside the genus

mycobacterium, the presence of *MAP*/*MAA* Hsp70 can be distinguished using these mAb from Hsp70 of other prokaryotic origin, without cross-reaction with eukaryotic (host) 70 kD heat shock proteins.

Both the intact protein as well as the dominant linear epitopes initially identified by the monoclonal antibodies are also recognized by cattle vaccinated with recombinant Hsp70 protein. Whether or not these calves were experimentally infected with *MAP* did not alter the antibody response to these epitopes. Similar results were obtained with goat kids although in the C-term region the dominant epitope in P590-603 was not recognized, yet a different epitope P605-618 was recognized.

In goats like in calves the experimental exposure to *MAP* concurrent with vaccination did not substantially influence the major B cell responses to vaccination with Hsp70. Experimental *MAP* infection alone did not lead to measurable induction of Hsp70 specific immune responses.

While relatively few data are available regarding B cell epitopes of the *MTb*-complex Hsp70 protein, B cell epitopes of *MLE* Hsp70 have been studied in more detail by Peake et al. [19]. One dominant B cell core epitope sequence EEADVRNQAE was described in the C-terminal 142 amino acids fragment of *MLE* Hsp70, which is identical between *MAP* and *MLE*. Although we did not find specific hybridoma's, the EEADVRNQAE containing peptide (P505-514) was recognized by sera of goats vaccinated with the *MAP* Hsp70 protein. Also the peptide AMEKLGQDSQALGQAIYEATQAASK was identified in the study of Peake et al. as a surface exposed epitope on *MLE* Hsp70 [19]. This peptide is represented in our study by *MAP* Hsp70 peptide QALGQAIYEATQAE (P575-588) and this peptide is also exclusively recognized by Hsp70 vaccinated goats which were not exposed to *MAP*. The differences observed in Balb/c responses may be explained by substantial differences in experimental procedures, as we vaccinated with the whole recombinant protein in DDA adjuvants and therefore may direct the immune response to different epitopes not present in the immunogen used by Peake at al.

In these as well as previous studies [17] it has been shown that in natural cases of paratuberculosis, both in pre-clinical as in clinical stages, very little Hsp70 specific antibody is formed. This is in contrast with the Hsp60 protein to which a pronounced antibody response is generated in natural disease. In contrast to the lack of induction of antibody responses the Hsp70 protein does induce a cell mediated response in natural cases of *MAP* [16]. In addition, also in experimental infections no antibody response against the Hsp70 protein can be detected [15]. However following vaccination with *MAP* Hsp70 formulated with DDA adjuvant a dominant antibody response is mounted against the protein. Following a short measurable induction of cell mediated immunity to Hsp70, we have very little evidence of a substantial prolonged period of activation

of Hsp70 specific cell mediated immunity [7, 15].

Apparently, during natural infection immune responses are skewed away from antibody responses towards cell mediated immune responses as far as the Hsp70 protein is concerned. It is likely that pathogen derived Hsp70 maybe encountered in lesional debris of dead mycobacteria and apoptotic bodies from infected host cells, either free or bound by specific antibodies to exposed antigens and thus effectively taken up and processed by antigen presenting cells. In the context of local mycobacterial infection, especially in early stages of paratuberculosis, adaptive immune responses have a Th1 signature and responses to various antigens may be skewed in this direction under these conditions.[6] The data presented in this study indicate that the Hsp70 protein is accessible to antibodies on intact *MAP* bacteria in suspension as well as incorporated in tissue and that the presence of the Hsp70 protein in the cell wall appears more pronounced in *MAP* as compared to *MAA*, despite high sequence similarities of *MAP* and *MAA* Hsp70 protein. As has been demonstrated for the smaller 16kD α-crystallin heat shock protein with respect to *MTb* [23], Hsp70 appears to be present in the intact mycobacterial cell wall of *MAP*. Furthermore it has been shown that local application of specific monoclonal antibodies to the 16kD α-crystallin confers protection to early stage tuberculous infection in a murine model of tuberculosis [23]. Thus antibodies specific for Hsp70 may contribute to protective immunity in mycobacterial infections which other studies have also indicated (reviewed in [12]). The generalized local skewing of immune responses may therefore not constitute the optimal protective response. Especially in paratuberculosis these Th1 directed responses in early stages of infection are easily detected however most animals do not recover from infection but become chronically infected, pointing towards insufficient protective immunity. An early adequate antibody response to surface exposed antigens may therefore be a feature of protective immune responses.

In conclusion this study demonstrated that at least two dominant linear B cell epitopes are present in the Hsp70 molecule. These are accessible to antibodies under a number of denaturing and non-denaturing as well as native conditions. On the one hand this provides options to use these antibodies in diagnostic specimen as an alternative to Ziehl-Neelsen staining. More importantly this study provides indications that the Hsp70 protein is present in the bacterial cell wall of *MAP* and it may be argued that vaccination induced antibodies, apparently not produced in the presence of *MAP* only, indeed bind intact bacteria and possibly alter their cellular fate following uptake by macrophages and other antigen presenting cells thus playing a role in the protective effect shown after Hsp70/DDA subunit vaccination against bovine paratuberculosis [15].

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5

Chapter 5

Subpopulations of bovine WC1^{+} γδ T cells rather than CD4^{+}CD25^{high}Foxp3^{+} T cells act as immune regulatory cells *ex vivo*

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SUMMARY

Regulatory T cells (Treg) are regarded essential components for maintenance of immune homeostasis. Especially $CD4^+CD25^{high}$ T cells are considered to be important regulators of immune reactivity. In humans and rodents these natural Treg are characterized by their anergic nature, defined as a non-proliferative state, suppressive function and expression of Foxp3. In this study the potential functional role of flow cytometry-sorted bovine white blood cell populations, including $CD4^+CD25^{high}$ T cells and γδ T cell subpopulations, as distinct *ex vivo* regulatory cells was assessed in co-culture suppression assays. Our findings revealed that despite the existence of a distinct bovine $CD4^+CD25^{high}$ T cell population, which showed Foxp3 transcription/expression, natural regulatory activity did not reside in this cell population. In bovine co-culture suppression assays these cells were neither anergic nor suppressive. Subsequently, the following cell populations were tested functionally for regulatory activity: $CD4^+CD25^{low}$ T cells, $WC1^+$, $WC1.1^+$ and $WC1.2^+$ γδ T cells, NK cells, $CD8^+$ T cells and $CD14^+$ monocytes. Only the $WC1.1^+$ and $WC1.2^+$ γδ T cells and $CD14^+$ monocytes proved to act as regulatory cells in cattle, which was supported by the fact that these regulatory cells showed IL-10 transcription/expression. In conclusion, our data provide first evidence that cattle $CD4^+CD25^{high}Foxp3^+$ and $CD4^+CD25^{low}$ T cells do not function as Treg ex vivo. The bovine Treg function appears to reside in the γδ T cell population, more precisely in the $WC1.1^+$ and the $WC1.2^+$ subpopulation, major populations present in blood of cattle in contrast to non- ruminant species.

INTRODUCTION

Immunoregulation comprises complex mechanisms involving the activities of various immune cell subtypes, among which natural regulatory T cells (Treg). Disbalance between responses that control the infection and counteracting responses that prevent chronic inflammation can lead to insufficient clearance of pathogens i.e. chronic infection and/or chronic inflammation. Regulatory T cells, especially $CD4^+CD25^{high}$ T cells, capable of suppressing immune responses in vitro and/or in vivo, were shown to express the transcription factor Foxp3 in the human and rodent systems [13]. Transfer of T cells depleted of $CD4^+CD25^+$ T cells, in contrast to transfer of the total T cell population, into athymic nude mice caused spontaneous development of T cell-mediated autoimmune responses. Thus the function of $CD4^+CD25^+$ T cells as natural Treg was defined [35]. Subsequently, $CD4^+CD25^+$ natural Treg were shown to function in controlling and regulating the immune system in infectious diseases [19], self-tolerance and autoimmune diseases [2]. Other cell types like suppressor

monocytes shown to have a regulatory/suppressor function in the human immune system in tissue injury and during inflammation [6] e.g. allergic inflammation [31]. In the bovine immune system a role for monocytes has been suggested in the control of γδ T cell responses [25], probably mediated by IL-10 secretion [23]. Literature suggests immunomodulation by γδ T cells [7, 32] and in addition potential age related differences in immune regulatory roles of $WC1^+$ γδ T cell subsets in ruminants [16, 33]. A functional regulatory/suppressive role of $WC1^+$ γδ T cells, comprising $WC1.1^+$, $WC1.2^+$ [12, 21] and $WC1.3^+$ subsets, and $WC1^-$ γδ T cells has not been shown thus far ex vivo. In humans an immunoregulatory role is suggested for Vδ2 T cells induced in the presence of BCG infected dendritic cells (DC) [22] and tumor-infiltrating Vδ1 T cells suppressing T and DC function [27].

The aim of the present study was to identify potential regulatory cells in cattle *ex vivo* with special emphasis on $CD4^+CD25^{high}$ T cells, as potential natural Treg in comparison to $WC1^+$ γδ T cells, $CD8^+$ T cells, NK^+ and $CD14^+$ subpopulations. Their regulatory potential was investigated functionally using co-culture assays [40] and by analysis of Foxp3, IL-10 and TGF-β transcription by quantitative RT-PCR and intracellular staining for Foxp3 and IL-10 protein expression as additional regulatory cell characteristics.

MATERIALS AND METHODS

Animal and human cell donors

Seven adult Holstein-Frisian cows (A-G, age >2 years) were used in the current study. The cows were housed under conventional conditions, and were checked daily for general health. Two healthy human volunteers donated blood to serve as positive controls in an intracellular staining assay to determine cross reactivity of an anti-murine/rat/human Foxp3 mAb with bovine cells. The use of animals was approved by the Ethical Committee of Utrecht University and performed according to their regulations.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) from human (n = 2) and bovine donors (A-G) were isolated from aseptically drawn heparinized blood samples by Histopaque 1.077 (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation. From PBMC of cow A a γδ T cell line ($W15B = N24^+/WC1.2^+$) was obtained by culturing these cells for more than 10 weeks in the presence of 100 U recombinant human IL-2/mL according to a published protocol [10].

Antibodies and flow cytometry

Unlabeled primary mouse mAb against bovine cell surface markers that were used in this study include: anti-CD25 (CACT108A, IgG2a), anti-CD14 (MM61A, IgG1), anti-CD21 (GB25A, IgG1), anti-γδ TCR (N24 ; GB21A, IgG2b), anti-CD3 (MM1A, IgG1), anti-Workshop cluster 1 (WC1; IL-A29, IgG1), anti-WC1.1 (BAQ159A, IgG1), anti-WC1.2 (CACTB32A, IgG1) from Veterinary Medical Research & Development (VMRD, Pullman, WA, USA); anti-CD335 (AKS1; NKp46, IgG1) from AbD Serotec (Kingston, NH, USA); anti-CD8 (IL-A105, IgG2a) kindly provided by J. Naessens (International Livestock Institute (ILRI) Nairobi, Kenya). As murine isotype controls biotinylated CD107 (IgG1), OX8, (IgG1), UD17 (IgG2a) and OX40 (IgG2b) from Hybridoma Center (Utrecht University) were used. The following conjugated antibodies against cell surface markers, intracellular cytokine and nuclear protein that were used in this study include: anti-bovine CD4 (IL-A11, IgG2a)-FITC, kindly provided by J. Naessens (ILRI); anti-bovine IL-10 (MCA2111B; CC320, IgG1)-biotin from AbD Serotec; anti-mouse/rat/human Foxp3 (150D, IgG1k)-Alexa Fluor 647® and murine IgG1k isotype control (MOPC-21, IgG1k)-Alexa Fluor 647® (Biolegend, San Diego, CA, USA) kindly provided by G. Lay (CEO Biolegend); anti-human CD4 (SK3, IgG1)-Cy-Chrome and anti-human CD25 (2A3, IgG1)-PE from Becton Dickinson (BD pharmingen, San Diego, CA, USA), kindly provided by Y. Vercoulen en B. Prakken (Wilhelmina Children's Hospital, Utrecht, The Netherlands). The following were secondary antibodies that were used: goat anti-mouse-PE, goat anti-mouse-FITC and streptavidin-PE from BD pharmingen.

For phenotype analysis and FACS sorting, cells were single and double-color surface stained in predetermined optimal concentrations according to methods described previously [18]. Data were acquired on a FACScan™, FACSCalibur™ or Vantage™ SE flowcytometer (BD) and analyzed using CELLQuest™ (BD) and WinMDI 2.8 software.

FACS sorting of bovine cell subpopulations

All bovine cell subpopulations were purified by FACS on a BD Vantage™ SE flowcytometer based on their characteristic forward and side scatter properties in combination with fluorescence intensity after surface staining. Isotype controls were used as technical controls to set fluorescence thresholds. Percentages of low frequency cell types (0.3-3% of all PBMC) like $CD4^{+}CD25^{high}$ (trace population), $WC1.1^{+}$ and $WC1.2^{+}$ γδ T cells were initially determined for 7 cows (A-G) on multiple independent occasions. Subsequently the cells were isolated from 2-6 × 10^{8} PBMC per cow per experiment. Purities of cell subpopulations as determined in 125 separate FACS sort experiments was 93.5 ± 6.0%.

Quantitative real time Reverse Transcriptase–Polymerase Chain Reaction (qRT-PCR) for bovine Foxp3, IL-10 and TGF-β

To determine a possible regulatory profile of the PBMC subpopulations $CD3^+$, $CD4^+$, $CD8^+$ T cells, $N24^+$ γδ T cells, $CD21^+$ B cells, $CD14^+$ monocytes and $NKp46^+$ cells (cow A-C) mean expression of Foxp3, IL-10 and TGF-β was assessed by qRT-PCR. In addition a similair assessment was performed on a panel of subpopulations of $CD4^+$ T cells and γδ T cells, being $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, $CD4^+CD25^-$, $CD4^-CD25^-$ cells (cow A-C) and $N24^+$, $WC1.1^+$ and $WC1.2^+$ γδ T cells (cow A and C). Sorted cells were used to isolate RNA with the RNeasy Mini Kit and the RNase-Free DNase set (Qiagen Benelux BV, Venlo, The Netherlands). For reverse transcription of mRNA the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) was used. According to the manufacturer's recommendations 5 ng of cDNA was used per PCR reaction. Bovine specific primer sequences were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA) and published bovine cytokine mRNA sequences (GenBank). As a housekeeping/ reference gene the β2-microglobulin gene was chosen. Primers (Invitrogen, Breda, The Netherlands**)** used in this study include β2-microglobulin forward (50 nM): TTACCTGAACTGCTATGTGTA-TGG; β2-microglobulin reverse (300 nM): GCTGTACTGATCCTTGCTGTTG (Gen-Bankno: X69084), IL-10 forward (300 nM): TGACATCAAGGAGCACGT-GAA; IL-10 reverse (300 nM): TCTCCACCGCCTTGCTCTT (GenBankno: U00799), TGF-β forward (300 nM) : TTCTTCAACACGTCCGAGCTC; TGF-β reverse (300 nM): AGCGCCAGGAATTGTTGCTAT (GenBankno: M36271), Foxp3 forward (300 nM): CACAACCTGAGCCTGCACAA; Foxp3 reverse (300nM): TCTTGCGGAACTCAAACTCATC (GenBankno: DQ322170).

QRT-PCR was performed with the ABI Prism 7000 Real-Time PCR Cycler (Applied Biosystems) using iTaq SYBR Green Supermix with ROX (Bio-Rad). All PCR reactions were set up in 96-well microAmp plates (Applied Biosystems) using predetermined forward and reverse primer concentrations in a reaction volume of 25 µL. After 10 min dissociation at 95 °C, the reactions were cycled 40 times at 95 °C for 15 s and 60 °C for 1 min. Melting point analysis was done after the last cycle to verify the amplification specificity. A twenty minutes temperature gradient was performed after the last cycle by cooling samples to 60 °C and increasing the temperature to 95 °C at 0.5 °C/min. A single product at a specific melting temperature was found for each target gene. Specificity of the PCR products, based on the predicted sizes according to the designed primer sets, was also confirmed by gel electrophoresis (2% agarose).

All samples were tested in triplicate and the mean cycle threshold (Ct) was used for

further calculations. Each run included a non-template control to test for contamination of assay reagents. Real-time PCR efficiencies (E) were calculated, using different cDNA concentrations of total PBMC to produce a standard curve. The relative expression ratio (R) of a target gene was calculated based on the PCR efficiency and the Ct deviation of a tested sample versus the control (total PBMC) [28], and expressed in comparison to β2-microglobulin using the ABI PRISM 7000 sds software version 1.1 and quantification software REST© MCS (Relative Expression Software Tool) [29]. Relative expression ratio calculated as 2-log ratio were converted in fold change in gene expression.

Intracellular staining (ICS) for Foxp3 and IL-10

Since no bovine Foxp3 specific antibodies were available, cross specificity of the anti-murine/rat/human Alexa Fluor 647®-Foxp3 mAb (clone 150D - IgG1k - Biolegend) [34] with bovine Foxp3 was investigated. As an isotype control the mouse mAb Alexa Fluor 647® (clone MOPC-21 - IgG1k - Biolegend) was used. Intracellular Foxp3 staining of human $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, $CD4^+CD25^-$ and $CD4^-CD25^-$ cells gated from PBMC prelabeled with CD4Cy-Chrome/CD25PE and bovine sorted bovine $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, $CD4^+CD25^-$ and $CD4^-CD25^-$ cells sorted from PBMC prelabeled with CD4FITC/CD25PE was performed according to the protocol of the manufacturer (Biolegend) with minor modifications. Cells, 1×10^6, were aliquoted into 5 mL tubes (BD/Falcon™, NJ, USA) and pelleted to be fixed and permeabilized. Incubation with Foxp3 Fix/Perm solution (Biolegend) was extended for 120 min at 4 °C after the standard 20 min at room temperature, furthermore incubation with Foxp3 Perm solution (Biolegend) was extended for 30 min at 4 °C after the standard 15 min at room temperature. Finally cells were resuspended in staining buffer and analyzed for Foxp3 expression using a FACSCalibur™ with CELLQuest™ software (BD).

Expression of intracellular IL-10 was investigated in bovine isolated PBMC stimulated with Con A (Sigma-Aldrich) (5 μg/mL) for 6 h and in the presence of brefeldin A (Sigma-Aldrich) (10 μg/mL) during the last 5 h. PBMC were single stained by unlabeled WC1, CD21, CD3, CD14, or NKp46 followed by goat anti–mouse-FITC (BD Pharmingen). Fixation and permeabilisation of PBMC were performed in Cytofix/Cytoperm and Perm/Wash solution according to the manufacturer's protocol (BD). Cells were incubated in Perm/Wash containing a predetermined optimal concentration (1/1000, IgG1, 1 mg/mL stock) of biotinylated anti-bovine IL-10 mAb MCA2111B (CC320, AbD Serotec). As an isotype IgG1 control mAb mouse anti-chicken biotinylated CD107 (1/750, IgG1, 1 mg/mL stock, Hybridoma Center, Utrecht

University) was used. As a second step streptavidin-PE (1/1000, BD) for PBMC was used. Finally, cells were washed twice and analyzed for IL-10 expression using a FACSCalibur™ with CELLQuest™ software (BD).

Bovine co-culture suppression assay

In a co-culture suppression assay [40], described for human use and adjusted for bovine purposes, the suppressive function of the following sorted potential regulatory cell subpopulations was tested; $CD4^+CD25^{high}$, $CD4^+CD25^{low}$ and $CD8^+$ T cells, $CD14^+$ monocytes, $NKp46^+$ cells, $WC1^+$, $WC1.1^+$ and $WC1.2^+$ γδ T cells, (cow A-G) and the $WC1^+$ γδ T cell line W15B (cow A).

Their impact on proliferation of sorted $CD4^+CD25^-$ responder T cells (Tresp) activated by plate-bound mouse anti-bovine CD3 mAb (MM1A, 3 µg/mL, inducing approximately 40-50% of the potential maximal Tresp response) in the presence of irradiated sorted $CD4^-CD25^-$ APC was studied. In assay 3×10^4, 6×10^4 and 9×10^4 potential regulatory cells in combination with 3.5×10^4 $CD4^+CD25^-$ Tresp (respectively 0.9:1, 1.7:1 and 2.6:1) and 7×10^4 irradiated (3000 cGy) $CD4^-CD25^-$ APC were co-cultured in a 96 well roundbottom microtiter plate (Corning Costar Corp., Acton, MA, USA). As controls all subpopulations of cells used were cultured as individual populations (Treg, Tresp or APC) and in all possible combinations of double/triple co-cultures. All tests were performed in triplicate in RPMI 1640 tissue culture medium (Gibco®-Invitrogen, Breda, The Netherlands) supplemented with 10% FCS (Bodinco BV, Alkmaar, The Netherlands), 50 IU/mL penicillin, 50 µg/mL streptomycin, 2 mM L-glutamine, 5×10^{-5} M β-mercapto-ethanol (Flow Laboratories, Irvine, UK) and 0.5 µg/mL amphotericin B (Sigma-Aldrich) (complete medium (CM)) at 37 °C and 5% CO_2 in a humidified incubator for 5 days. Finally 0.4 µCi ^{3}H-thymidine (Amersham, Buckinghamshire, UK) was added to each well and cells were cultured for an additional 18 h. Subsequently, cells were harvested onto glass fiber filters and incorporation of ^{3}H-thymidine was measured by micro-Betaplate liquid scintillation counting (EG & G® Wallac, Turku, Finland) and expressed as average cpm + 1SD.

Statistical analysis

All experiments were performed in duplicate or triplicate and results shown are representative of two or three independent experiments. Differences between overlay histograms of Foxp3 stainings were calculated using Kolmogorov Smirnov (two sample K-S test, testing goodness of fit between distributions, p-values < 0.05 were considered statistically significant) statistics as part of CellQuest software (Becton Dickinson). Power calculations for determining animal sample size via transcriptional

analysis of fold changes in gene expressions were performed by Episcope 2.0 software. For all other comparisons, a paired two-tailed t test was used to determine significance differences, p-values < 0.05 were considered statistically significant.

RESULTS

Quantification of bovine $CD4^+CD25^{high}$, $CD4^+CD25^{low}$ T cells and $WC1.1^+$, $WC1.2^+$ γδ T cell populations by flowcytometry

Independent samplings in 7 animals (A-G) showed that in average 27.8 ± 9.3% of all bovine $CD4^+$ T cells were $CD25^+$ (Fig. 1a, gate R2) (i.e. 6.0 ± 2.0% of all PBMC), 23.1 ± 3.7% of all $CD4^+$ T cells were $CD4^+CD25^{low}$ T cells (Fig. 1a, gate R3) (i.e. 5.0 ± 0.8% of all bovine PBMC) and 1.9 ± 0.6% of all $CD4^+$ T cells were $CD4^+CD25^{high}$ T cells (Fig. 1a, gate R4) (i.e. 0.4 ± 0.1% of all bovine PBMC). Of bovine PBMC 7.3 ± 2.0% were γδ T cells and 5.5 ± 1.4% were $WC1^+$ T cells (data not shown). Mean $WC1.2^+$ percentages (Fig. 1b, gate R6) of 48.8 ± 18.2% of all $WC1^+$ γδ T cells (2.7 ± 1.0% of all PBMC) and mean $WC1.1^+$ percentages (Fig. 1c, gate R7) of 36.7 ± 6.9% of all $WC1^+$ γδ T cells (2.0 ± 0.4% of all PBMC) were measured.

Differential transcription of IL-10, TGF-β and Foxp3 in sorted bovine leukocyte subpopulations

In total PBMC and sorted subpopulations: $CD3^+$, $CD4^+$, $CD8^+$, $N24^+$, $CD21^+$, $CD14^+$ and NK^+ cells, isolated from cows A, B, and C (Fig. 2a), mean transcription levels of Foxp3, IL-10 and TGF-β were determined by qRT-PCR, at 3 or more independent

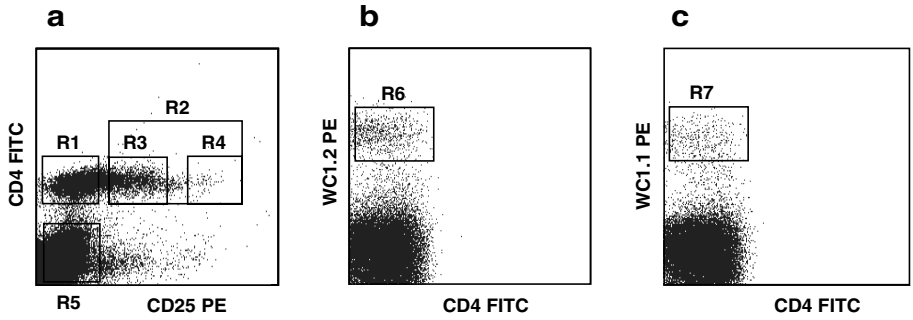

Fig. 1. CD4CD25, $WC1.2^+$ and $WC1.1^+$ expression on stained bovine PBMC. (a) Representative dotplot of CD4FITC/CD25PE double-color surface stained PBMC (cow A) and gate regions for sorting and/or frequency analysis of $CD4^+CD25^-$ (gate R1), total $CD25^+$ (gate R2), $CD4^+CD25^{low}$ (gate R3), $CD4^+CD25^{high}$ (gate R4) and $CD4^-CD25^-$ (gate R5) cells. (b) Representative dotplots of $WC1.2^+$, (gate R6) respectively (c) $WC1.1^+$ (gate R7) γδ T cell subsets stained PBMC and gate regions for sorting and frequency analysis.

occasions, and representative results were depicted as fold change in gene expression. Specificity of the PCR products, based on the predicted sizes according to the designed primer sets, confirmed by gel electrophoresis, showed expected products of 171 bp for β2-microglobulin, 141 bp for TGF-β, 112 bp for IL-10 and 88 bp for Foxp3 (Fig. 2b).

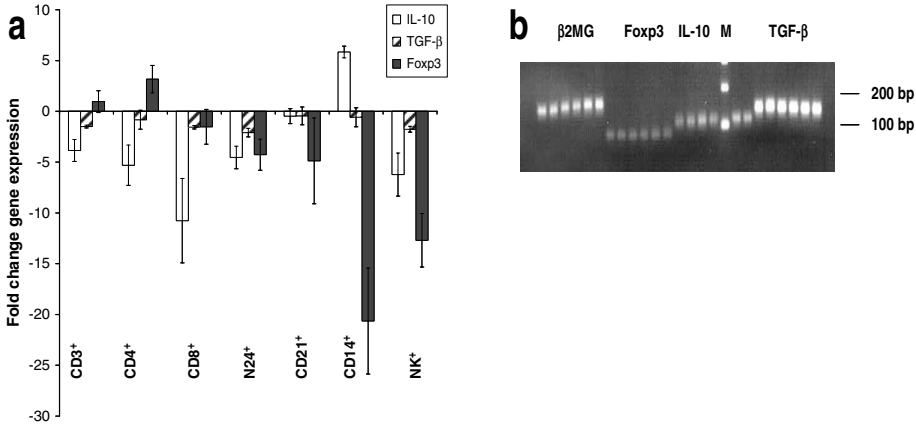

Fig. 2. Transcription of IL-10, TGF-β and Foxp3 in 7 sorted bovine cell subpopulations as determined by qRT-PCR. (a) PBMC were isolated from cows A-C, respectively PE stained and sorted $CD3^+$, $CD4^+$, $CD8^+$, $N24^+$, $CD21^+$, $CD14^+$, $NKp46^+$ cells were tested. Results show mean fold changes in gene expression (± 1SEM) of triplicate samples from three representative experiments of specific cell populations compared to total PBMC, calculations based on REST© (Relative Expression Software Tool) software [29] relative to the gene expression of β2-microglobulin. (b) MW sizes of products of qRT-PCR (6 independent PCR), for β2-microglobulin (171 bp), Foxp3 (88 bp), IL-10 (112 bp) and TGF-β (141 bp) (cow A) performed on bovine PBMC derived samples on a 2% agarose gel and compared to a MassRuler™ low range DNA ladder.

The transcription in the specific cell populations compared to that in total PBMC, (mean fold change expression in 3 animals), $CD3^+$ T cells showed a fold change in Foxp3 gene expression of 1.0 and $CD4^+$ T cells showed a fold change in Foxp3 gene expression of 3.2. In $CD14^+$ cells a 5.8 fold change in IL-10 gene expression was observed. In all other cell subpopulations IL-10, TGF-β and Foxp3 transcription showed a -0.5 to -20.6 fold change in gene expression compared to that found in total PBMC.

In sorted $CD4^+CD25^{high}$ T cells from all three animals, a 51.2 fold change in Foxp3 gene expression than in total PBMC was observed, in $CD4^+CD25^{low}$ cells a 12.5 fold change, in $CD4^+CD25^-$ a 1.5 fold change and in $CD4^-CD25^-$ a -1.7 fold change in Foxp3 gene expression was measured (Fig. 3a). In all CD4CD25 subsets mean

changes in IL-10 and TGF-β gene expression were measured ranging from a 1.2 to -5.4 fold change in gene expression compared to total PBMC (Fig. 3a). Finally, in the γδ T cell subpopulations of cow A and cow C (Fig. 3b, cow B had to be culled during the experiments due to an infectious disease), IL-10 mRNA was transcribed in all $WC1^+$ cells as well as in the $WC1.1^+$ and $WC1.2^+$ subpopulations ranging from a 7.3-25.9 fold change in IL-10 gene expression compared to that found in total PBMC. A mean change in Foxp3 and TGF-β expression was only detected in low amounts (ranging from 1.2 to -5.0) compared to that found in total PBMC (Fig. 3b).

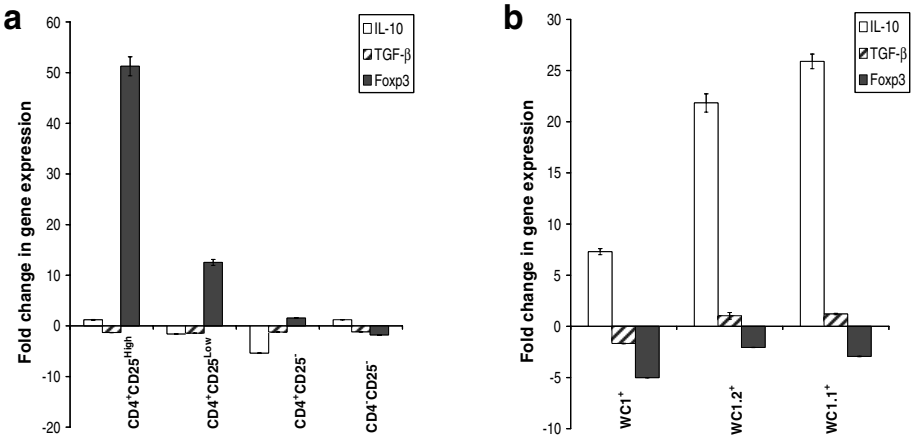

Fig. 3. Transcription of IL-10, TGF-β and Foxp3 in CD4CD25 and γδ T cell sorted bovine cell subpopulations as determined by qRT-PCR. (a) $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, $CD4^+CD25^-$, and $CD4^-CD25^-$ cells were FACS sorted (CD4FITC/ CD25PE) from cow A-C and (b) $WC1^+$, $WC1.2^+$ and $WC1.1^+$ PE stained γδ T cells were sorted from cow A and C. Results show mean fold changes in gene expression (± 1SEM) of triplicate samples from three representative experiments of specific cell populations compared to total PBMC, calculations based on REST© (Relative Expression Software Tool) software [29] relative to the gene expression of β2-microglobulin.

Intracellular expression of Foxp3, but not IL-10, in bovine $CD4^+CD25^{high}$ T cells

Flowcytometric analysis of human PBMC surface double stained for CD4 and CD25 revealed that only $CD4^+CD25^{high}$ and not $CD4^+CD25^{low}$ (Fig. 4a) or $CD4^+CD25^-$ and $CD4^-CD25^-$ (data not shown) showed intracellular staining by the crossreactive anti-Foxp3 mAb compared to an isotype control.

Bovine CD4 and CD25 surface double stained PBMC sorted for $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, $CD4^+CD25^-$ T cells and $CD4^-CD25^-$ cells after ICS with anti-human/ mouse/rat Foxp3-647 mAb showed staining in $CD4^+CD25^{high}$ T cells and not in $CD4^+CD25^{low}$ (Fig. 4a) or $CD4^+CD25^-$ and $CD4^-CD25^-$ (data not shown) cells after

intracellular staining by the cross reactive anti-Foxp3 mAb compared to an isotype control.
Bovine PBMC, Con A stimulated and brefeldin A treated, stained by mouse anti-chicken CD107 as an isotype control mAb irrelevant for the bovine system showed low staining compared to anti-bovine IL-10 mAb in a representative experiment (Fig. 4b). Furthermore intracellular IL-10 staining was shown in $CD14^+$ monocytes, low intracellular IL-10 staining in $CD3^+$ T cells and $WC1^+$ γδ T cells and no IL-10 staining in $CD21^+$ or $NKp46^+$ cells (Fig. 4c).

Bovine $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells are non-anergic and lack suppressive properties; $WC1.1^+$, $WC1.2^+$ γδ T cells and $CD14^+$ monocytes show suppressive properties

Inhibition of proliferation of a fixed number of anti-CD3 stimulated bovine $CD4^+CD25^-$ responder T cells (Tresp) + irradiated APC ($CD4^-CD25^-$), by increasing numbers of the potential regulatory cell (sub)populations: $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, $CD8^+$ T cells, the γδ T cell subsets $WC1^+$, $WC1.1^+$, $WC1.2^+$ and $WC1.2^+$ T cell line W15B, NK^+ cells, and $CD14^+$ cells (cow A-G) was determined in a bovine co-culture assay (Fig. 5a-i).
Proliferation ranging from 77 000-247 000 cpm was observed when $CD4^+CD25^-$ Tresp cells were cultured in the presence of plate-bound aCD3 and irradiated APC confirming the potency of the bovine readout system. When increasing numbers of $CD4^+CD25^{high}$ T cells were cultured in the presence of plate-bound aCD3 and irradiated APC (without Tresp) a dose dependent proliferation ranging from 87 000 – 211 000 cpm was shown (Fig. 5a) indicating that these cells are non-anergic. Addition of increasing numbers of these $CD4^+CD25^{high}$ T cells to bovine $CD4^+CD25^-$ Tresp cells in a co-culture assay showed a dose dependent significant increase of proliferation ranging from 49-72% ($p < 0.0005$) compared to the proliferation of Tresp + APC only (Fig. 5a).
Significantly increased proliferation was also measured after addition of $CD4^+CD25^{low}$ T cells (43-65%, $p < 0.003$) (Fig. 5b) and $CD8^+$ T cells (122-287%, $p < 0.0007$) (Fig. 5c). The $NKp46^+$ cell population (Fig. 5d) and $WC1^+$ γδ T cell subpopulation (Fig. 5e) showed no significant effect ($p > 0.05$) on proliferation of Tresp + APC.
Dose dependent inhibition of proliferation of Tresp was caused by the $WC1^+$ γδ T cell line W15B (14-36%, $p < 0.003$), $WC1.2^+$ ($WC1\text{-}N3^+$) γδ T cells (38-64%, $p < 0.0003$) and $WC1.1^+$ ($WC1\text{-}N4^+$) γδ T cells (32-44%, $p < 0.0009$) (Fig. 5f-h). Furthermore, the $CD14^+$ cell population caused dose dependent inhibition (67-97%, $p < 0.000007$) of proliferation of Tresp + APC (Fig. 5i) (p values reflect comparison of proliferation of potentially Treg + Tresp (2.6:1) + APC vs Tresp + APC).

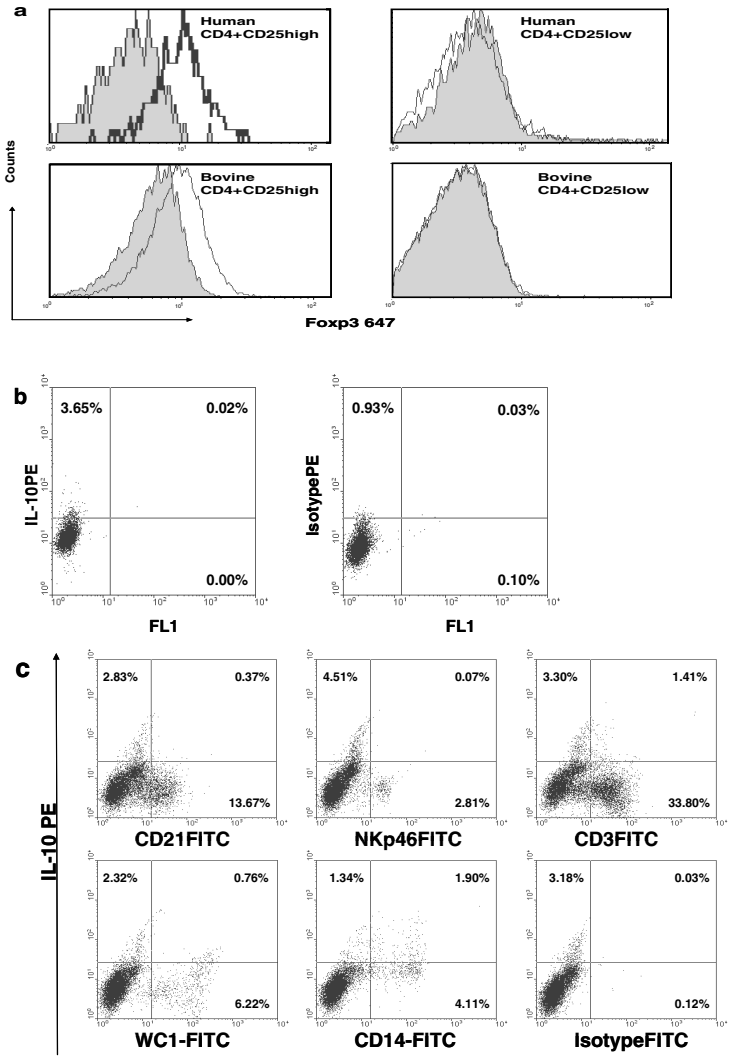

Fig. 4. Intracellular staining of bovine Foxp3, IL-10, and human Foxp3.
(a) Histograms showing intracellular Foxp3 fluorescence intensity of gated human $CD4^{+}CD25^{high}$, $CD4^{+}CD25^{low}$ and FACS sorted bovine $CD4^{+}CD25^{high}$, $CD4^{+}CD25^{low}$ T cells after ICS with anti-human/mouse/rat Foxp3 mAb (150DAlexa 647, unfilled histogram) compared to a murine IgG1k isotype control mAb (MOPC-21Alexa 647, filled histogram). (b) Dotplots representing intracellular staining of IL-10 in bovine PBMC gated for live cells after Con A stimulation + Brefeldin A, stained with intracellular biotinylated anti-bovine IL-10 (IgG1) and StreptavidinPE as a second step and the relevant isotype control biotinylated anti-chicken CD107 (IgG1). (c) Dotplots representing intracellular staining of IL-10 in bovine PBMC gated for live cells after Con A stimulation + Brefeldin A, surface stained with anti-bovine CD21, NKp46, CD3, WC1, CD14 and goat anti-mouseFITC as a second step in combination with intracellular biotinylated anti-bovine IL-10 and StreptavidinPE as a second step.

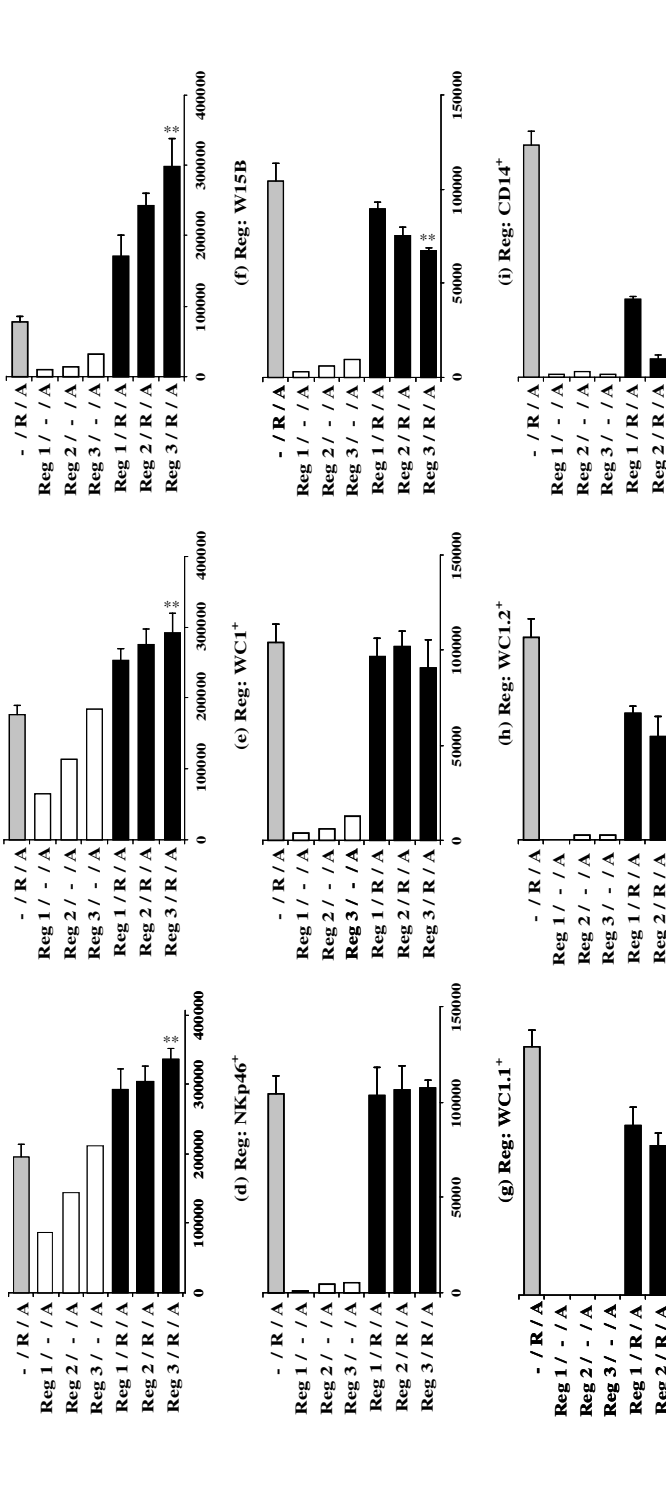

Fig. 5. Ex vivo regulatory functions of 9 bovine cell populations in a co-culture assay. As a read out system (grey bars) proliferation of 35 000 $CD4^-CD25^-$Tresp cells (population R1 in Fig. 1, indicated as R along the Y-axis), isolated from peripheral blood (cow A as a representative example) combined with 70 000 irradiated $CD4^-CD25^-APC^-$(population R5 in Fig. 1, indicated as A along the Y-axis) is shown. In white bars control results of co-culturing 30 000 (indicated as Reg 1 along the Y-axis), 60 000 (indicated as Reg 2 along the Y-axis) or 90 000 (indicated as Reg 3 along the Y-axis) potential regulatory cells (a) $CD4^+CD25^{high}$, (b) $CD4^+CD25^{low}$, (c) $CD8^+$, (d) $NKp46^+$, (e) $WC1^+$, (f) W15B ($WC1^+$ T cell line), (g) $WC1.1^+$, (h) $WC1.2^+$ and (i) $CD14^+$ monocytes combined with 70 000 irradiated $CD4^-CD25^-APC$ are shown. In black bars results are shown of co-cultures with the 9 potential regulatory cells (30 000-90 000 cells indicated as Reg 1-3 along the Y-axis) in combination with $CD4^-CD25^-$Tresp (R) and irradiated $CD4^-CD25^-APC^-$(A). The data are representative experiments and are presented as dose dependent (30 000, 60 000 and 90 000) potential Treg proliferation or ratio dependent (potential Treg: Tresp = 0.9:1, 1.7:1 and 2.6:1, R1-R3:R) as the mean of proliferation on day 5 (+ 1 SD) in cpm. The stimulus used was plate-bound anti-bovine CD3 at a sub maximal concentration of 3 µg/mL. (p values reflect comparison of R1 + R (2.6:1) + A versus R + A, ** $p \leq 0.01$).

DISCUSSION

To our knowledge this is the first report describing the identification and functional characterization of sorted ex vivo bovine $WC1^+$ γδ T cell subsets, WC1.1 and WC 1.2, and $CD14^+$ cells as leukocytes with regulatory function. In addition it was shown that in cattle in contrast to humans and rodents the $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cell subpopulations do not have regulatory capacity ex vivo.

Regulatory cells are regarded essential components in maintenance of homeostasis of the immune system capable of suppressing other immune responses in vitro and/or in vivo. In rodents approximately 5-15% of the $CD4^+$ cells identified in the spleen are $CD25^+$ cells and 6-10% of all $CD4^+$ T cells demonstrate regulatory function [36]. The percentage of circulating $CD25^+$ cells identified in humans is approximately 10-15% of all $CD4^+$ T cells and approximately 1-3% of all human $CD4^+$ T cells demonstrates regulatory function [34] as tested in a co-culture assay. The percentage of circulating $CD25^+$ cells identified in cattle was approximately 19-37% of all $CD4^+$ T cells, and the percentage of $CD4^+CD25^{high}$ T cells found in cattle (1.3-2.5% of all $CD4^+$ T cells) was similar to that in humans. The two main characteristics of $CD4^+CD25^{high}$ natural Treg are their functional regulatory (suppressive) activity and their anergic state (non-proliferative) upon stimulation e.g. with plate-bound anti-CD3. In the mouse both the $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells seem to be regulatory while in humans this is confined to the $CD4^+CD25^{high}$ T cell population [3]. The anergic nature of $CD4^+CD25^{high}$ Treg was proven by stimulation via cross linking of the TCR with an anti-CD3 mAb. Addition of low concentrations of IL-2 resulted in minor proliferation of natural Treg in these species. In contrast our functional studies using bovine cells showed that the $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ populations are non-anergic (proliferative) and has no regulatory/suppressive effect.

The expression of Foxp3 is regarded as a hallmark of natural Treg ($CD4^+CD25^{high}Foxp3^+$) and is considered as critically important for the development and function of natural Treg in humans and rodents [43]. Deficiency of Foxp3, a disorder called scurfy in mice [8] and IPEX in humans [5] leads to a fatal autoimmune lympho-proliferative disease as a consequence of chronic T cell activation while overexpression of Foxp3 in the mouse leads to a reduction in the mature T cell population and decreased T cell function, all proving a regulatory disbalance. Foxp3 was also identified in murine $B220^+$ B cells [8] however these cells displayed no suppressor function in vitro in contrast to certain human $CD8^+LAG\text{-}3^+CD25^+Foxp3^+CCL4^+$ T cells that did display suppressor function [14] indicating that the links between Foxp3 and suppression and between suppression and CD4/CD25 expression may not be absolute. The present

study showed that the bovine Foxp3 gene is highly transcribed in $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells, while transcription was low or almost absent in $CD4^+CD25^-$ or $CD4^-CD25^-$ T cells, consistent with the situation in humans and rodents. Low expression of intracellular bovine and moderate expression of human Foxp3 protein was detected in $CD4^+CD25^{high}$ T cells, but none could be detected in $CD4^+CD25^{low}$, $CD4^+CD25^-$ T cells or $CD4^-CD25^-$ human or bovine cells. Foxp3 staining in bovine $CD4^+CD25^{high}$ T cells proved to be weaker, probably due to less specificity of the crossreactive anti-human/mouse/rat Foxp3 mAb for bovine Foxp3 as compared to human cells and/or lower amounts of intracellular Foxp3 present in bovine versus human cells.

In humans two different isoforms of Foxp3 are known and the ectopic expression of the Foxp3Δ2 (lacking exon 2) failes to induce the development of suppressor T cells but leads to Foxp3 expression without regulatory T cell development [1]. Human IPEX-like-3 patients show moderate to low, full length, Foxp3 expression, so the affected $CD4^+CD25^{high}$ T cell regulation may result from mutations in other genes, questioning the role of Foxp3 as the "master regulator" of human natural Treg development and function [11]. Seo et al. identified the bovine Foxp3 gene and sequenced the bovine Foxp3 protein, and found a high similarity with other homologs of Foxp3 [38]. Since the mAb 150D (Biolegend) used in the current study, recognizes a Foxp3 epitope in the exon 2 region, a positive ICS shows the expression of exon 2 and no expression of Foxp3Δ2. The exclusive high presence of Foxp3 transcription in cattle $CD4^+CD25^{high}$ T cells which lack regulatory function argues against a prominent role for Foxp3 as driving force in development and function of 'natural Treg' in cattle. Foxp3 may be an important but not unique marker [17] that defines all cells with regulatory activity in most species however plays a functional role following activation of conventional T cells [34].

In conclusion, the presence of bovine $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells was demonstrated; Foxp3 mRNA was detected in both celltypes and protein expression in $CD4^+CD25^{high}$ T cells only. In functional assays these cells failed to confirm being regulatory T cells in cattle. Our data therefore indicate that co-expression on T cells of $CD4^+CD25^{high}$ and endogenous Foxp3, at least in cattle, is not exclusive for functional bovine regulatory T-cells, as has recently also been described in humans [41]. This indicates that the bovine immune system may be governed by different regulatory mechanisms as compared to rodents and humans.

Apart from $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells, different cell types have been suggested as potential regulatory cells (i.e. $CD4^+$, $CD8^+$ T cells, $CD14^+$ cells and $WC1.1^+$, $WC1.2^+$ γδ T cells) in humans, rodents [6, 20, 26, 31, 39] and cattle [7, 16,

25, 33, 38] but has until now not been proven functionally ex vivo.

In the present study ex vivo functional evidence of the regulatory potential of the bovine WC1.1$^+$, WC1.2$^+$ γδ T cell subsets and CD14$^+$ monocytes has been generated in the co-culture suppression assay. Furthermore it was shown that NK cells and CD8 T cells do not show regulatory functions ex vivo.

Activation of γδ T cells studied by Sathiyaseelan et al. [37] and Baldwin et al. [4] showed that bovine γδ T cells could be activated to a low degree in response to CD3 crosslinking. We observed a similar response when, WC1$^+$, WC1.1$^+$ and WC1.2$^+$ γδ T cells + irradiated APC were tested in a co-culture assay (Fig. 5), proliferation ranging from 2 000-13 000 cpm by 90 000 cells which is low compared to the proliferation of CD4$^+$CD25$^-$ T responder cells + irradiated APC (ranging from 77 000-247 000 cpm by 35 000 cells). Crosslinking of CD3 may not be the optimal stimulation for γδ T cell proliferation but it does stimulate suppressive activity as shown in our co-culture experiments. Finally it is possible that γδ T cells are already activated in vivo or require less stringent (or none at all) activation requirements for suppressive function.

Suppression as a biproduct of sequestration of IL-2 by natural Treg has been suggested in the past, and although IL-2 is a vital cytokine maintaining and activating natural Treg, it is not sequestration but rather inhibition of IL-2 production in Tresp cells [36]. Sathiyaseelan et al. [37] showed that the sequestration of IL-2 by γδ T cells does not seem a likely explanation for the observed suppressive effect as addition of IL-2 in combination with CD3 crosslinking didn't show a substantial increase in γδ T cell replication in bovine PBMC cultures. Sequestration of growth factors can not formally be excluded, although in the presented experiments we found dose dependent decreased proliferation which is not in accordance with sequestration of IL-2 by γδ T cells. First because these cells are not likely to proliferate to a large extent as argued above (in contrast to the responder cells, that eventhough in comparable or lower numbers were shown to be readily activated, thus to produce IL-2). Second because it is likely that activation of the γδ T cells would have contributed to the total proliferation, not in accordance with the observed decreased proliferation.

In view of the apparent discrepancy in suppressive activity between sorted WC1.1$^+$ / WC1.2$^+$ and WC1$^+$ γδ T cells other cell populations like WC1.3$^+$ γδ T cells or other WC1$^+$ subpopulations, as yet unidentified within the WC1$^+$ γδ T cell population, may influence the potential regulatory or stimulatory cells within the WC1$^+$ γδ T cell population. Furthermore potentially regulatory T cells present in the WC1$^+$ populations may influence each others function.

WC1.1$^+$ cells described in this study as immune suppressors showed to be IFN-γ secretors [33], after specific defined stimulation, that can act as innate immune cells

potentially by directing adaptive immune responsiveness. These functions seem contradictory, however cells producing IFN-γ and IL-10 simultaneously have been described as a potential regulatory subset of $CD4^+$ T cells maintaining a balance between Thl and Th2-type cells [15]. Besides IFN-γ has been reported to act in a suppressive fashion [24]. Different ways of stimulation may result in differential activities of relevance in adaptive versus innate immune functioning by $WC1.1^+$ cells. Also further subdivision of $WC1.1^+$ and $WC1.2^+$ cell populations can not be excluded.

Since a role for cytokines like IL-10 and TGF-β [42] has been implicated in the regulatory mechanisms of murine and human natural Treg, T_R1 and T_H3 regulatory T cells, we assessed the production of these cytokines by the different bovine celltypes after stimulation [9, 30]. In our study bovine $CD4^+CD25^{high}$ T cells produced low amounts of IL-10 and TGF-β specific mRNA in comparison to the levels in the total bovine PBMC population. Similar low amounts of IL-10 and TGF-β transcription were found in $CD4^+CD25^{low}$, $CD4^+CD25^-$ and $CD4^-CD25^-$ cells. Intracellular staining of bovine PBMC confirmed that no or very little IL-10 was present in $CD4^+CD25^{high}Foxp3^+$ T cells, in agreement to what was found before in the murine system where expression of Foxp3 was proven not to be correlated to IL-10 expression [17]. However, IL-10 transcription was clearly increased in sorted $WC1.1^+$, $WC1.2^+$ γδ T cells and in CD14 monocytes, in addition we found actual expression of this cytokine in both $CD14^+$ and $WC1^+$ γδ T cells which is in accordance with earlier publications [10, 33].

In contrast Foxp3 transcription/ expression were very low or absent in these celltypes.

In conclusion, we have functionally characterized $WC1.1^+$ ($WC1\text{-}N4^+$) and $WC1.2^+$ ($WC1\text{-}N3^+$) γδ T cell subpopulations and $CD14^+$ monocytes, in which IL-10 transcription and expression could be detected but no Foxp3 transcription, as suppressive/regulatory bovine cells. The exact mechanism of the suppression observed was not adressed in the present study. In contrast, $CD4^+CD25^{high}Foxp3^+$ and $CD4^+CD25^{low}$ T cells did not perform the regulatory function shown in other species. The fact that γδ cells are a major T cell population in cattle, varying in numbers in age with as yet undefined function, calls for further analysis of the mechanism of their regulatory function in health and disease in cattle.

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Chapter 6

Summarizing **d**iscussion

SUMMARIZING DISCUSSION

Prevention of paratuberculosis by *MAP* Hsp70 vaccination

The current vaccines against *Mycobacterium avium* subspecies *paratuberculosis*, experimental as well as commercially available, are based on killed or live attenuated whole bacterium preparations. These vaccines induce substantial reduction of the number of clinically affected animals in herds, but do not protect against infection and shedding of *MAP*, interfere with tuberculosis diagnostics in cattle [27] and often cause severe local tissue reaction [42]. Vaccination with DNA encoding heat shock proteins (Hsp) proved to be succesful for treatment of murine tuberculosis (Hsp65 and Hsp70) [31] and ovine paratuberculosis (Hsp60) [44]. In addition mycobacterial Hsp70 has shown to exert a potent adjuvant effect in vaccination against both infectious agents and human tumor cells [30].

In our and other previous studies *MAP* Hsp70 protein showed to be an immunogenic/immunodominant antigen in different stages of bovine paratuberculosis [25]. It is therefore possibly an interesting subunit vaccine candidate to be tested for protective capacity against bovine paratuberculosis (chapter 2). Based on the current insights in transmission of bovine paratuberculosis the most susceptible individuals, neonates, are born in an infectious environment [47]. Since a classical prime-boost (preventive) *MAP* Hsp70 vaccination before experimental challenge would not represent the natural sequel of events, vaccination and challenge were performed at the same time. The *MAP* Hsp70 vaccinated group of animals enabled comparison of development of potential differential immune responses in experimentally infected versus uninfected animals.

The current study (chapter 2) demonstrated that recombinant *MAP* Hsp70 combined with DDA as an adjuvant can be successfully used as a subunit vaccine against bovine paratuberculosis. Vaccination reduced shedding of bacteria in feces significantly during the first 2 years following experimental infection. Unfortunately, our study was too short to evaluate the probable protective effect of the vaccination in terms of prevention of progression to the clinical stage of the disease, which is a hallmark of the whole bacterin vaccine. However the whole bacterin vaccine does not reduce shedding of bacteria in feces. It also does not enable differentiation between vaccinated and infected animals within infected herds, important for efficacy of possible prophylactic (preventive) treatment strategies.

The current dogma states that in early stages of paratuberculosis (and other mycobacterial infections) protective Th1 type cell-mediated immune responses driven by IFN-γ responses [7, 36] predominate, whereas in the clinical stage of the disease the Th2 type humoral responses prevail. This possibly indicates a switch from Th1

to Th2 type immune reactivity related to disease progression. Although the cause of this potential switch is unknown. Factors potentially of influence are intracellular multiplication and killing (via NO release) of *MAP* in macrophages possibly occurring at the same time. In addition during this process cytokines like IL-18 [48], chemokines like MIP-2, its receptor CCR2 [49], and prostaglandins may play an important role in the Th1-to-Th2 switch in paratuberculosis. In our previous studies we demonstrated a switch from Th1- to Th2-type T-cell reactivity in animals naturally infected with *MAP*, that appeared to be associated with a loss of Th1-like T cells both at the site of infection and in blood [23]. Whether the thymic involution observed in later stages of paratuberculosis contributes to clinical signs of disease by means of altered T cell-generation, as compared to age matched healthy controls, is suggested but still unknown [8].

It has not been proven that Th1 responses detected in healthy animals after being chronically infected with *MAP* fully protects these animals, as shedding may still occur and part of the animals may ultimately die when reaching the clinical stage. In fact the only proven disease association is the decreased antigen specific Th1 responsiveness in animals with clinical paratuberculosis. *MAP* antigen specific serological responses [26, 54] especially IgG1(Th2) / IgG2(Th1) isotype differentiation to assess changes in Th1/Th2 balance were studied. Experiments in cattle indicated that there is no uniform association with increased antibody responses during the progression from the asymptomatic stage to the clinical stage of bovine paratuberculosis but rather a loss of type 1 reactivity [26]. Similar serological studies in tuberculosis and leprosy patients have however, yielded contradictory results on whether immunoglobulin sub-isotypes reflect changes in the Th1-Th2 balance [16, 46].

Our study (chapter 2) demonstrated that in spite of the fact that the *MAP* Hsp70 protein was selected because of its ability to induce cell mediated immunity in natural infection, clear differences in IFN-γ production between vaccinated and infected versus non-vaccinated and infected animals was not found. The observed antigen-specific IFN-γ production was related to *MAP* challenge, rather than to *MAP* Hsp70 vaccination. Prominent and prolonged production of antibodies to *MAP* Hsp70 was restricted to the vaccinated groups. Increase in *MAP* Hsp70 specific antibodies, correlating with decreased shedding of bacteria, indicated that antibodies per se are not detrimental in the context of paratuberculosis infection. During the first 2 years after experimentally or natural infection with *MAP* little anti-*MAP* Hsp70 antibody could be detected in spite of the fact that we showed the presence of Hsp70 on the surface of *MAP*. This clearly shows that the response to *MAP* Hsp70 in natural infection as compared to that after vaccination together with DDA as an adjuvant differs [1] [29].

MAP Hsp70 vaccination may thus hamper the use of current serologic diagnostic assays at these early stages of infection [20], as during vaccination the antibodies develop early and the classical serological diagnosis method is usually not possible in this period and commonly used only in animals older than 3 years. The clear difference in *MAP* Hsp70 specific antibody production however enables differentiation between vaccinated and non-vaccinated animals even within infected herds, which is clearly an advantage in the application of the *MAP* Hsp70 vaccine. Little direct or long term side effects were observed after vaccination with *MAP* Hsp70/DDA.

Studies with experimentally infected calves have also challenged the dogma of antibody formation in later stages of infection. They demonstrated that humoral immune responses may occur very early after inoculation [53]. Other studies, using experimental infection through oral dosing and natural infection, suggest that the humoral response does not dominate until later in the infection cycle [35]. Our and other studies show that the differences in humoral responses to *MAP* and *MAP* Hsp70 depend on the antigen dosage and treatment, adjuvant, timing of exposure, and routes of entry in these studies [13].

To our knowledge this is the first study describing a heat shock protein-based subunit vaccine approach to bovine paratuberculosis, which leads to significantly decreased shedding of bacteria into the feces.

Definition of *MAP* Hsp70 T and B cell epitopes in outbred populations

Subunit vaccination like that with *MAP* Hsp70 or even smaller subunits implies the use of antigenic preparations with limited numbers of epitopes, that may even induce differential responsiveness. For that reason we studied the recognition of *MAP* Hsp70 by the immune system of outbred animals and hence fine specificity of responses in the T cell compartment (chapter 3).

After *MAP* Hsp70 immunization T cell responses were detected, indicating activation of Th1 type T cells, with specificity for up to 9 epitopes (chapter 3). Maximal *MAP* Hsp70 T cell responses were observed 14-21 days after secondary immunization, rapidly decreasing thereafter. This is in contrast with the long B cell memory responses observed after *MAP* Hsp70 vaccination (1 to 2 years). Furthermore based on recognition of at least 1 of the 5 most immunodominant *MAP* Hsp70 specific T cell epitopes, promiscuity of responses was detected in 82% of the animals (15 different MHC haplotypes) experimentally infected with *MAP*. This indicates relevance of the epitopes during infection, and may partly explain the efficacy of *MAP* Hsp70 subunit vaccination in an outbred population.

Alignment studies of the complete Hsp70 sequences of different mycobacterial

species showed high homology (and low homology to *E. coli)*. The *MAP* Hsp70 500-589 aminoacid region, however, containing the 5 most dominant peptides, was 100% homologous between *MAP* and *Mycobacterium avium* subsp. *avium (MAA)*, while homology with *Mycobacterium bovis (MBO)*, *Mycobacterium tuberculosis* (*MTU)* and *Mycobacterium leprae* (*MLE*) was 85 to 89% and homology with *E. coli* was only 30%. Of the variable aminoacids, substitutions observed compared to the *MAP*/*MAA* sequence, in this region 54-64% (*MLE-MBO/MTU*) were part of at least one of the 5 identified immunodominant *MAP* Hsp70 peptides. These results indicate that this is a relatively rapidly evolving region within the Hsp70 gene of *MBO*, *MTU* and *MLE* mycobacterial strains compared to the *MAP* and *MAA* strains.

The reason for studying recognition of *MAP* Hsp70 by the immune system of outbred animals and defining B cell epitopes of Hsp70 (chapter 4) was a result of the pronounced and prolonged antibody production observed after MAP Hsp70 vaccination that coincided with decreased shedding of bacteria in experimentally infected animals (chapter 2). Analysis of immunogenic regions of the *MAP* Hsp70 protein using murine monoclonal antibodies specific for *MAP* Hsp70 with *MBO*, *MTU*, *E.coli* and *Bos taurus* 70kD heat shock proteins as controls revealed that at least two dominant linear B cell epitopes are present in the *MAP* Hsp70 molecule (chapter 4), accessible under a number of denaturing and non-denaturing as well as native conditions. Furthermore these epitopes were found to be immunogenic in vaccinated calves and goats. This study also provided indications that the Hsp70 protein is present in the bacterial cell wall of *MAP*. It may be argued that antibodies, induced by vaccination, may bind intact bacteria and play a direct role in the protective effect shown after Hsp70/DDA subunit vaccination and may contribute to protection against paratuberculosis. T cell-dependent Ig production involves multiple steps in T and B cell collaboration [3, 21]. Interaction between different types of cells is crucial for a potent immune response to an antigen like *MAP* Hsp70. *MAP* Hsp70 receptor mediated uptake by antigen presenting cells has been observed suggesting recombinant *MAP* Hsp70 based vaccines may be used for the succesful immunization of cattle [28]. Balb/c mice immunized either with recombinant *MAP* Hsp70 or whole-cell extract of *MAP* [4] readily showed dominant B cell responses against Hsp70. Differences in routes and modes of exposure to recombinant protein at immunization gives an immune response different from that induced upon natural exposure to the pathogen i.e. no Hsp70 antibody titers for a prolonged period after infection. In addition immunization of mice with whole-cell extracts of *MAP* led to the generation of monoclonal antibodies against isocitrate lyase (*MAP*1643) and Hsp70 (*MAP*3840) predominantly. This indicates that these are proteins which are immunogenic and abundantly present in *MAP* [4]. Vaccination

induced antibodies, apparently not produced in the presence of *MAP* only, and were shown to bind intact bacteria. As a consequence their cellular fate following uptake by macrophages may be altered and this may play a role in the protective effect shown after Hsp70/DDA subunit vaccination against bovine paratuberculosis [24].

***MAP* Hsp70 and a model for *MAP* immune responses**

Based on data from numerous laboratories a model for interaction of *MAP* and the immune system of cattle was proposed by Coussens (2004) [13]. After initial *MAP* infection, repopulation of the gut mucosal tissue with IFN-γ activated macrophages [38] was due to upregulation of adhesion molecules and other factors at sites of infection [2]. Mycobacterial Hsp70 is known to bind specifically to innate receptors present on macrophages [9] and dendritic cells [28], leading to production of pro-inflammatory signals, which may in turn lead to IFN-γ production by T and NK cells. We have indications (chapter 4) that the Hsp70 protein is present in/on the bacterial cell wall of *MAP* and may bind specifically to innate receptors present on macrophages and dendritic cells. The observation that all animals in the *MAP* Hsp70 vaccination study (chapter 2) had high numbers of monocytes, gradually decreasing with age, adds to the model according to Coussens.

An early development of a proinflammatory T-cell population (probably $CD4^+$ T cells) [5], within lymph nodes draining sites of infection followed by expansion of non-cytotoxic suppressor cell populations capable of producing IL-10 is suggested. These suppressor cell populations, may be of the $\gamma\delta^+$ TCR subtype although experience with *MAP* and other pathogens would suggest that a $CD4^+$ T cell is the more likely candidate [6, 50]. In a previous study we showed an increased frequency of $\gamma\delta^+$ T cells and a loss of $CD4^+$ T cells in ileum lamina propria lymphocytes during progressive bovine paratuberculosis [23]. The $CD4^+$ T cells showed decreased proliferative responses, assays to measure suppressive functioning of these $\gamma\delta^+$ T cells and $CD4^+$ T cells were not performed. It is proposed that this suppressor population requires either contact with antigen, close contact with proinflammatory cells, or contact with products of these cells to produce significant amounts of IL-10 [13]. This hypothesis suggests that in circulation, suppressor cells may function relatively mild and do little to limit IFN-γ, IL-1, and IL-6 production by proinflammatory cells. However, if *MAP* antigens are added, proinflammatory T cells continue to produce IFN-γ, IL-1, and IL-6 mRNAs until the suppressor population has been stimulated and produces enough IL-10 to quiet the proinflammatory response. Which is supported by data demonstrating that PBMCs from Johne's disease-positive cows produce significant amounts of IFN-γ mRNA in vitro without further antigen stimulation [14].

Intestinal tissue damage is probably due to the chronic inflammatory immune response, IL-1α production, and release of reactive oxygen produced by activated macrophages or neutrophils.

In vivo activation of a suppressor (immunomodulating) population restricting local proinflam-matory and cytotoxic immune reactivity would be helpful in limiting tissue damage. At the same time it could allow expansion of *MAP* infection. As a consequence PBMC responses to *MAP* will be quite variable depending on the balance between proinflammatory and suppressor cell populations. Furthermore, cytotoxic immunoregulatory cells likely $\gamma\delta^+$ T cells or $CD8^+$ T cells [10, 11] are proposed to develop in very-late-stages of infection and it is suggested that these are largely responsible for the T-cell anergy often observed in PBMCs of clinically infected cows. Until now only superantigen (staphylococcus enterotoxin C1) stimulated $CD4^+$ T cells were tested and proved to be suppressor T cells in a bovine co-culture assay [45] but *MAP* antigens are not yet tested.

Finally, in this proposed model, there is not actually an active switch from a proinflammatory to a predominately IgG1 (Th2) response that may develop at any time, depending on the dose of bacteria, and their route of entry as suggested in chapter 2 and 4. The IgG1 response seems to be the result of the altering balance between proinflammatory and suppressor cells activities as infection progresses and the proinflammatory cells are depleted. It is at this time that infection might be classified as both clinical and terminal [13].

Immunomodulation, adjusting immune responses, e.g. immunopotentiation, immuno-suppression, or induction of immunologic tolerance is described in several diseases [33, 39]. Different sources of Hsp70 induces different kinds of immunomodulation. Mycobacterial Hsp70 increases IL-10 production and decreases TNF-α production which protects rats from experimentally induced arthritis [15]. Furthermore virulent strains of *T. gondii* inhibits NO production by producing HSP70 after which the parasite is known to evade host proinflammatory responses in mouse tissues [17, 34]. Finally after injury/local insults (e.g. cardiac dysfunction) the overproduction of TNF-α, leading to tissue damage and organ failure, is shown to be suppressed by human Hsp70 [32].

Given the slowly progressing nature of paratuberculosis and the concurrently changing immune responsiveness, resulting in anergy in clinically ill animals, it is likely that immune regulatory mechanisms change in course of time. Changes in regulatory mechanisms are also thought to be involved during *MAP* Hsp70 vaccination induced immune responsiveness.

Heat shock proteins are reported to activate regulatory cells in several species and

diseases [18, 51, 52]. Extracellular heat shock proteins are shown to have a range of immunoregulatory activities, which can downregulate pathogenic processes suggesting a potential role for heat shock proteins as therapeutic agents [40, 41].

Immune regulation in cattle

Since in cattle (ruminants) little is known about regulatory cells, functional and phenotypical characteristics of bovine *ex vivo* regulatory cells were studied (chapter 5). Our data provided first evidence that cattle $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells do not function as regulatory T cells [19] *ex vivo*, are non-anergic but still $Foxp3^+$. The bovine regulatory cell function, as defined by ex vivo suppressive capacity appears to reside in $CD14^+$ monocytes and in the γδ T cell population, more precisely in the $WC1.1^+$ and the $WC1.2^+$ subpopulation, major populations present in blood of cattle in contrast to non-ruminant species. We described the identification and functional characterization of sorted *ex vivo* bovine $WC1^+$ γδ T cell subsets, WC1.1 and WC 1.2, and $CD14^+$ cells as leukocytes with regulatory function and showed that IL-10 transcription was clearly increased in sorted $WC1.1^+$, $WC1.2^+$ γδ T cells and in CD14 monocytes (chapter 5). In addition we found actual expression of IL-10 in $CD14^+$ and $WC1^+$ γδ T cells which is in accordance with earlier publications [12, 43]. In contrast, Foxp3 transcription / expression was very low or absent in these cell types indicating that the bovine immune system may be governed by different regulatory mechanisms as compared to rodents and humans. The $WC1.1^+$ γδ cells described in this study as immune suppressors have been described as IFN-γ secretors [43], which can act as innate immune cells potentially by directing adaptive immune responsiveness, after specific defined stimulation. The suppressive and proinflammatory functioning of $WC1.1^+$ γδ cells seems contradictory, however cells producing IFN-γ and IL-10 simultaneously have been described as a potential regulatory subset of $CD4^+$ T cells maintaining a balance between Thl and Th2-type cells [22]. Besides IFN-γ has been reported to act in a suppressive fashion [37]. Different ways of stimulation may result in differential activities of relevance in adaptive versus innate immune functioning by $WC1.1^+$ cells. Also further subdivision of $WC1.1^+$ and $WC1.2^+$ γδ T cell populations can not be excluded.

Further research to determine the involvement of antigen specific regulation (*MAP* Hsp70) by $CD4^+$ or γδ T cells, via a mechanism unknown until now, has to be performed. A regulatory role of these cells by modulating the chronic paratuberculosis infection towards a more protective form of defense would be an interesting option to study. Knowledge concerning the involvement and interactions of *MAP* Hsp70 in the specific cell mediated immune reactions and bovine APC interactions will increase

insight in possible prevention or treatment of bovine paratuberculosis.
Furthermore, γδ cells being a major T cell population, probably consisting of more subpopulations besides those currently described in cattle. They travel abundantly outside of the main blood circulation and vary in numbers depending on animals age. Their as yet undefined functions, at least harbouring populations with regulatory potential, calls for further analysis of the mechanism of these regulatory function in health and disease in cattle.

Chapter conclusion

In conclusion, our study showed that *MAP* Hsp70 vaccination induces protection, as determined by decreased shedding in infected animals. Vaccination seems to result in antibody responsiveness rather than T cell reactivity, suggesting a role for antibodies in protection. Further analysis of T and B cell epitopes indicated promiscuity of at least some of the detected T cell epitopes, in addition the existence of dominant B cell epitopes, support the applicability of *MAP* Hsp70 as a vaccine in outbred animals.
Finally γδ T cells (in addition to $CD14^+$ cells) were identified as potential regulatory cells in cattle. Their role in paratuberculosis as potential antigen specific regulatory T cell needs to be determined.

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Summary

SUMMARY

Heat shock proteins (Hsp) known to be of vital importance in prokaryotic and eukaryotic cells are evolutionary conserved molecules that have shown to be immunogenic/ immunodominant antigens in different stages of bovine paratuberculosis and other mycobacterial diseases. In paratuberculosis it was shown that immune responses to recombinant *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) Hsp70 protein after natural or experimental infection were predominantly cell mediated which is in accordance with the dogma that protective immunity to intracellular mycobacterial pathogens is cell-mediated in origin.

Efficacy of vaccination with *MAP* Hsp70 was studied in a longitudinal study with experimentally infected animals and humoral and cellular immune responses were measured for almost 2 years **(Chapter 2)**. The priming of animals with *MAP* Hsp70 using DDA as an adjuvant induced an immune response, which was clearly different from that after encounter of the protein during infection. Antigen-specific IFN-γ production was observed in this study, although significant differences, high in immunized groups and low in infected groups, of IFN-γ production were measured in the different treatment groups. However these were related to infectious challenge rather than to Hsp70 treatment. A prominent and prolonged production of antibodies to *MAP* Hsp70 was restricted to the immunized groups. It correlated well with the observed significantly reduced shedding of bacteria in the immmunized and challenged group, suggesting a role for antibodies in protection. Challenged and uninfected animals showed low or absent total anti-*MAP* Hsp70 IgG which was significantly different compared to immunized animals. This clear divergence/discrepancy in antibody production enables differentiation between vaccinated and non-vaccinated animals even within infected herds which may clearly be an advantage in the application of the vaccine. Little direct or long term side effects (like local swelling/inflammation) were observed after vaccination with *MAP* Hsp70/DDA.

As the use of subunit vaccines implicates using antigenic preparations with potentially less epitopes it was studied to which extend outbred animals are able to respond to such a subunit vaccine. Hence fine specificity of responses in the T cell compartment (chapter 3) and the B cell compartment (chapter 4) were studied.

T cell responses studied in *MAP* Hsp70 immunized animals **(Chapter 3)** indicated activation of Th1 cells with specificity for up to 9 epitopes. Maximal *MAP* Hsp70 specific T cell responses were observed 14-21 days after secondary immunization that decreased rapidly thereafter. This is in contrast with the long B cell memory responses observed after *MAP* Hsp70 vaccination (1 to 2 years). Based on recognition of at least 1 of the 5 most immunodominant *MAP* Hsp70 specific T cell epitopes it

was concluded that at least part of the epitopes was promiscuous as responses were detected in 82% of the animals (15 different MHC haplotypes) experimentally infected with *MAP*. This indicates relevance of the epitopes during infection, and may partly explain the efficacy of *MAP* Hsp70 subunit vaccination in an outbred population. Although alignment studies of the complete *MAP* Hsp70 sequences of different mycobacterial species showed high homology (and low homology to *E. coli),* for the *MAP* Hsp70 500-589 aa region containing the 5 most dominant peptides, there was total homology between *MAP* and *Mycobacterium avium* subsp. *avium (MAA)*, while homology with *Mycobacterium bovis (MBO)*, *Mycobacterium tuberculosis* (*MTU)* and *Mycobacterium leprae* (*MLE*) was 85 to 89% and homology with *E. coli* was only 30%. Of the variable aminoacid (aa) substitutions observed compared to the *MAP*/*MAA* sequence, in this region 54-64% were part of at least one of the 5 identified immunodominant *MAP* Hsp70 peptides. These results indicate that this is a relatively rapid evolving region within the Hsp70 gene of *MBO*, *MTU* and *MLE* mycobacterial strains compared to the *MAP* and *MAA* strains.

The main immune response after immunization with *MAP* Hsp70 was a pronounced and prolonged antibody production, that coincided with decreased shedding of bacteria in experimentally infected animals. As already mentioned above, for application of subunit vaccines using antigenic preparations with potentially less epitopes, it was studied to which extend outbred animals are able to respond to such a subunit vaccine and hence fine specificity of responses in the B cell compartment (chapter 4) was determined. This part of the study aimed at fine tuning/identification of immunogenic regions of the *MAP* Hsp70 protein by use of murine monoclonal antibodies (mAb specific for *MAP* Hsp70) and 70kD heat shock proteins of *MBO*, *MTU*, *E.coli* and *Bos taurus* as controls. The linear B cell epitopes of *MAP* Hsp70 were mapped using synthetic peptides of *MAP* Hsp70 **(Chapter 4).**

Based on recognition by mAb it was demonstrated that at least two dominant linear B cell epitopes recognized by mAb are present in the Hsp70 molecule as they were accessible under a number of denaturing, non-denaturing, as native conditions. Furthermore these epitopes were found to be immunogenic in vaccinated outbred calves and goats. This study also provided indications that the Hsp70 protein is present in the bacterial cell wall of *MAP* and it may be argued that antibodies, induced by vaccination, may bind intact bacteria and play a direct role in the protective effect shown after *MAP* Hsp70/DDA subunit vaccination thus potentially contributing to protection against paratuberculosis.

Given the slowly progressing nature of paratuberculosis and the concurrently changing immune responsiveness, resulting in anergy in clinically ill animals, it may indicate

that immune regulatory mechanisms change in course of time as well as after *MAP* HSP70 vaccination. In several species and diseases a role for regulatory cells and their potential activation by heat shock proteins is reported. Since in cattle (ruminants) little is known about regulatory cells, functional and phenotypical characteristics of bovine regulatory cells were studied *ex vivo* **(Chapter 5)**. Our studies provided first evidence that cattle $CD4^{+}CD25^{high}$ and $CD4^{+}CD25^{low}$ T cells do not function as regulatory T cells (Treg) *ex vivo*, are non-anergic but still express Foxp3. The bovine regulatory cell function, as defined by ex vivo suppressive capacity appears to reside in $CD14^{+}$ monocytes and in the γδ T cell population, more precisely in the $WC1.1^{+}$ and the $WC1.2^{+}$ subpopulation, major populations present in blood of cattle in contrast to non-ruminant species.

The exact mechanism of the *ex vivo* suppression observed and Ag specific regulation (*MAP* Hsp70) has to be addressed in future studies. So far the potential role of the regulatory cells identified in this study could not be investigated in *MAP* infected animals. The fact that γδ cells are a major T cell population in cattle, abundantly migrate through tissues outside of the main blood circulation, and vary in numbers with age calls for further analysis of the mechanism of their regulatory and potentially other functions in health and disease in cattle.

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

Tijdens dit promotieonderzoek werd vastgesteld dat een nieuw vaccin leidt tot duidelijke vermindering van het uitscheiden van bacteriën door koeien met paratuberculose. Dit resultaat zou een belangrijke stap kunnen zijn in de strijd tegen deze bacteriële ziekte. In een twee jarig experiment werd het vaccin getest op werkzaamheid en werden de reacties van het afweersysteem gemeten. Door activatie van het afweersysteem van koeien vermindert de bacteriële uitscheiding en lopen gevoelige jonge kalveren minder kans om geïnfecteerd te worden.

Paratuberculose is een sluipende ziekte die na een infectie van kalveren vaak jarenlang onopgemerkt meegedragen wordt, de bacterie verstopt zich veelal langdurig in cellen (intracellulair), en pas na uitscheiding van de bacteriën kan de ziekte definitief vastgesteld worden. Dieren worden na 3-6 jaar werkelijk ziek dat leidt tot de dood van het dier. Deze wereldwijd voorkomende ziekte in runderen, blijkt zich snel te verspreiden binnen de runder populatie.

Paratuberculose heeft een grote economische invloed door verminderde melkproductie, vermagering door chronische diarree en uiteindelijk afvoer van de besmette dieren. In Nederland een schadepost van miljoenen maar wereldwijd van miljarden Euro's.

Een rol van de paratuberculose bacterie tijdens het ontstaan van de ziekte van Crohn (een darmaandoening bij mensen), wordt veelvuldig gesuggereerd maar is niet bewezen. Toch lijkt er voldoende reden te zijn om ook blootstelling van mensen te minimaliseren, door controle van deze ziekte bij koeien.

Stress eiwitten (zoals Hsp70) worden aangemaakt als cellen in problemen zijn, bijvoorbeeld doordat er een infectie is en koorts ontwikkeld wordt, waardoor belangrijke eiwitten in de cel kapot kunnen gaan. Stress eiwitten beschermen oa eiwitten tegen afbraak waardoor de cel goed blijft functioneren. Stress eiwitten staan er bekend om dat zij van vitaal belang zijn in eencelligen (zoals bacteriën) en eukaryote cellen (zoals lichaamscellen), en zijn eiwitten die gedurende de evolutie maar weinig verandert zijn. Deze eiwitten geven ondanks het feit dat ze al erg lang in vorm ongewijzigd voorkomen echter nog steeds aanleiding tot een reactie (afweer respons om het organisme te beschermen) van het afweersysteem als deze het eiwit (antigeen) herkent in verschillende ziekte-fasen van runder paratuberculose en andere mycobacteriële ziekten.

Een recombinant eiwit is een eiwit dat op een laboratorium veelal in een bacterie geproduceerd wordt, verkregen door erfelijk materiaal (DNA) van een organisme in deze bacterie over te brengen. In paratuberculose is aangetoond dat afweer tegen een recombinant *Mycobacterium avium subspecies paratuberculosis* (*MAP* bacterie =

ziekteveroorzaker van paratuberculose) Hsp70 na een natuurlijke of experimenteel veroorzaakte besmetting (door de onderzoeker toegediend) voornamelijk werkt via een specifieke afweercel, de T cel genoemd. Deze T cellen (helper T cellen) kunnen nadat ze het juiste epitoop (stukje van eiwit/antigeen) herkent hebben van de bacterie signaalstoffen uitscheiden (cytokinen genoemd, zoals IFN-γ) en zo geinfecteerde cellen cellen stimuleren om de bacterie in de cel op te ruimen. Ook zijn er T cellen (cytotoxische T cellen) die geïnfecteerde cellen doden zodat de bacterie vrij komt en door andere cellen kan worden opgeruimd. Als laatste groep zijn er T cellen (regulatoire T cellen = Treg) die andere T cellen afremmen als de afweer reactie lang genoeg heeft geduurd en de bacterie is opgeruimd. De werking van deze T cellen wordt de cellulaire afweer genoemd, en is in overeenkomst met het denkbeeld (dogma) dat beschermende afweer tegen deze *MAP* bacterie werkt via deze T cellen.

We hebben de werkzaamheid van *MAP* Hsp70 vaccinatie, in combinatie met een chemische stof (DDA) dat de werking van het vaccin versterkt, bestudeerd in een studie waarin de door ons bacterieel besmette dieren bijna 2 jaar lang werden onderzocht. Gekeken werd of de bescherming via T cellen (cellulaire afweer) of via een ander type cel van het afweer systeem, de B cel (humorale afweer) werkt. Deze humorale bescherming ontstaat als B cellen antilichamen produceren die aan de bacteriën binden als een soort van vlag markering, waarna de bacteriën herkent kunnen worden door cellen die de bacteriën opruimen (macrofagen).

De dieren werden (experiment beschreven in **Hoofdstuk 2)** in 4 groepen verdeeld, een onbehandelde controle groep, een *MAP* Hsp70 gevaccineerde groep, een *MAP* bacterie geinfecteerde groep en een *MAP* Hsp70 gevaccineerde + *MAP* bacterie geinfecteerde groep. De afweer reactie die optreedt na vaccinatie met het *MAP* Hsp70 eiwit bleek duidelijk anders dan de afweer reactie die gevonden wordt als het eiwit na een infectie wordt herkend door het afweer systeem.

De productie door T cellen van het cytokine IFN-γ, na herkenning van het bacteriële antigeen, bleek vooral te maken te hebben met de experimentele infectie dmv de bacterie en niet met de vaccinatie, wat dus aangaf dat de verwachtte cellulaire afweer als vorm van bescherming na vaccinatie niet de belangrijkste lijkt te zijn.

Wel duidelijk te meten was een sterke en langdurige humorale respons in de vorm van een productie van antilichamen, die duidelijk was in de gevaccineerde groep dieren en de gevaccineerde + geinfecteerde groep, maar laag was in de controle dieren of de groep van *MAP* geinfecteerde dieren. Deze sterke en langdurige productie van antilichamen kwam goed overeen met de gevonden verminderde uitscheiding van bacteriën in de gevaccineerde + geïnfecteerde groep tov de geïnfecteerde groep. Dit suggereert een beschermende rol voor antilichamen in gevaccineerde dieren. Dit duidelijke verschil

in antilichaam produktie tussen gevaccineerde en niet gevaccineerde dieren, zelfs in geïnfecteerde kuddes, is duidelijk een voordeel in het gebruik van dit vaccin. Hierdoor is het dus mogelijk onderscheid te maken tussen dieren die een afweer respons vertonen doordat zij gevaccineerd zijn of doordat zij geïnfecteerd zijn. Verder werden zo goed al geen bijwerkingen (zoals zwellingen of ontstekingen) geobserveerd na vaccinatie met *MAP* Hsp70/DDA.

Het gebruik van een subunit vaccin (een onderdeel van de bacterie, bijvoorbeeld een eiwit) zoals het door ons gebruikte *MAP* Hsp70 kan als gevolg hebben dat bepaalde dieren (die bijna allen genetisch/door erfelijk DNA materiaal verschillend zijn) wel op de in het subunit vaccin aanwezige stukjes eiwit (ook wel epitopen genoemd) reageren en anderen niet. Een goed vaccin geeft een sterke beschermende reactie in zo veel mogelijk genetisch verschillende dieren. De precieze epitopen herkenning door T cellen en B cellen werd hierop onderzocht.

T cell responsen werden onderzocht in *MAP* Hsp70 gevaccineerde dieren **(Hoofdstuk 3)** en een specifieke respons werd gemeten op 9 epitopen van het *MAP* Hsp70 eiwit. De hoogste T cel respons respons werd 14-21 dagen na een tweede vaccinatie gemeten, deze repons duurde echter maar kort (weken) en nam snel weer af. Dit is in tegenstelling tot de B cel respons die minimaal 1 tot 2 jaar gemeten kan worden na vaccinatie.

Van de 9 *MAP* Hsp70 epitopen bleken er 5 het sterkst herkent te worden in een groep van genetisch verschillende dieren, 82% van deze dieren reageerden op een van de 5 geteste epitopen. Dit geeft aan dat de epitopen aanwezig in het vaccin dus geschikt zijn om een afweer reactie op te wekken in een groep van genetisch verschillende dieren en dit verhoogt de bruikbaarheid als vaccin sterk en verklaart waarschijnlijk gedeeltelijk de goede werking van het vaccin. De DNA volgorde van het genetisch materiaal en de volgorde van de bouwstenen die een eiwit vormen (aminozuren) kunnen bepaald worden van verschillende mycobacteriële bacterie stammen (zelfde familie van bacteriën) en krijgen een nummering oplopend in volgorde.

Tussen de verschillende soorten bacteriën bestaat er vaak een grote overeenkomst in eiwitvolgorde en tov andere bacterie soorten vaak een lagere overeenkomst. Wij vonden dat de overeenkomst in eiwit volgorde van het Hsp70 eiwit (en specifiek het gedeelte waar de 5 epitopen liggen die het sterkst herkent worden) tussen *MAP* en mycobacteriële familiesoorten zoals de veroorzakers van lepra (*Mycobacterium leprae* (*MLE*)), tuberculose (*Mycobacterium tuberculosis* (*MTU)*) en runder tuberculose (*Mycobacterium bovis* (*MBO*)) vaak erg hoog is (85-99%) en tussen *MAP* en andere bacteriën (*E. coli)* vaak maar laag (30%). Hoewel de stress eiwit volgorde gedurende de evolutie niet veel verandert tussen de verschillende mycobacteriële familiesoorten bleek dat van de aanwezige verschillen in de eiwit volgorde 54-64%

onderdeel uit te maken van de 5 gevonden *MAP* Hsp70 epitopen. Dit lijkt aan te geven dat er binnen het Hsp70 eiwit toch een gedeelte bestaat dat relatief snel verandert in volgorde.

De belangrijkste respons na vaccinatie bleek een sterke en langdurige antilichaam productie te zijn die overeenkwam met de verminderde uitscheiding van bacteriën door experimenteel geïnfecteerde dieren.

Zoals hierboven reeds vermeld, is het belangrijk dat als een subunit vaccin ontwikkeld wordt, waar waarschijnlijk maar een beperkt aantal epitopen in aanwezig is, om te onderzoeken welk percentage van dieren binnen een groep van genetisch verschillende dieren reageren met dit vaccin. Dus werden ook de specifieke B cel epitopen onderzocht van *MAP* Hsp70, dmv antilichamen. Deze antilichamen werden verkregen door muizen in te spuiten met het *MAP* Hsp70 eiwit waarna de muizen vele verschillende antilichamen produceerden die ieder een specifiek gedeelte van het eiwit herkennen. De B cel epitopen werden ook getest door stukjes van het eiwit (peptiden) na te maken en te testen welke peptiden een reactie opwekten in gevaccineerde en/of geinfecteerde dieren **(Hoofdstuk 4)**. Gebaseerd op de herkenning door antilichamen bleken er minimaal twee B cel epitopen in het *MAP* Hsp70 vaccin aanwezig te zijn. Ook deze epitopen bleken een reactie op te wekken in gevacineerde kalveren en geiten. Deze studie geeft ook aanwijzingen dat het Hsp70 eiwit van *MAP* aan de buitenkant van de bacterie celwand beschikbaar is. Hierdoor kunnen antilichamen, door vaccinatie verkregen, de bacterie binden en waarschijnlijk een directe rol spelen in het beschermende effect. Door ons gevonden na *MAP* Hsp70/ DDA subunit vaccinatie van geïnfecteerde dieren.

De langzame ontwikkeling van de ziekte totdat dieren klinisch zijn en geen beschermende afweer reactie meer vertonen, door een waarschijnlijk wisselende afweer reactie, zou een rol kunnen spelen tijdens infectie maar ook tijdens vaccinatie.

In verschillende diersoorten en ziekten is bekend dat regulatoire cellen, eventueel geactiveerd door stress eiwitten, een rol spelen. In runderen is nog maar erg weinig bekend over regulatoire cellen. Daarom zijn wij begonnen met het identificeren van cellen dmv eiwitten op het oppervlak van deze cellen die herkent worden door antilichamen, waarna experimenteel bepaald kan worden of deze cellen aanwezig zijn en in welke hoeveelheden. Deze cellen kunnen we daarna isoleren uit het dier en testen wat voor speciale eigenschappen zij bezitten **(Hoofdstuk 5)**. Wij vonden dat cellen met dezelfde oppervlakte eiwitten (aangegeven via een cluster of differentiation (CD) naamgeving dat een specifiek molecuul/eiwit aangeeft waardoor cellen ingedeeld kunnen worden) als tot nu toe gevonden in muis, rat en mens, ook in runderen gevonden kunnen worden. Deze runder $CD4^{+}CD25^{high}$

and $CD4^{+}CD25^{low}$ T cellen blijken echter niet te functioneren als regulerende cellen (cellen die de afweer reactie van andere T cellen afremmen). Deze cellen bleken in tegenstelling tot regulerende cellen uit de muis, rat en mens proliferatief te zijn (in staat om te delen) maar wel het Foxp3 eiwit in de kern te produceren zoals in de andere diersoorten is aangetoond. Van dit Foxp3 eiwit wordt aangenomen dat het aanwezig is in dit type regulatoire cellen en van belang is voor de regulatoire werking. Als runder regulatoire cellen werden $CD14^{+}$ monocyten en een specifieke T cell populatie, de γδ T cellen geïdentificeerd, deze cellen bleken experimenteel in staat te zijn om de deling van andere T cellen te remmen. Van de gevonden γδ T cellen bleek weer een specifiek populatie, de $WC1.1^{+}$ and the $WC1.2^{+}$ T cellen, regulatoir te zijn, in het bloed van runderen zijn dit soort T cellen verhoogd aanwezig in vergelijking met andere diersoorten.

Het exacte werkings mechanisme van de remming door γδ T cellen is nog onbekend, zo is ook de regulatie van *MAP* Hsp70 specifieke T cellen nog onbekend en zal in de toekomst onderzocht worden. Het feit echter dat γδ T cellen een belangrijke populatie zijn in runderen, zich vanuit de bloedbaan verplaatsen naar de weefsels en variëren in aantal afhankelijk van de leeftijd van het rund vraagt om verdere analyse van de regulatoire en mogelijk andere functies in gezonde en zieke runderen.

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DANKWOORD

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Curriculum vitae

CURRICULUM VITAE

Aad Hoek werd geboren op 21 augustus 1963 in Rotterdam. In 1983 behaalde hij het MTS-A diploma afstudeerrichting Werktuigbouwkunde van de Eerste Christelijke MTS in Rotterdam. Ook werd in 1983 begonnen aan een Hoger Laboratorium Onderwijs (HLO) studie Medische Microbiologie aan het van 't Hoff Instituut in Rotterdam. Van september 1987 tot mei 1988 verrichtte hij zijn eerste wetenschappelijke stage op de afdeling Immunologie van de Erasmus Universiteit te Rotterdam onder leiding van prof. dr. W. van Ewijk en dr. P. Brekelmans. In mei 1988 studeerde hij af als ingenieur op het stage-onderzoek "Immortalisatie van epitheliale cellen uit de thymus van de muis" met als vraagstelling "Reguleren stromale cellen de T-cel differentiatie in de thymus". Van september 1988 tot januari 1992 werkte hij als research analist op de afdeling Immunobiologie van het Rijks Instituut voor Volksgezondheid en Milieuhygiene (RIVM) in Bilthoven. Onder leiding van prof. dr. A.D.M.E. Osterhaus werd onder andere aan idiotypische netwerken, $CD5^+$ B cellen en een nieuw rabies virus vaccin gewerkt. Vanaf februari 1992 tot nu werkt hij als research analist op de afdeling Immunologie van de faculteit Diergeneeskunde, Universiteit Utrecht aan fundamenteel en toegepast immunologisch onderzoek van ziekten in runderen en andere diersoorten. Van september 1991 tot augustus 2000 werd in deeltijd de studie Biologie gevolgd aan de Universiteit Utrecht. Binnen deze studie werd van december 1996 tot september 1997 op de afdeling Immunologie, Faculteit Diergeneeskunde, een wetenschappelijke stage uitgevoerd met als titel BLAD: Bovine Leukocyte Adhesion Deficiency (consequences for immunological responsiveness of β_2-integrin deficiency in cattle) onder leiding van dr. V.P.M.G. Rutten van de afdeling Immunologie, Faculteit Diergeneeskunde. De studie Biologie werd in augustus 2000 afgerond met een onderzoeksreferaat getiteld "Bovine paratuberculosis (the contribution of molecular biology to *Mycobacterium avium* subspecies *paratuberculosis* research)" onder leiding van dr. V.P.M.G. Rutten en dr. A.P. Koets (afdeling Immunologie, Faculteit Diergeneeskunde) en prof. dr. W.P.M. Hoekstra van de afdeling Microbiologie, Faculteit Biologie van de Universiteit Utrecht. In augustus 2000 behaalde hij zijn doctoraal examen Biologie (Moleculaire Microbiologie en Immunologie). Onder leiding van dr. A.P. Koets (tegenwoordig Departement Gezondheidszorg Landbouwhuisdieren, divisie Epidemiologie en Departement Infectieziekten en Immunologie, divisie Immunologie, Faculteit Diergeneeskunde), prof. dr. V.P.M.G. Rutten (tegenwoordig Departement Infectieziekten en Immunologie, divisie Immunologie, Faculteit Diergeneeskunde en Department of Tropical Veterinary Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa) en prof. dr. W. van Eden (Departement Infectieziekten en Immunologie, divisie Immunologie, Faculteit Diergeneeskunde) werd in september

2002 begonnen als Assistent in Opleiding, aan het promotieonderzoek zoals beschreven in dit proefschrift. In november 2003 en 2004 werden door hem twee posterprijzen gewonnen op de veterinaire wetenschapsdagen van de Faculteit Diergeneeskunde met de onderwerpen “Role of γδ T cells in bovine paratuberculosis” respectievelijk “Elimination of *Trypanosoma (megatrypanum) Theileri (laveran 1902)* from bovine cell cultures”.

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