Towards the use of CD1 presented mycobacterial lipids in vaccines

The research described in this thesis was conducted from October 2007 to October 2011 at the Division of Immunology, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, the Netherlands.

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Front cover: GMM is inserted into CD1b molecule (adapted from Batuwangala et al., The Journal of Immunology, vol 172 pp.2382-2388, 2004 with the reprint permission from JI. Copyright 2004. The American Association of Immunologist, Inc.)

Back cover: Mycobacterial cell wall that comprises thick different lipid layers

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Towards the use of CD1 presented mycobacterial lipids in vaccines

Op weg naar het gebruik van CD1 gepresenteerde mycobacteriële lipiden in vaccins
(met een samenvatting in het Nederlands)

Hướng tới việc sử dụng kháng nguyên lipit từ mycobacteria được trình diện qua phân tử CD1 trong vắc-xin
(với bản tóm tắt tiếng Việt)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
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Nguyễn Thị Kim Anh

geboren op 28 juni 1974 te Hà Bắc, Vietnam

Promotoren: Prof. dr. W. van Eden
Prof. dr. V.P.M.G. Rutten
Co-promotor: Dr. I. van Rhijn

For my parents

Contents

Abbreviations

Ag	Antigen
APC	Antigen presenting cell
APA	Alanine-proline-rich antigen
α-GalCer	α-galactosylceramide
BCG	Bacillus Calmette-Guérin
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFP	Culture filtrate protein
DC	Dendritic cells
DDA	Dimethyldioctadecylammonium bromide
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
ELISA	Enzyme-linked immunosorbent assay
ESAT-6	Early secretory antigenic target 6kDa
FCS	Fetal calf serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMM	Glucose monomycolate
HSP	Heat shock protein
IFN	Interferon
IL	Interleukin
KLH	Keyhole limpet hemocyanin
LAM	Lipoarabinomannan
MAP	Mycobacterium avium subspecies paratuberculosis
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MPT	Mycobacterium tuberculosis protein
MSD	Meso Scale Discovery
NKT cells	Natural killer T cells
PBS	Phosphate buffered saline
PE	Phycoerythrin (Fluorescent dye)
PE-biotin	Phosphatidylethanolamine-N (cap biotinyl)
PIM	Phosphatidylinositol mannosides
TB	Tuberculosis
TCR	T cell receptor for antigen
TH1	Helper T cell type 1
TLC	Thin layer chromatography
TNF	Tumor necrosis factor

Chapter 1

General introduction

1. Mycobacterial infections

Several species in the genus Mycobacterium are well known pathogens responsible for serious chronic diseases. *M. tuberculosis* and *M. bovis* infection cause tuberculosis, a serious disease that primarily affects the lungs, *M. leprae* infection in humans leads to severe lesions at the extremities, and *M. avium subspecies paratuberculosis* (MAP) infection gives rise to a chronic wasting intestinal disease in cattle and potentially contributes to the immunopathogenesis of Crohn's disease in humans. Mycobacteria are known for their lipid rich cell wall, which is responsible for their survival in harsh environmental circumstances as well as in host cells. Many mycobacterial protein antigens have been studied extensively in the context of presentation by classical MHC molecules, especially antigens specific for the *Mycobacterium tuberculosis* complex like ESAT6 and CFP10. Immunity mediated by non conventional T cells against lipid antigens abundantly present in the mycobacterial cell wall has had much less attention. This thesis focuses on both host and pathogen aspects of recognition of lipid molecules of mycobacteria in cattle, as natural hosts of some of the pathogenic mycobacteria.

1.1. Pathogens

Mycobacterium is a genus of the family Mycobacteriaceae, suborder Corynebacterineae, order Actinomycetales, and phylum Actinobacteria. Among more than 100 species of this genus, important pathogens causing serious diseases in mammals are *M. tuberculosis, M. bovis, M. leprae* and *M. avium subspecies paratuberculosis*, as well as many non tuberculous mycobacteria surviving in the environment. *Nocardia sp.* and *Rhodococcus sp.* are closely related to the mycobacteria have a similar cell wall constitution.

Mycobacteria have special characteristics that they do not share with either Gram-negative or Gram-positive bacteria groups when being detected by staining. All mycobacteria are acid fast because of their lipid rich cell wall but do not retain the crystal violet of the Gram stain well. However, because mycobacteria lack an outer membrane, which is a hallmark of Gram-negative bacteria, they are usually classified as Gram-positive bacteria. Except for *M. marinum*, which has been desribed to be able to move inside the macrophages, other mycobacteria are non-motile (Ryan and Ray, 2004).

Uniquely, mycobacterium species have a thicker cell wall than any other known bacteria. The cell wall comprises a very rich hydrophobic mycolate layer, an arabinogalactan polysaccharide layer, a peptidoglycan layer, and a plasma membrane with lipoarabinomannan (LAM), and phosphatidylinositol mannoside (PIM). Lipid, over 60% of the mycobacterial cell wall, plays a very important role in the pathogenesis of *M. tuberculosis*. It has been associated with resistance to antibiotics and to reactive oxygen species and thus survival inside macrophages. Mycolic acid, the most abundant component of the cell envelope, is responsible for many of the unique microbiological characteristics of mycobacteria (Ehrt & Schnappinger, 2007).

Medically, mycobacteria can be classified based on diagnosis and treatment purposes into several major groups such as the M. tuberculosis complex including *M. tuberculosi*s and *M. bovis* which cause tuberculosis (TB), *M. leprae* which causes leprosy, M. avium complex including MAP which cause disseminated infections but not lung infection (Ryan and Ray,

2004), and *M. ulcerans* which causes Buruli ulcer – a skin and subcutaneous tissue infection (Table 1).

Table 1. Main mycobacterial infections

Microorganism	Mycobacteriosis
M. bovis	Tuberculosis
M. tuberculosis	Tuberculosis
M. avium	Non-tuberculous mycobacteriosis
M. avium subsp paratuberculosis	Johne's disease (Crohn's disease)
M. leprae	Leprosy
M. ulcerans	Buruli ulcer

M. tuberculosis, first discovered in 1882 by Robert Koch, an aerobic pathogen, is the etiologic agent of tuberculosis in humans. It is closely related to *M. bovis* causing tuberculosis in both cattle and man. Drinking unpasteurized milk of cows is the most common route of transmission of *M. bovis* to humans, but aerosol exposure leading to lung infection is also known to occur.

Mycobacterium avium subspecies paratuberculosis, abbreviated as *M. paratuberculosis* or MAP, is the causative reagent of Johne's disease in cattle and other ruminants, and may play a role in the induction of Crohn's disease in humans.

Since *M. bovis* as well as MAP infections in cattle are considered excellent natural models of mycobacterial infection, which can be used for vaccine development, further emphasis in this thesis is on CD1 presented lipid specific immunity in cattle against these pathogens.

1.2. Human and bovine tuberculosis

Human tuberculosis is the leading cause of death worldwide. One third of the global population is infected with *Mycobacterium tuberculosis*. This pathogen causes active tuberculosis in about nine million people and more than 1.7 million deaths occur worldwide annually (World Health Organization, 2009). The prevalence of tuberculosis has not diminished even though it has been longer than 75 years since BCG, the only vaccine against this disease, has been developed and applied (Hoft, 2008). *M. tuberculosis* infects mainly humans and non-human primates (Alfonso et al., 2004; Washko et al., 1998), and is efficiently transmitted between members of these species. Very rarely *M. tuberculosis* infection occurs in cattle (Ocepek et al., 2005), pigs (Fourie et al., 1950), dogs (Erwin et al., 2004), elephants (Mikota et al., 2000), and birds (Washko et al., 1998), but these species are to be considered as end hosts. It has been shown that man can be the source of infection for elephants, and *vice versa*, especially in Asia where they live closely together and where elephants may come into contact with large crowds with high TB prevalence, for example at ceremonial occasions. No cases of cow-to-cow or cow-to-humans transmission of *M. tuberculosis* have been recorded (O'Reilly and Daborn, 1995).

M. bovis has a much wider natural host range and infects most mammals. It occurs in both developing and developed countries and it can be transmitted from cattle to humans via the respiratory route or by ingestion. The milk-borne infection seems to be effectively eradicated in developed countries by applying pasteurization. In Europe, *M. bovis* has been eradicated effectively in cattle with the exception of UK and Ireland, where the infection is thought to be maintained in badgers as a wildlife reservoir. Retrospective estimates suggest that in the early 20th century, before the implementation of test-and-slaughter policies to remove infected animals and before milk pasteurization became widespread, one third of TB cases in humans in the UK were caused by *M. bovis* (Young, 2008). Currently, in developing countries where raw-milk or dairy products are the main source of infection, like for instance Ethiopia, more than 30% of TB in humans may be caused by *M. bovis* (Yimenu, 2008). *M. bovis* can also transmit via the respiratory route, so pulmonary disease is likely to develop in those who are in close contact with *M. bovis* infected cattle or other animals on farms, or in slaughter-houses. Although *M. tuberculosis* is nowadays the main cause of human tuberculosis in the developed world, *M. bovis* has been found as a common cause of TB among AIDS patients (Park et al., 2010).

1.3. Paratuberculosis in cattle

Paratuberculosis or Johne's disease is a chronic granulomatous enteritis caused by *Mycobacterium avium subsp. paratuberculosis* (MAP). This disease is prevalent in domestic animals and principally affects ruminants like cattle, sheep, and goats. It results in considerable economic losses worldwide, especially in the dairy sector due to gradual production losses and occasional fatal cases (Johnson-Ifearulundu et al., 1999; Rosseels, 2008). In cattle, the two main signs of the disease observed are chronic diarrhea and cachexia (Rosseels, 2008). This infection also affects wildlife ruminants like deer, bisons, goats, camels, and llamas, on all continents (Cocito et al., 1994). Neonatal and juvenile animals are at the highest risk for acquiring MAP infection (Cocito et al., 1994; Harris and Barletta, 2001).

MAP infections are widely spread in domestic livestock and the bacteria are present in retailed pasteurized milk as well as in water supplies and may convey to humans (Bull et al., 2003; Hermon-Taylor et al., 2000). The main route of transmission in a herd is via the fecal-oral route. Hygienic schemes and culling programs for bacteria shedding animals have been implemented for years but these were insufficient to eradicate the disease. In addition, diagnostic tools available at present are not powerful enough for early and specific detection of this pathogen (Rosseels, 2008).

It is still debatable if there is a relation between Johne's disease and Crohn's disease. A study using a *Mycobacterium avium subsp. paratuberculosis* specific PCR reported a significantly higher rate of MAP in patients with Crohn's disease compared to the control group without Crohn's disease (Bull et al., 2003). However, the involvement of MAP in Crohn's disease in humans remains uncertain due to the difficulties related to specificity and sensitivity in detecting this pathogen.

In early stages of paratuberculosis, there are strong cell-mediated immune responses that limit the proliferation of the etiological agent (Cocito et al., 1994). In addition, humoral

responses have been observed in early course of the experimental Johne's disease (Waters et al., 2003) and become more pronounced when the disease progresses.

1.4. Animal models for mycobacterial diseases

Animal models have become standard tools for human infectious disease studies. Using the natural host in infection trials, highly relevant information on host-pathogen interactions can be obtained (Harris and Barletta, 2001). Cattle are natural hosts for *M. bovis*, which causes bovine tuberculosis and is also pathogenic for humans. The preferential locations in the lungs and associated lymph nodes and the type of lung granulomas that develop in cattle closely resemble the situation in human tuberculosis. For these reasons we used cattle as an animal model to investigate the immune responses of the host to the CD1-restricted lipid antigens and potential novel vaccine candidates against these mycobacterial zoonotic diseases. Using cattle as a model serves both human and animal welfare and has as several additional advantages. Larger amounts of blood can be collected to perform in depth studies of correlates of protection and macroscopic and histopathological assessment of lesions post mortem may be conducted. Furthermore, testing a vaccine that targets newborns and children up till five years can be carried out in a calf model that has comparable immune function and disease development (Endsley et al., 2009).

In many studies on immune responses to infection with *M. tuberculosis*, mice are used. Although this experimental animal contributed to understanding of TB aspects such as immunopathogenesis, host genetic influence on infection, and host/pathogen interactions, the manifestations of the disease in humans and mice are not very similar. For example in mice the infection always becomes systemic instead of being mostly limited to the lungs and associated lymph nodes, as is the case in humans and cattle. Moreover, in terms of CD1-restricted immunity, the absence of group 1 CD1 genes in mice has important consequences for evaluating the contribution of CD1 antigen presentation in this model for human TB. Bovine tuberculosis presents with similar clinical aspects as human tuberculosis e.g. infection route, course of the disease, latency, pathology, as wel as with respect to CD1-retricted T cell responses and gamma delta T cell responses (Van Rhijn et al., 2008). Although both humans and cattle are immunocompetent at birth, the only existing vaccine, BCG, although protective initially, does not protect in both species from tuberculosis (Buddle et al., 2005).

Several models, including small animals like chickens (Larsen, 1972; Valente et al., 1997), guinea pigs (Francis, 1943), hamsters (Harding, 1959), mice (Chandler, 1962, Lominski et al., 1956), and rabbits (Mokresh et al., 1989, Mokresh, 1990; Rankin, 1958), have been developed for MAP infections. Except for mice, all other small-animal models are somewhat limited in their use for studying MAP infections because they do not consistently reproduce disease symptoms in experimentally infected animals (Harris and Barletta, 2001). Cattle, the natural host of MAP is the most suitable species to address the disease experimentally. An important advantage is that the disease occurs worldwide and natural infected animals can be obtained easily. However, a strong disadvantage is the slow progress of this mycobacterial disease in which clinical symptoms may only appear four years or more after infection.

2. Immunity to Mycobacteria

Mycobacteria can occupy many different niches in the host, and that may be the reason why the immune system often fails to protect the host from serious disease. During infection mycobacteria are found extracellularly and intracellularly mainly in macrophages and dendritic cells. In macrophages they may live in specialized phagolysosomes or free in the cytoplasm (van der Wel et al., 2007). Macrophages are the main cell type to be infected during tuberculosis. The mycobacterium inhibits the maturation of the phagolysosome and is able to multiply in this organelle. Even though macrophages usually become activated upon contact with bacteria, during mycobacterial infection macrophages become deactivated as indicated by reduction of expression of IFN-gamma-inducible genes (Hussain et al., 1999). In addition, the antigen processing and synthesis of MHC class II is diminished in macrophages infected with *M. tuberculosis* (Noss et al., 2000). Thus mycobacteria can reside and multiply in macrophages for prolonged periods of time while dampening the immune response.

Infected macrophages may be activated by helper T cells to assist in killing their phagolysosomal content, they may be killed altogether by cytotoxic T cells, or T cells may assist in the formation of a granuloma around infected macrophages. Even though the intracellular location of mycobacteria shields them from antibodies, it has recently been suggested that humoral responses may play a role in protection against *M. tuberculosis* (Glatman-Freedman, 2006). It is not clear which kinds immune responses are the most helpful to the host. Below, three types of T cell responses will be discussed: classical MHC-restricted T cells, γδ T cells, and CD1 restricted T cells.

2.1. MHC-restricted immunity

The classic major histocompatibility complex (MHC) molecules are MHC class I and MHC class II. Both MHC class I and class II present peptides to γδ T cells. MHC-restricted T cells confer immunological memory to the host upon vaccination or infection.

MHC class I molecules are found on all somatic cells, whereas MHC class II molecules are expressed primarily on professional antigen presenting cells including macrophages, B cells, and dendritic cells. MHC class I consists of a polymorphic heavy chain consisting of α1, α2, and α3 domains forming a heterodimer with β2-microglobulin (Figure 1). The antigen binding groove is situated between the two alfa-helices of the α1 and α2 domains and the β pleated sheet. The α3 domain interacts with the CD8 co-receptor on T cells. The antigen binding groove of MHC class II is open at both ends and allows binding of longer peptides than the groove of MHC class I which has two closed ends. MHC class I binds peptides that result from processing of cytosolic proteins by the proteasome and that are transported to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) system, which is called the cytosolic or endogenous pathway. In contrast, MHC class II presents peptides derived from extracellular antigen via the so called endocytic or exogenous pathway.

A couple of immunogenic MHC-presented mycobacterial antigens have been described, some of which are currently being developed or used in vaccination and diagnosis like the secretory protein Ag85 complex (Ag85A, Ag85B, and Ag85C), TB10.4, early secretory

antigenic target 6kDa (ESAT-6), culture filtrate protein (CFP-8, CFP-10, CFP-15, CFP-11, CFP-29, CFP-31, CFP-32), Mycobacterium tuberculosis protein (MPT-64, MPT-51), and Mtb8.4 (Sable et al., 2007; Deenadayalan et al., 2010; Kumar et al., 2010)

Several different T cell populations are required to control mycobacteria, of which conventional CD4+ and CD8+ αβ T-cells play a major role in adaptive immunity against tuberculosis (Kaufmann et al., 2010). The present dogma is that helper T cell type 1 (T_H1) cells producing IFNγ critically contribute to host resistance against mycobacteria in tuberculosis and paratuberculosis. The CD8+ T cells are cytolytic and can detect and destroy *M. tuberculosis* infected macrophages. The peptide antigens are presented to CD8+ T cells via MHC class I. Granulysin, a compound of CD8+ cytolytic granules, has direct microbicidal effects against both extracellular and intracellular mycobacteria (Stenger et al., 1998). Although CD4+ T cells are thought to be the most important subset for *M. tuberculosis* protective immunity, CD8+ T cells are considered to contribute as well. Mycobacterium-specific CD4+ T cells which are mainly differentiated into T_H1 produce IFNγ, IL-2, and TNF-α that can activate intracellular antimicrobial properties of macrophages and other cells, as well as initiate protective granulomatous inflammatory responses (Kaufmann et al., 2010). T_H2 cells producing cytokines (e.g. IL-5, IL-10, and IL-13) that are essential for antibody-mediated responses are scarcely present (Mosmann et al., 1996; Munk, 1995).

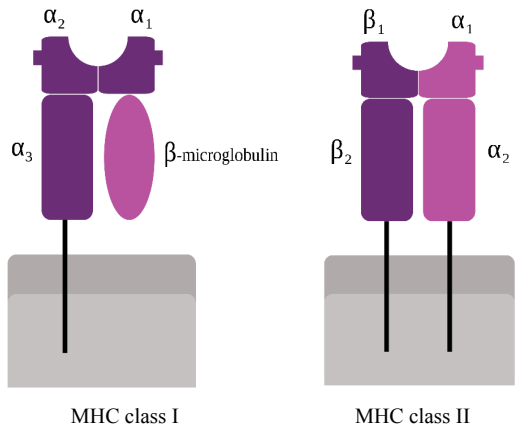

Figure 1. Schematic of the structure of MHC class I and class II

2.2. γδ T cells

The γδ T cell subset expresses T cell receptors composed of dimers of γ and δ chains. Although γδ T cells are in theory able to generate at least as many different T cell receptors as αβ T cells, their repertoire *in vivo* is dominated by specific subsets that recognize a limited number of antigens (Carding, 2002).

In humans and mice only 1-5% of the lymphocytes circulating in blood are γδ T cells but in epithelium-rich tissues such as the skin, intestine and reproductive tract up to 50% of the lymphocytes can be γδ T cells (Carding, 2002). In sheep and cattle this lineage of T cells

constitutes 30-75% of the lymphocytes in the blood as well as in tissues (Allison, 2002).

Unlike αβ TCRs that recognize peptide antigens bound to MHC, γδ TCR can directly recognize soluble proteins and non-peptide antigens. For instance, pyrophosphomonoester, alkylamine, and aminobisphosphonate antigens are recognized by human γδ T cells expressing Vγ9Vδ2 TCRs (Tanaka et al., 1995). Immunological roles of γδ T cells include immediate response to pathogenic invasion, recognition of stress induced self ligands, and modulation of inflammation. From some experimentally induced bovine tuberculosis studies, γδ T cells are suggested to play a role in the initiation of immune responsiveness (Cassidy et al., 2001). However, in another study, removal of γδ T cells in vivo had no effect on the course of bovine tuberculosis (Kennedy et al., 2002).

The role of γδ T cells in the pathogenesis of paratuberculosis is also controversial. In progressive bovine paratuberculosis, CD4+ T cell-directed cytotoxic effects of γδ T cells may actually contribute to the progression of the disease (Chiodini, 1993). It has been indicated that bovine γδ T cells are important sources of IFNγ and IL-2 in innate immunity (Cassidy et al., 2001; Asai et al., 2003; Wilson et al., 2002). Prominent contribution of γδ T cells at the subclinical stage of MAP in cattle is suggestive for a role in the innate immunity when specific cellular responses begin to decline (Badi et al., 2010).

There is limited evidence whether γδ T cells are suitable targets for vaccination strategies. Even though primate Vγ9Vδ2 have been shown be effectively primed during infection (Shen et al., 2002), this is most likely not the case for all γδ T cell subsets.

2.3. CD1-restricted immunity

Besides protein antigens that are recognized by MHC-restricted γδ T cells, lipid antigens can also be recognized by γδ T cells via presentation by CD1 molecules. In contrast to the classic MHC molecules, CD1 proteins are non polymorphic. The non polymorphic nature of CD1 molecules implies that, unlike MHC proteins, the whole population of a species expresses the same CD1 proteins. For vaccine development this has the advantage that a CD1 binding antigen will be presented in every individual of the population.

2.3.1. Introduction to CD1

CD1 is an MHC class I-like protein that presents lipid antigens to T cells. Thus, it is defined as the third lineage of antigen presenting molecules beside MHC class I and MHC class II. Similar to MHCclass I, the CD1 structure comprises 3 extracellular domains α1, α2, and α3 with a transmembrane and cytoplasmic domain at the carboxy terminus. The α3 domain is non-covalently associated with β2-microglobulin. However, the binding groove of CD1 is narrower and deeper than that of MHC, and because of the hydrophobic amino acids that line the groove, it can bind the hydrophobic tails of lipids (Figure 2).

2.3.2. CD1 genes and cellular expression

CD1 genes have been intensively studied in humans and mice. In humans, the CD1 gene

family is composed of five non-polymorphic genes: CD1A, CD1B, CD1C, CD1D, and CD1E encoding four protein molecules CD1a, CD1b, CD1c, and CD1d that are expressed on the cell surface (Porcelli and Modlin, 1999). CD1e localizes to the late endosomes of both immature and mature DCs and is not expressed on the cell surface. Based on structure, cellular expression pattern, and function, CD1a, CD1b, CD1c and CD1e are grouped together and referred to as CD1 group 1 molecules, while CD1d molecules form CD1 group 2.

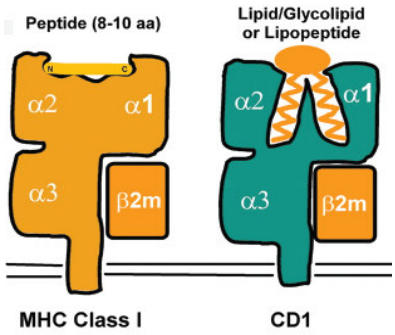

Figure 2. MHCI and CD1 structure (Adapted from Porcelli, 2005)

In contrast to humans and other mammalian species, mice and rats lack CD1 group 1 genes and have two CD1D genes (CD1.1 and CD1.2) (Chen et al., 1999). Some researchers investigated CD1 in other species such as mice (*Mus musculus*) (Bradbury et al., 1988), rats (*Rattus norvegicus*) (Ichimaya et al., 1994), cottontail rabbits (*Sylvilagus floridanus*) (Calabi et al., 1989), guinea pigs (*Cavia porcellus*) (Dascher et al., 1999), sheep (*Ovis aries*) (Chun et al., 1999; Eguchi-Ogawa et al., 2007), pigs (*Sus scrofa*) (Ferguson et al.,1996; Rhind et al., 1996), dogs (*Canis familiaris*) (Looringh van Beeck et al., 2008), and cattle (*Bos taurus*) (Van Rhijn et al., 2006). However, not all of these genes have been shown to lead to functional transcripts or proteins yet. CD1 genes have also been discovered in chickens (Maruoka et al., 2005; Miller et al., 2005; Salomonsen et al., 2005). The number of CD1 genes in the various genomes varies between 2 and 12. It has been determined that cattle have one CD1E, one CD1A, and 5 CD1B genes of which only CD1b1, CD1b3, and CD1b5 are functional and expressed on the cell surface (Van Rhijn et al., 2006). In the same publication it was shown that the two bovine CD1D genes have structural features that strongly suggest that they can not be translated into proteins expressed on the cell surface. No CD1C gene has been identified. In chapter 6 of this thesis, we will investigate the details of the CD1 expression and function in this species.

2.3.3. CD1 presented antigens

Both group 1 and group 2 CD1 molecules present foreign and self lipids. In general, CD1 presented antigens are chemically very diverse and include mycolic acids, sphingolipids, polyisoprenoids, polyketides, polyacylated carbohydrates, lipopeptides, and phospholipids. Most of the known group 1 CD1 presented foreign lipids are derived from mycobacteria and include mycolates like free mycolic acids, glycerol monomycolate, trehalose monomycolate, and glucose monomycolate, that are presented by CD1b to activate clonally diverse T cells

(Figure 3). Mycobacteria synthesize glucose monomycolate (GMM) from mycolic acids by utilizing glucose from the host as a sugar source during the infection (Enomoto et al., 2005). In addition to the CD1b-presented mycolates, the mycobacterial lipopeptide dideoxymycobactin and mycobacterial polyketides are presented by CD1a and CD1c respectively. Lipid antigens from *M. tuberculosis* are listed in Table 2 together with the CD1 isoforms responsible for their presentation (Beckman et al., 1994; Sieling et al., 1995; Moody et al., 1997; Moody et al., 2000; Fischer et al., 2004; Gilleron et al., 2004; Matsunaga et al., 2004; Moody et al., 2004)

Table 2. Mycobacterial lipids presented by CD1 molecules

Antigen	CD1 isoform
Mycolic acid	CD1b
Glucose monomycolate (GMM)	CD1b
Sulfolipid	CD1b
Phosphatidylinositol mannosides (PIM's)	CD1b, CD1d
Mannosylated lipoarabinomannan (Man-LAM)	CD1b
Mannosyl-β1-phosphomycoketides (MPM)	CD1c
Mannosyl phosphodolichol (MPD)	CD1c
Didehydroxymycobactin (DDM)	CD1a

In addition to stimulating T cells via presentation by CD1b, mycolates are involved in the formation of granulomas and inflammation. GMM isolated from *Nocardia asteroides* exhibits strong granuloma-forming activity in lungs, spleen, and liver in mice (Han et al., 1998). The production of tumor necrosis factor (TNF) has been shown to be induced when mice were intravenously injected with trehalose dimycolate and GMM from *N. rubra* containing C36-48 mycolic acids or *Rhodococcus terrae* that constitutes C56-60 mycolic acids (Natsuhara et al., 1990). Inflammatory reactions occurring in the lung of mice after live BCG inoculation were similar to those observed in mice inoculated intravenously with GMM extracted from *Mycobacterium bovis* BCG (Silva, 1985). Some of these previously described effects may be mediated by Mincl, a recently identified receptor for trehalose dimycolate (Schoenen et al., 2010; Ishikawa et al., 2009).

The CD1d molecule is known to present self and exogenous lipids. Of the exogenous lipids α-galactosylceramide, a highly bioactive glycolipid originally isolated from the marine sponge *Agelas mauritianus* is the best known (Kawano et al., 1997; Burdin et al., 1998; Spada et al., 1998). It has been reported more recently that NKT cells recognize several bacterial antigens presented by CD1d such as α-glucuronosylceramide, a glycosphingolipid from *Sphingomonas spp.* (Kinjo et al., 2005; Mattner et al., 2005), α-galactosyldiacylglycerol from *Borrelia burgdorferi* (Kinjo et al., 2006), and lipophosphoglycan from *Leishmania donovani* (Amprey et al., 2004)

2.3.4. CD1 molecular structure

The crystal structures of human CD1a, CD1b, CD1c and CD1d, mouse CD1d, and bovine

CD1b3 have been described in complex with ligands (Figure 4). CD1 antigens have one or two hydrophobic tails coupled to a hydrophilic head group (Figure 3). CD1 molecules have a hydrophobic ligand binding groove that embeds the long hydrophobic alkyl chain of lipid antigens. The head group is solvent-exposed and recognized by T cell receptors.

To bind antigenic lipids that differ in size and chemical composition the antigen binding grooves of CD1 molecules differ in size and shape (Moody et al., 2005). Among CD1 molecules, CD1a has the smallest binding groove composed of the two channels A' and F' (Zajonc et al., 2003). The structure of CD1b with its antigen binding groove can accommodate very long alkyl chains (Girardi et al., 2010; Gadola et al., 2002; Batuwangala et al., 2004). In human CD1b the A'and F'channels are connected by the T'tunnel, which runs along the bottom of the binding groove. The bovine CD1b3 structure has A', C, and F'pockets that are similar with human'CD1b, whereas the T tunnel is absent. However, the bovine CD1b3 antigen binding groove is very big and is capable to bind mycobacterial mycolates (Girardi et al., 2010). The unusual structure of CD1b with the large tunnels A', T' and F, and the C' portal allows presentation of long-chain mycolic acid (C80) and GMM antigens that are unlikely to be presented by other CD1 molecules (Moody et al., 2005) (Figure 4).

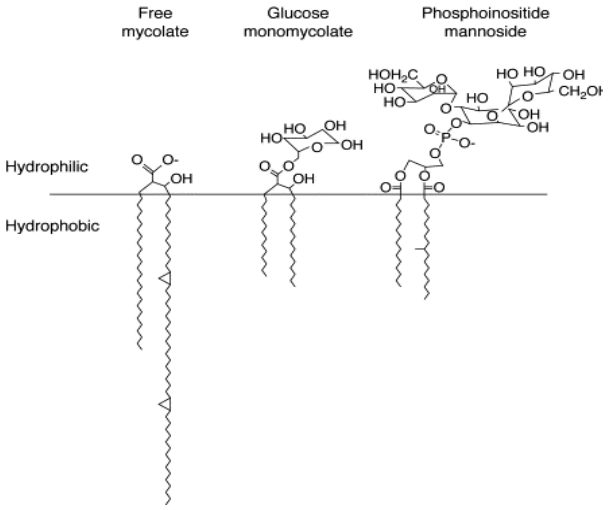

Figure 3. Mycobacterial lipids with hydrophilic head group and hydrophobic tails (Adapted from Porcelli, 2005).

CD1 expression on APC

Group 1 CD1 is expressed mainly on thymocytes and professional APCs like B cells, Langerhans cells and myeloid dendritic cells. In fact, multiple CD1 isoforms can be expressed on the same APC (Dougan et al., 2007). Group 1 CD1 expression was found to be upregulated during infectious disease. In dermal DCs of *Mycobacterium leprae* infected patients that developed the tuberculoid form of the disease, CD1a, CD1b, and CD1c were expressed, while expression was absent in patients with the lepromatous form of the disease, which is characterized by ineffective cellular immunity (Sieling et al., 1999).

Group 2 CD1 (CD1d) has a wide tissue distribution and in addition to expresion on professional APCs and thymocytes, it is expressed by many non-hematopoietic cells, including epithelial cells, monocytes, macrophages, and DC (Exley et al., 2000; Yue et al., 2005; Skold et al., 2005; Spada et al., 2000; Marschner et al., 2005; Kitamura et al., 1999; Nishimura et al., 2000; Bleicher et al., 1990; Balk et al., 1994; Somnay-Wadgaonkar et al., 1999; Kim, 2000; Roark et al., 1998).

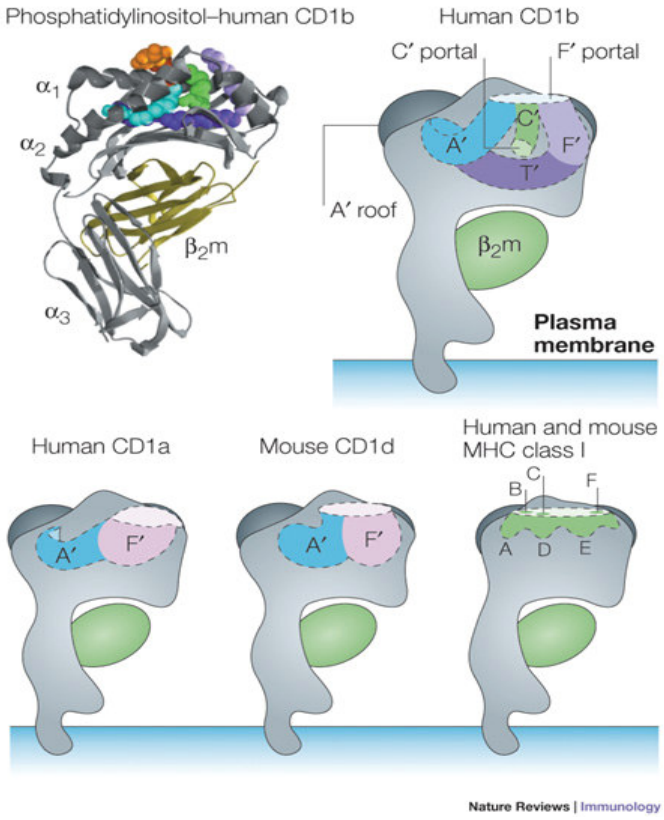

Figure 4. CD1 pockets and portals (Adapted from Moody et al., 2005).
The grooves of the human and murine CD1 proteins are deeper than those of MHC class I molecules, and comprise up to four pockets namely A', C', F', and T'. Ligands can be loaded into the CD1 grooves through the F'portal. The phospatidylinositol is inserted into A'and C'pockets, and the spacer molecules can be inserted into the T'tunnel and F'pocket.

2.3.5. CD1 intracellular loading and trafficking

In contrast to humans, who have single copies of five known CD1 genes, several mammal species have variations in the number of genes and specific isoforms. The heterogeneity of the CD1 gene family among species raises certain fundamental questions regarding the function of the individual isoforms. Studies regarding human CD1 describe that each isoform has a

unique trafficking pattern through the endosomal system of APCs (Dascher and Brenner, 2003b) (Figure 5). In humans, CD1a traffics in the recycling pathway of the early endocytic system (Sugita et al., 1999), while trafficking of CD1b resembles that of MHC class II to late endosomal and lysosomal compartments (Sugita et al., 1996). CD1c traffics through both early and late endosomes (Briken et al., 2000; Sugita et al., 2000; Sugita et al., 1996). The trafficking pathways of human and murine CD1d are slightly different. The intracellular location of murine CD1d molecules is more in the lysosomal compartment, whereas the human CD1d is excluded from that compartment (Sugita et al., 2004).

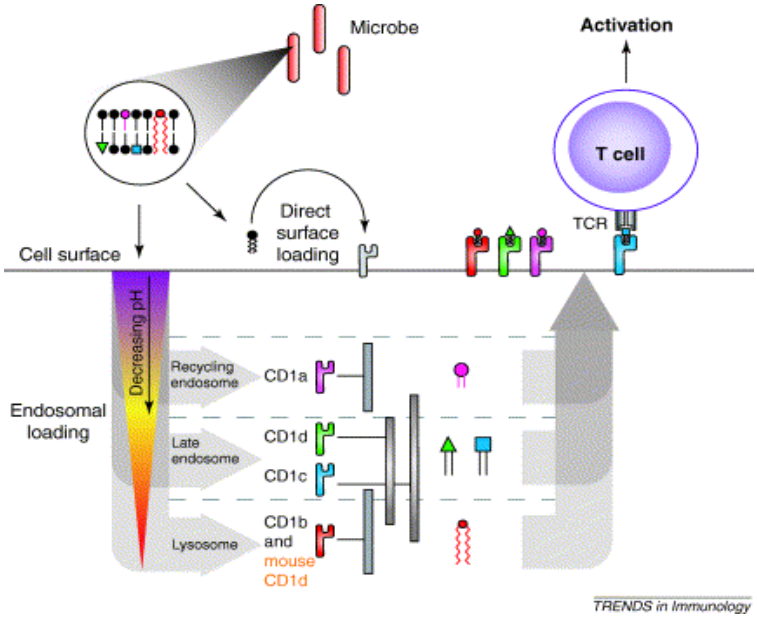

Figure 5. Model of CD1 and lipid antigen traffic through an antigen-presenting cell (Adapted from Dascher and Brenner, 2003).

Lipids from membrane fragments or dead bacteria are taken up by the antigen presenting cell or shed by intracellularly replicating bacteria. These lipids will be encounterd by different CD1 isoforms that traffic through different parts of endosomal network. Lipid loading into CD1 may be facilitated by microsomal triglyceride transfer protein (Zeissig et al., 2010), CD1e (de la Salle et al., 2005), acid pH (Cheng et al., 2006), or saposins (Yuan et al., 2007). After the lipid antigen is loaded into the CD1 molecules, the CD1-antigen complexes traffic to the cell surface where specific T cells can recognize the antigens presented by CD1 molecules. Some antigens need processing for T cell recognition (Prigozy et al., 2001). Some antigens are loaded directly on the cell surface without an internalization process (Moody et al., 2002).

2.3.6. Lipid-specific T cells

Besides CD4+ and CD8+ T cells that are stimulated by MHC class II and MHC class I, CD1-

restricted T cells play significant roles in anti-mycobacterial immunity. Studies in humans and animal models suggested that in acquired immunity against *M. tuberculosis*, MHC-restricted CD4+ and CD8+ T cells play a dominant role and are supported by other T cell subsets including, γδ T cells and CD1-restricted αβ T cells (Boom, 1996; Boom et al., 2003). CD1-restricted T cells may be of importance in cytotoxic responses against intracellular pathogens (Stenger et al., 1997; Stenger S. 2001; Dascher and Brenner, 2003a), or T_H1 type immune responses (Sieling et al., 2000).

Group 1 CD1 restricted T cells:

In humans, group 1 CD1-reactive T cells can express either αβ or γδ TCRs (Porcelli et al., 1998). The majority of CD1 restricted T cells that have been described are αβ T cells however. The phenotype of CD1 restriced αβ T cells is often thought to be limited to CD4-CD8- double negative (DN) or CD8+ but that is a bias that may have been introduced by the fact that most early experiments used CD4-depleted T cell subsets as a starting point. More recently also CD4+ group 1 CD1 restricted T cells have been described (Sieling et al., 2000; Bastian et al., 2008). In vitro studies of the cytolytic activity of group 1 CD1 restricted T cells demonstrated that both subsets lysed *M. tuberculosis* infected macrophages. The DN T cells were found to lyse *M. tuberculosis* infected macrophages using FAS-FasL mediated mechanism and do not affect the viability of mycobacteria, while CD8+ T cells utilized a perforin/granzyme-mediated killing mechanism and resulted in killing of the mycobacteria as well (Sieling et al., 2000; Bastian et al., 2008; Cooper et al., 1997). Granulysin is also produced by CD1c-restricted γδ T cells, which use both perforin- and Fas-mediated cytotoxicity (Spada et al., 2000).

Group 2 CD1 restricted T cells- natural killer T cells:

Natural killer T cells (NKT cells) are CD1d-restricted T lymphocytes that have contrasting functions including both promoting the cell-mediated immunity (in tumours and infectious diseases) and suppressing the cell-mediated immunity (in autoimmune diseases and allograft rejection). The activity of NKT cell can be deleterious in some diseases like allergy and atherosclerosis (Godfrey et al., 2010). The existence of different NKT subsets might be responsible for the contradictory functions. Several classifications have been assumed based on the presence of invariant TCR and CD1d dependency (Eberl et al., 1999; Godfrey et al., 2004). It is widely accepted now that there are two main subsets of CD1d restricted NKT cells: invariant and non-invariant NKT cells. Invariant NKT cells express a semi-invariant T cell antigen receptor (TCR). The human iNKT TCR is composed of Vα24-Jα18 segments paired with Vβ11 (V: variable, J: joining). In mice, Vα14-Jα18 segments are paired with either Vβ8.2, or Vβ7, or Vβ2. The specific TCR αβ-chain combinations in humans and mice confer NKT cells with specificity for glycolipid antigens presented by CD1d (Borg et al., 2007). Both human and mouse iNKT cells can be identified by the use of α-galactosylceramide (α-GalCer)-loaded CD1d tetramers. Human and mouse iNKT cells are reactive to α-GalCer when presented by either human or mouse CD1d molecules (Brossay et al., 1998), showing high conservation. Non-invariant NKT cells are present in humans and mice and they can recognise hydrophobic antigens such as small non-lipidic aromatic molecules (Van Rhijn et al., 2004), sufatide (Jahng et al., 2004), and lysophospholipids (Chang et al., 2008).

Microbial lipid antigens that are presented to NKT cells are listed in table 3 (Brigle and Brenner, 2010).

Table 3. CD1d-presented antigens

Source	Antigen
Mycobacteria	Phosphatidylinositol mannosides
Sphingomonas spp.	α-Glucuronosylceramide
Borrelia burgdorferi	α-Galactosyldiacylglycerol
Leishmania donovani	Lipophosphoglycan
Mammalian (self)	Phosphatidylinositol
	Phosphatidylethanolamine
	Isoglobotrihexosylceramide (iGb3)
Synthetic or marine sponge	α-Galactosylceramide (α-GalCer)

3. Vaccines and vaccination against mycobacterial infections

The immense public health impact of human tuberculosis is now widely recognized. A global plan to reduce the annual death toll by the year 2015, targeting at the big step of eradication worldwide by 2050 was announced at the World Economic Forum in Davos, Switzerland in 2006 (World Health Organization, 2006). Vaccination is expected to make a major contribution to this goal (Young and Dye, 2006). Furthermore, vaccination is still the lowest cost and preferred strategy for the prevention of mycobacterial infection, as it has been successfully used to eradicate many other infectious diseases (Endsley et al., 2009).

The strong economic impact of paratuberculosis including early culling of infected cattle, reduced milk yield and weight loss, as well as potential human food -born infection, demand an effective control program. Despite testing and culling programs, the incidence of paratuberculosis has not decreased in many countries of Europe, and still increases in the Netherlands, Finland, Italy, and Scotland (Over et al., 2011). Vaccination may provide a more effective method in efforts to reduce the impacts of this disease.

Clear knowledge about the biology of the pathogen and immunological mechanisms of host protection should provide essential information for the design of vaccines against tuberculosis and paratuberculosis. So far, heterologous prime boost strategies using combinations of BCG and a mycobacterial protein are thoroughly being investigated. Alternatives that go beyond induction of MHC-restricted memory T cells, like activation of CD1-restricted αβ T cell and γδ T cell populations are occasionally being targeted (Young and Dye, 2006). Lipid antigens presented by CD1 have been considered as potential new candidates in the development of vaccines and immunotherapeutic agents of infectious diseases. Combining lipid antigens with protein components may create more protective subunit vaccine formulations to elicit effector functions of different T cell subsets (Dascher and Brenner, 2003a). However, many mechanistic aspects of CD1-restricted immunity have not yet been well established. Cattle as the natural host of mycobacterial infections including tuberculosis and paratuberculosis provide ideal natural models to study efficicacy of new vaccine candidates and test underlying

immunological mechanisms to the benefit of both humans and cattle.

3.1. Vaccines for human and bovine TB:

Human TB: WTO estimated 9 million incident cases of TB in 2007 and most of those cases were in Asia (55%) and Africa (31%) (World Health Organization, 2009). TB is in most cases a latent disease, and only approximately 10% of infected cases develop into active tuberculosis disease (Young and Dye, 2006; Kaufmann, 2005). To achieve the goal of eradication of *M. tuberculosis*, the new vaccine generation should have the capability to attack dormant mycobacteria contained inside macrophages (Kaufmann, 2005). Over almost a century many vaccination approaches to prevent *M. tuberculosis* infection have been applied. Different vaccine candidates including attenuated mycobacteria, subunits, live vectors, heterologous prime/boost combinations, mucosal vaccination, and immunotherapy have been tested (Hoft, 2008). Most of the new vaccines candidates have been assessed as replacements or enhancement of the available BCG vaccine which is of limited efficacy. BCG, the only applicable vaccine, can reduce the incidence of severe forms of disseminated disease in infants, but is less effective against the major infectious form of active pulmonary TB that occurs in adults. Live vector vaccines, or heterologous prime/boost combination vaccines can trigger protective T cell response to key antigens as shown in humans (McConkey et al., 2003; Vuola et al., 2005). However, these types of vaccines/combinations have not proven to be more protective against tuberculosis than BCG (Hoft, 2008). As for TB prevention, heterologous prime-boost vaccinations that are being tested are several combinations like DNA vaccines with recombinant viruses, two different recombinant viruses, or recombinant viruses in combination with BCG. Because BCG or heterologous prime/boost combinations that include BCG have never been surpassed in efficiency by any BCG-less approach, it is likely that in the future, BCG will not be replaced, but rather be implemented in an improved, combined vaccination strategy.

Bovine TB remains a relevant problem in developing countries where the control of bovine TB in cattle and buffalos is very limited or absent (Hogarth et al., 2006), as well as in some developed countries where infection cycles persist due to presence of *M. bovis* in wildlife reservoirs (Hope, 2008). Vaccination of cattle against bovine tuberculosis would be the preferred strategy for the control of disease in developing countries where a test and cull control program is not affordable (Hogarth et al., 2006). Advances in the understanding of protective immune responses to *M. bovis* infection in cattle, new molecular biological techniques, and the availability of the *M. bovis* genome have provided opportunities for the rational development of improved tuberculosis vaccines. A number of new tuberculosis vaccines including attenuated *M. bovis* strains, killed mycobacteria, protein and DNA vaccines are under development and many are being assessed in cattle with promising results (Vordermeier and Hewinson, 2006).

Development of diagnostic tests and vaccines against bovine TB and human TB has always been interconnected. As mentioned previously, BCG is currently the only applicable vaccine against human TB, and new vaccine or vaccination strategies to prevent human TB are likely based around BCG (Hope J.C. 2008). Combined vaccination regimens combining BCG with subunit vaccines, similar to what is currently being tested for use in humans, are being

considered for bovine TB prevention. Combinations of the proteins HSP65, HSP70, and APA (alanine-proline-rich antigen) followed by BCG in prime-boost vaccination strategies may provide a potential for use (Hope, 2008). Other combinations, like BCG followed by adenovirus expressing Ag85A showed improved protective efficacy as compared to BCG since fewer visible pathology and increased numbers of animals without visible lesions were observed (Vordermeier et al., 2009). Even though the combined vaccination has still not proven to be applicable in the field, these studies can be considered to use cattle as a target species as well as a model for human tuberculosis. Therefore we think it is appropriate to investigate the host immune response to lipid antigens as potential vaccine candidate for prevention of bovine TB as well as a model for human TB.

3.2. Vaccines for bovine paratuberculosis

Vaccines for paratuberculosis have been produced since 1926 but they have shown undesirable side effects and induce non-optimal levels of protection. Up to date, the existing vaccines produced from whole killed or live-attenuated bacteria can decrease and slow down the rate of appearance of clinical symptoms, but none of them protect animals from infection efficiently (Rosseels, 2008).

Gudair, a vaccine containing a killed (heat-inactivated) suspension of *Mycobacterium avium paratuberculosis* combined with a mineral oil adjuvant, is the only licensed vaccine against paratuberculosis in goats and sheep. Vaccine injection site lesions (Reddacliff et al., 2006) and interference with serodiagnostic test for paratuberculosis (Rosseels, 2008) are serious constraints of this vaccine in field trials. Up to present, the Gudair vaccine is not licenced for use in cattle.

Subunit vaccines have been considered as a strategy to prevent MAP infection (Rosseels, 2008). A recombinant MAP Hsp70 vaccine was shown to induce cell-mediated responses in paratuberculosis infected cattle at different stages of disease (Koets et al., 1999; Koets et al., 2001; Koets et al., 2002), reduced shedding of MAP in feces (Koets et al., 2006), and it induced antibody production against MAP Hsp70 (Santema et al., 2010). In contrast to the inactivated whole cell vaccine (Gudair® Vaccine, Australia), the Hsp70/DDA vaccine does not interfere with current tuberculosis diagnostics (Santema et al., 2009). Contrary to the Gudair vaccine, no side effects other than minor swelling were recorded.

Apart from protein antigens, glycolipids are abundantly present in mycobacteria. These antigens are presented to T cells by the CD1 family. It has been widely accepted that the glycolipid antigens elicit strong cell-mediated immune response, but they are not yet used as subunit vaccine candidates. Immune responses against mycobacterium-derived lipid antigens will provide insight into non-classical processes of presentation, recognition and into potential protective efficacy when cattle are immunized with vaccine containing glycolipid antigens (like GMM). Understanding of immunological mechanisms is the basis for future vaccine strategies against mycobacterial infections. In this thesis, studies are reported regarding immunogenicity of glycolipid antigens presented by CD1 as well as the expression of representatives of this family of molecules in cattle.

4. Scope of the thesis

The research reported in this thesis addresses the question whether vaccination with mycobacterial lipids has merits in addition to vaccination strategies aiming at protein specific protective immunity to mycobacterial infection. The studies were conducted in cattle, *Bos taurus*, natural hosts of the important pathogens *Mycobacterium bovis* and *M. avium subsp paratuberculosis*.

Immunity against MHC-presented peptides has long been known to play a critical role in protection against mycobacteria. CD1-presented bacterial lipids have much later been discovered as targets of the adaptive immune system. Mycobacterial cell walls consist of thick and complex lipid layers and the present research focuses on these lipids and their presentation in the context of the CD1 system.

Little was known about the CD1 system in cattle; hence part of our investigations focused on genetics and expression patterns of bovine CD1 as a basis for understanding its function in lipid specific immune responses and vaccination. Like in humans and unlike in mice, which express only CD1d, group 1 and group 2 CD1 genes have been identified in cattle. We studied the expression and antibody recognition of bovine CD1 molecules in **chapter 5**. Previously, the CD1D gene structure was suggested to be non functional due to mutations in the start codon and intron splice site. We investigated its unusual gene structure, describe the unexpected expression of the CD1d molecule, and performed in vivo studies using α-galactosylceramide in cattle (**Chapter 6**).

We studied the immunity to lipid antigens extracted from *M. bovis* and MAP in cattle naturally infected with these pathogens (**Chapter 2**). To further understand the immune responses to glycolipid antigens and their capacity to generate memory responses, we immunized animals with vaccines that contained free or protein-conjugated GMM, a glycolipid extracted from mycobacteria in parallel with protein containing vaccines as classical controls stimulating both cell-mediated and humoral responses (**Chapter 3** and **Chapter 4**). The overall aim of this thesis is to assess the value of glycolipids as subunit vaccines in the battle against mycobacterial infections.

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Chapter 2

Low cross-reactivity of T-cell responses against lipids from *Mycobacterium bovis* and *M. avium paratuberculosis* during natural infection

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Abstract

Although CD1 proteins are known to present mycobacterial lipid antigens to T cells, there is little understanding of the in vivo behavior of T cells restricted by CD1a, CD1b and CD1c, and the relative immunogenicity and immunodominance of individual lipids within the total array of lipids that comprise a bacterium. Because bovines expressmultiple CD1 proteins and are natural hosts of *Mycobacterium bovis* and *Mycobacterium avium paratuberculosis* (MAP), we used them as a new animalmodel of CD1 function. Here, we report the surprisingly divergent responses against lipids produced by these two pathogens during infection. Despite considerable overlap in lipid content, only three out of 69 animals cross-react with *M. bovis* and MAP total lipid preparations. The unidentified immunodominant compound of *M. bovis* is a hydrophilic compound, whereas the immunodominant lipid of MAP is presented by CD1b and was identified as glucose monomycolate (GMM). The preferential recognition of GMM antigen by MAP-infected cattle may be explained by the higher expression of GMM by MAP than by *M. bovis*. The bacterial species-specific nature of the CD1-restricted, adaptive T-cell response affects the approach to development of lipid based immunodiagnostic tests.

Introduction

Mycobacterial diseases are highly prevalent in humans and cattle. Human tuberculosis, caused by *M. tuberculosis*, and bovine tuberculosis, caused by *Mycobacterium bovis*, are widespread. Infections with *Mycobacterium avium paratuberculosis* (MAP), the causative agent of Johne's disease or bovine paratuberculosis, cause substantial economic losses in the dairy cattle industry [1]. Effective vaccines against mycobacteria are not available for humans and animals despite large efforts in discovery, production, and testing of improved *M. bovis* BCG strains and mycobacterial protein antigens.

The protective effect of a vaccine is based on priming of the adaptive immune system and the ensuing immunological memory, which is dependent on the development of T-cell memory. Immunization with protein antigens presented by MHC molecules is a very effective way of generating T-cell memory. However, an important disadvantage of immune responses that are dependent on a single or a few proteins is that immune evasion in the form of single mutations may occur. In addition, in outbred populations, vaccine efficacy of a subunit vaccine can be limited at the individual level by the lack of binding capacity of the antigen to the specific set of MHC alleles present in that individual.

The CD1 family of proteins presents lipids, glycolipids and lipopeptides to T cells. The group 1 CD1 isoforms (CD1a, CD1b, CD1c) are largely conserved in mammalian evolution, suggesting that they have an important natural function in the immune response. Targeting the CD1 system for vaccine development has the advantage that the presented antigens are not gene products and are therefore not subject to rapid mutation. Also, the CD1 system shows very limited polymorphism, minimizing interindividual differences within an outbred population in the capacity to present a certain antigen [2]. In addition, CD1- restricted T cells have been shown to be able to express molecules that are important in immune response to mycobacterial infections, including g-interferon and granulysin [3, 4]. In fact, guinea pigs,

immunized with total lipid extract of *M. tuberculosis*, developed smaller granulomatous lesions upon challenge with *M. tuberculosis* [5] than control animals, although the bacterial burden was only slightly reduced.

However, a basic unresolved question is whether CD1- restricted T cells, once activated by either natural infection or vaccination, lead to durable adaptive antigen-specific immune responses. Most evidence suggests that CD1d-restricted NKT cells are rapidly but transiently activated such that repeated exposure to antigen can cause anergy, but does not generally lead to priming or durable expansion in ways that lead to protection at time periods long after the initial antigen exposure. Indeed, most models of CD1d-restricted NKT cells emphasize that these cells are innate lymphocytes that, upon stimulation, have a strong, transient effect that is not subject to vaccination. However, unlike CD1d, the three group 1 CD1 isoforms (CD1a, CD1b, CD1c) show inducible expression on dendritic cells that is regulated by Tolllike receptors, and they present more diverse classes of antigens. Accordingly, T cells restricted by group 1 CD1 proteins may have a more diverse pool of precursors that could be primed to become antigen specific memory cells. Thus, answering the question against which antigens CD1-restricted adaptive T-cell responses develop is a fundamental question to the field and of practical importance for vaccine development.

The known CD1-presented mycobacterial lipid antigens are diverse and include mycobacterial mycolates [6–8], diacylglycerols [9], polyisoprenoid lipids [10], sulfotrehalose-containing lipids [11] and lipopeptides [12]. Of note, all these mycobacterial lipids are presented by group 1 CD1 proteins (CD1a, CD1b and CD1c). Because mice do not have orthologs of group 1 CD1 molecules [13] and other non-human models are limited, there have been few studies of the natural functions, targets or frequency of the responding T cells in vivo. Cattle express CD1a, CD1e and multiple CD1b molecules, but lack CD1c and CD1d [14, 15], and this, in combination with a predisposition to mycobacterial infection, allows study of the strength, antigen specificity and duration of mycobacteria-specific T-cell responses to lipids. Because of its close resemblance to human tuberculosis, bovine tuberculosis is an excellent model for human tuberculosis [16].

The in vitro generation of T-cell lines has been extremely useful for identifying CD1-presented antigens but has real limitations for understanding the role of natural antigens during infection in vivo and the phenotype and functions of the T cells that recognize these antigens in vivo. For example, many lipid-reactive T cells have been enriched by selection for cells that lack CD4 and CD8 or by culturing them with CD1-expressing antigen-presenting cells derived from MHC mismatched donors. Such manipulations may cause the observations to represent relatively rare T cells or identify lipid antigens of subdominant antigenicity. Also, many long-term T-cell clones show a drift toward a Th1 phenotype and so may not accurately reflect functions that normally occur *in vivo.*

Data obtained from non-manipulated material obtained from *in vivo* models of mycobacterial infections can provide valuable additional insights into antigen specificity and phenotype of T cells *in vivo*. Because non-human models that express orthologs of CD1a, CD1b or CD1c are currently limited to guinea pigs [5, 17], most *ex vivo* data are obtained in humans. A limited number of studies describe lipid-reactive T cells expanded in the acute phase of

human infection, using total lipid extracts or antigens that had previously been identified using T-cell clones [10, 11, 18]. From among the various mycobacterial non-protein antigens known to date, the particular antigens that dominate the response against a certain mycobacterial species or strain are unknown. However, identifying the antigens and T cells that generate the strongest adaptive responses are the key pieces of information necessary to use CD1 technology to develop diagnostic tests and vaccines.

In vitro, CD1-restricted T cells have been shown to precisely discriminate detailed structures of mycobacterial lipids such as mycolic acids, glucose monomycolates (GMM), sulfotrehaloses, dideoxymycobactins and mycoketides. Because these lipids vary naturally among pathogenic and environmental strains of mycobacteria, these results suggest that lipid-reactive T cells might respond differently to individual species of mycobacteria. For example, GMM is made using glucose derived from the host and is not known to be made by mycobacteria growing in the environment, leading to the hypothesis that this antigen is a marker that distinguishes productive infections from environmental growth [8]. An example of a mycobacterial species-specific lipopeptide is the MAP-specific lipopentapeptide L5P [19]. Differing species of mycobacteria also share many classes of lipids with a common basic design and even though some of these have species-specific molecular characteristics, cross-reactive T cells are known to exist [20]. These considerations suggest that mixtures of mycobacterial lipids could be used to distinguish mycobacterial infections from infection by non-mycobacterial species, but species-specific immunodiagnosis requires further work to define the immunodominant antigens within a given species.

To provide evidence for priming of adaptive, CD1-restricted T cells during natural infection and to identify immunodominant antigens, we describe the *ex vivo* T-cell responses against lipids during bovine tuberculosis, caused by acute infection with *M. bovis*, and paratuberculosis, caused by chronic infection with MAP. We found significantly elevated immune responses against lipids among animals naturally infected with either *M. bovis* or MAP as compared with controls, providing evidence for antigen specific T-cell responses, primed by in vivo infection. Further, we found that these two mycobacterial species cause mycobacterial species-specific T-cell responses against lipids, and showed that it is possible to determine immunodominance of lipids in freshly isolated T cells.

Results

M. bovis and MAP infection generate strong antilipid T-cell responses in vivo

Adult animals suffering from a fecal culture-confirmed infection with MAP, resulting from natural oral exposure (n538) and MAP culture- negative controls or animals obtained from farms that have not resulted in any positive MAP cultures during the last 5 years (called ‘‘MAP negative controls’’, n526) were tested for T-cell reactivity against MAP total lipid extract from the strain MAP 280105. In MAP-infected animals, the stimulation index, defined as the proliferative response obtained with antigenic stimulation divided by the response obtained with incubation with medium alone, was significantly higher (median 6.9) against total extracts from MAP compared with MAP-negative controls (median 1.6; p50.01) (Fig. 1A). Similarly, *M. bovis*-infected animals (n531) show significantly higher responses to *M.*

bovis 4913C total lipid extract (median 20.3) than bovine purified protein derivative (PPD) skin test-negative controls (n513; median 4.6; p50.001) (Fig. 1B). To compare the strength of the antilipid response with an antiprotein response, we also performed stimulations with ESAT6 protein, an immunogenic protein expressed by members of the *M. tuberculosis* complex, including *M. bovis*. *M. bovis*-infected animals (n512) show a significantly higher response to ESAT6 (median 7.9) than bovine PPD skin test-negative controls (n511; median 1.8; p50.00003) (Fig. 1C). The strength of the anti-ESAT6 response was in all cases lower than the antilipid response. Of note, two skin test-negative animals with a relatively high response against *M. bovis* total lipid extract (animal 25 and animal 2) do not respond to ESAT6. We conclude that natural infection of cattle stimulates a robust and durable immune response to lipid extracts, which is stronger but less specific than the response against the *M. bovis* protein ESAT6.

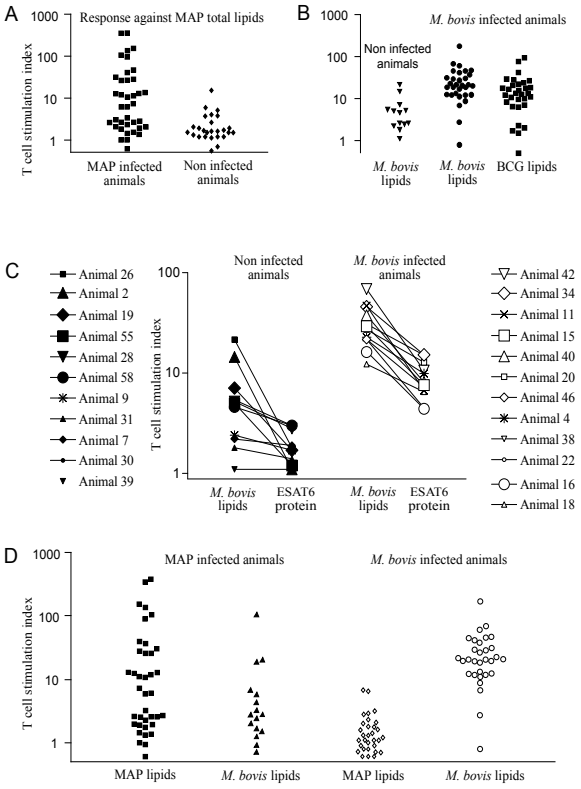


Figure 1. T-cell responses of MAP- and *M. bovis*-infected and non-infected animals to total lipid extracts. (A) Freshly isolated PBMC of MAPinfected animals or age-matched controls from MAP-free farms were stimulated with MAP total lipid extract and with *M. bovis* total lipid extract. (B) *M. bovis*-infected cattle and buffalo and non-infected controls from the same herd were stimulated with *M. bovis* total lipid extract. (C) *M. bovis* infected buffalo and non-infected controls from the same herd were stimulated with M. bovis total lipid xtract and recombinant, pure ESAT6 protein. Each symbol represents one individual animal. (D) Cross reactivity between lipid extracts was determined by stimulating PBMC of singly infected animals with total lipid extracts of MAP and *M. bovis*. The T-cell stimulation index was calculated as described in the Materials and methods section.

Low cross-reactivity of T-cell responses against lipids from M. bovis and MAP

It is known that *M. tuberculosis* and *M. leprae* have lipids in common that generate cross-reactive T-cell responses in humans [20]. We studied whether the same is true for *M. bovis* and MAP in cattle, or whether mycobacterial species-specific lipids are targets of the T-cell response. *M. bovis*-infected animals were tested for reactivity against MAP lipids, and MAP-infected animals were tested for reactivity against *M. bovis* lipids (Fig. 1D). Of the 16 MAP-infected animals tested, only three (7.8%) responded with a stimulation index of 10 or higher to *M. bovis* lipids. None of the 31 *M. bovis*-infected animals responded with a stimulation index of greater than 10 to MAP lipids. The low cross-reactivity prompted a search for specific lipid antigens of MAP and *M. bovis*.

Along with the tests for cross–species reactivity, we determined whether the response was pecific for the mycobacterial strain used and for any possible effects of the medium in which it was grown. First, we tested whether animals with reactivity against *M. bovis* 4913C lipids also responded to lipids from the related vaccine strain, *M. bovis* BCG. *M. bovis*-infected animals showed an increased response to *M. bovis* BCG lipids (po0.001) with response rates similar to that of WT *M. bovis* (Fig. 1B), although the stimulation index against *M. bovis* lipids was higher (median 20.3) than against BCG lipids (median 13.2). Stimulation with lipid extracts of *M. bovis* 4913C grown in 7H9 supplemented with oliec acid dextrose complex (OADC) or grown in 7H9 supplemented with 1% D-glucose or *M. bovis* ATCC 19210 grown in 7H9 supplemented with OADC or grown in 7H9 supplemented with 1% D-glucose resulted in highly comparable T-cell responses in *M. bovis*-infected animals (data not shown). In addition, we compared lipid extracts of MAP strain ATCC 19698 grown in 7H9 medium supplemented with OADC and mycobactin J (MycJ) and MAP strain 280105 grown in Watson–Reid medium supplemented with MycJ, which resulted in highly comparable T-cell responses in MAP-infected animals (data not shown).

To determine the value of lipid-based T-cell assays as a diagnostic tool, we compared our results with the reference diagnostics, consisting of skin testing for *M. bovis* infections and culture-based protocols for MAP infections, as described in the Materials and methods. If a stimulation index of 10 or higher was considered a positive response to lipids, the sensitivity and specificity of this were calculated to be 87 and 85% for *M. bovis*-infected animals tested for reactivity against *M. bovis* lipids, and 47 and 96% for MAP-infected animals tested for reactivity against MAP lipids. The sensitivity of the T-cell assay was calculated as the percentage of animals with a T-cell stimulation index of 10 or higher of the total number of animals with a positive test result, using the reference diagnostics. Specificity refers to the percentage of animals with a T-cell stimulation index lower than 10 of the total number of negative animals using the reference diagnostics.

Differing immunodominant substances in MAP and M. bovis total lipid extracts

Thin layer chromatography (TLC) analyses performed on lipid extracts of several MAP, BCG and *M. bovis* strains cultured on independent occasions and in different media revealed that the overall composition of the total lipid extracts of each bacterial species was imilar and reproducible (Fig. 2A). Of note, much more GMM, resolving as a doublet, was present in MAP.

It is known that for the production of GMM, glucose needs to be present in the culture medium. D-glucose was present in all media that we used for mycobacterial culture in concentrations between 0.2% (7H9 supplemented with OADC) and 1.2% w/v (Watson-Reid supplemented with OADC). Lipid extracts of MAP consistently contained more GMM than *M. bovis* and *M. bovi*s BCG, independently from the type of medium and the glucose concentration present in the medium (Fig. 2A).

To determine which constituents of the MAP and *M. bovis* total lipid extract were responsible for the observed T-cell stimulation, total lipid extracts were fractionated on open silica columns using chloroform, mixtures of chloroform and acetone, acetone, methanol and water as eluents. A TLC analysis of the MAP fractions confirmed the expected separation of compounds into distinct classes (Fig. 2B). Infected animals were tested for T-cell reactivity against each fraction. Of the *M. bovis* lipid fractions, we noted that each of four animals tested showed similar profiles of reactivity and that the water eluate was the only fraction

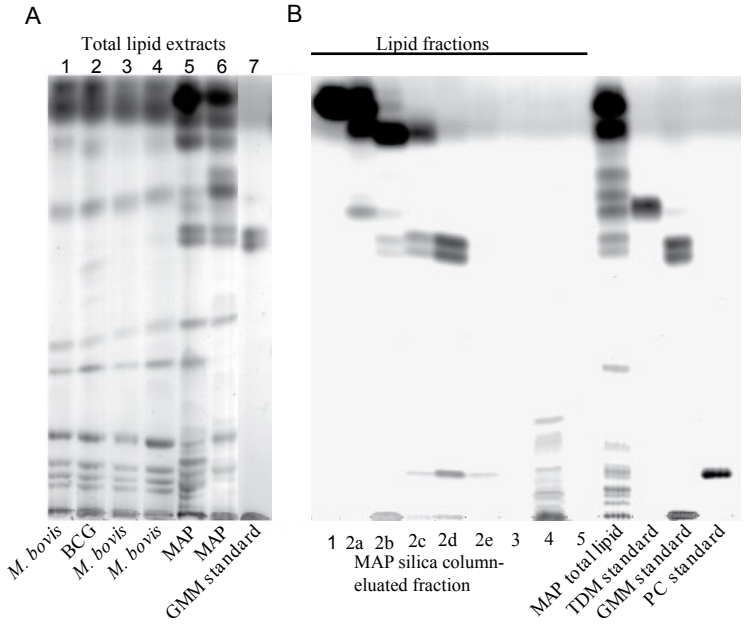


Figure 2. TLC analysis of lipid extracts. (A) An aliquot of 10 mg of total lipids, extracted from bacterial cultures by chloroform/methanol extraction, was applied to a silica TLC plate and developed as described in the Materials and methods section. Lane 1: *M. bovis* ATCC 19210 grown in 7H9/OADC; lane 2: BCG Pasteur grown in 7H9/OADC; lane 3: *M. bovis* 4913C grown in 7H9/OADC; lane 4: *M. bovis* ATCC 19210 grown in 7H9/D-glucose; lane 5: MAP ATCC 19698 grown in 7H9/OADC/MycJ; lane 6: MAP 280105, grown in Watson–Reid medium/MycJ; lane 7: purified *M. phlei*-derived GMM. Additional lanes between lanes 6 and 7 that were present on the original TLC plate but not relevant for this figure were electronically removed. (B) An aliquot of 4mg of MAP total lipids was loaded on a silica column and eluted with increasingly hydrophilic eluents (fraction 1: chloroform; fraction 2a–e: chloroform:acetone mixtures in ratios of 9:1, 8:2, 7:3, 6:4 and 5:5 v:v; fraction 3: acetone; fraction 4: methanol; fraction 5: water). A fixed percentage of each fraction was loaded on a TLC plate and developed and visualized. Trehalose dimycolate (TDM), GMM and phosphatidylcholine (PC) were used as standards.

to which T cells consistently reacted at high levels (Fig. 3A, left panel). Each of three cattle infected with MAP tested showed a similar pattern of reactivity to fractions, and this pattern was different from that observed in *M. bovis*-infected cattle. The immunodominant fractions of MAP were more hydrophobic compounds eluting from the silica column in chloroform:acetone mixtures in ratios of 8:2, 7:3, and 6:4 v:v (Fig. 3A, right panel). Because TLC analysis had shown that each of these stimulatory fractions was highly enriched for the doublet corresponding to GMM (Fig. 2B), animals that responded to them were subsequently tested for reactivity against pure GMM standard purified from *M. phlei* (Fig. 4A, left panel). The purity and identity of this GMM standard was confirmed by mass spectrometry (Supporting Information Fig. 1b, lower panel). Cross reactivity of T cells against GMM from different sources is expected because when mycolic acid derivatives are presented by CD1 to T cells, the length variation, as well as the variable meromycolate modifications, are buried in the CD1 molecule and not directly recognized by the T-cell [7, 8, 21]. T cells from each of the animals that recognized GMM-containing MAP fractions also recognized the GMM standard, and the GMM standard is as potent as the MAP fraction that contains GMM.

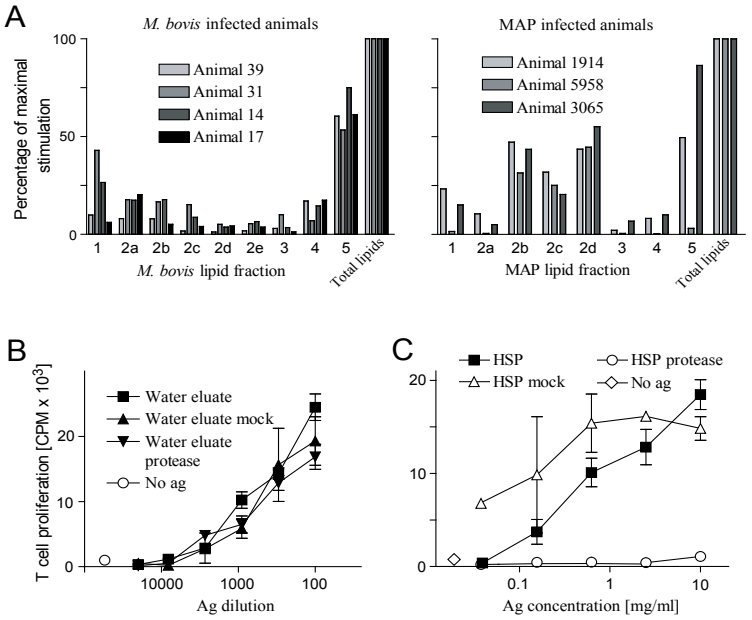


Figure 3. T-cell responses against subfractions of MAP and *M. bovis* lipid extracts. (A) *M. bovis* (left panel) and MAP (right panel) infected animals that respond to total lipid extracts were tested for reactivity against fractions eluted from a silica column loaded with total lipid extract. The following eluents were used: fraction 1: chloroform; fraction 2a–e: chloroform:acetone mixtures in ratios of 9:1, 8:2, 7:3, 6:4 and 5:5 v:v; fraction 3:acetone; fraction 4: methanol; fraction 5: water. [3H]thymidine incorporation by freshly isolated PBMC was measured to determine T-cell proliferation. (B) Protease treatment of MAP water eluate did not diminish its antigenicity. (C) A control protein antigen, HSP70, that was proteasetreated in parallel with the water eluate, was effectively destroyed as shown by its greatly diminished T-cell stimulatory effect on T cells from healthy animals that had been immunized with this protein.

From this we conclude that GMM is an immunodominant lipid of MAP. *M. phlei* GMM was not recognized by *M. bovis*-infected animals, indicating that the development of this response in vivo is specific for the species of the pathogen (Fig. 4A, right panel). Although GMM-containing fractions produced the most potent response, two of the three MAP-infected animals also responded to compounds present in the water eluent from the silica column.

Bovine T-cell responses to GMM are CD1b-restricted

The only known mechanism by which T cells respond to GMM is via presentation by CD1 proteins. In humans, GMM is presented to T cells by CD1b, and only this isoform is known to have a groove large enough to accommodate the large (C80) mycolic acids. Cattle express three CD1b molecules: CD1b1, CD1b3 and CD1b5. Therefore, we hypothesized that this response might be dependent on one or more of the bovine CD1b orthologs. The BCD1b3.1 monoclonal antibody developed against human CD1b and is considered to be a broadly cross-reactive anti-CD1b antibody because it recognizes guinea pig CD1b [17] and bovine CD1b3 [14]. This antibody but not an isotype control antibody blocked the response to GMM, implicating bovine CD1b as necessary for the response (Fig. 4B). Similarly, reactivity against the MAP fractions eluting in chloroform:acetone 8:2, 7:3 and 6:4v:v from the silica column could be blocked with the BCD1b3.1 antibody.

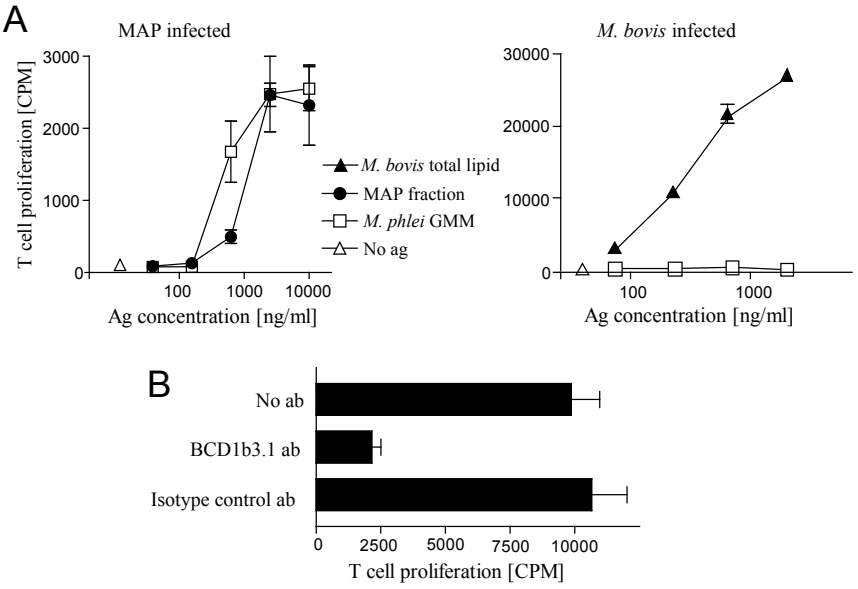


Figure 4. Pure GMM is recognized by MAP-infected animals and presented by CD1b. (A) The GMM-containing mixture of compounds eluting from a MAP lipid-loaded silica column with a 7:3 v:v chloroform:acetone mixture, and pure GMM from M. phlei were used to stimulate freshly isolated PBMC of MAP (left panel) and M. bovis (right panel) infected animals. [3H]thymidine incorporation was measured after 3 days of incubation to determine T-cell proliferation. Representative data of two animals are shown. (B) Reactivity against the 7:3 v:v chloroform:acetone mixture-eluted compounds could be blocked with the BCD1b3.1 antibody that recognizes bovine CD1b, but not with and isotype control antibody

Partial analysis of the silica column water eluates

TLC analysis of the water eluents from silica columns of *M. bovis* and MAP lipid extracts revealed no spots other than some material at the origin. Weak staining at the origin was obtained with the general cupric acetate staining and with ninhydrin staining for amide groups, but not with the phosphate-specific Dittmer reagent. Because the water eluate represents the most hydrophilic fraction of the total lipid extract we considered the possibility that these fractions contain contaminating bacterial proteins. To test this, a bicinchoninic acid protein assay was performed on the samples. The standard curve was generated using serial dilutions of BSA and the detection limit of the assay was determined to be 3.5 mg/mL. Using this method, we measured absorbances corresponding with 13, 60, 86 and 40 mg/mL protein in the undiluted water eluates of MAP 280105, MAP ATCC 19698, *M. bovis* AN5 and *M. bovis* 4913C, respectively. Because this type of protein assay is not equally sensitive for all amino acids, and because lipids, biogenic amines and other substances are known to produce positive signal as well, we also performed an HPLC-based analysis of the total amino acid content after acid hydrolysis (Table 1). These data show that the maximum protein content was 27, 20 and 11 mg/ mL in the undiluted water eluates of MAP ATCC 19698, *M. bovis* AN5 and *M. bovis* 4913C lipids, respectively. Of note, the two different *M. bovis* water eluates contained only a partly overlapping amino acid content.

Table 1. Amino acid content water eluates of total lipid extract-loaded silica columns

Amino acid	Silica column water eluate from lipids from:		
	MAP ATCC 19698	*M. bovis* AN5	*M. bovis* 4913C
	Amino acid concentration (mM)		
Asn+Asp	35	22	7
Glu+Gln	77	28	14
Cys	2	4	8
Ser	12	20	8
His	4	3	3
Gly	24	40	15
Thr	6	0	0
Arg	2	0	2
Ala	14	23	6
Tau	4	3	37
Tyr	1	1	0
Val	5	15	0
Phe	3	6	1
Ile	3	7	4
Leu	8	18	4
Orn	4	0	0
Lys	37	17	2
Protein (mg/mL)[a)]	27	20	11

[a)] Protein content was calculated based on the MWof the residuals of the determined set of amino acids, excluding Tau and Orn, on a mg/mL basis.

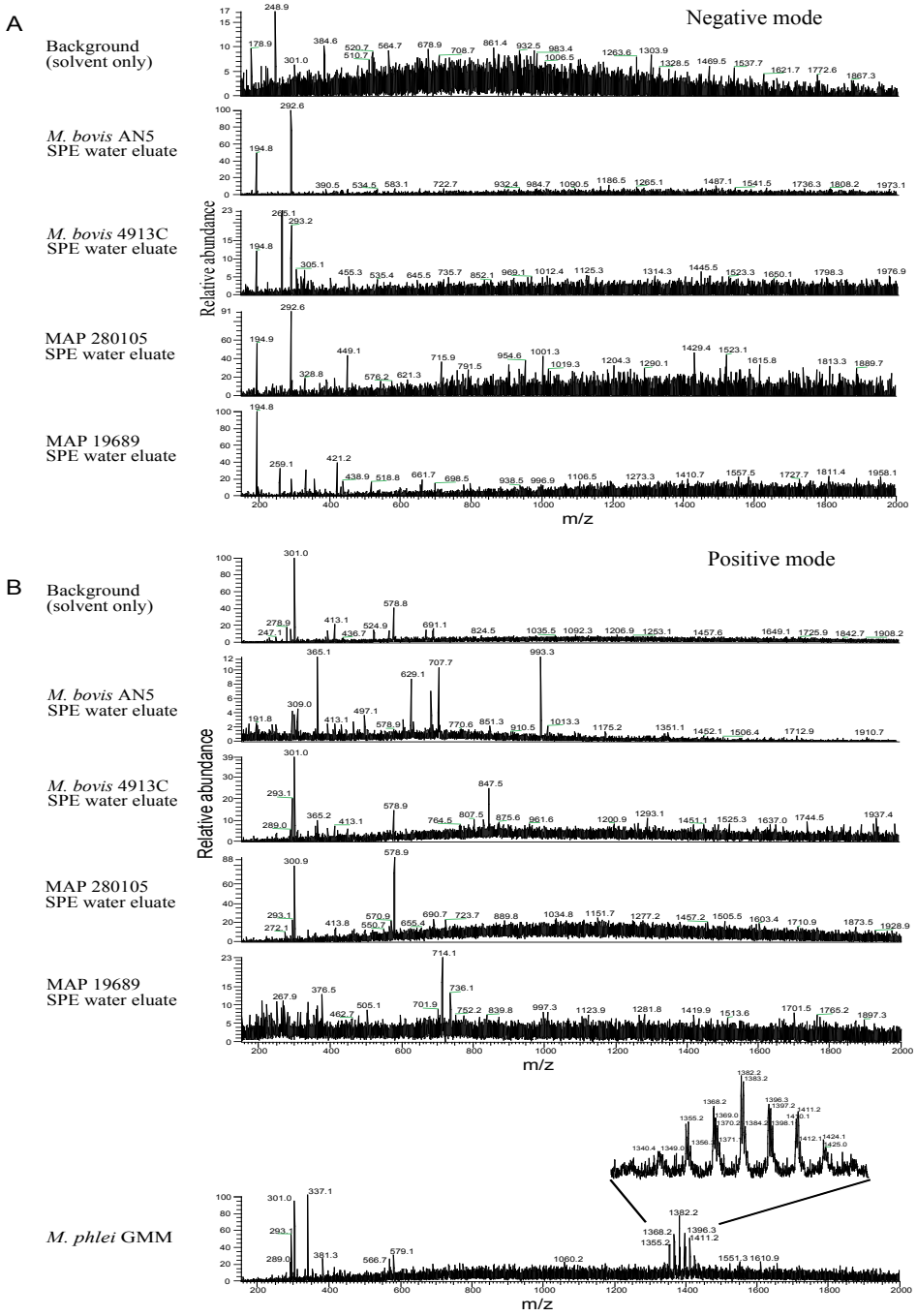
Positive-mode and negative-mode mass spectra were collected using nanoelectrospray ionization mass spectrometry (Supporting Information Fig. 1). In all T-cell assays performed on *M. bovis* infected animals, the response to the water-eluted compounds of the lipid extracts

of two different *M. bovis* strains was always comparable and the same was true for the water-eluted compounds of total lipid preparations of two different MAP strains when used to stimulate T cells from MAP-infected animals. Therefore, we hypothesized that the chemical nature of the antigenic substance in the water-eluted fractions derived from different strains of one mycobacterial species is likely to be identical. For this reason we scanned the mass spectra for peaks shared between preparations derived from one mycobacterial species, but we could not identify any shared ions. To test whether the strong T-cell responses against the silica column water eluates were dependent on proteins, we performed experiments using proteases. When the water-eluting compounds from *M. bovis* or MAP were pretreated with either of two broad spectrum proteases, proteinase K and pronase, they retained their ability to stimulate T cells as the same level as mock-treated samples (Fig. 3B). In contrast, pronase and proteinase K greatly reduced or abolished recognition of the protein antigen HSP70 by T cells of animals that had been vaccinated with this antigen (Fig. 3C). Thus, the antigenicity of the water eluates from silica columns loaded with M. bovis and MAP lipids was not dependent on proteins.

Discussion

Almost all of the reported *ex vivo*, polyclonal T-cell responses are directed against proteins or peptides. Here, we show that mycobacterial lipids are strongly recognized by *ex vivo* T cells of bovines that suffer from infections with MAP or *M. bovis*. Even though mycobacterial lipid extracts of different species share many lipid compounds and are known to be able to induce crossreactive T-cell responses when responses against *M. tuberculosis* and *M. leprae* are compared [20], we found little cross reactivity between the lipid extracts of MAP and *M. bovis* in infected cattle. Reactivity to the lipid preparations was exclusively detected in infected animals, showing that the observed results reflected adaptive, in vivo primed responses, and not some general immunostimulatory effect of the lipids leading to an innate-like immune response. Although slightly weaker, reactivity against BCG lipids was detected in the same animals in which reactivity against *M. bovis* lipids was detected. Similar responses were expected because of the close genetic relationship between *M. bovis* and BCG and this suggests that future efforts to generate species-specific immunodiagnostic reactions for such closely related organisms will rely on identifying species specific lipids, such as trehalose sulfoglycolipids, rather than administering more complex mixtures of ipids.

The chemical nature of the immunostimulatory compound(s) present in the highly immunogenic silica column water eluates of the total lipids could not be determined. Mass spectrometric analysis did not lead us to clear candidate antigenic compounds in the water eluates. It is possible that the antigenic compounds are difficult to detect by TLC and mass spectrometry and have therefore escaped detection. It is unlikely that proteins are responsible for the observed responses against the water eluates because protease digestion did not diminish the immunogenicity of the water eluates. Also, based on direct measurement of the protein and amino acid content of the undiluted water eluates, we calculated that the dilutions of the water eluates that effectively stimulated T cells in our in vitro assays contained 10–50 ng/mL protein, which is about 100-fold below the usually reported optimal concentration of a protein antigen. Last, the methods used for the extraction of total lipids, of which the water eluate is a subfraction, is known to lead to low protein contamination [5].



Supporting information Figure 1. Mas spectrometry was performed to collect the positive-mode and negative-mode mass spectra of water eluates from *M. bovis* and MAP (A and B panels), and to confirm the purity and idientity of *M. phlei* GMM standard (lower panel).

In animals infected with MAP we identified GMM as an immunodominant lipid. Even though *M. bovis* is known to produce GMM as well, animals infected with *M. bovis* did not recognize GMM; so it seems that in vivo, *M. bovis* has less GMM response-priming capacity than MAP. TLC analysis of the total lipid extracts of our bacterial cultures showed that in vitro MAP produces much more GMM than *M. bovis*, and that these differences could not be related to the culture medium used. It is likely that similar quantitative differences in GMM content in vivo play a role in the differential capacities of these two mycobacterial species to prime an anti-GMM T-cell response in vivo. Recent studies show that GMM is produced by the action of a mycolate transferase known as antigen 85A, suggesting that this gene and other potentially redundant mycolyl transferases are now candidates for controlling immunogenicity *in vivo* [22].

The immunogenicity of GMM in humans has been previously demonstrated in a T-cell line derived from a skin biopsy of a leprosy patient after extensive in vitro culture. GMM has been shown to be recognized weakly by non-manipulated ex vivo T cells of tuberculosis patients [18], also in a CD1-restricted fashion. The data presented here show that also in naturally infected cattle, GMM is immunogenic and presented by CD1b. From this we conclude that the previously reported presentation of GMM to the LDN5 cell line is not a rare event, but rather a phenomenon that takes place in several mycobacterial infections in at least two different host species. However, the quantity of GMM produced by the infecting mycobacterium may be crucial for effective CD1 presentation of GMM and CD1-restricted T-cell activation, as suggested by the lack of GMM reactivity by *M. bovis*-infected animals.

Our work shows that the identification of potential lipid or other non-protein subunit vaccine candidates in bovines is possible by in vitro stimulation of freshly isolated PBMC. The i*n vitro* use of T cells that have been primed *in vivo* by a natural infection provides insights into immunogenicity and immunodominance of substances that have not yet received as much attention as protein antigens.

Materials and methods

Bacteria

MAP clinical isolate 280105, isolated in The Netherlands, and ATCC strain 19698 were grown in open liquid cultures using Watson–Reid medium (containing 1% D-glucose) supplemented with 0.5 mg/mL MycJ (Allied Monitor, Fayette, MO, USA), or Middlebrook 7H9 medium (Difco) supplemented with Middlebrook OADC (BD BBL) and 0.5 mg/mL MycJ. *M. bovis* clinical isolate 4913C (isolated from cattle in South Africa), ATCC strain 19210, the strain AN5 and *M. bovis* BCG Pasteur were grown in 7H9 medium with OADC or in 7H9 medium with 1% D-glucose. *M. phlei* was grown in 7H9 medium supplemented with 0.5 mg/mL Tween-80 and 1% D-glucose. 7H9 medium does not contain glucose, but supplementation with OADC as we did leads to a final concentration of 0.2% D-glucose; thus, all bacteria used had been grown in the presence of glucose. The lipid content of the different strains grown in the different media was compared to assess the variation between strains and the effect of different media (Fig. 2A).

Antigen preparation

Bacterial cultures were subjected to centrifugation for 20 min at 4000 g and the pellet was washed once with distilled water. The wet pellets were extracted for 2 h at room temperature in chloroform:methanol 1:2 v:v and 2:1 v:v consecutively. The total lipid extracts were dried in a rotating evaporator at room temperature and re-dissolved in chloroform and quantification and analysis was done by TLC. Fractionation of the MAP total lipid extracts was performed by loading on a silica solid phase extraction column with a bed weight of 2 g (Supelco) and consecutive elution with three bed volumes (12 mL) of each of the following eluents: chloroform, 9:1, 8:2, 7:3, 6:4, 5:5 v:v mixtures of chloroform and acetone, acetone, methanol and then water. The GMM standard was isolated from *M. phlei* by preparative TLC. Preparative TLC plates (200 mm) were resolved in 60:30:6 v:v chloroform:methanol:water, dried and sprayed with water to visualize bands. Individual bands were scraped and silica dust was extracted in 2:1 v:v chloroform:methanol. The identity and purity of GMM was confirmed as described in the ‘‘lipid analysis’’ section below.

HSP70 was prepared as previously described [23]. Pronase is a mixture of endopeptidases and exopeptidases (carboxypeptidases and aminopeptidases) that cleave denatured and native proteins down into individual amino acids. Proteinase K is an endopeptidase that has a preference for cleavage between an aliphatic, aromatic or hydrophobic and any other amino acid; however, it will digest any peptidic bond if added in excess and over long incubation periods. Pronase and proteinase K were used to digest water eluates from silica columns loaded with MAP and *M. bovis* lipids, and HSP70 in protease buffer (10mM CaCl, 10mM HEPES buffer, 25mM ammoniumbicarbonate) for 4 h at 40^0C, followed by 10 min of inactivation at 85^0C. Mock treatment of antigens was performed in the same buffer and at the same temperatures, but without addition of the proteases. Recombinant ESAT6 protein was described previously [24].

Lipid analysis

For analytical TLC, approximately 25 mg of lipids were loaded on a 2020 cm Silica 60 TLC plate (Merck) and developed in 60:16:2 v:v:v chloroform:methanol:water. Plates were sprayed with 3%w/v cupric acetate in 8%v/v phosphoric acid, dried and baked for 1 h at 140^0C. Alternatively, plates were sprayed with 0.2% w/v ninhydrin and 3%v/v acetic acid in water-saturated butanol for detection of free amino groups, or with Dittmer–- Lester reagent for the detection of phosphates. Total amino acid content was analyzed by HPLC (Jasco) after acid hydrolysis in 6M HCl for 16 h and pre-column ortho-phthalaldehyde derivatization. Nanoelectrospray ionization mass spectrometry (ThermoFinnigan LCQ Advantage) was performed on fractions dissolved in a 1:1 v:v water:methanol mixture and on purified GMM in a 1:2 v:v chloroform:methanol mixture using borosilicate glass pipettes pulled to a final orifice of 1–2 mm and an internal stainless steel electrode.

Animals

Adult Holstein–Frisian cattle infected with MAP were obtained from dairy cattle farms in The Netherlands with a known history of MAP infections. Diagnosis of MAP infection was

performed using a fecal culture system [25] at the National Veterinary Health Service, Deventer, The Netherlands. Samples were checked for bacterial growth every 4wk and considered negative if after a culture period of 16 wk no bacterial growth was observed. Bacteria were confirmed to be MAP based on mycobactin dependence of the culture and the confirmation of the presence of the MAP-specific IS900 insertion sequence by PCR [26]. This culture-based protocol is not highly sensitive at the level of an individual animal, but highly specific, allowing confirmed MAP culture-positive animals into the study. Because all animals included in our study were more than 2 years of age, and because infection with MAP takes place in very young calves [1], all MAP-infected animals used can be considered chronically infected. Control animals originated from farms with unknown MAP status if they were confirmed to be MAP culture-negative, or from Dutch dairy farms, which have participated in a national paratuberculosis control program for at least 5 years during which no test-positive animals were found. On these farms all animals older than 2 years of age are checked for paratuberculosis infection on a yearly basis by the National Veterinary Health Service using an ELISA (Pourquier) and pooled fecal culture. All animals from The Netherlands were considered tuberculosis- free as bovine tuberculosis is not endemic in The Netherlands.

Tuberculosis-infected dairy cattle were collected during natural outbreaks in Belgium and in South Africa (Afrikaner, Jersey, Nguni). In addition, two bovine tuberculosis-infected free-ranging African buffalo herds were sampled in the Hluhluwe- iMfolozi Park in South Africa. The diagnosis of bovine tuberculosis was determined at the level of the individual animal by single or comparative skin testing using bovine and avian PPD. Animals were considered positive when the induration caused by bovine PPD was at least 2mm bigger than the induration caused by avian PPD, or at least 6mm if the single tuberculin test was used. This skin test, based on the same principles as the Mantoux test for human tuberculosis, depends on adaptive, mycobacterial protein-specific T-cell responses, and turns positive in bovines 3 or 4wk after infection with *M. bovis* [27, 28]. In addition, the diagnosis of bovine tuberculosis was confirmed at herd level by post-mortem examination and *M. bovis* culture from visible lesions from at least one animal from the herd. For some control experiments, we used healthy animals that had been immunized with recombinant HSP70 protein in dimethyl dioctadecyl ammoniumbnomide (DDA), as described earlier [29]. In all cases 20–100mL of heparinized blood was drawn from the jugular vein. Experiments were conducted according to the regulations of the Veterinary and Agrochemical Research Centre CODA CERVA, KwaZulu Natal parks, and approved by the Animal Ethical Committee of the University of Utrecht, The Netherlands (protocol number 2007.II.06.152/Vervolg1).

T-cell assays

PBMC were isolated by standard Ficoll–Hypaque gradient centrifugation of heparinized blood that was drawn from the jugular vein. For biological testing lipids were evaporated to dryness under nitrogen and sonicated with T-cell medium. T-cell medium was made by supplementing 500mL of RPMI medium with 50mL fetal calf serum (Hyclone), penicillin (Gibco), streptomycin (Gibco), 20mM HEPES (Gibco) and 4mL 1N NaOH solution. T-cell activation was measured by incubating $2x10^5$ PBMC with antigens in series of dilutions. Proliferation was measured after coculture for 3 days with antigen, followed by a 7 h pulse of 1 mCi of [^{3}H]thymidine before harvesting and counting b emissions. Stimulation indices

were calculated by dividing the number of counts per minute obtained with the optimal antigen dilution by the number obtained with incubation with medium alone. Anti-human CD1b (BCD1b3.1), which is known to cross react with bovine CD1b3 [14], and an IgG1 isotype control (P3), were used for blocking studies at 20 mg/mL.

Statistical analyses

Logarithmic transformation of stimulation indices of T-cell proliferation assays was performed to achieve homogeneity of variance and to better approximate a normal distribution. To test the differences in T-cell reactivity to total lipid extracts between infected and non-infected groups of animals, a two-sided, nonpaired t-test was used.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

Abbreviations: GMM: glucose monomycolate MAP: Mycobacterium avium paratuberculosis MycJ: Mycobactin J TLC: thin layer chromatography

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Chapter 3

The mycobacterial glycolipid glucose monomycolate induces a memory T cell response comparable to a model protein antigen and no B cell response upon experimental vaccination of cattle

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Abstract

Glycolipids are presented to T cells by human group 1 CD1 proteins, but are not used as subunit vaccines yet. Experimental immunizations with pure mycobacterial glucose monomycolate (GMM) and keyhole limpet haemocyanin (KLH) in cattle, a species which, unlike mice, expresses group 1 CD1, showed that GMM was equally efficient as KLH in generating T cell responses in blood, but not in the draining lymph node. Also, KLH induced strong antibody responses whereas GMM did not. These data suggest that non-overlapping T cell populations are targeted and demonstrate the potential of glycolipids as a special class of subunit vaccine candidates.

Introduction

It has been fully established that proteins, including haptenized and glycosylated proteins, are the main targets of the adaptive immune system. More recently, non-protein antigens, including glycolipids, have been shown to be recognized by T cells in vitro. Unlike protein antigens, glycolipids are presented to T cells by the CD1 family of proteins. CD1d presents a limited set of glycolipids to specialized T cells, invariant NKT cells. CD1a, CD1b, and CD1c proteins, collectively called group 1 CD1 proteins, present a more diverse set of lipids to T cells with T cell receptors (TCR) that do not seem to be invariant or otherwise different from regular T cells. Interestingly, not all CD1 isoforms are present in all animal species, which is illustrated by the fact that mice only express CD1d molecules, and no group 1 CD1 molecules. Guinea pigs, cattle, rabbits, pigs, dogs, and humans express at least one group 1 CD1 molecule but the exact numbers and isoforms expressed vary substantially [1–8].

Many of the known lipid-reactive human T cell lines and clones are derived from individuals suffering from a mycobacterial infection, or have been derived in vitro by repeated stimulation of T cells from healthy donors with mycobacterial extracts. This has led to the identification and characterization of a number of CD1-presented mycobacterial antigens including mycolates [9–11], diacylglycerols (LAM) [12], polyisoprenoid lipids [13], sulfotrehalose-containing lipids [14] and lipopeptides [15]. Of note, all mycobacterial antigens appear to be presented by group 1 CD1 (CD1a, CD1b, and CD1c). The mechanism of immune activation by the mycobacterial glycolipid glucose monomycolate (GMM) has been described in great detail [11,16,17]. GMM is known to be presented to human T cells by CD1b. The TCR of the human T cell line LDN5 recognizes GMM of different mycobacterial species that only differ in their lipid tails. The co-crystal of human CD1b with GMM shows that both acyl chains are buried in the antigen binding groove, leaving the glucose moiety exposed on the surface of the CD1 molecule available for recognition by the TCR, explaining why one TCR can recognize GMM from different sources [18].

Using the CD1 system for vaccine development would have the advantage that the molecules it presents are not subject to rapid mutations, like certain antigenic viral proteins. Also, the CD1 system has very limited polymorphism [19], minimizing inter-individual differences in the capacity to present a certain antigen within an outbred population. In addition, CD1-restricted T cells have been shown to be able to express molecules that are very effective in

fighting mycobacterial infections [20–22]. Last, many currently used diagnostic tests for mycobacterial infections make use of mycobacterial purified protein derivative (PPD) or related protein preparations. These tests turn positive upon vaccination with BCG or killed bacteria because of priming of T cells that recognize proteins that are present in those vaccines and in the PPD. It is unlikely that immunization with CD1-presented glycolipids interferes with PPD-based diagnostic testing, which would be another advantage of potential lipid-based vaccines.

Even though it has been shown that CD1d-restricted NKT cells support the development of memory B cells, NKT cells themselves express a natural memory phenotype cannot be primed in the sense that immunization with a CD1d-presented antigen improves the strength and the kinetics of subsequent challenges [23–25]. To address the question whether group 1 CD1-restricted T cells can be primed, guinea pigs have been immunized with total lipid extract of *Mycobacterium tuberculosis* [26,27], but no other species have been studied so far. Total lipid extracts are likely to stimulate the CD1 system by several mechanisms, including TLRmediated adjuvant activity [28], in addition to providing cognate CD1 antigens that are directly presented to T cells. Using pure antigens without known adjuvant activity facilitates the analysis of the adaptive immune response and limits the influence of immune stimulation via mechanisms other than the TCR and/or BCR.

The aim of the current study was to determine whether immunization with a pure mycobacterial glycolipid can prime the adaptive immune system and generate T cell memory, and whether this interferes with the PPD-based diagnostic skin test for tuberculosis. To address these questions, we immunized cattle with the highly purified glycolipid glucose monomycolate (GMM) in the synthetic adjuvant dimethyl dioctadecyl ammonium bromide (DDA). As a control immunogen we used the protein keyhole limpet haemocyanin (KLH) in the same adjuvant. Cattle were chosen for this experiment because cattle are the natural host of several mycobacterial pathogens and therefore a target species for vaccine development. Infections with *Mycobacterium bovis*, causing bovine tuberculosis, and *Mycobacterium avium ssp. paratuberculosis*, the causative agent of Johne's disease or bovine paratuberculosis, cause substantial economic losses in the cattle industry [29]. Also, cattle express CD1b molecules [2], which present GMM to T cells from cattle suffering from mycobacterial infection (Van Rhijn et al., manuscript submitted).

Materials and methods

Bacteria and antigens

Mycobacterium phlei and *Nocardia farcinica* were grown in 7H9 culture medium (Difco) supplemented with 10% glucose and 0.5 mg/ml Tween-80 (Sigma–Aldrich). Bacteria were spun down and washed once with distilled water. The wet pellets were extracted for 2 h at room temperature in chloroform:methanol 1:2 and 2:1 (v:v) consecutively. The total lipid extracts were dried in a rotating evaporator at room temperature and redissolved in pure chloroform. For the preparation of GMM, the total lipid extracts were fractionated by loading on a silica solid phase extraction column (Supelco) and consecutive elution with three column volumes of chloroform, followed by three column volumes of 15%, 30%, 40%, 50%, 60%,

70%, and 80% acetone in chloroform, and finally with pure acetone. Quantification and overall analysis was done by thin layer chromatography (TLC) using GMM standards that were analyzed by nanoelectrospray ionization mass spectrometry (Thermo Finnigan LCQ Advantage). After loading the lipids, the TLC plates were resolved in chloroform:methanol:water 60:16:1.5 (v:v) and dried at room temperature. TLC plates were sprayed with 3% cupric acetate in 8% phosphoric acid, dried and baked at 150 °C for 1 h. The fraction containing pure GMM was dried and redissolved in chloroform for storage.

KLH, concanavalin A (conA), and nervonic acid were obtained from Sigma–Aldrich. Phosphatidylinositol, and phosphatidylcholine were from Avanti Lipids.

Animals and immunization

For this study twelve Holstein-Friesian, 2-week-old bull calves were purchased from documented tuberculosis free and paratuberculosis unsuspected dairy herds in The Netherlands. The bulls were group housed and conventionally reared using milk replacer, concentrate and roughage. At the age of 3 months, following a 10-week pre-immunization period, seven animals were immunized subcutaneously with KLH in the left shoulder and with GMM in the other shoulder. Each dose contained either 100 μg GMM or 100 μg KLH (Sigma– Aldrich) in 0.75 ml PBS/5% BSA, and 0.75 ml of a 20 mg/ml suspension of DDA (Sigma– Aldrich) in PBS. GMM was dried under a stream of nitrogen to remove organic solvent and sonicated in PBS/5% BSA. The remaining five animals received two doses of adjuvant only, containing the same components, except for KLH and GMM. A second immunization was performed 1 month after the primary immunization. Two of the GMM/KLH-immunized animals were euthanized at the end of the experiment and their left and right prescapular lymph nodes were collected. Experiments were approved by the Animal Ethical Committee of the University of Utrecht, The Netherlands (protocol numbers DEC 0409.0801 and DEC 2007.II. 06.152).

In order to compare humoral responses of animals suffering from an infection with a GMM producing bacterium and animals exposed to GMM by immunization, sera of animals suffering from clinical paratuberculosis, caused by natural exposure to *M. avium paratuberculosis*, were included in this study. Diagnosis of MAP infection was performed using a faecal culture system [30] at the National Veterinary Health Service, Deventer, The Netherlands.

T cell proliferation assays

T cell assays were performed from 6 weeks before the first immunization till 4.5 months after the second immunization. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by histopaque ficoll (Sigma–Aldrich) and lymph node cells were isolated by cutting the lymph nodes in small pieces and passage through a cell strainer (BD Falcon). T cell proliferation assays were performed in round bottom 96-well plates (200,000 cells/well). GMM was used in series of dilutions after drying under a stream of nitrogen gas to remove organic solvent and sonicating to be dissolved in T cell medium consisting of RPMI supplemented with 10% FCS (Hyclone), penicillin and streptomycin (Gibco), 20 mM HEPES (Gibco), and 4 ml 1N NaOH. For antibody blocking assays, 20 μg/ml of the anti-human CD1b monoclonal antibody BCD1b.3 or the isotype control P3 was continuously

present during the T cell culture. Proliferation was measured after culture for three days in the presence of serial dilutions of KLH, GMM, or concanavalin A (ConA) in a 37 °C, 5% CO2 humidified incubator, followed by a 7 h pulse of 1 μCi of [3H]thymidine before cells were harvested and counted for β-emissions. Stimulation indices (SI) were calculated by dividing the number of counts per minute obtained with the optimal antigen dilution by the number obtained by incubation with medium alone (Table 1).

Enzyme-linked immunosorbent assays

To assess serological responses, blood was collected in vacutainer tubes (BD), centrifuged, and the serum was collected and stored at −20 °C. Antigen-specific immunoglobulins were measured using enzyme-linked immunosorbent assays (ELISA). Lipids were dried under nitrogen to remove chloroform and sonicated in methanol. Polysorb plates (Nunc, Denmark) were coated with 5 μg/well *N. farcinica* GMM or control lipids (PI, PE, or nervonic acid) and dried overnight at room temperature in a fume hood. Costar high-binding 96-well plates (Corning) were used to coat KLH (0.1 μg/well) by overnight incubation at 4 °C. After blocking with blocking reagent (Roche) for 1 h, 1:30 dilutions of serum in PBS, or PBS only as a negative control, were added to the plates and incubated overnight at 4 °C. Plates coated with GMM were washed with washing buffer consisting of PBS containing/0.05% Tween-20 (Sigma– Aldrich), and plates coated with KLH were washed with washing buffer consisting of PBS/ 0.25% Tween-20. Biotinylated mouse anti-bovine IgG total (Sigma–Aldrich), diluted 1:50,000 in blocking reagent, was added and incubated for 1 h, followed by three washes with washing buffer, and a 1-h incubation with a 1:4000 dilution of avidine PO (Dako) in blocking reagent. For isotype-specific, antigen-specific ELISA, unlabelled mouse anti-bovine IgG1, IgG2, IgM, or IgA (Prionics), diluted 1:4000 in blocking reagent were added after serum incubation and incubated overnight at 4 °C, followed by three washes with washing buffer and a 1-h incubation with polyclonal rabbit anti-mouse (1:2000) conjugated to HRP (Roche). After three washes with washing buffer and two with PBS, ABTS (Roche) was used to develop green colour which was measured spectrophotometrically at the wavelength of 405 nm. OD values of the wells that were incubated without serum were subtracted from the values obtained with serum.

Skin testing

A single intradermal comparative cervical tuberculin test was conducted according to the regulations (EU directive 64/432/EEC) at the end of the experiment, 4 months after the last immunization. In short, 0.1 ml bovine tuberculin (2000 IU) and 0.1 ml avian tuberculin (2000 IU) (Central Veterinary Institute, Lelystad, The Netherlands) were injected intradermally in the neck of each animal. At 72 h post-injection the skin-fold thickness was measured and corrected for skin-fold thickness measured at time of application. Animals are considered to test positive for *M. bovis* if, after 72 h, the increase in skin thickness at the site of application of bovine tuberculin is more than 4 mm larger than for avian PPD. If the reaction to bovine PPD is between 2 and 4 mm greater than the reaction to avian PPD, this is considered an indeterminate result. Animals showing differences less than 2 mm are considered negative.

Statistics

Following Levene's test for homogeneity of variance, the post-immunization T cell profileration data and the ELISA data of the two treatment groups were analyzed with oneway analysis of variance (ANOVA) using SPSS 15.0 software. Differences with a P value < 0.05 were considered significant.

Table 1. Proliferation data, expressed in counts per minute (CPM), thar form the basis of the stimulation indices presented in Figure.1.

Animal#	Immunization	Symbol in Fig. 1	CPM GMM[a]	sd	CPM KLH	sd	CPM medium	sd	CPM ConA	sd
0041	*M. phlei* GMM/KLH	■	19838	19581	22300	24425	320	538	185909	94281
0472	*M. phlei* GMM/KLH	●	18863	22569	16863	12853	204	1120	167182	83289
2629	*M. phlei* GMM/KLH	▼	4233	1411	2687	1588	278	289	205727	43388
3051	*M. phlei* GMM/KLH	✖	2660	2542	2665	2301	357	359	240182	55587
4815	*N. farcinica* GMM/KLH	★	1340	1149	1410	1267	267	232	138136	96760
5083	*M. phlei* GMM/KLH	◆	9603	7198	9338	5663	467	638	169818	66562
9596	*M. phlei* GMM/KLH	▲	4915	1799	4413	2388	595	758	237364	59488
0177	adjuvant only	△	451	419	599	425	197	186	171545	75306
3050	adjuvant only	▽	2190	1996	1519	691	803	598	210545	54959
4014	adjuvant only	◇	668	376	2206	784	290	256	244273	73157
6597	adjuvant only	○	2605	2497	1913	816	725	630	199636	43058
7408	adjuvant only	□	2026	1395	4491	3007	526	764	203636	54397

[a] Average of CPM obtained upon in vitro stimulation of PBMC with the same GMM that was used for immunization, or with *M. phlei* GMM for stimulation of PBMC of adjuvant only immunized animals.

Results

Immunization with GMM is as efficient in priming T cell responses as a protein antigen

To study the efficiency of T cell priming upon immunization with a glycolipid antigen as compared with a known immunogenic model protein, cattle (n = 7) were immunized with GMM and KLH in DDA. To reduce the effects of inter-individual differences, while preventing any possible interaction of the two antigens when mixed, the animals received the two antigens separately, in the right and the left shoulder, on the same day. Another group of animals (n = 5) of the same age and sex, housed together with the GMM/KLH immunized group, was immunized with adjuvant only and served as a control group. Before the first immunization and from 1 week till 4.5 months after the second immunization, animals were tested every other week for T cell reactivity against GMM and KLH. The GMM specific proliferative T cell response that was detected in freshly isolated PBMC after immunization with GMM/KLH was significantly higher than in adjuvant only immunized animals ($P < 0.001$) (Fig. 1A). A similar pattern was observed for KLH when GMM/KLH immunized and adjuvant only immunized groups were compared (Fig. 1B). Of note, the strength of the response against KLH and against GMM was comparable. Before immunization there were no significant differences between the groups (not shown).

In order to be able to assess whether a cross-reactive T cell response to GMM would develop, the animals were not immunized with an identical GMM preparation, but rather with GMM

from *M. phlei* (n = 6) or *N. farcinica* (n = 1), which differ in their mycolic acid structure. The purity and the size of the GMM preparations was confirmed by TLC and mass spectrometry (Fig. 2). Cross-reactive T cell responses, measured by using GMM from *N. farcinica* to stimulate T cells from animals that were immunized with *M. phlei* GMM and vice versa, were clearly detected, though they were in general slightly weaker than the response against the GMM that was used for immunization (Fig. 1A). The restriction element for human GMM specific T cells is CD1b. To confirm whether the GMM-specific T cell response in cattle was restricted by bovine CD1b, we successfully performed antibody blocking assays using the antihuman CD1b antibody BCD1b.3 that has been used for this purpose in the human system, and which is known to cross-react with bovine CD1b [2] (Fig. 1C).

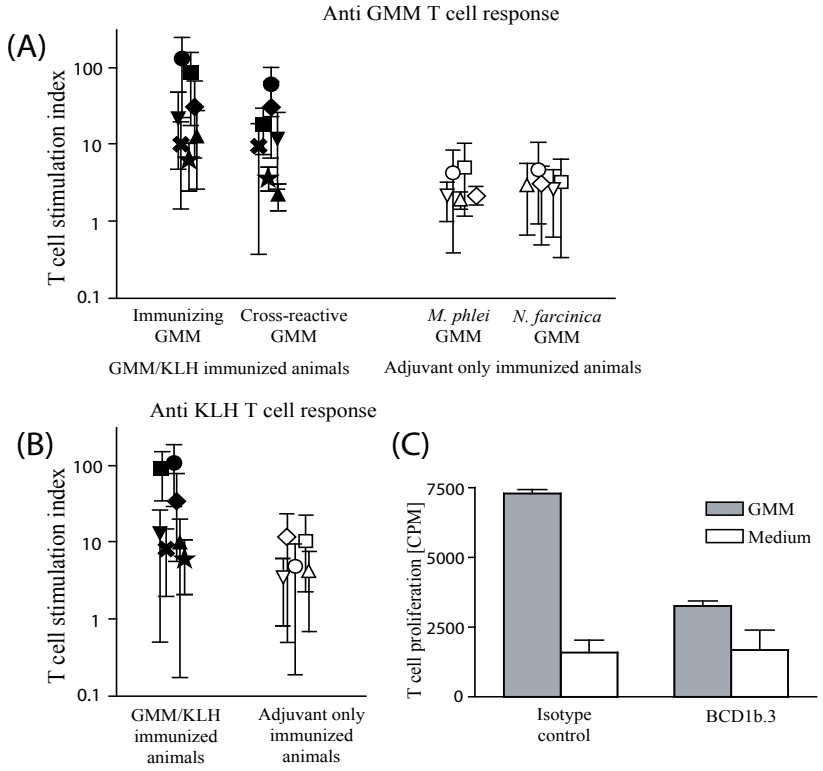

Fig. 1. T cell responses against GMM and KLH, induced by immunization. (A) Freshly isolated PBMC of GMM/KLH-immunized animals and adjuvant only immunized animals were stimulated with GMM extracted from *N. farcinica* and *M. phlei*. The cross-reactive T cell stimulation shown is measured by stimulating the T cells with the GMM that was not used for immunization. (B) In the same assay, the T cell response against the protein antigen KLH was determined. The T cell stimulation index was calculated by dividing the [3H]thymidine incorporation of the antigen-stimulated PBMC by the [3H]thymidine incorporation of PBMC cultured in medium without lipids (Table 1) after a 8-h [3H]thymidine pulse performed after a three-day incubation. Each symbol represents the mean stimulation index (±standard deviation) of eight independent experiments performed post-immunization on one animal. Black symbols represent the GMM/KLH-immunized animals and white symbols represent adjuvant only immunized animals. (C) PBMCs of a GMM/KLH-immunized animal were incubated with GMM or medium only in the presence of the cross-reactive anti-human CD1b monoclonal antibody BCD1b.3, or the isotype control P3. Bars represent the mean CPM of triplicate wells (±standard deviation).

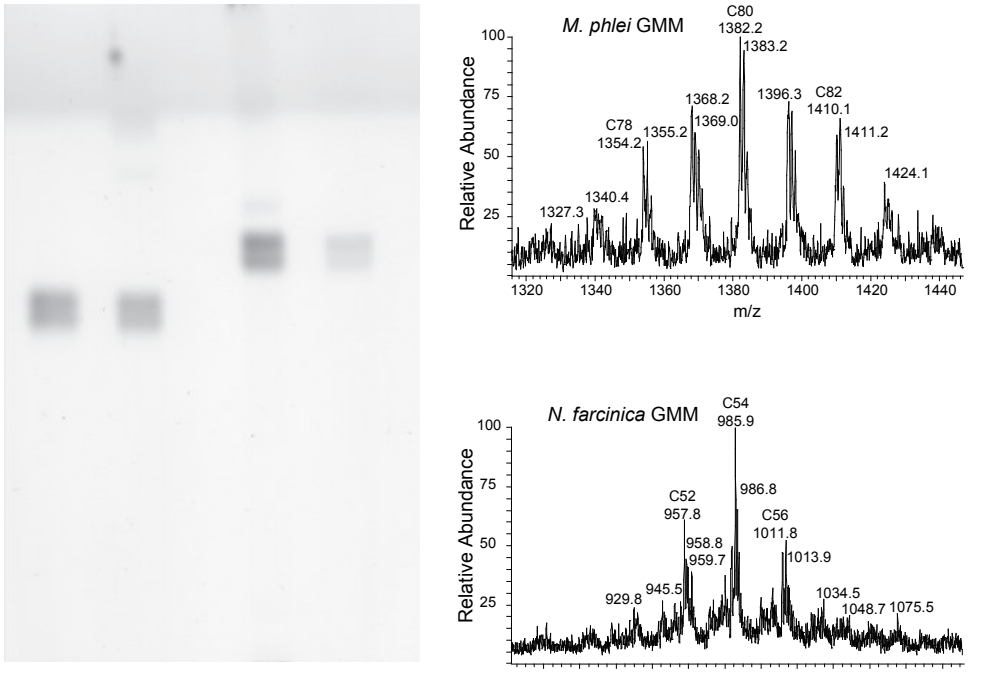

Fig. 2. TLC and mass spectrometric analysis of *M. phlei* and *N. farcinica* GMM. Ten micrograms of GMM was applied to a silica TLC plate. The plate was developed in 60:16:2 chloroform:methanol:water (v:v), sprayed with 3% cupric acetate in 8% phosphoric acid, dried, and baked for 1 h at 140 °C. Lane 1: *N. farcinica* GMM standard; lane 2: purified *N. farcinica* GMM; lane 3: M. phlei GMM standard; lane 4: purified *M. phlei* GMM. Positive mode spectra were collected by electrospray ionization mass spectrometry.

These results indicate that, similar to protein antigen, immunization with GMM antigen raises a T cell response that can be detected in PBMC and that GMM is recognized by T cells regardless of the bacterial source of the GMM.

Immunization with GMM does not cause a positive intradermal skin test for bovine tuberculosis

Four months after the second set of GMM/KLH immunizations, all animals were subjected to the standard comparative intradermal skin test for bovine tuberculosis. Bovine and avian PPDs were applied intradermally and the difference in the increase in skin thickness after 72 h was calculated. In one out of seven GMM/KLH-immunized animals and one out of five adjuvant only immunized animals the increase in skin thickness at the bovine PPD application site was between 2 and 4 mm larger than at the avian PPD application site, qualifying them as indeterminate responders. All other animals were negative. KLH was determined. The T cell stimulation index was calculated by dividing the [3H]thymidine incorporation of the antigen-stimulated PBMC by the [3H]thymidine incorporation of PBMC cultured in medium without lipids (Table 1) after a 8-h [3H]thymidine pulse performed after a three-day incubation. Each symbol represents the mean stimulation index (±standard deviation) of eight independent experiments performed post-immunization on one animal. Black symbols represent the GMM/

KLH-immunized animals and white symbols represent adjuvant only immunized animals. (C) PBMCs of a GMM/KLH-immunized animal were incubated with GMM or medium only in the presence of the cross-reactive anti-human CD1b monoclonal antibody BCD1b.3, or the isotype control P3. Bars represent the mean CPM of triplicate wells (±standard deviation).

Unlike KLH, immunization with GMM does not induce a strong antibody response

Antibodies against GMM and KLH in serum were examined by ELISA. Initial testing for antigen-specific total IgG showed that GMM/KLH-immunized animals had generated a strong response to KLH as compared to adjuvant only immunized animals ($P < 0.01$) (Fig. 3A). The situation was different for anti-GMM responses: GMM/KLH-immunized animals as well as adjuvant only immunized animals showed a very weak IgG response against GMM and the difference between groups was not significant ($P > 0.05$) (Fig. 3A). To obtain a more complete view of the antibody responses, ELISAs for antigen specific IgG1, IgG2, IgM, and IgA were performed separately. We detected weak anti-GMM responses of the IgM and IgA isotype (Fig. 3B and C). The IgA response against GMM was significantly higher in GMM/KLHimmunized animals than in adjuvant only immunized animals ($P < 0.05$), whereas the difference in IgM response was not ($P > 0.05$). Also, anti-KLH responses of the IgM and IgA isotype were weak. The differences between GMM/KLH immunized and adjuvant only immunized groups were not statistically significant ($P > 0.05$ for both isotypes) (Fig. 3D and E). High serological responses of the IgG1 and IgG2 isotype were detected against KLH in the GMM/KLH immunized group ($P < 0.01$ for both isotypes) (Fig. 3F and G). Because we did not detect any anti-GMM IgG responses in any of the immunized animals, and no positive control bovine sera with known anti-GMM reactivity were available, we included sera of animals suffering from advanced paratuberculosis in the study. GMM is an immunodominant glycolipid antigen during paratuberculosis, a disease caused by infection of cattle with *M. avium paratuberculosis* (Van Rhijn et al., manuscript submitted). Interestingly, strong anti-GMM IgG1 and IgG2 responses were detected in sera from paratuberculosis infected animals, but no increased IgM and IgA (Fig. 3H). No anti-KLH antibody response of any of the isotypes could be detected in these clinical paratuberculosis sera (Fig. 3I). To rule out the possibility that the anti-GMM response of the sera from paratuberculosis infected animals was reflecting an antigen-independent false positive signal in the ELISA, we showed that the same sera showed no response against the non-relevant lipids phospatidylinositol, phosphatidylcholine, and the long chain fatty acid nervonic acid when tested in parallel with GMM (Fig. 3J). In addition, sera from animals acutely infected with enteropathogenic *Escherichia coli* K88, a bacterium that does not produce GMM, did not show a response against GMM (Fig. 3J).

T cells responses against GMM were not detected in draining lymph nodes, while KLH-specific responses were

Protein-specific T cells can readily be detected in lymph nodes, where they can provide help to B cells, and they are usually highly abundant in the lymph nodes that drain an area of immunization or infection. To assess whether this is also the case for CD1-presented lipid antigen, the left and right prescapular lymph nodes that drained the area of immunization of

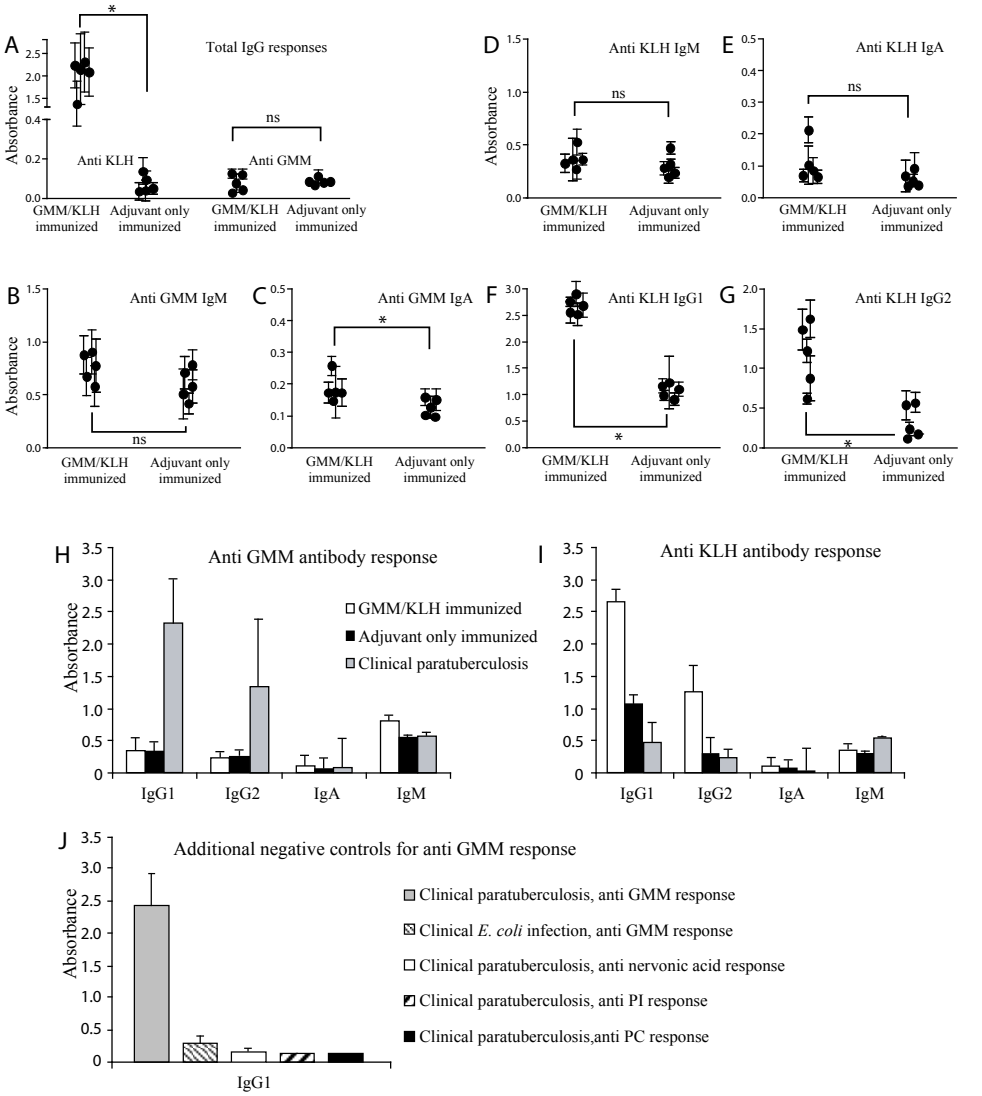

Fig. 3. Serological responses of GMM/KLH-immunized animals against GMM and KLH. (A) Sera of GMM/KLH-immunized animals and adjuvant only immunized animals were tested for total IgG responses against KLH and GMM in an ELISA. (B and C) Anti-GMM IgM and IgA were determined using an isotype-specific ELISA. (D–G) The anti-KLH serological response was studied in more detail by determining the individual IgM, IgA, IgG1 and IgG2 responses. Each dot in panel A–G represents the mean absorbance (±standard deviation) of eight independently obtained sera post-immunization from one animal. (H and I) Sera of five animals suffering from advanced paratuberculosis were tested for anti-GMM and anti-KLH antibody responses of the IgM, IgA, IgG1, and IgG2 isotype and compared with the values obtained from the GMM/KLH immunized group and the adjuvant only immunized group. In panel H and I, the anti-GMM IgG1 and IgG2 data as well as the data from the animals suffering from paratuberculosis are based on a single serum per animal; all other data are based on eight independently collected sera per animal. (J) IgG1 responses against GMM, phosphatidylinositol (PI), phosphatidylcholine (PC), and nervonic acid, a C24:1 fatty acid, were determined in sera from five animals suffering from an *E. coli* K88 infection and five animals suffering from advanced paratuberculosis by ELISA.

two GMM/KLH-immunized animals that were euthanized at the end of the experiment were collected and used for T cell proliferation assays. As expected, a very strong anti-KLH response was elicited in both lymph nodes. The response in the left lymph node, where the KLH immunizations were applied, was stronger than in the right lymph node. Interestingly, no anti-GMM T cell response could be detected at all in any of the lymph nodes (Fig. 4A). An experiment performed on the same day on PBMC from the same animals showed clear T cell responses against KLH and GMM of comparable strength, though lower than the KLH responses measured in lymph nodes (Fig. 4B).

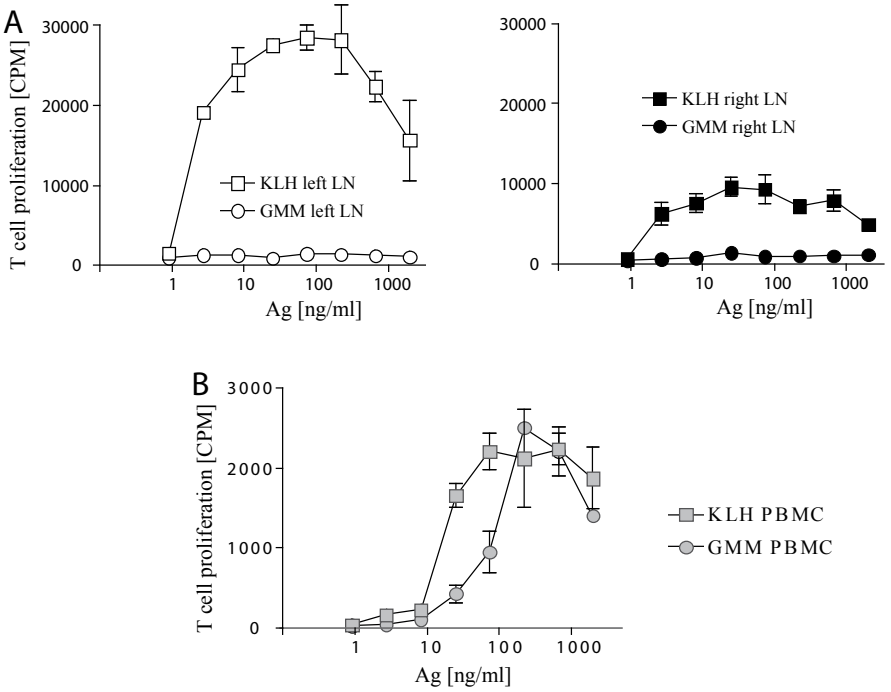

Fig. 4. Comparison between T cell responses in draining lymph nodes and PBMC. T cell proliferation assays were performed 4.5 months after the second set of GMM/KLH immunizations using cell suspensions from the left (KLH immunization side) and right (GMM immunization side) prescapular lymph nodes (A), and from PBMC (B). The results shown here were obtained with material from one animal. Highly comparable results were obtained in a second animal (not shown).

Discussion

Immunization of cattle with a pure glycolipid, GMM, as we describe here induces memory T cell responses but minor antibody responses. Because the GMM that we used was highly purified and did not contain any detectable protein, the observed T cell responses against GMM are probably not caused by protein contaminations present in the GMM preparations. This is supported by the fact that T cells from animals immunized with GMM of one bacterial species recognized GMM from a different bacterial species. So, even if a very low protein

contamination would have been present in the GMM preparation used for vaccination, the subsequent in vitro recognition of GMM purified from a different bacterial species cannot be explained by a memory response against protein because even if the preparations contain proteins, they cannot be identical because they would be from different bacterial species.

Even though the anti-GMM T cell response was highly comparable to the T cell response against the control protein KLH in strength and duration, these cells are likely to perform different functions in vivo. Unlike KLH-specific T cells, we showed that T cells that respond to GMM were not detectable in lymph nodes, including the lymph node draining the vaccination site, which is one possible explanation for the big differences in antibody response against these antigens. Another explanation for this is that B cell help is most efficient when the B cell presents the antigen directly to the T cell. In humans and cattle, GMM is presented by CD1b, which is not expressed by B cells. Therefore, cognate B cell help, in the strict sense that the B cell internalizes the antigen that is recognized by its B cell receptor and presents it directly to T cells, cannot take place in the case of GMM. Recently, in an immunization experiment in mice, using a haptenized, CD1d-binding α-galactosyl ceramide analogue, a normal, class-switched antibody response was generated [31]. In this case, cognate B cell help was provided by NKT cells, which was possible because B cells express CD1d. Taken together it seems that, unlike CD1d-restricted NKT cells, CD1b-restricted T cells elicited by immunization with GMM are not capable to provide B cell help. The published in vitro studies are consistent with our observations in the sense that group 1 CD1-restricted T cells are less dependent on fully mature DC with the B7 costimulatory molecules, such as found in lymph nodes, for their activation [28,32] and they express a unique set of effector molecules which is not characteristic for T cells providing B cell help [20,21,33].

The lack of development of antibodies against GMM upon immunization is not due to the absence of a B cell repertoire for this antigen because we have shown that sera of animals suffering from an infection with *M. avium paratuberculosis* do contain antibodies against GMM. A possible explanation for the generation of antibodies during infection and not by immunization may be that during infection in vivo, fragments of bacterial cell walls, that contain other compounds including a variety of proteins, may function like a conjugate vaccine. Also, mycobacteria are known to possess strong adjuvant activity.

The question arises whether the lack of generation of an antibody response will be a major impediment for the use of glycolipids as subunit vaccines. In the case of mycobacteria, it has been suggested that anti-lipid antibodies may provide protection [34,35], but others doubt whether an antibody response provides any protection at all. It is generally accepted that a strong cellular immune response is beneficial for the host. For the protection against other infectious agents, antibodies are required. Antibodies against complex glycosylations can be generated by means of conjugate vaccines, and it might be possible to accomplish the same for glycolipids. Regardless of the question whether the generation of antibody responses against a glycolipid is possible and necessary, it may be worth to use glycolipids in a single or chemically diverse multi-subunit vaccine because group 1 CD1-restricted T cells have some unique features that are not shared by T cells induced by immunization with a regular protein. Thus, a broader range of the immune system will be primed by including a glycolipid in a mixture of antigens. Proteins are known to be able to induce Th1, Th2, cytotoxic, and regula-

tory T cell responses, but it seems that glycolipids do not induce the same effector T cell populations. We have shown here that glycolipid-specific T cells do not support the generation of strong antibody responses and others have reported that the phenotype of lipid-specific cytotoxic T cell clones does not overlap with peptide-specific cytotoxic T cells [20,21]. Also, it is possible that regulatory T cells can exclusively be found in the peptide-specific, MHC class II-restricted T cell population, and if so, immunization with pure glycolipids opens a possibility to avoid the stimulation of regulatory T cells.

We have shown that immunization with GMM does not cause the standard intradermal tuberculosis test in bovines to turn positive. This is likely due to the absence of lipids and glycolipids in purified protein derivative (PPD). If it appears in the future that vaccines exclusively consisting of lipids and/or glycolipids provide a reasonable level of protection against mycobacterial infectious diseases, a major advantage of using them, instead of the currently used vaccines based on attenuated mycobacteria, like *M. bovis* BCG, which provides some protection against tuberculosis, or killed mycobacteria, like the Gudair vaccine against bovine paratuberculosis, is that they do not cross-react with PPD-based diagnostic tests.

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Chapter 4

Immune response of cattle immunized with a conjugate of the glycolipid glucose monomycolate and protein

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Abstract

Strong anti glycolipid IgG responses can occur in humans and animals, but contrary to anti protein responses and anti glycoprotein responses, the exact mechanism of induction is unknown. We have previously shown that experimental immunization with the glycolipid glucose monomycolate (GMM) causes the development of specific T cell responses, but not of anti GMM antibodies. However, cattle naturally infected with Mycobacterium avium ssp. paratuberculosis produce high levels of anti GMM IgG. In the present study, we tested whether vaccination with GMM conjugated to a protein mimics natural infection in its capacity to induce the production of antibodies against GMM. Cattle were immunized (n = 5 per group) with GMM conjugated to a protein, or GMM and protein non-conjugated and administered at contralateral locations, or carrier only. Although immunization with the GMM–protein conjugate vaccine and the non-conjugated vaccine induced protein specific antibody responses, GMM specific antibodies were not detected in either of the groups. In conclusion, the generation of isotype-switched anti lipid antibodies appears to require more than providing peptide epitopes for T helper cells to support glycolipid specific B cells in antibody production.

Introduction

Mycobacterial infections cause enormous health problems world wide. A distinguishing feature of mycobacteria is their thick cell wall, rich in lipids and glycolipids, which form a strong physical barrier. It has been suggested that anti lipid antibodies may contribute to protection against mycobacterial infection (Lang and Glatman-Freedman, 2006). Antibody responses to lipoarabinomannan are thought to be relevant in the protection against *Mycobacterium tuberculosis* in humans and mice (Costello et al., 1992, Fischer et al., 2004; Hamasur et al., 2003).

Similar to proteins, lipid antigens have the capacity to elicit T cell proliferative responses in a number of species including humans (Moody et al., 2000), mice (Kinjo et al., 2006), guinea pigs (Hiromatsu et al., 2002), and cattle (Nguyen et al., 2009; Van Rhijn et al., 2009). We have recently described the cell-mediated and humoral immune responses in cattle upon vaccination with the glycolipid glucose monomycolate (GMM), an abundant component of the mycobacterial cell wall (Nguyen et al., 2009). A vaccine containing GMM as the only antigen elicited T cell responses but no antibody responses while a vaccine with a pure protein as the only antigen generated both T cell and antibody responses (Nguyen et al., 2009). In general, the generation of antibodies of the IgG isotype requires T cell help. It has recently been shown in mice that presentation of α-galactosylceramide and related compounds by CD1d on B cells can attract strong T cell help (Barral et al., 2008; Leadbetter et al., 2008; Galli et al., 2003). However, in humans and cattle, GMM is presented by CD1b, which is not expressed by B cells (Van Rhijn et al., 2006; Girardi et al., 2010). In addition, it has been suggested that GMM-specific T cells do not home to the lymph nodes, where B cell maturation takes place (Nguyen et al., 2009). However, *M. avium ssp. paratuberculosis* infected cattle are known to mount an antibody response to GMM (Nguyen et al., 2009) which could possibly be caused by fragments of bacterial cell walls that function as a conjugate vaccine because they contain cell wall lipids and proteins. We therefore hypothesized that a conjugate of GMM with

a protein may provide T cell help for B cells to produce antibodies against surface exposed glycolipids on mycobacteria, analogous to the glycoconjugate vaccines that have successfully been designed to induce antibodies against polysaccharides.

The aim of the current study was to investigate if GMM specific antibodies are generated when GMM is coupled to protein and used in an experimental vaccine. Keyhole limpet haemocyanin (KLH) is an immunogenic model antigen in cattle as we have shown previously (Nguyen et al., 2009). As an alternative protein component of the experimental conjugate vaccine we used the ubiquitous 70 kD heat shock protein (Hsp70) of *M. avium ssp. paratuberculosis*, the bacterium that causes a strong GMM antibody responses during infection. Hsp70 is known to induce αβ T cell responses and humoral responses (Koets et al., 1999; Koets et al., 2001; Bonorino et al., 1998). We immunized cattle with GMM–KLH or GMM–Hsp70 conjugate vaccines, or non-conjugated vaccines of which the GMM and protein components were delivered in contralateral shoulders, or vaccine without immunogenic protein or GMM, and evaluated both cell mediated and antibody mediated immune responses.

Materials and methods

2.1. Conjugate vaccines

2.1.1. Antigens

M. phlei was cultured in 7H9 medium (Difco) supplemented with 1% glucose and 0.05% Tween-80 (Sigma–Aldrich). Bacteria were washed in distilled water, spun down and extracted in chloroform:methanol 1:2 and 2:1 consecutively for 2 h at room temperature. This total lipid extract was fractionated on a LC-Silica Packing column (Supelco) and GMM was prepared as described (Nguyen et al., 2009). KLH (Sigma–Aldrich) and Hsp70 (production described previously by Koets et al., 2001) were biotinylated using d-biotinoyl--aminocaproic acid N-hydroxysuccinimide-ester (Roche) in DMSO according to the supplied protocol. Free biotin was removed from biotinylated proteins by Econo-Pac 10DG column.

2.1.2. Vaccine preparation

The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (PE-biotin) from Avanti Polar Lipids were used to prepare liposomes (as described in Barral et al., 2008). Mixtures of DOPC, PE-biotin, and GMM (88:2:10, m:m:m) or DOPC and PE-biotin(98:2, m:m) were prepared in glass tubes and dried under a stream of nitrogen, followed by high speed vacuum to remove all traces of solvent. Liposomes were made by cryofracture of the dried lipid mixtures in Tris–HCl 25 mM, NaCl 50 mM, pH 8. Liposomes were incubated with 150 μg of 50 mg/ml silica micro spheres per vaccine dose (100 nm; Kisker GbR), washed once in PBS and once in blocking buffer (PBS, 1% BSA, 1% FCS), followed by incubation with 1 μg/ml streptavidin (Sigma) for 20 min at room temperature and another wash with blocking buffer. At this point, the production of “GMM only” vaccines (microspheres with GMM containing liposomes) and the “carrier only” vaccines (microspheres with liposomes that lack GMM) was completed. For the KLH and Hsp70-containing vaccines, a subsequent incubation with 300 μg biotinylated

protein was performed, followed by two washes in FACS buffer. All vaccines were resus pended in 1.5 ml PBS for injection. The contents of the vaccines are summarized in Table 1.

Table 1. Vaccine components and treatment of groups.

Vaccine	Components
GMM–KLH conjugate	GMM/KLH/microspheres/DOPC/PEbiotin/streptavidin
GMM–Hsp70 conjugate	GMM/Hsp70/microspheres/DOPC/PEbiotin/streptavidin
GMM only	GMM/microspheres/DOPC/PEbiotin/streptavidin (no protein)
KLH only	KLH/microspheres/DOPC/PEbiotin/streptavidin (no GMM)
Hsp70 only	Hsp70/microspheres/DOPC/PEbiotin/streptavidin (no GMM)
Carrier only	Microspheres/DOPC/PEbiotin/streptavidin (no GMM, no protein)
Group (n = 5 per group)	Vaccines given (shoulder) GMM–KLH conjugate (left); carrier only (right)
GMM–KLH conjugate	
GMM–Hsp70 conjugate	GMM–Hsp70 conjuage (left); carrier only (right)
GMM–KLH non-conjugated	GMM only (left); KLH only (right)
GMM–Hsp70 non-conjugated	GMM only (left); Hsp70 only (right)
Carrier only	Carrier only (left); carrier only (right)

To demonstrate the presence of the lipid species, aliquots of vaccines were loaded on a TLC plate and resolved in chloroform:methanol:water 60:16:1.5 (v:v:v), dried, and sprayed with 3% cupric acetate in 8% phosphoric acid, dried, and baked at 150 °C for about 1 h to visualize GMM, DOPC, and PE. The presence of the proteins in the vaccines was assessed by dot blot and Western blot analyses. Aliquots of Hsp70-containing vaccines were boiled in Laemmli loading buffer and separated on a 14% polyacrylamide gel, along with prestained protein ladder (Fermentas), and transferred to nitrocellulose membrane. Recombinant Hsp70 was detected using mouse anti Hsp70 (clone KoKo.B02) (Santema et al., 2010) goat anti mouse IgG AP (Southern Biotech) and NBT/BCIP (Roche). KLH is too large to be efficiently transferred to a nitrocellulose membrane by blotting, so KLH-containing vaccines and a KLH standard of know concentration were denatured by boiling with laemmli buffer, spotted directly to the nitrocellulose membrane, blocked, and incubated with polyclonal rabbit anti KLH (Gene Tex), followed by mouse anti rabbit IgG AP (Sigma) and NBT/BCIP (Roche).

2.2. Animals and immunization

Twenty five Holstein–Friesian calves (3 weeks of age) were purchased and housed for two weeks before the first immunization, which was considered to be experimental day 0. Blood was drawn from the jugular vein in heparinized and uncoated Vacutainer tubes (BD) 7 days before the first immunization, two blood samplings, at day 14 and 28, were performed between the first and second immunization, which took place at day 35, and three samplings took place after the second immunization, on day 49, 70, and after 75 days. To investigate the stimulation of humoral and cellular responses of calves to lipid–protein conjugates, 5 animals were immunized subcutaneously with GMM–KLH conjugate vaccine and 5 animals were immunized with GMM–Hsp70 conjugate vaccines. There were two non-conjugated vaccine control groups: one group received a GMM only vaccine in one shoulder and KLH only vaccine in the contralateral shoulder, and another group received GMM only vaccine in one shoulder and Hsp70 only vaccine in the contralateral shoulder. One additional control group

received the carrier only in both shoulders. The exact contents of the vaccines given to each of the groups are listed in Table 1. Sera of animals suffering from clinical paratuberculosis and of animals that were vaccinated with inactivated whole cell mycobacteria (Gudair, CZ Veterinaria, Spain) were included in this study. Paratuberculosis caused by natural exposure to *M. avium ssp. paratuberculosis* was diagnosed by a fecal culture at the National Veterinary Health Service (GD Deventer, The Netherlands). Experiments were approved by the Animal Ethical Committee of the University of Utrecht, The Netherlands.

2.3. Enzyme-linked immunosorbent assays

To detect antigen-specific immunoglobulins, Polysorb plates (Nunc, Denmark) were coated with GMM (5 μg/well, dissolved in methanol, and dried overnight in a fume hood), and Costar high binding 96-well plates were used to coat proteins (0.1 μg/well, dissolved in PBS, and coated overnight at 4 °C). All plates were blocked for 1 h with blocking buffer (Roche), before serum was added in three-fold dilutions, starting at 1:30, and plates were incubated overnight at 4 °C. Plates were washed 3 times with PBS–0.25% Tween-20 or PBS–0.5% Tween-20 respectively before adding biotinylated mouse anti bovine total IgG (Sigma–Aldrich) diluted 1:50,000 in blocking buffer for 1 h, followed by 3 washes, incubation with by Avidin PO (BD Pharmigen) 1:1000 in blocking buffer, and 3 washes with PBS without Tween. ABTS (Roche) was used as PO substrate and the ELISA plates were read by spectrophotometer at 405 nm.

2.4. T cell proliferation assays

PBMC were isolated using Histopaque-1077 (Sigma–Aldrich) ficoll centrifugation. Lipid and protein antigens were added to 96-well round bottom plates in series of dilutions, starting at 15 μg/ml. GMM was dried in stream of nitrogen to remove organic solvent, and sonicated in RPMI 1640 (Gibco) with 25 mM HEPES supplemented with 10% FCS (Hyclone), penicillin and streptomycin (Gibco), and 2 mM glutamax (Gibco). PBMC (2 × 105/well) were added to the plates and incubated in a 5% CO2 incubator at 37 °C for 3 days. Proliferation was measured by a 7 h pulse of 1 μCi of [^{3}H] thymidine before cells were harvested and β-emission counted. Stimulation indices represent the counts/min of triplicate wells stimulated with antigen divided by the counts/min of triplicate wells stimulated with medium without antigen.

2.5. Statistics

ANOVA and two-tailed Student's t-test from GraphPad Prism 4.0 and Microsoft Office Excel 2003 were used. Differences between groups are considered significant if $P \leq 0.05$.

3. Results and discussion

3.1. Protein can be efficiently conjugated to GMM in a vaccine

To be able to investigate the effect of GMM conjugation to protein on the anti GMM antibody response, we used a method of non covalent association of protein and lipid to microspheres (Barral et al., 2008). For a valid comparison between the non-conjugated and conjugate vac-

cines, it is crucial that the amount of GMM is equal in both types of vaccine, as well as the amount of protein. The amounts of the other vaccine components like the carrier lipids DOPC and PE, and the microspheres were standardized for all types of vaccines. The quantity of GMM in conjugate and non-conjugated vaccines was analyzed by TLC (Fig. 1A) and showed a comparable amount of this antigen in both types of vaccines, which was estimated to be 100 µg per dose. The quantity of KLH and Hsp70 was analyzed by dot blot and Western blot respectively (Fig. 1B and C). Equal amounts of each protein were present in conjugate and non-conjugated vaccines, which were estimated to be 20 µg per dose. Of note: all vaccines, including carrier only, were prepared with carrier lipids, streptavidin, and microspheres as described in Section 2 and summarized in Table 1.

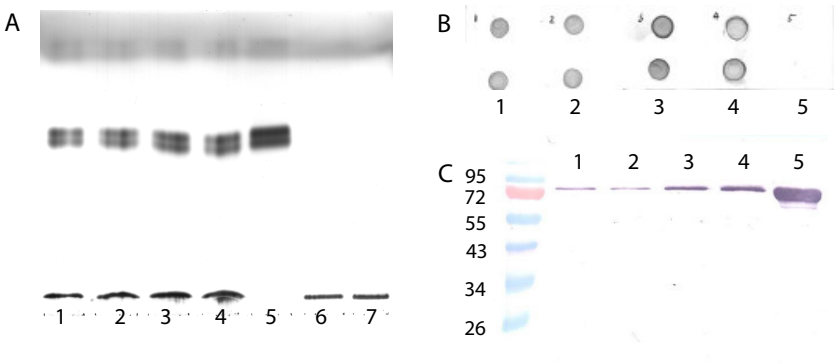


Fig. 1. Protein and lipid content of vaccines. Upon finalization of the conjugated and non-conjugated vaccines, the amount of GMM in the vaccines was analyzed by TLC (A). Of each GMM-containing vaccine, 25% of a vaccine dose was loaded in lane 1–4. Lane 1: GMM–KLH conjugate vaccine; lane 2: GMM–Hsp70 conjugate vaccine; lane 3–4: GMM only vaccine; lane 5: pure GMM standard (50 µg); lane 6: DOPC (25 µg); lane 7: PE (25 µg). KLH in KLH-containing vaccines was analyzed by dot blot (B). KLH was detected by polyclonal rabbit anti KLH antibody. In 1 and 2, each duplicate dot contains 6% of a vaccine dose. 1: GMM–KLH conjugate vaccine; 2: KLH only vaccine; 3: biotinylated KLH (6 µg); 4: non-biotinylated KLH (6 µg); 5: Hsp70 as a negative control (6 µg). Hsp70 was detected in Hsp70-containing vaccines by Western blotting, using mouse anti Hsp70 antibody for detection (C). Lane 1: 3% of a GMM–Hsp70 conjugate vaccine; lane 2: 3% of a Hsp70 only vaccine; lane 3: 6% of a GMM–Hsp70 conjugate vaccine; lane 4: 6% of a Hsp70 only vaccine; lane 5: 6 µg biotinylated Hsp70.

3.2. GMM–protein conjugate vaccine does not induce anti GMM antibodies

One group of animals (n = 5 in all groups) was immunized with GMM–KLH conjugate vaccine in one shoulder and carrier only in the contralateral shoulder; one group was injected with GMM only vaccine in one shoulder and KLH only vaccine in the contralateral shoulder and one group of animals received carrier only in both shoulders. Total serum IgG antibody against GMM and KLH was examined by ELISA (Fig. 2). As compared to the control vac cination, carrier only, GMM specific antibodies were not detected in either of the two groups after immunization (Fig. 2B, D and F). Some animals showed a weak IgG response against GMM before the first immunization, which disappeared several weeks later (Fig. 2F), which can be explained by the presence of maternal antibodies. As expected, a strong IgG antibody response against KLH was seen in animals immunized with KLH separately (Fig. 2E) as compared to carrier only immunized animals ($P < 0.05$). Although weaker responses were detected in 2 out of 5 animals that were immunized with GMM–KLH conjugate vaccines, the

anti KLH antibody response of this whole group was also significantly higher than the carrier only immunized group ($P < 0.05$) (Fig. 2A).

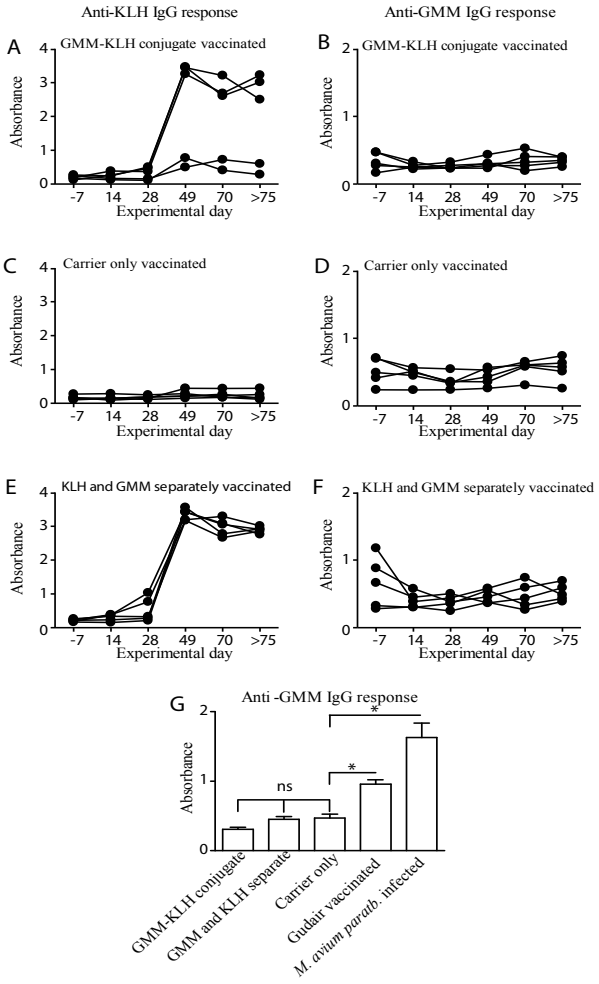
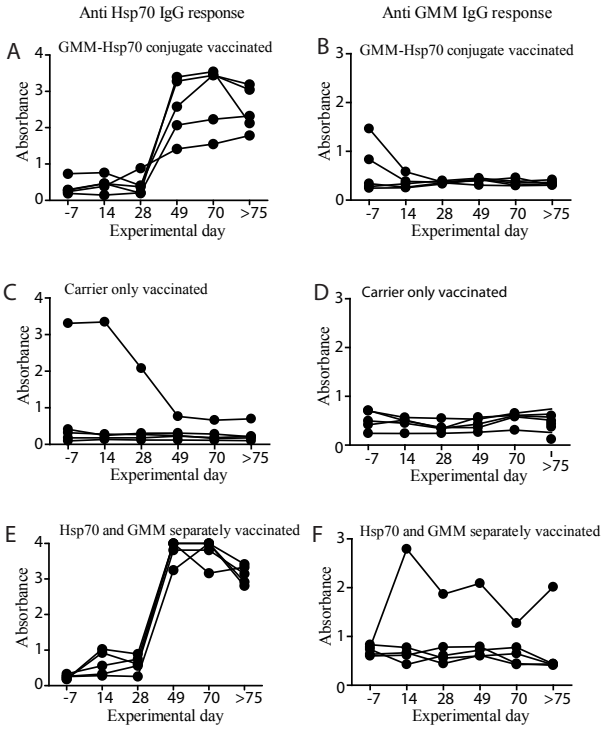


Fig. 2. Antibody responses against lipid and protein vaccine components. (A–F) Total IgG responses against KLH (left panels) and *M. phlei* GMM (right panels) of conjugate GMM–KLH vaccinated animals (A and B); carrier vaccinated animals (C and D); and non-conjugated GMM and KLH vaccinated animals (E and F) was determined by ELISA. Animals received the first immunization on day 0 and the second one on day 35 of the experiment. Symbols connected with a line represent data from one individual animal. (G) GMM specific IgG in animals two weeks after the second immunization with the three experimental vaccines, three weeks after the second immunization with the Gudair vaccine, and in animals chronically infected for 2–4 years with *M. avium ssp. paratuberculosis*. Each bar represents data from 5 animals. Statistically significant differences ($P \leq 0.05$) are marked with *.

In addition, we also included two groups immunized with GMM–Hsp70 conjugate and non-conjugated vaccines. A similar pattern was seen when we used Hsp70 instead of KLH (Supplemental Fig. 1A, B, D and E).

In the group of animals that received non-conjugated GMM and Hsp70 vaccines, one animal showed an anti GMM IgG response (Supplemental Fig. 1F). In addition, one animal in the group that was injected with carrier only showed a strong anti Hsp70 IgG response from day 7 till day 14 of the experiment, which dropped at 49 days after the first immunization (Supplemental Fig. 1C).



Supplemental Fig. 1. Antibody responses against lipid and protein vaccine components. ELISA was performed to test for the anti Hsp70 total IgG (left) and anti *M. phlei* GMM total IgG (right) responses of conjugate GMM/ Hsp70 vaccinated animals (A and B); carrier vaccinated animals (C and D); and GMM and Hsp70 separate vaccinated animals (E and F). Animals received the priming immunization on day 0 and the boost on day 35 of the experiment.

From these experiments it becomes clear that vaccination with a GMM–protein conjugate does not recapitulate the anti GMM antibody inducing capacity of a natural infection. When tested in parallel with the sera of the GMM–KLH or the GMM–Hsp70 conjugate vaccine immunized animals, a markedly higher IgG reactivity against GMM was seen in animals that were naturally infected with *M. avium ssp. paratuberculosis* ($P < 0.05$). To investigate whether infection with a live bacterium is necessary to induce GMM antibodies we also tested sera of animals that were vaccinated subcutaneously with the Gudair vaccine, which consists of inactivated (non-replicating) *M. avium ssp. paratuberculosis*. Though lower than the responses of animals infected with live M. avium ssp. paratuberculosis, Gudair vaccinated animals did show a significant anti GMM IgG response compared to the carrier vaccinated group ($P < 0.05$) (Fig. 2G). From this we conclude that additional mycobacterial components rather than factors associated with active infection with the pathogen can stimulate the immune system to induce anti GMM antibody response.

3.3. Immunization induces weak T cell responses against GMM

In addition to the evaluation of the antibody responses, we examined T cell activity against

GMM and proteins in animals that were immunized with GMM–protein conjugate vaccine, non-conjugated vaccine and carrier only. T cell proliferation assays were carried out before immunization and after immunization until 1.5 months after the second immunization using freshly isolated PBMC. A higher, but non significant response against GMM was detected in animals that were immunized with GMM-containing conjugate or non-conjugated vaccines compared to carrier only vaccinated animals (Fig. 3A and C). Lack of significance of T cell responses could be due to the limited number of blood samplings after the second immunization as compared to our previously published GMM vaccination studies (Nguyen et al., 2009).

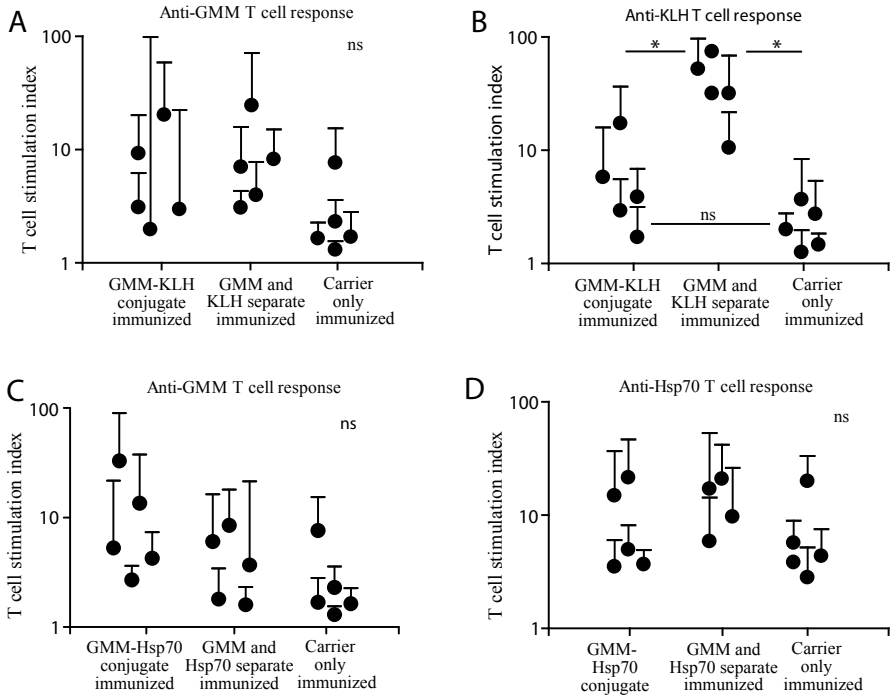


Fig. 3. T cell responses against lipid and protein vaccine components. (A–D) PBMC freshly isolated from blood were stimulated for three days with *M. phlei* GMM (A and C), KLH (B), and Hsp70 (D). Each symbol represents the average value of 5 independent experiments carried out on one animal post immunization. The T cell stimulation index was calculated by dividing the counts/min of the antigen stimulated wells divided by counts/min of medium (without antigen) after a 7-h pulse of [3H] thymidine. Statistically significant differences ($P \leq 0.05$) are marked with *.

3.4. Non-conjugated vaccine induces stronger anti protein T cell and antibody responses than conjugate vaccine

Because the amount of protein in the conjugate and non-conjugated vaccines was equal, we expected that the anti protein antibody responses and the anti protein T cell responses would be comparable. However, as noted in Section 1 on antibody responses, all animals that received KLH in a non-conjugated vaccine responded with a strong anti KLH IgG responses, but only three out of five animals that received GMM–KLH conjugate vaccine showed a

comparably strong response (Fig. 2A and E). The difference in anti KLH IgG response between the conjugate and non conjugate vaccine immunized groups was however not statistically significant. Also in animals immunized with non-conjugated vaccine containing Hsp70, higher IgG response to Hsp70 were detected than in animals receiving the GMM–Hsp70 conjugate, and this case the difference between the groups was significant ($P < 0.05$) (Supplemental Fig. 1A and E).

The differences between conjugate and non-conjugated vaccines in the generation of anti protein antibody responses were mirrored by a difference in anti protein T cell responses. Even though the total amount of antigens administered was equal, the difference in anti KLH T cell responses between non-conjugated KLH and conjugate GMM–KLH groups was significant ($P < 0.05$) (Fig. 3B). No significant differences were detected in T cell proliferation of animals that were immunized with Hsp70 in non-conjugated or conjugate vaccines compared to animals vaccinated with carrier only (Fig. 3D).

Our data show that conjugation of GMM to a protein down regulates or prevents an optimal antibody response against that protein. One possible explanation is that GMM masks or changes epitopes of the protein. Why antibodies against GMM itself are not generated upon vaccination is not clear. During natural infection with M. avium ssp. paratuberculosis or vaccination with inactivated bacteria (the Gudair vaccine), proteins and GMM are also "conjugated" in the bacterial cell wall, yet strong anti GMM antibody responses develop. Additional stimulatory components present in mycobacteria, like TLR agonists, may be responsible for the difference between conjugate vaccine and exposure to bacterial cell walls. Inclusion of these compounds in a GMM–protein conjugate vaccine may improve the impaired T cell responses that were observed using the conjugate vaccine, and this could possibly overcome the lack of development of anti GMM IgG antibodies (Pasare and Medzhitov, 2005; Banchereau and Steinman, 1998).

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Chapter 5

The bovine CD1D gene has an unusual gene structure and is expressed, but does not stimulate an NKT-like cytokine release response *in vivo*[*]

Running title: Bovine CD1D function *in vivo*

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Abstract

Whereas CD1d and NKT cells have been proposed to have highly conserved functions in mammals, data on CD1d protein expression and NKT cell function in species other than humans and rodents are lacking. Upon stimulation with the CD1d-presented synthetic antigen α-galactosylceramide, human and rodent NKT cells release large amounts of cytokines. We have previously described the bovine CD1 locus, which contains CD1A, CD1B, CD1D, and CD1E genes. The two bovine CD1D genes have structural features that suggest that they can not be translated into functional proteins expressed on the cell surface. Here we provide evidence that the intron/exon structure and the signal peptide of bovine CD1D is different from all other known CD1 genes, but that it can be translated into a protein that is expressed on the cell surface and is recognized by the CC43 and CC118 monoclonal antibodies. To test whether the bovine CD1D gene product performs the conserved function of presenting α-galactosylceramide to T cells in vivo, we injected cattle intravenously with 0.1 μg/kg, 1 μg/kg, 10 μg/kg of α-galactosylceramide and determined serum cytokine levels. This treatment did not lead to an increase in body temperature and serum cytokine levels of the animals. Our data show that the antigen specificity and/or cellular functions of the human and mouse CD1d and NKT system are not present in cattle.

Introduction

Due to their quick, strong responses and the availability of the strong synthetic antigen α-galactosylceramide (α-GalCer), invariant NKT cells are currently targeted in anti cancer treatment, vaccine development, and immunotherapy for autoimmune diseases. CD1d can bind α-GalCer in its hydrophobic binding groove, and recognition of this complex by the T cell receptor of invariant NKT cells triggers the release of large amounts of cytokines, including IL-2, IL-4, IL-10, IL-8, IL-13, IL-21, GM-CSF, IFN-γ , TNF-α , MIP-1 α and MIP-1 β (1-3). CD1d and the invariant T cell receptor of NKT cells are so conserved that mouse CD1d can present α-GalCer to human invariant NKT cells and *vice versa* (4, 5).

With the availability of multiple mammalian genomes it has become clear that CD1D genes are widespread. With the exception of marsupials, not a single mammalian genome has been reported to lack CD1D genes altogether (6). However, whether there is a consistent causal relationship between a CD1D gene in the genome and the development of a functional invariant NKT cell population in a species is unknown. Despite the absence of data directly addressing this question, it is thought that most mammals have a functional CD1d and invariant NKT cell system, with the notable exception of ruminants (7). Even though CD1D genes are present in the ruminant genomes and are being transcribed, all of the studied ruminant CD1D genes have been shown to have mutations that eradicate the start codon and an intronic splice site, suggesting that functional protein might be absent (7, 8). CD1d proteins have not been detected in ruminants to date. Because the CD1d and invariant NKT system is such a prominent part of the immune system of humans and mice, two species belonging to different orders of mammalia, it is often assumed that the system has been broadly conserved during evolution, and is also functional in the other CD1D gene containing orders. Therefore, a naturally occurring genetic distortion of the ruminant CD1D genes as described previously (7) and the ensuing suggestion that ruminants lack invariant NKT cells was unexpected and

needs further investigation.

All MHC class I-like molecules, including CD1, consist of a heavy chain, which contains the three extracellular α domains, a transmembrane domain, and a cytoplasmic tail. Upon translation and translocation into the endoplasmic reticulum, the signal peptide is cleaved off. The mature heavy chain forms a heterodimer with the β2 micoglobulin molecule. The MHC class I-like molecules also share a highly comparable intron/exon structure. The start codon and signal peptide lie on one exon and each of the three α domains, as well as the transmembrane domain and the cytoplasmic tail are located on separate exons. Surprisingly, we found that the bovine CD1D gene, which is known to be transcribed is also translated in vivo. This is possible because a sequence that is intronic in mouse and human is an exon in cattle, and encodes an alternative start codon and peptide with signal peptide function. However, despite the expression of this CD1D gene product, we also show that intravenously applied α-GalCer in cattle has no effect on serum cytokine levels and body temperature, arguing against the existence of bovine invariant NKT cells and a functional bovine antigen presenting molecule for α-GalCer.

Materials and Methods

Animals

Three groups of three Holstein-Friesian calves of 4 months old, weighing approximately 120 kg each, were treated by intravenous injection of 0.1 μg/kg, 1 μg/kg, 10 μg/kg of α-GalCer in 5 ml sterile PBS in the jugular vein. α-GalCer was dried under a stream of N2 gas to remove organic solvents and sonicated at 50ºC in PBS. Serum was collected once before and at 2, 4, 8, 16, and 30 hours after α-GalCer injection, and stored at -20ºC. The rectal temperature was measured at the same time points as serum collection, and one day before treatment at the same hour as the post- α-GalCer time points. Experiments were approved by the Animal Ethical Committee of the University of Utrecht, the Netherlands.

Holstein or Holstein cross calves of 6 months old were infected via the intratracheal route with 2000 CFU *Mycobacterium bovis* (strain AF2122/97). Serum samples were collected 9 weeks post-infection. Disease was confirmed by post-mortem performed 9 weeks post-infection by the presence of visible pathology typical of bovine tuberculosis and the culture of the *M. bovis* from tissues.

Dairy-cross calves, 8-10 week old, were experimentally infected with 105 TCID50 bovine viral diarrhoea virus (BVDV; strain UK1362727) via intranasal inoculation. Serum samples were collected 8 days post-infection at which point animals were pyrexic, leukopenic and viraemic. Work was carried out in accordance with UK legislation pertaining to care and use of animals under experimentation.

Bovine IFN-γ, IL-1β, IL-4, IL-10, IL-12, and MIP-1β detection

Simultaneous detection of IFN-γ , IL-1β, IL-4, IL-10, IL-12, and MIP-1β was performed in sera using a custom bovine multiplex cytokine/chemokine assay developed in collaboration

with MSD (9). Multiplex 96-well plates were supplied with each of the commercially available target capture antibodies: bovine IFN-γ (Mabtech, Stockholm, Sweden), IL-4 (Endogen, Rockford, IL, USA), IL-10 and IL-12 (AbD-Serotec), IL-1β, and human cross-reactive MIP-1β (MSD) pre-spotted onto spatially separated locations in each well. Incubations were performed at room temperature. Plates were blocked with MSD assay buffer prior to addition of 25 µl/well test sera or standards. The standards were serially diluted in MSD dilution buffer with top concentration of standard shown in parentheses: IFN-γ (Endogen, 100 ng/mL); IL-1β (bovine IL-1β calibrator (MSD), 20 ng/mL); IL-4 (bovine IL-4 calibrator (MSD), 2 ng/mL); IL-10 (IAH, 30 U/mL); IL-12 (IAH, 1000U/mL), and MIP-1β (human MIP-1β calibrator (MSD), 10 ng/mL). Following a 2 h incubation, plates were washed and then incubated for a further 2 h with a combined cocktail of biotinylated secondary detection antibodies (IFN-γ , IL-1β, IL-4, IL-10, IL-12, MIP-1β, (all from MSD) and Streptavidin-Sulfotag (MSD) in MSD dilution buffer. Minimum detectable levels are as follows: IFN-γ 0.046 ng/mL; IL-1β 0.043 ng/mL; IL-4 3.19 pg/mL, IL-10 0.018 U/mL, IL-12 1.83 U/mL and MIP-1β 1.32 pg/mL. After a final wash, plates were coated with MSD Buffer-T and luminescence signal measured on a MSD-6000 instrument. Serum IFN-γ levels were also measured using the sandwich ELISA provided with the Bovigam® assay (Prionics AG, Zurich, Switzerland). Using this method the minimum detectable level of IFN-γ was 0.016 ng/mL.

Molecular cloning of bovine CD1D transcript, transfections, and flow cytometry

Bovine CD1D cDNA (accession number BT029852) was obtained from BACPAC resources, Oakland, CA, and cloned into pcDNA3.1. 293T cells and K562 cells were transfected with bovine CD1D cDNA in pcDNA3.1 using fugene-6-reagent (Roche). 293T cells were harvested 48 hours after transfection and cell surface stained with antibodies raised against bovine thymocytes (CC43) and bovine intestinal epithelial cells (CC118) (10), and anti-human CD1d clone CD1d42 (11), followed by goat anti mouse-PE. K562 cells were selected on 1 mg/ml G418 for several weeks. K562 transfected with human CD1d have been described previously (12).

Cellular assays

The human invariant NKT cell lines J3N.5 and J24L.17 were described previously (13). K562 cells transfected with human or bovine CD1d (5 x 104/well) were mixed with 10-fold dilutions of α-GalCer that was sonicated in tissue culture medium consisting of RPMI supplemented with 10 % fetal calf serum and penicillin/streptomycin. Upon addition of invariant NKT cells (5 x 104/well), the cultures were incubated for 24 h at 37°C and 5% CO2. Culture supernatants were tested for the presence of IFN-γ by ELISA.

Statistical analyses

Statistical analysis was performed with R statistical software (2.10.1, www.R-project.org). Linear mixed effects models were used to analyze the effect of α-GalCer treatment on serum cytokine levels, which included treatment group and time, and their interaction as fixed factors, and animal as random factor.

Results

Transcription and translation of boCD1D genes previously considered pseudogenes

We have previously described two bovine CD1D genes and provided evidence for transcription of one of them, boCD1D1 (7). However, at that time we considered the transcript to be non-functional because the sequence that is homologous to the part that encodes the murine and human signal peptide starts with a mutated start codon in cattle (Figure 1A). In addition, the part that is homologous to the intron that is present between the signal peptide-encoding exon and the α 1 domain exon in mice and man, does not contain functional splice sites in cattle. Consistent with this, no transcripts with this intron properly spliced out could be cloned and were absent in the databases, while numerous transcripts were identified in which the intron was present. Upon re-evaluation of this transcript we found that there is an ATG present right after the region corresponding to the region that encodes the signal peptide in the murine and human transcript (Figure 1A).

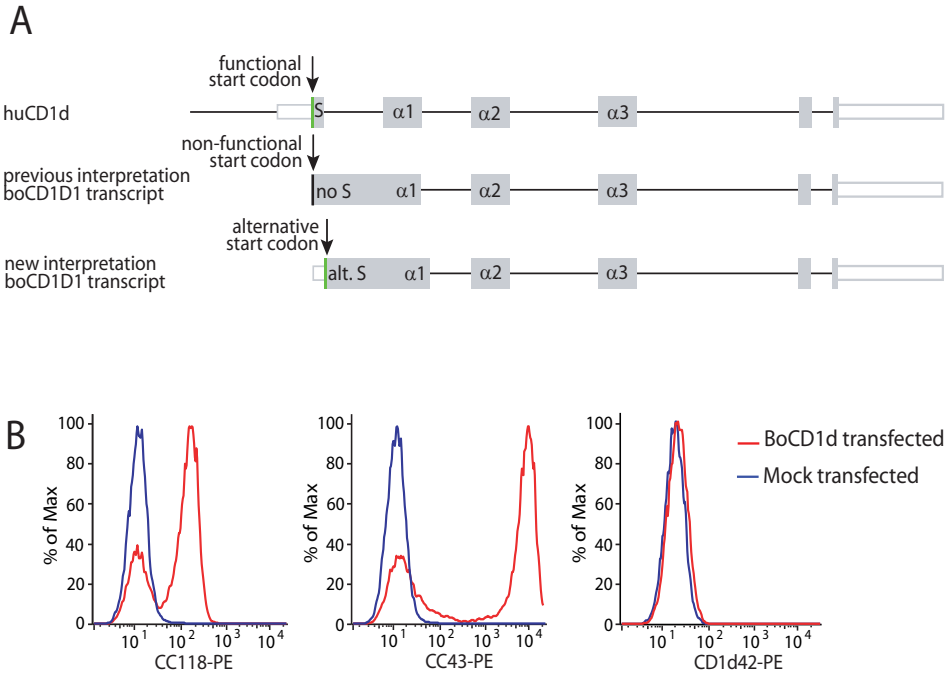

Figure 1. Structure of human and bovine CD1d transcripts In the human CD1d transcript (accession number NM_001766), the intron (black horizontal lines)/exon (gray boxes) structure shows a pattern that is typical for all MHC class I-like molecules (A, top). The bovine CD1D1 transcript (accession numbers DQ192544 and BT029852) has previously been shown to contain a nonfunctional start codon (black) at the same position as where the human functional start codon lies (green) (A, middle). Here we show that the bovine CD1D1 transcript contains a functional start codon in the part that is homologous to the human intron before the α1 domain (A, below). S: Signal peptide encoding sequence; Alt. S: Alternative signal peptide encoding sequence. 293T cells transfected with the bovine CD1D1 transcript or mock transfected were cell surface stained with the CC43, CC118, and CD1d42 antibodies (B).

We hypothesized that if translation starts at this ATG, the sequence which is intronic in humans and mice might be translated into an alternative peptide, possibly with signal peptide capacities and continue into a functional CD1d protein. To test this hypothesis we transfected 293T cells with a bovine CD1D1 transcript. Sequencing confirmed that the insert contains 45 bp 5' of the location of the murine and human start codon, which is the mutated start codon in cattle, and this 5' stretch did not contain any additional ATGs. We stained transfected and mock transfected 293T cells with the available anti human CD1d and anti bovine CD1 antibodies of which only CC43 and CC118 recognized the surface of the transfected cells, but not the mock transfected cells (Figure 1B). CC43, which was raised against bovine thymocytes, and CC118, which was raised against bovine intestinal epithelial cells, have previously been suggested to recognize bovine CD1 based on staining pattern and the fact that they immunoprecipitate a heterodimer with MW consistent with a class I-like molecule (10). However, CC43 and CC118 do not recognize boCD1a and boCD1b3 (7). The identification of the bovine CD1D gene product as the target molecule for these antibodies is consistent with all published data.

The fact that this molecule translocates to the cell surface implies that a signal peptide-like function is performed by the sequence that is homologous to the human and murine intron. Indeed, the 99-aa long peptide before the α 1 domain contains a hydrophobic segment consistent with signal peptide function of random sequences (14). The SignalP program (available at www.cbs.dtu.dk) does not predict a signal peptidase cleavage site, suggesting that the peptide results in a membrane anchor.

The bovine CD1D gene product does not present α-GalCer to human invariant NKT cells

The highly conserved structure and function of the human and mouse CD1d and invariant NKT cell system is illustrated by the fact that human CD1d presents α-GalCer to murine invariant NKT cells and vice versa. To test whether the bovine CD1D gene product can perform a similarly conserved function in vitro, we tested whether it can present α-GalCer to human invariant NKT cell clones. For this purpose we transfected the human myelogenous leukemia cell line K562 with the bovine CD1D transcript and compared its antigen presenting capacity to that of K562 cells transfected with human CD1d in an IFN-γ release assay. Figure 2A shows that two different human invariant NKT cell clones release IFN-γ when stimulated by K562 cells transfected with human CD1d and α-GalCer, but not when stimulated with K562 cells transfected with bovine CD1D transcript and α-GalCer, despite strong cell surface expression of the bovine CD1D gene product (Figure 2B). Of note, using K562 cells transfected with human CD1d, half maximum response is obtained around 10 nM α-GalCer, and responses are detectable around 1 nM α-GalCer, which is consistent with the published potency of α-GalCer for invariant NKT cells.

Serum cytokine response upon intravenous application of α-GalCer in cattle

The failure to present to human invariant NKT cells led us to focus on bovine T cells. To investigate whether the bovine CD1D gene product performs the conserved function of presenting α-GalCer to T cells in vivo, we injected three groups of three calves of four months of age intravenously with 0.1 μg/kg, 1 μg/kg, 10 μg/kg of α-GalCer and determined serum

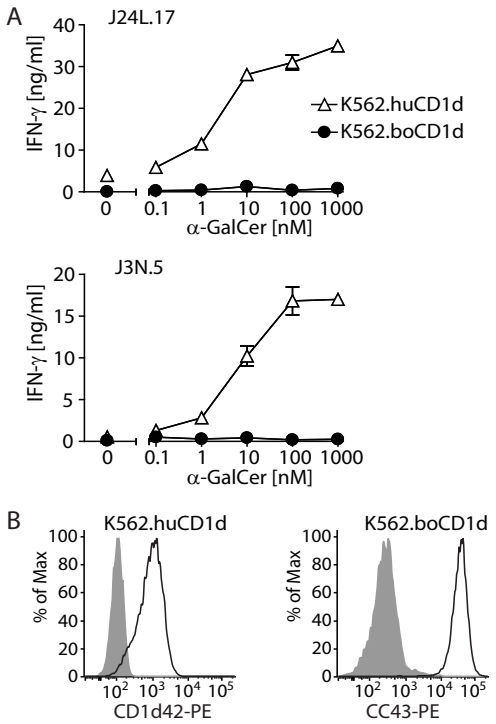

Figure 2. The bovine CD1D gene product does not stimulate human NKT cells K562 cells (5 x 104/well) stably transfected with human CD1D or bovine CD1D1 transcript, were incubated with the indicated concentrations of α-GalCer and the human NKT cell clones J24L.17 or J3N.5 (5 x 104/well). Supernatants were harvested after 24 hours and tested for the presence of IFN-γ by ELISA (A). The cell surface expression of human CD1d and bovine CD1D1 gene product on the K562 cells at the day that the cellular assay was performed was confirmed by flow cytometry (B). This experiment has been replicated independently, with essentially the same results.

IL-1β, IL-4, IL-6, IL-10, IL-12, IFN-γ , and MIP-1β levels using the Meso Scale Discovery (MSD) technique. From each animal, serum was collected just before (time point 0), and at 2, 4, 8, 16, and 30 hours after injection (Figure 3A-G). To show that the MSD technique that we used was adequate for the detection of cytokines in bovine sera, and to provide a rough indication of physiological cytokine level ranges during acute phase of infection, we included sera of animals with acute viral (bovine viral diarrhea virus) or bacterial (*Mycobacterium bovis*) infections (left part of each panel), as well as a wide concentration range of recombinant standards. For IL-4, IL-10, IFN-γ , and MIP-1β at least one of the infected control animals showed a much higher serum cytokine level than the α-GalCer treated animals (left part of each panel). Please note that the scale of the y-axes for IL-4, IL-10, IFN-γ for the control animals versus the α-GalCer treated animals is different. These results, in addition to the standard curves obtained with recombinant cytokines, shows that the detection method for serum cytokine levels was adequate.

There were no statistically significant increases in serum cytokine levels in any of the α-GalCer treated groups. For each cytokine, in each animal, the increase from the cytokine

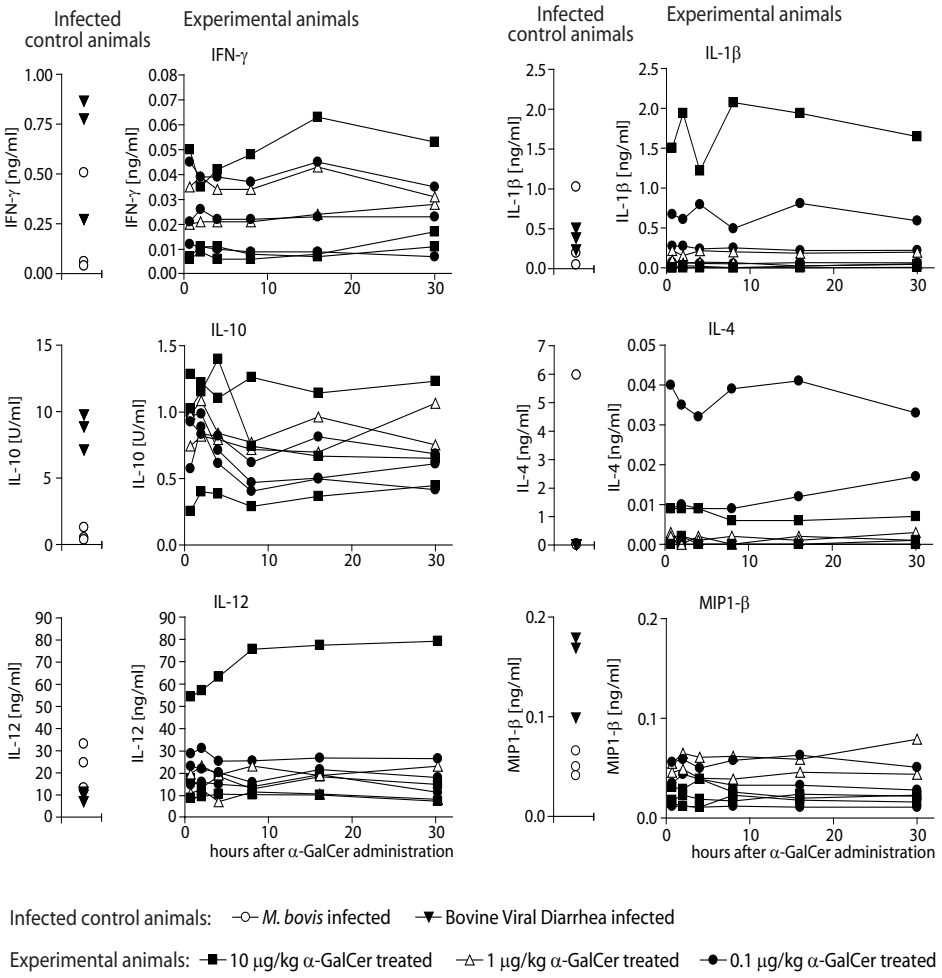

Figure 3. Serum cytokine responses in cattle upon α-GalCer treatment Three groups of three calves were treated with 0.1 μg/kg (n=3), 1 μg/kg (n=2), 10 μg/kg (n=3) α-GalCer intravenously and serum cytokine levels were determined before (time point 0) and at the indicated time points after treatment. Symbols connected with a line represent data from one individual animal. As technical controls, animals suffering from M. bovis (n=3) or bovine viral diarrhea virus (n=3) were included.

before treatment (time point 0) was never more than 1.5-fold, which we consider a fairly stable cytokine level (right part of each panel). Each animal, represented by symbols connected by a line, apparently has a certain level of each cytokine before injection of α-GalCer (time point 0), and this levels remains fairly constant in time, but may be different from other animals. Comparable intravenous α-GalCer injections in mice typically show a 20-fold increase in cytokine levels, with the peak of the response, depending on the cytokine, around 4-16 hours after intravenous injection (15-17). By testing the capacity to activate human NKT cells in vitro, the α-GalCer used in this in vivo experiment was confirmed to be bioac-

tive, resulting in a half maximum response around 10 nM α-GalCer, and detectable responses around 1 nM α-GalCer, which is consistent with the published potency of α-GalCer for invariant NKT cells (Figure 2A).

Using the standard sandwich ELISA as provided with the Bovigam assay, the IFN-γ data were replicated with essentially identical results (data not shown).

Body temperature upon intravenous application of α-GalCer

In human clinical trials and animal models where α-GalCer is administered, a raise in body temperature has sometimes been observed (18-20). To test whether cattle react to administration of α-GalCer with a raise in body temperature, the rectal temperature was measured just before (time point 0) and at 2, 4, 8, 16, and 30 hours after α-GalCer treatment, simultaneously with serum collection (Figure 4). Because body temperature follows a circadian rhythm, body temperature of the animals was also measured at the same hour, a day before α-GalCer treatment ("8 calves untreated" in Figure 4).

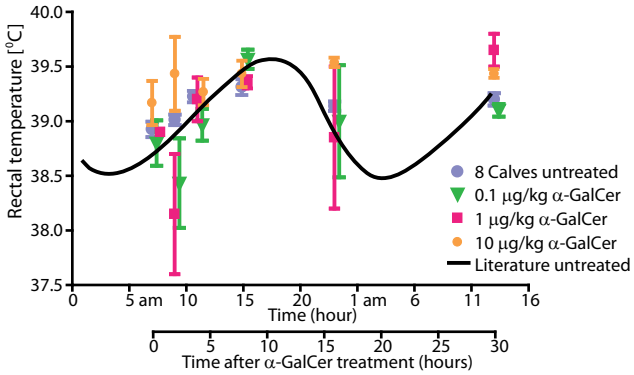

Figure 4. Body temperature of cattle treated intravenously with α-GalCer. The body temperature of calves treated with 0.1 µg/kg (n=3), 1 µg/kg (n=2), and 10 µg/kg (n=3) α-GalCer was measured at the indicated time points after treatment. The circadian rhythm of the body temperature of healthy calves, as predicted from literature, is shown as a black line. One day before α-GalCer treatment the body temperature of the calves was measured at the same hour as the post- α-GalCer time points (in blue: "8 Calves untreated").

Treatment with α-GalCer did not lead to a significant increase in body temperature. This was true when each of the three dose groups was compared to the untreated group, and also when all treated animals were compared as one group to the untreated situation. To be able to visualize how the temperature data we collected fit in a normal circadian rhythm, the expected course of body temperature as based on published data is shown in Figure 4 as a black line (21).

Discussion

Ruminants were thought to be the only group of mammals lacking functional CD1D genes (7). Here we have demonstrated that, despite an intron/exon structure that is uncharacteristic

for CD1 genes and a highly unusual signal peptide, transfection of the bovine CD1D gene transcript leads to cell surface expression of the gene product in vitro. Expression on bovine thymocytes and B cells in vivo, which matches the CD1d expression pattern in humans and mice, is implied by the staining patterns of the CC43 and CC118 antibodies (10), which we have shown here to recognize the bovine CD1D product.

Despite the expression of a CD1D gene product, we provide data that suggest that cattle do not have functional invariant NKT cells. Universal defining features of the classical CD1d and invariant NKT system across animal species are that 1) the antigen presenting element CD1d and the invariant NKT TCR are highly homologous, 2) α-linked glycolipids for which α-GalCer is a model, are presented and recognized, and 3) this leads to the release of large amounts of cytokines. We have shown that in cattle, intravenous administration of α-GalCer does not lead to a rise in body temperature or serum cytokine levels, which is a hallmark of the activation of the CD1d and invariant NKT system. This may imply that the bovine CD1D gene product can not present α-GalCer, or that there is no distinct T cell population with the invariant NKT TCR, or that they do not release cytokines upon stimulation,. Even though we do not have a definitive answer to the question at what level the system is impaired, we conclude from this experiment that in cattle the classical CD1d and invariant NKT system is not functional at a detectable level.

In support of this main conclusion we have previously shown that even though the murine CD1d-α-GalCer complex stimulates human invariant NKT cells and vice versa and can be used to visualize these cells using flow cytometry, human and murine CD1d-α-GalCer tetramers do not stain a T cell population in bovine PBMC (7). However, this experiment could not distinguish between the human and murine CD1d-α-GalCer complex having a low or no affinity for bovine invariant NKT cells and bovine invariant NKT being absent in the material we analyzed. Using highly responsive human invariant NKT clones, we have shown here that the bovine CD1D gene product does not stimulate human invariant NKT cells. This result can be interpreted in two ways, which are both consistent with invariant NKT cells being absent or non functional in cattle: either the bovine CD1D gene product can not bind and present α-GalCer, or it does, but unlike murine CD1d, it is not sufficiently conserved to present α-GalCer to human invariant NKT cells. Another independent set of data suggesting that cattle may not have a functional CD1d and invariant NKT system is that a set of bovine TCR α chain sequences that use the V segment that is homologous to the segment used by murine and human NKT cells, does not contain an invariant NKT TCR α chain, even though the same approach led to the identification of the equine and porcine invariant NKT TCR α chain (8).

In humans and mice there are also minor populations of T cells other than invariant NKT cells that respond to CD1d. These cells recognize lipids without α-linked sugars like phospholipids (22), sufatides (23), or small aromatic molecules (24) and are called type II NKT cells (25). It is possible that in ruminants this type of NKT cells is present and that in more general terms the evolutionary conserved function of CD1D is to present lipids to T cells, and that the presentation of α-GalCer to invariant NKT cells is a special case of that function that is not as widely present as often assumed.

We have previously described the CD1D genes of other ruminants (African buffalo, sheep, bushbuck, bongo, N'Dama cattle, and roe deer) (8), and because they all show an alternative start codon at the beginning of the sequence that is homologous to the first intron in human and mice, and lacks consensus splice sites, it is likely that CD1D in these species is expressed in a similar fashion as in cattle as we have described in the current paper. We conclude that the CD1D gene ruminants, with its unusual intron/exon structure and signal peptide is expressed, but not part of a classical, functionally conserved CD1d and invariant NKT system.

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Footnotes

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Non standard abbreviations: CD1: Cluster of Differentiation 1; NKT cell: Natural Killer T cell; α-GalCer: α-galactosylceramide

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Chapter 6

In vivo and *in vitro* expression of bovine CD1 proteins and dissection of the specificities of the anti bovine CD1 antibodies

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Summary

Lipid antigens are presented to T cells by CD1 molecules. Humans express one gene of each of the five known CD1 isoforms, but for unknown reasons many animals express multiple genes of one isoform, while genes for other isoforms may be lacking. CD1b is known to present mycolic acid containing mycobacterial glycolipid antigens. In cattle, three CD1b genes are transcribed: CD1b1, CD1b3, and CD1b5. These CD1b molecules differ in their antigen binding domains and in their cytoplasmic tails, suggesting that they may bind different antigens and/or that they may traffic differently in the cell. In the current study, we describe the transcription of these bovine CD1b variants, as well as CD1d and a newly discovered CD1a molecule, in DCs and B cells from different tissues. Determining the isoform specificity of previously only partly characterized anti CD1 antibodies allowed us to determine cell surface protein expression of each isoform. Our study suggests that CD1b1 and CD1b3 are more broadly expressed than CD1b5, which can only be detected in pseudoafferent lymph DCs. Pseudoafferent lymph DCs express all CD1b genes,but no transcription is detected in lymph nodes. Even though B cells transcribe CD1b1 and CD1b3, there is no evidence of CD1b protein expression at the cell surface. Like in other species, bovine B cells express CD1d, confirming that the typical CD1 group 1 and group 2 expression patterns are largely conserved

Introduction

Functionally and structurally, the CD1 family of proteins can be divided in two groups. In humans, all group 1 CD1 proteins (CD1a, CD1b, and CD1c) are highly expressed on immature thymocytes and immature and mature dendritic cells (DCs). In addition, in humans, CD1a is present on Langerhans cells and CD1c on B cell subsets. Of note, group 1 CD1 genes are lacking in mice. Group 2 CD1 (CD1d) has a much broader expression pattern in humans and mice. It is expressed at a low level on many cell types, and some cases of minor cell populations with high levels of CD1d expression have been described, such as mantle zone B cells in the lymph node and marginal zone B cells in the spleen (Dougan et al., 2007), and Ito cells in the liver (Winau et al., 2007). The reason for the differential expression of group 1 and group 2 CD1 proteins is most likely related to their differential function. Group 2 CD1 protein (CD1d) is the restricting element for invariant NKT cells, a cell population with a very limited and highly conserved T cell repertoire that can quickly release large amounts of cytokines. Recognition of CD1d presented self or foreign lipids and stimulation with cytokines are physiologically relevant ways of NKT cell stimulation. Group 1 CD1 proteins are best known for their ability to present exogenous lipid antigens to T cells with a diverse T cell repertoire, though recently CD1a has been shown to be recognized by T cells without the addition of exogenous antigen (de Jong et al., 2010). Most of the known group 1 CD1-presented antigens are from mycobacterial origin. CD1-restricted T cells that recognize such a mycobacterial antigen are very efficient in killing antigen-pulsed or mycobacterium-infected target cells and therefore CD1 presented lipids are potentially interesting as subunit vaccine candidates. Of note, mammalian species vary widely in the numbers of group 1 CD1 genes that are present in their genomes. Humans have one gene for each of CD1a, CD1b, and CD1c, but dogs have eight CD1a genes (Looringh van Beeck et al., 2008), and guinea pigs have five CD1b genes and four CD1c genes (Dascher et al., 1999).

Humans are the only species in which the cellular expression pattern of group 1 CD1 molecules has been studied extensively. Less complete studies have been performed on rabbits (Hayes and Knight, 2001), dogs (Looringh van Beeck et al., 2008), and guinea pigs (Dascher et al., 1999). It is unknown to which extent the know expression patterns of group 1 and group 2 molecules are conserved among species. Also, it is unknown whether multiple genes of the same isoform have specific expression patterns and functions. Of the two murine CD1D genes, CD1D1 is clearly the most broadly and strongly expressed molecule responsible for the NKT cell selecting functions in mice (Chen et al., 1999, Chiu et al., 2002). Among the four guinea pig CD1b molecules, one seems to be differentially expressed on different types of dendritic cells (Hiromatsu et al., 2002). Also, among the multiple canine CD1a molecules, two are expressed in skin and thymus, and one is expressed in thymus only.

Recently, we have used cattle (*Bos taurus*) as a model for the study of CD1-presented mycobacterial lipid antigens (Nguyen et al., 2009; Nguyen et al., 2011). In the bovine CD1 locus one functional CD1A gene, three functional CD1B genes (CD1b1, CD1b3, and CD1b5), and one functional CD1E gene have been described (Van Rhijn et al., 2006). In addition there are two CD1B pseudogenes (CD1B2 and CD1B4) and two CD1D genes that were previously thought to be pseudogenes but that were subsequently shown to be expressed at the cell surface [Nguyen, 2011, submitted]. No CD1C gene is present in the bovine genome.

Cattle are sensitive to natural infection with *M. bovis* and *M. avium paratuberculosis*, and we have shown that lipid antigens are recognized during these infections. In addition, immunizations with a glycolipid that was already known to be presented by human CD1b during leprosy and tuberculosis, glucose monomycolate, showed that this compound is also immunogenic in cattle. In humans, this lipid is presented by the only human CD1b isoform. In cattle T cell recognition of glucose monomycolate could be blocked by the monoclonal antibody BCD1b.3, which is known to recognize bovine CD1b3, but whether it also recognizes other bovine CD1b molecules is unknown.

Since the description of the bovine CD1 locus, only the CD1a, CD1b3, and CD1d1 transcripts were cloned and the recognition by some existing monoclonal antibodies of these two proteins was demonstrated (Van Rhijn et al., 2006)[Nguyen, 2011, submitted]. A considerable number of monoclonal antibodies have been raised against ovine and bovine thymocytes and intestinal epithelial cells in the past, and their target molecule was suggested to be CD1 based on expression on thymocytes, and on immunoprecipitation of two protein chains with masses corresponding approximately with a class I-like heavy chain and B2M, and in one case based on recognition of human CD1b (CC20) (Howard et al., 1993b, Howard et al., 1993a, Howard and Naessens, 1993, Parsons and MacHugh, 1991, Parsons et al., 1991, O'Reilly and Splitter, 1989, MacHugh et al., 1988, Hopkins et al., 2000, Rhind et al., 1996).

To be able to interpret ex vivo antibody staining patterns, we dissect the specificity of the monoclonal antibodies CC14, CC20, CC122, SBU-T6 (also called 20.27), BCD1b.3, CC118, and CC43 using recombinant proteins in Elisa and transfected cell lines. In addition, using gene-specific PCR, we describe the differential transcription of bovine CD1b1, CD1b3, CD1b5, CD1d, and CD1a, and a newly discovered bovine CD1a molecule, which we named CD1a2, on sorted DCs, B cells, and in vitro cultured DCs.

Materials and methods

Animals, tissue samples, and cDNA synthesis

For flow cytometry and nucleic acid extractions, bovine tissue samples (prescapular lymph node, thymus, peripheral blood drawn from the jugular vein, and pseudoafferent lymph) were collected from a Holstein-Frisian bull of three months of age. The procedure for obtaining pseudoafferent lymph has been described previously (Van Rhijn et al., 2007). At the time of tissue collection and blood sampling, these animals showed no clinical signs of disease. PBMC were prepared using a standard Ficoll density gradient. Single cell suspensions of bovine tissues were used without further purification. RNA isolation and cDNA synthesis was performed on 2*104 sorted cells. RNA was isolated with the Qiagen RNAEasy kit, followed by first strand cDNA synthesis with Multiscribe reverse transcriptase.

Identification of novel bovine CD1 genes

The first description of the set of bovine CD1 genes was based on the bovine genome assembly Btau3.1 (Van Rhijn et al., 2006). In that assembly most CD1 genes were assigned to "chromosome fragments". At the time of writing the current manuscript the Btau4.0 assembly was available and we repeated the searches for CD1 genes as described (Van Rhijn et al., 2006). In short, BLAST searches were performed with the α1 and α2 domains of all human CD1 isoforms, and the results were analyzed in phylogenetic trees to be able to assign the correct CD1 isoform to the resulting bovine genes.

Molecular cloning of bovine CD1b1 and CD1b5 transcripts

Full length CD1b3 had been cloned previously (Van Rhijn et al., 2006). Bovine CD1b1 and CD1a2 were obtained as ESTs (EH164202 and BC149754, respectively) that were identified by performing a BLAST search with the predicted CD1b1 and CD1a2 transcript and confirmed by sequencing (IMAGE clones 8452304 and 8436847 respectively, from Geneservice UK). No full length CD1b5 transcript was present in the databases. Based on the genomic sequence of CD1b5, primers for CD1b5 were designed:
CD1b5FullLengthFor: 5'-AGTTCTACTTCCCATTGAAATGCTGCTTCTG-3';
CD1b5FullLengthRev:5'-GTAATTGCTCTAAATGGGAAAGAAGACACG-3',
as described previously (Girardi et al., 2010). PCR was performed with PFU Turbo polymerase (Stratagene) according to the protocol of the manufacturer under the following cycling conditions: an initial denaturation of 7 min. at 95°C, followed by 35 cycles of 30 sec. at 95°C, 45 sec. at 58°C, 1 min. at 72°C, followed by a final elongation step of 7 min. at 72°C. PCR products were cut from an agarose gel, purified, and ligated in a Topo4blunt vector for sequencing and in pcDNA3.1 for expression.

Monoclonal antibodies, facs staining, and cell sorting

Bomac cells were transiently transfected with cloned full length wild type CD1b1, CD1b3, and CD1b5 in pcDNA3.1 using Fugene-6 reagent (Roche) according to the manufacturer's protocol, and analysed 48 hours after transfection. K562 cells transfected with bovine CD1d

and human CD1a, and C1R cells transfected with bovine CD1b and CD1a were described previously (Girardi et al., 2010, de Jong et al., 2010). The anti bovine CD1 antibodies CC20 (IgG2a), CC43 (IgG2b), and CC122 (IgG1) were kindly provided by Dr. C. J. Howard, Compton, UK; 20.27 SBU-T6 (IgG1) was obtained from the European Collection of Cell Cultures; CC14 (IgG1) and CC118 (IgG1) were kindly provided by Dr. J. C. Hope, Compton, UK; BCD1b.3 (IgG1) was provided by Dr. D. B. Moody, Brigham and Women's Hospital, Boston, goat anti mouse FITC and goat anti mouse PE was obtained from Becton Dickinson. PE-labelled anti Ig light chain antibody IL-A59 were used as DC and B cell markers respectively (both from Serotech).

DC culture

DC were cultured as described (Hope et al., 2003). In short, PBMC were prepared using Histopaque ficoll (Sigma-Aldrich). The CD14+ cells were positively selected with anti human CD14 MicroBeads (Miltenyi Biotec) on a MACS separator. The CD14+ cells were then cultured in 24-well plates at a density of 106 cells/ml in RPMI/10%FCS supplemented with bovine IL-4 and bovine GM-CSF. Immature DC cells were analyzed by flowcytometry and CD1 gene-specific PCR.

Gene-specific PCR

To be able to confirm transcription of CD1A1, CD1A2, CD1B1, CD1B3, CD1B5, and CD1d, we designed the following gene-specific primers: CD1a1For: 5'-CCATTTCCTTCAAAGTCATCTGTGTCC -3'; CD1a1Rev: 5'-AAGAGGAAATGTGGGCAGGTATCAC -3'; CD1a2For: 5'-CTCATCATTTTATAGCCAATTCTTTGC -3'; CD1a2Rev: 5'-AAGAGGAAATGTGGGCAGGTATCAC -3'; CD1b1For: 5'-TTCACTTGCGGAATGCAGGATCGA-3'; CD1b1Rev: 5'-GCACAAAACTTCTGCCCCCTGAA-3'; CD1b3For: 5'-TGATGATGAGGTGACTGAGCTGGT-3'; CD1b3Rev: 5'-GCTGTCTGGTGCAGGCACACAA-3'; CD1b5For: 5'-TTCAGTGATGAGGAGGTGGCTGAGA-3'; CD1b5Rev: 5'-AGTAATGAATACACAAAACCACTGTGCATCA-3'; CD1dFor: 5'-GGCAGCATACACTTCTGGTTTATCG-3'; CD1dRev: 5'-CAGGCCCAGGACTGGGGCCACTGG-3'. The two CD1D genes that are present in cattle are so highly homolous that we could not design primers to distinguish them. The forward primers were located in the α1 domains and the reverse primers in the α2 domains because most of the differences between CD1 sequences are located there. The specificity of these gene-specific PCRs was confirmed by using vectors containing full length cloned CD1b1, CD1b3, CD1b5, and CD1a as a template for each of these gene specific PCRs. Using the same PCR conditions as described for the cloning of CD1b5, but with an annealing temperature of 70°C for CD1b1, CD1b3, and CD1b5, and 62°C for CD1a, PCR products could only be detected using the vector DNA with the cloned gene for which the primer set was designed as a template, and not using the other CD1 genes as a template.

Recombinant CD1 protein design and expression

Single chain bovine CD1b1, CD1b3, and CD1b5 constructs were generated as described previously for human CD1d (Im et al., 2004) by linking the sequence for the entire bovine β2-microglobulin (B2M) at the N-terminal sequence of the α1 domain of the CD1 proteins by the insertion of a sequence encoding a flexible peptide linker (GGGSGGSGSGGGA). Sequence elements encoding the BirA enzymatic biotinylation site (HVGLNDIFEAQKIEWHEGH), and a hexahistidine (HHHHHH) tag were added to the C terminus of the α3 domain of the CD1 proteins. In addition, we used the CD1b5 splice variant lacking part of the α3 domain. The amino acid sequences of the fusion proteins are shown in Supplementary Figure 1. The supernatants of HEK 293 T cells transiently transfected with these constructs were collected and purified on Ni-NTA columns. The size and concentration of the recombinants proteins were determined using SDS-PAGE and Coomassie dye.

PNGase-F treatment and ELISA

All ELISAs were performed on Costar high bind 96-well plates. For ELISA, coating of single chain recombinant proteins (0.5 μg/well) was followed by washing, blocking with PBS/1%BSA/0.05% tween 20 (Merck), incubation with detecting antibody, washing, incubation with polyclonal rabbit anti mouse IgG-HRP (Roche), washes with PBS/0.05% tween and PBS, and incubation with ABTS solution (Roche). ELISA plates were read at 405 nm. Bovine single chain CD1b3 was deglycosylated using the PNGaseF kit from New England Biolabs. The native deglycosylation reaction was performed in G7 reaction buffer for five days at 37°C. For mock treated protein, PNGase was omitted, but otherwise treated equally. The same samples that were used for ELISA were analyzed on a denaturing 14% SDS-page gel followed by Coomassie staining, together with samples that were deglycosylated in glycoprotein denaturing buffer, NP40 buffer, and G7 reaction as a control for maximum deglycosylation obtained under denaturing conditions.

Results

Identification of two new CD1A genes and comparison of transcripts of multiple bovine CD1B and CD1A genes

Blast searches with the α1 and α2 domains of all human CD1 isoforms in the Btau4.0 assembly of the bovine genome revealed that, like in the previous versions, the CD1 locus had not been assembled yet, contained big gaps, and some of the CD1 genes are found on “chromosome fragments”. We found a novel CD1A gene that is predicted to be translated into protein. We called the newly identified CD1A gene CD1A2, and the gene that was previously named CD1A was renamed CD1A1. The most notable differences between the three CD1A genes is in their predicted cytoplasmic tails.

It is likely that many of the “new” CD1 genes in a new assembly of the bovine genome are part of sets of two allelic forms of CD1 genes that happen to be present in the animal used for sequencing. The small variations between the genes may not have been recognized in the previous assembly.

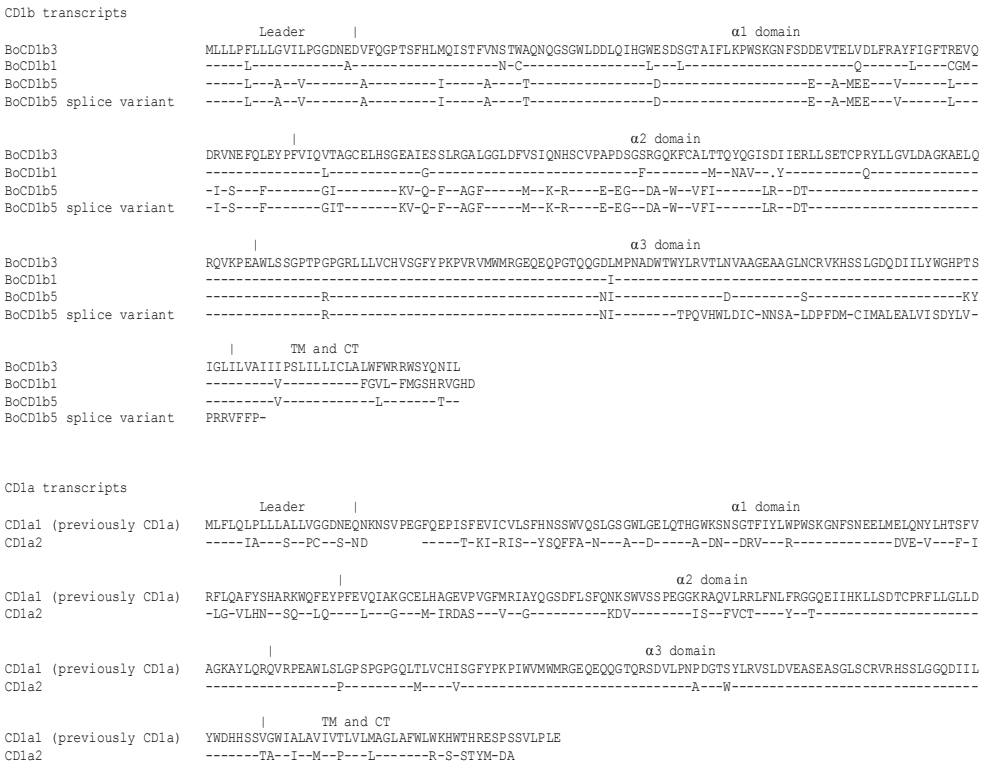

Figure 1. Sequences of multiple bovine CD1b and CD1a molecules Bovine CD1b and CD1a cDNA sequences were translated and aligned. The accession numbers of the sequences included in this figure are: BoCD1b3: DQ192542; BoCD1b1: EH164202; Bocd1b5 splice variant: (JN033695); Bocd1b5: GU325785; BoCD1a(1): DQ192541; BoCD1a2: BC149754. TM: Transmembrane part; CT: Cytoplasmatic tail; - identical residue.

Cloning of full length CD1b5 (GU325785) was described earlier (Girardi et al., 2010). During the cloning of full length CD1b5, a splice variant was obtained that lacks part of the α3 domain (JN033695). This splice variant was also present in the EST database (EH160235, which is a partial CD1b5 sequence), IMAGE clone 8444381, from Geneservice UK, which became apparent when we obtained the clone and sequenced the complete insert. Full length CD1b5 transcript was absent from the databases. Figure 1 shows an alignment of the predicted amino acid sequences of the full length bovine CD1b transcripts and the CD1b5 splice variant, (upper panel), and the two full length bovine CD1a transcripts (lower panel). Note that the cytoplasmic tails of CD1b3 and CD1b5 are very similar to each other, with possibly a YXXZ motif and a dileucine motif, but the cytoplasmic tail of CD1b1 is very different and does not seem to contain any known motifs. The cytoplasmic tails of CD1a1 and CD1a2 are very different from each other and from the cytoplasmic tail of human CD1a, and do not contain any known motifs.

Characterization of the specificity of monoclonal antibodies using recombinant proteins and transfectant cells

Full length cloned CD1b1, CD1b3, and CD1d transcripts were used to transiently transfect the bovine macrophage cell line Bomac (Figure 2A). Mock transfected and transfected cells were stained with the unlabeled monoclonal antibodies CC14, CC20, CC43, CC118, CC122, SBU-T6, and BCD1b3, followed by goat anti mouse-PE. CD1b3 was recognized by all of these antibodies except CC43 and CC118. CD1b5 was only recognized by SBU-T6 and BCD1b3. CD1d was recognized by CC43, CC18, and SBU-T6. Transfections of Bomac cells with CD1b5 did not lead to any detectable antibody staining. However, it was not clear whether this was caused by inefficient transfection and/or translation or lack of recognition by antibodies. Also, we did not detect any intracellular staining of CD1b5 (data not shown).

As an alternative approach, we produced tagged fusion proteins of bovine β2M and CD1. The cloned and sequenced full length CD1b1, CD1b3, and CD1b5 transcripts, and the CD1b5 splice variant were used as starting material for the cloning and production of soluble β2M-CD1 single chain proteins (Supplementary Figure 1).

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                                |      Leader      |                                                β2M
scBoCD1b5 splice variant        MARFVALVLLGLLSLSGLDAIQRPPKIQVYSRHPPEDGKPNYLNCYVYGFHPPQIEIDLLKNGEKIKSEQSDLSFSKDWSFYLLSHAEFTPNSK
scBoCD1b5 full lenght           MARFVALVLLGLLSLSGLDAIQRPPKIQVYSRHPPEDGKPNYLNCYVYGFHPPQIEIDLLKNGEKIKSEQSDLSFSKDWSFYLLSHAEFTPNSK
scCD1b3                         MARFVALVLLGLLSLSGLDAIQRPPKIQVYSRHPPEDGKPNYLNCYVYGFHPPQIEIDLLKNGEKIKSEQSDLSFSKDWSFYLLSHAEFTPNSK
scBoCD1b1                       MARFVALVLLGLLSLSGLDAIQRPPKIQVYSRHPPEDGKPNYLNCYVYGFHPPQIEIDLLKNGEKIKSEQSDLSFSKDWSFYLLSHAEFTPNSK

                                        |   linker     |                                                α1 domain
scBoCD1b5 splice variant        VKHVTLEQPRIVKWDRDLGGGGSGGSGSGGGGS EDAFQGPTSFHLIQISTFANSTWTQNQGSGWLDDLQIHGWDSDSGTAIFLKPWSKGNFSI
scBoCD1b5 full lenght           VKHVTLEQPRIVKWDRDLGGGGSGGSGSGGGGS EDAFQGPTSFHLIQISTFANSTWTQNQGSGWLDDLQIHGWDSDSGTAIFLKPWSKGNFSI
scCD1b3                         VKHVTLEQPRIVKWDRDLGGGGSGGSGSGGGGSNEDVFQGPTSFHLMQISTFVNSTWAQNQGSGWLDDLQIHGWESDSGTAIFLKPWSKGNFSI
scBoCD1b1                       VKHVTLEQPRIVKWDRDLGGGGSGGSGSGGGGS ADVFQGPTSFHLMQISTFVNNTCAQNQGSGWLDDLQIHGLESDLGTAIFLKPWSKGNFSI

                                                |                                                α2 domain
scBoCD1b5 splice variant        EELFRVYFIGFTLEVQDIVSEFQFEYPFVIQGITGCELHSGKVIQSFLRAGFGGLDFMSIKNRSCVPEPEGGSDAQWFCVFITQYQGILRIIDT
scBoCD1b5 full lenght           EELFRVYFIGFTLEVQDIVSEFQFEYPFVIQGITGCELHSGKVIQSFLRAGFGGLDFMSIKNRSCVPEPEGGSDAQWFCVFITQYQGILRIIDT
scCD1b3                         VDLFRAYFIGFTREVQDRVNEFQLEYPFVIQVTAGCELHSGEAIESSLRGALGGLDFVSIQNHSCVPAPDSGSRGQKFCALTTQYQGISDIIEF
scBoCD1b1                       QDLFRAYLIGFTCGMQDRVNEFQLEYPFVIQLTAGCELHSGEAIGSSLRGALGGLDFVSIQNHSCVPAPDSGFRGQKFCALMTQNAVIS YIEF

                                                |                                                α3 domain
scBoCD1b5 splice variant        PRYLLGVLDAGKAELQRQVKPEAWLSSGPTPRPGRLLLVCHVSGFYPKPVRVMWMRGEQEQPGTQQGNIMPNADWTWTPQVHWLDICGN
scBoCD1b5 full lenght           PRYLLGVLDAGKAELQRQVKPEAWLSSGPTPRPGRLLLVCHVSGFYPKPVRVMWMRGEQEQPGTQQGNIMPNADWTWYLRVTLDVVAGEAAGLS
scCD1b3                         PRYLLGVLDAGKAELQRQVKPEAWLSSGPTPGPGRLLLVCHVSGFYPKPVRVMWMRGEQEQPGTQQGDLMPNADWTWYLRVTLNVAAGEAAGLN
scBoCD1b1                       PQYLLGVLDAGKAELQRQVKPEAWLSSGPTPGPGRLLLVCHVSGFYPKPVRVMWMRGEQEQPGTQQGDIMPNADWTWYLRVTLNVAAGEAAGLN

                                            |    Bir tag    | His tag
scBoCD1b5 splice variant        ALLDP              GHVGLNDIFEAQKIEGHHHHHH
scBoCD1b5 full lenght           SLGDQDIILYWGHPKYI   GHVGLNDIFEAQKIEGHHHHHH
scCD1b3                         SLGDQDIILYWGHPTSIGLIGHVGLNDIFEAQKIEGHHHHHH
scBoCD1b1                       SLGDQDIILYWGHPTSIGLIGHVGLNDIFEAQKIEGHHHHHH
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Supplementary Figure 1. Alignment of single chain CD1 proteins used in this study.

ELISA plates were coated with the recombinant proteins and recognition of the proteins by the aforementioned panel of seven monoclonal antibodies was tested (Figure 2B). Recombinant CD1b1 protein is recognized by SBU-T6 and BCD1b3, but not by the other antibodies; recombinant CD1b3 protein is recognized by all antibodies, except CC118 and CC43, and recombinant CD1b5 protein is only recognized by SBU-T6, and the naturally occurring splice variant is not recognized by any of the antibodies.

CD1 gene expression by B cells and DCs

Because the considerable differences among the antigen binding domains and cytoplasmic

tails of the bovine CD1 molecules will likely have implications for their antigen capture and presenting capacities, we set out to determine which CD1 genes are transcribed by professional APCs in cattle. In addition, these data will clarify whether cellular expression patterns are conserved among species. Long before it was known that cattle do not have a

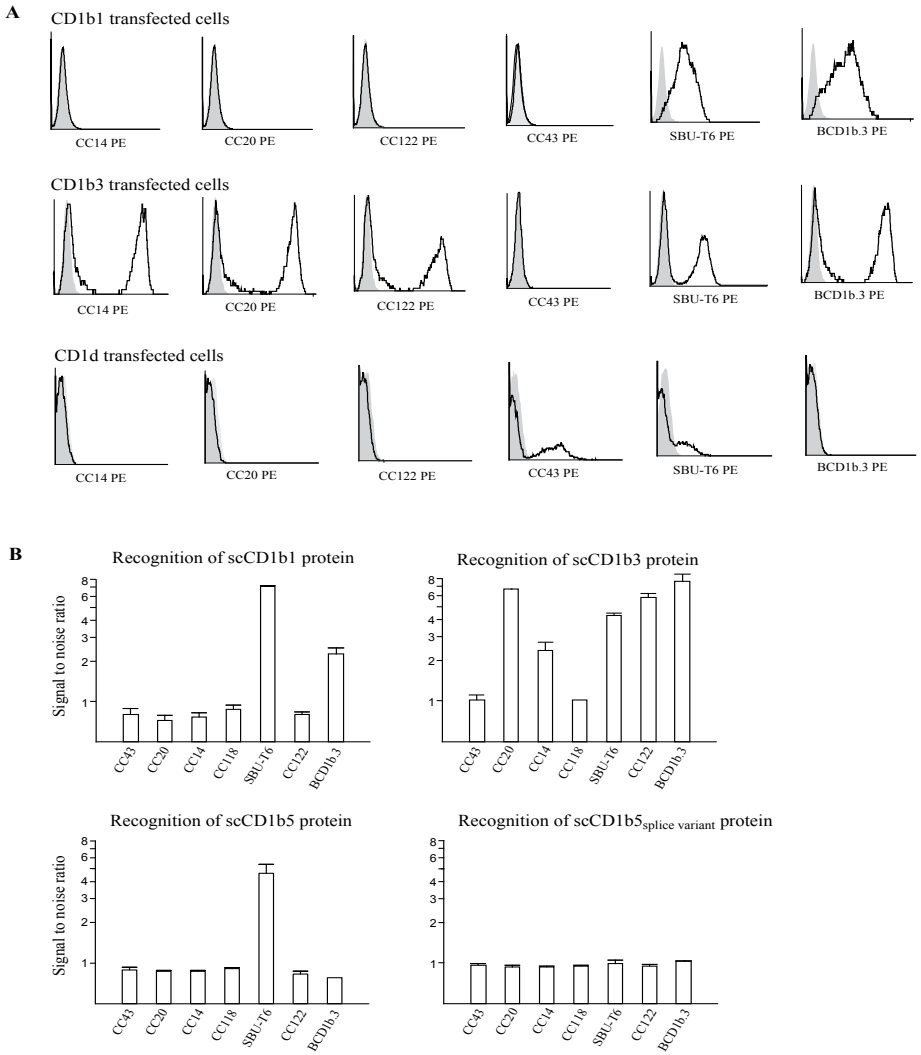

Figure 2 Antibody recognition of transfected cells and recombinant proteins. Bomac cells were mock transfected (thin black line, grey shade) or transiently transfected with bovine CD1b1, CD1b3, and CD1d (thick black line) and stained with the monoclonal antibodies CC14, CC20, CC43, CC118, CC122, SBU-T6, and BCD1b3 (A). Single chain recombinant proteins of CD1b1, CD1b3, CD1b5, and a splice variant of CD1b5 with part of α3 domainn deleted, were coated on Elisa plates and tested for reactivity with the monoclonal antibodies CC14, CC20, CC43, CC118, CC122, SBU-T6, and BCD1b3. The signal to noise ratio is defined the means of the duplicate OD values obtained from wells coated with single chain CD1 proteins divided by the means of the OD values obtained by wells without single chain CD1 protein (B).

CD1C gene, it has been suggested that the SBU-T6 antibody might recognize the bovine and ovine homolog of CD1c (Dutia and Hopkins, 1991). The antibody SBU-T6 has later been characterized as a "pan-CD1" antibody, recognizing most or all bovine and ovine CD1 molecules. Indeed, it has previously been shown to recognize boCD1b3 and boCD1a (Van Rhijn et al., 2006), the only boCD1 molecules cloned at that time, and it has been used to immunoprecipitate ovine CD1e (Rhind et al., 1999), and in the previous paragraph we show

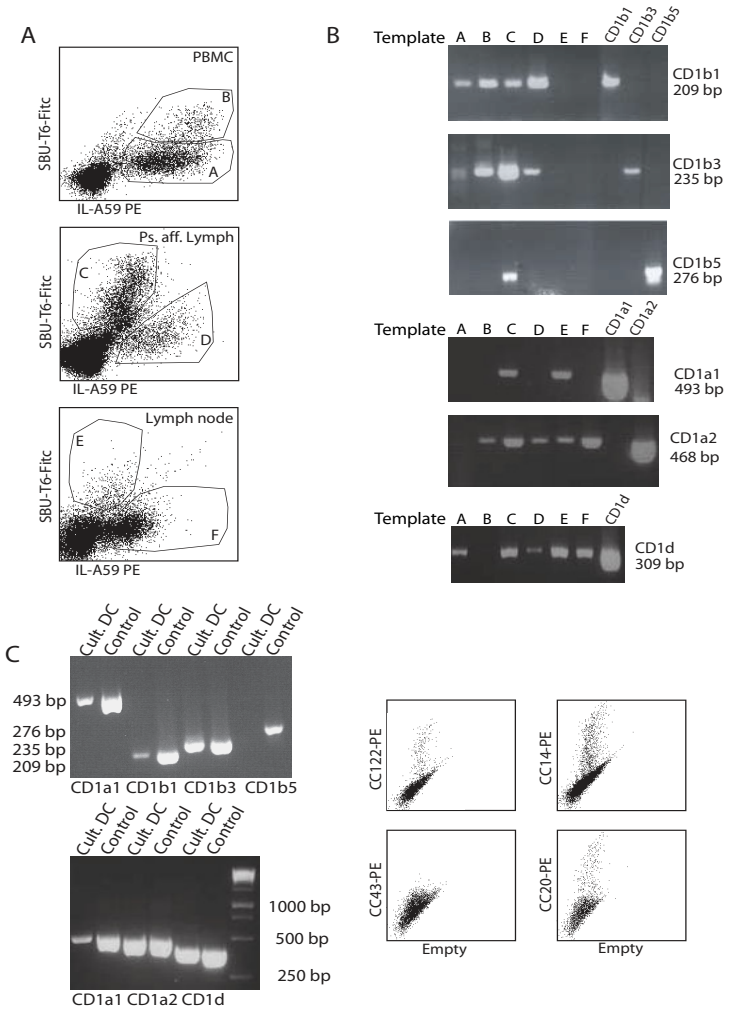

Figure 3. Transcription of individual CD1 genes by bovine APCs. Cells from PBMC (A, top panel), pseudoafferent lymph (A, middle panel), and prescapular lymph node (A, lower panel) were sorted based on expression of the Ig light chain (IL-A59), and CD1 (SBU-T6). The cDNA synthesized from the sorted cell popluations was subected to CD1 gene-specific PCR (B). Immature DCs were derived in vitro by treatment of monocytes with GM-CSF and IL-4 (C). The CD1 expression of the DCs was confirmed and cDNA derived from the DCs was subjected to CD1 gene-specific PCR. The specificity control material included in these studies consisted of vector with the designated cloned CD1 transcript.

that it also recognizes boCD1d, boCD1b1 and boCD1b5, confirming its pan-CD1 specificity. In fact, SBU-T6 also recognizes a canine CD1a and CD1b molecule (Looringh van Beeck et al., 2008), and two guinea pig CD1b molecules (Hiromatsu et al., 2002). To check for CD1 gene transcription by bovine B cells and DCs, we sorted SBU-T6 expressing cells from fresh PBMC, pseudoafferent lymph, and lymph node (Figure 3). Cells that were double positive for SBU-T6 and IL-A59 (Ig light chain), were considered CD1-expressing B cells, and cells that were SBU-T6 positive and IL-A59 negative were considered CD1-expressing DCs (Figure 3A). Alternatively, we used CD172a (MyD1) as an independent marker for DC sorting.

Gene specific PCR primers were designed and validated on cloned CD1b1, CD1b3, CD1b5, CD1a1, CD1a2, and CD1d transcripts. The primers amplify one gene of one isoform, and do not cross react with other CD1 genes. Application of these gene specific PCRs to cDNA synthesized from RNA from sorted APC populations showed that CD1b1 and CD1b3 had a much broader expression pattern than CD1b5, which was only found in DCs sorted from pseudoafferent lymph (Figure 3B). CD1b was not expressed on lymph node-derived cells. CD1a1 was only present on DCs, but CD1a2 had a broader expression pattern, including lymph node B cells. CD1d has a very broad expression pattern. PCR for β-actin showed that the amount of input cDNA of the sorted cell populations was similar (data not shown). In addition to these ex vivo cells, in vitro cultured DCs were characterized by facs and PCR (Figure 3C). Even though the percentage of DCs in these cultures was not very high, the DCs stained very strongly with CC20, CC14, and CC122, and weakly with CC43. The cultured DCs transcribe all the CD1 genes, except CD1b5.

The SBU-T6 epitope is dependent on glycosylation

We have recently reported the human cell line K562 transfected with bovine CD1d [Nguyen, 2011, submitted]. That cell line is recognized by CC43 and CC118 [Nguyen, 2011, submitted], but interestingly not by SBU-T6 (I. Van Rhijn, unpublished). Here we report that Bomac bovine macrophage cells transfected with bovine CD1d are recognized by CC43, CC118, and SBU-T6. One obvious difference between those experiments is the species from which the cell line was derived. To study whether this phenomenon was more generally true, we also checked SBU-T6 recognition of bovine CD1b3 in human C1R cells, knowing that bovine CD1b3 is recognized by SBU-T6 when transfected into Bomac cells. Clearly, bovine CD1b3 is not at all recognized by SBU-T6 when expressed by human C1R cells, but it is recognized by CC14, CC20, CC122, and BCD1b3 in this context (data not shown). Initially, this led us to the hypothesis that the epitope for SBU-T6 is formed by bovine CD1 in complex with bovine β2M, but not in complex with human β2M. An important argument against this hypothesis was the observation that SBU-T6 recognizes huCD1a on K562 cells, but not on C1R cells, which are both human cell lines (data not shown).

An alternative hypothesis is that the recognition of CD1 by SBU-T6 is influenced by the glycosylation status of the molecules. To test this we digested single chain CD1b3 under native conditions with PNGase F or mock treated it and tested its recognition by SBU-T6 and CC20 (Figure 4). Even though SDS-gel followed by Coomassie staining showed that the protein was approximately for 80% digested (A), the ELISA signal using SBU-T6 as a detecting antibody was greatly reduced, while the ELISA signal using CC20 as a detecting antibody

was barely affected (B). From this we conclude that a PNGase F sensitive glycosylation is part of the SBU-T6 epitope.

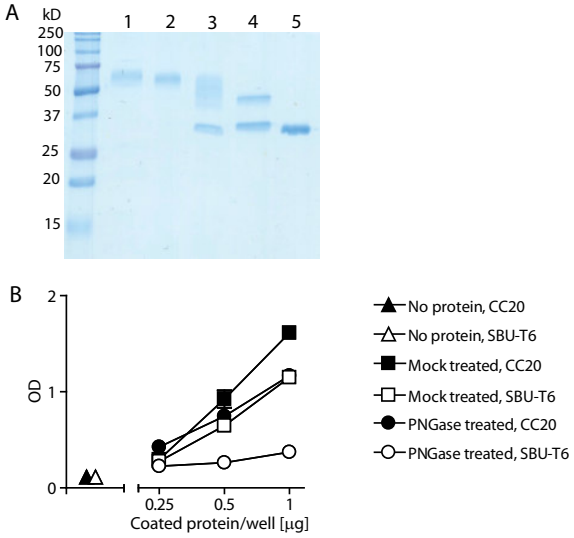

Figure 4. The influence of deglycosylation on CD1 recognition by SBU-T6. Single chain bovine CD1b3 was treated with PNGase F and analysed by SDS-Page followed by Coomassie stain (A). Lane 1: untreated protein; Lane 2: mock treated protein; Lane 3: PNGase F treated protein, non denaturing conditions during PNGase F treatment; Lane 4: PNGase F treated protein, denaturing conditions during PNGase F treatment; Lane 5: PNGase F enzyme only. The non denatured PNGase F treated protein and mock treated protein were coated on an elisa plate and analysed for the reactivity with SBU-T6 and CC20 (B).

Discussion

The widely variable numbers of CD1 genes among mammalian species is puzzling. We have recently shown that bovine group 2 CD1 (CD1d) is expressed, but does not seem to fulfill the model function of presenting α-galactosylceramide to NKT cells and thereby cause the release of cytokines [Nguyen, 2011 submitted]. One of the questions we had is whether group 1 and group 2 molecules, even when the numbers of genes, and possibly their functions are different, follow the group 1 and group 2 expression patterns that are known from humans and mice. Another question that we were interested in was whether the genes that belong to one CD1 isoform are differentially expressed in cattle.

We found that the bovine CD1 genes are differentially expressed. However, the general expression patterns of group 1 and group2 in cattle are conserved. Bovine B cells transcribe CD1d, and stain with CC43 and SBU-T6, so we conclude that the CD1d transcript is translated into CD1d protein on B cells. Unlike in humans, we have shown here that CD1b1 and CD1b3 are transcribed by B cells but they are known to not stain with the CD1b specific antibodies CC14, CC20 (Howard et al., 1993a, Howard and Naessens, 1993), BCD1b [Van Rhijn, unpublished], so there appears that the CD1b genes are transcribed but not translated into protein. CD1a2, again, unlike in humans, is transcribed by bovine B cells. We did not

have an antibody that distinguishes CD1a2 protein from CD1d protein on B cells, so we do not know whether bovine CD1a2 protein is present on B cells. In conclusion, the expression of CD1d on B cells is a conserved feature. CD1c is also expressed by human B cells, but cattle do not have a gene for CD1c. Remakably, bovine B cells transcribe CD1b1, CD1b3, and CD1a2, which human B cells do not.

We have shown here that CD1b transcription DCs in lymph nodes is not detectable while CD1b transcription in pseudoafferent lymph cells is high. This latter finding is in agreement with CD1b protein expression on (pseudo)afferent lymph DCs as reported by us and others (Van Rhijn et al., 2007, Dutia and Hopkins, 1991, Hope et al., 2003), and by the inability of lymph node cells to present the CD1b-presented antigen glucose monomycolate to T cells as compared to PBMC, which do present glucose monomycolate to T cells (Nguyen et al., 2009). From this we conclude that pseudoafferent DCs strongly downregulate their CD1b expression upon entry into the lymph node.

SBU-T6 is an antibody that recognizes all CD1 isoforms but not under all circumstances. The human cell lines K562 transfected with bovine CD1d and C1R with bovine CD1b3 are not recognized by SBU-T6 while Bomac cells transfected with these molecules are. We have found a strong effect of deglycosylation of CD1 on recognition by SBU-T6. It is possible that the epitope of SBU-T6 is a conformational epitope formed by CD1b protein and its glycosylation. Knowing this does not affect the interpretations and conclusions of the work presented here, but it has implications for interpretation of negative staining, especially of transfected cells.

Finally, our CD1 transcription data and and the data that definitively confirmed the specificity of the monoclonal antibodies CC14, CC20, CC43, CC118, CC122, and BCD1b3 allow us to fine-tune the interpretations of previously published histological and flow cytometric studies using these antibodies (Parsons et al., 1991, Dutia and Hopkins, 1991, Mackay et al., 1985, Howard et al., 1993b, O'Reilly and Splitter, 1989). Our studies also confirm the general conclusion that group 2 CD1 (CD1d) expression patterns are conserved among species, despite the fact that the function of bovine CD1d has not been confirmed to be equivalent to the function of human and murine CD1d, and that group 1 CD1 molecules are differentially expressed among professional APCs, and that there might be a discrepancy between group 1 CD1 transcription and protein expression.

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Chapter 7

Summarizing discussion

The work presented in this thesis addresses the potential of mycobacterial lipid vaccination against mycobacterial infections. Cattle, natural hosts of *M. avium subsp paratuberculosis* (MAP) and *M. bovis* were used throughout this thesis because they are an important target species, as well as an animal model for human tuberculosis. Since CD1 is likely to play a role in presentation of mycobacterial lipid antigens, the presence and expression of bovine CD1 genes and their function in natural infection was investigated. CD1 restricted lipid antigen specific T cell responsiveness was studied in naturally infected cattle and led to the identification of the glycolipid glucose monomycolate (GMM) as an immunodominant lipid compound of MAP. This immunogenic mycobacterial lipid was subsequently used in two different vaccine formulations to examine induction of specific immune responses.

1. CD1 expression in cattle

The CD1 lipid antigen presentation system is part of the immune system of vertebrates, and can be traced back as far as the origin of birds in the phylogenetic tree (Porcelli, 2005). The human CD1 family of antigen presenting molecules has been well characterized with CD1a, CD1b, CD1c (group 1), and CD1d (group 2). However this system in humans is not conserved with exactly the same family members among mammalian species. Observations from T cell responses against *M. tuberculosis* lipids in humans indicated that these were mainly mediated by presentation via CD1 molecules (Ulrichs et al., 2003). Although mouse models have been used very frequently for human TB vaccine research they are not a good model for CD1 based vaccine trials since only CD1d molecules exist in mice. Therefore, alternative animal models, like cattle, are preferred to assess lipid subunit vaccine candidates for mechanistic studies and potential protective effects in TB.

The bovine CD1 molecules were investigated in detail in this thesis to contribute to understanding of CD1-restricted immunity against mycobacterial lipid antigen (chapter 5 and chapter 6). Others have shown that CD1b in humans is responsible for presentation of GMM to T cells. In contrast to humans, who have only one CD1 gene of each isotype, our studies showed that cattle have at least two CD1a and five CD1b genes, and of these, two CD1a and three CD1b molecules are expressed at protein level. The crystal structure of the bovine CD1b3 molecules has been shown be big enough to fit GMM (Girardi et al., 2010). The reason for having multiple CD1 genes in cattle and other animals remains unknown. Among CD1a and CD1b isotypes, molecules are different in their antigen binding domains and in their cytoplasmic tails, suggesting that they may traffic differentially in the cell and may bind different sizes of antigens. In this study we showed the expression pattern of bovine CD1 molecules in antigen presenting cells (B cells and DC) that closely resembles the expression pattern in humans and meets the requirement for the presentation of mycobacterial lipid antigens to bovine T cells (**chapter 5**).

In previous studies, it was shown that the bovine CD1d gene is not likely to be translated into CD1d proteins (Van Rhijn et al., 2006). In addition, it is not clear whether NKT cells that are known to recognize lipid antigens presented by CD1d protein in other species, are present in cattle. Human and murine CD1d/NKT systems are structurally and functionally highly conserved, which is illustrated by the fact that even cross-species antigen presentation of α-galactosylceramide can take place (Benlagha et al., 2000), In **chapter 6** the conservation of

the bovine CD1d/NKT system was studied. In cattle in vivo application of α-galactoceramide did not lead to a rise in serum cytokine levels or in the body temperature, which are hallmarks of CD1d/NKT activation in other species. The presence of NKT cells in cattle remains debatable, but it is very clear from our observations that the CD1D gene of this species has an unusual intron/exon structure and signal peptide and is not likely to be part of a classical, functionally conserved CD1d/NKT system, even though the bovine CD1d molecule is expressed (**Chapter 6**). Whether or not the apparent lack of the conserved CD1d/NKT system in cattle has a direct or indirect effect on bovine immune responses against mycobacterial lipid antigen is unknown. Except for phosphatidylinositol mannoside that is presented by both CD1b and CD1d molecules, most of the mycobacterial lipd antigens originating from *M. tuberculosis* have been shown to be recognized by T cells by CD1a, CD1b, and CD1c presentation in humans (Beckman et al., 1994; Sieling et al., 1995; Moody et al., 1997; Moody et al., 2000; Fischer et al., 2004; Gilleron et al., 2004; Moody et al., 2004). Cattle have CD1a and CD1b that are appropriate to present mycobacterial lipid antigens to T cells. Cattle therefore provide a valuable animal model to study the role of group 1 CD1 in host defense against mycobacteria and to test the potential of CD1-restricted lipid vaccines.

In summary, CD1a and CD1b molecules, that have been shown to present mycobacterial lipid antigens in humans, are present and expressed in cattle. This thesis describes bovine CD1d expression for the first time and even though its function is not yet clear, it does not seem to include classic α -galactosylceramide presentation and activation of NKT cells. The expression of bovine CD1 molecules that we described in this thesis will provide basic knowledge for development of CD1-presented lipid antigens as subunit vaccine candidate.

2. Mycobacterial lipid specific immunity in naturally infected cattle

Cattle used in our studies are natural hosts for *M. bovis* as well as MAP. *M. bovis* is the pathogen causing tuberculosis in cattle and most other mammalian species including humans, whereas MAP causes bovine paratuberculosis, a chronic intestinal inflammation causing substantial economic losses worldwide. Understanding of the natural host's immune reactivity against mycobacterial lipids after natural infection may aid lipid subunit vaccine development. Moreover, naturally infected cattle can be a useful model for human mycobacterial diseases and vaccine development (Van Rhijn et al., 2008). To date, there is only little understanding of *in vivo* behaviour of T cells restricted by CD1a, CD1b, and CD1c during tuberculosis. However, the few studies on lipid reactivity *ex vivo* show that T cells from humans exposed to *M. tuberculosis* respond to *M. tuberculosis* total lipid extract and GMM, which suggests the generation of a CD1-restricted adaptive immune respons to this mycobacterial species (Ulrichs et al.,2003).

We show that mycobacterial lipids were strongly recognized in vitro by T cells of cattle that were naturally infected with MAP or M. bovis (**chapter 2)**. While glycolipid glucose monomycolate (GMM) was identified as an immunodominant lipid compound in MAP infected animals, and shown to be presented by bovine CD1b, this compound is produced in lower amounts by *M. bovis* and apparently not immunogenic in the setting of natural infection with *M. bovis*. Low cross-reactivity of T cell responses against crude lipid extracts from these two species of mycobacteria needs to be considered in development of mycobacterium species specific lipid vaccines.

Strong immune responsiveness against lipid antigens of mycobacteria may represent important mechanisms of defence against infection. This immune response to lipid preparations in natural infected animals demonstrates existence of memory responses in animals that were exposed to the pathogens before. As shown in this chapter, mycobacterial lipid antigens can elicit T cell responses and have a potential value as a subunit vaccine candidate.

3. Vaccination

An appropriate animal model to assess the function of the CD1-restricted lipid specific immune response *in vivo* is essential. Previous studies in guinea pigs showed T cell responses to glycolipid antigen in immunized animals (Hiromatsu et al., 2002). In another study, lipid antigens were shown to contribute to the protective immune response to *M. tuberculosis* as shown by the generation of CD1-restricted T cells in the guinea pig aerosol tuberculosis challenge model. Immunisation of animals with total lipid extracted from *M. tuberculosis* after the TB challenge showed reduction of granuloma sizes in the lung similar to what was observed in BCG-vaccinated animals (Dascher et al., 2003). However, the best animal model is the target species itself. Therefore, after studying the immune response against lipids in naturally infected cattle, we performed experimental immunizations in cattle.

As shown in chapter 2, MAP infected animals responded strongly to lipid antigens. Moreover, MAP contains GMM as its immunodominant lipid compound. Therefore, we immunized cattle with a vaccine consisting of GMM in DDA adjuvant to study the cell-mediated and humoral immune responses (**chapter 3**). Another group of animals was immunized with protein to be able to compare the strength of the responses. This study revealed that T cell responses against lipid antigens are comparable in strength to the T cell response against protein antigen.

An additional question to address when using lipid as a subunit vaccine against mycobacterial infection is whether antibody responses are generated. Antibodies are required to protect the host against many infectious agents. However, the role of the humoral response in protection against mycobacteria remains controversial. Recently, humoral responses have been suggested to provide protection against tuberculosis (Hamasure et al., 2004; Lang and Glatman-Freedman, 2006). Our immunization using GMM/DDA did not result in production of anti lipid antibodies in immunized animals, in contrast to what we observed in naturally infected animals where GMM specific antibodies were clearly present, and in contrast to the protein control vaccine (**chapter 3**). One possible explanation for this difference is that during the natural infection, the fragments of bacterial cell walls containing a variety of proteins and lipids, function as a conjugate vaccine. For that reason we investigated whether lipid specific antibodies were produced when cattle were immunized with GMM conjugated with protein. For that purpose, either KLH or HSP70 were non-covalently coupled with GMM as a vaccine (**chapter 4**). Unexpectedly, the conjugate vaccine did not generate antibodies against GMM. Our findings using the conjugate vaccines implied that simply adding peptide epitopes to support glycolipid specific B cells in producing antibodies is insufficient for anti-lipid antibody generation. Research into the mechanism in natural infected animals that generates glycolipid specific antibodies should therefore be performed.

Regarding the safety of lipid based vaccines, pure lipids may be more advantageous than whole bacteria. Animals vaccinated with GMM-containing vaccines did not show signs of pathology at the immunization sites. Furthermore, the standard comparative intradermal skin test for bovine tuberculosis did not show positive test results four months after the second GMM immunization, indicating that immunization with GMM does not interfere with bovine tuberculosis diagnostics.

4. Future perfectives

Is there a future for a lipid vaccine against mycobacterial infections?

Even though in this thesis we have identified mycobacterial lipids that are immunogenic in the setting of natural disease and vaccination, the efficacy of this lipid vaccination has not yet been addressed directly by a vaccination/challenge experiment.

Using selected immunodominant antigens in subunit booster vaccines administered after BCG vaccination has become the main approach in TB vaccine development recently (McShane, 2009). Most of the subunit vaccine candidates are recombinant virus or bacterial vector expressing secreted mycobacterial antigens like Ag85B and ESAT-6, or recombinant protein in combination with adjuvant. Lipid antigens or a combination of glycolipid and other vaccine constituents have not been used as subunit booster vaccine or in a heterologous prime-boost strategies against mycobacterial infections so far. A vaccine regimen that could target multiple T cell subsets with different classes of antigens is potentially more effective at preventing disease (Felio et al., 2009). In addition, the proper adjuvant for lipid antigen should be considered. The generation of long-lasting immunity is required for preventive vaccines against infectious diseases. Hence an important question for lipid vaccine approaches is whether or not responses are persisting.

The effector mechanisms of CD1b-restricted T cells are known to include the direct killing of infected target cells (Stenger et al., 1997). However, other effector mechanisms of CD1b restricted cells have not been elucidated yet. CD1d-restricted T cells contribute to both innate and adaptive immune responses since they have cytotoxic effector functions, helper and adjuvant-like functions, and interact with other cells types including macrophages, dendritic cells, NK cells, T cells, and B cells (Brigl, 2004). It is possible that CD1b-restricted T cells share some of these effector functions with CD1d-restricted T cells. Together with previous studies on CD1-restricted immune responses to mycobacteria in human CD1group 1 transgenic mice (Felio et al., 2009), or guineapigs (Hiromatsu et al., 2002; Dascher et al., 2003), our findings in cattle indicate that the group 1 CD1 presented molecules are appealing targets for the development of lipid subunit vaccines.

5. Concluding remarks

An efficacious vaccine against human TB is not available. Multiple drug-resistant strains are on the rise. The need for an improved vaccine against tuberculosis therefore is urgent. Among many efforts in the development of TB vaccines, subunit vaccines for use in combination with BCG have been proven to be a promising approach. Bovine TB is a zoonotic disease

that can spread to humans and therefore vaccination of cattle is similarly necessary in view of reduced risk of infections in humans, in addition to improving animal health and welfare in itself. Bovine TB has also been proven to be an excellent model for human TB, and in the past, vaccines and diagnostic tests for human TB have successfully been tested in cattle.

The findings presented in this thesis enhance the knowledge of CD1-restricted lipid specific T cells and anti lipid antibodies in cattle. Understanding of the interaction between the bovine immune system and mycobacteria provides the basis for potential future use of mycobacterial lipids in vaccines against mycobacterial infections in cattle as well as in humans.

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Nederlandse samenvatting

Vaccinatie wordt beschouwd als de belangrijkste strategie voor de controle van mycobacteriële infectieziekten, zoals tuberculose bij mens en dier veroorzaakt door M. tuberculosis respectievelijk M. bovis en paratuberculose bij herkauwers ten gevolge van infectie met M. avium subsp paratuberculosis. Deze ziekten veroorzaken naast gezondheidsproblemen ook aanzienlijke economische schade. BCG, het enige vaccin beschikbaar voor tuberculose in mens en rund, heeft beperkte effectiviteit. Ook de toepasbaarheid van vaccins tegen paratuberculose kent beperkingen.

Het onderzoek gepresenteerd in dit proefschrift, had als doelstelling de potentiële toepasbaarheid van mycobacteriële lipiden in vaccinatie tegen mycobacteriële infecties in beeld te brengen. Het rund, natuurlijke gastheer van M. avium subsp paratuberculosis (MAP) en M. bovis, werd in deze studies gebruikt, als belangrijk doeldier voor deze infecties en tevens als model voor humane tuberculose.

Op zoek naar vaccinkandidaten voor mycobacteriële vaccins heeft men zich tot nu toe vooral gericht op MHC gerestricteerde eiwit antigenen. Lipiden, in ruime mate aanwezig in mycobacteriën, kregen slechts in beperkte mate aandacht als potentiële vaccin kandidaten.
Aangezien CD1 moleculen in de mens mycobacteriële lipide antigenen presenteren, werden de aanwezigheid en expressie van runder CD1 genen en hun functioneren bij natuurlijke infectie in het rund onderzocht. T cel reactiviteit specifiek voor CD1 gerestricteerde lipide antigenen werd bestudeerd, hetgeen leidde tot de identificatie van het glycolipid glucose monomycolate (GMM) als een immuundominante lipide component van MAP. Dit immunogene mycobacteriële lipide werd vervolgens in twee verschillende vaccin formuleringen gebruikt om de inductie van specifieke immuunresponsen te onderzoeken.

Dit proefschrift bestaat uit 7 hoofdstukken. Algemene informatie over pathogene mycobacteriën, gastheer immuniteit tegen mycobacteriën, MHC- en CD1-gerestricteerd, en vaccins en vaccinatie evenals een algemene opzet van het onderzoek worden gegeven in **hoofdstuk 1**.

De immuunrespons na natuurlijke infectie met mycobacteriën van een natuurlijke gastheer, het rund, tegen mycobacteriële lipiden wordt beschreven in **hoofdstuk 2**. Het begrijpen van deze aspecten van de afweer zal het ontwikkelen van een lipide subunit vaccin ondersteunen. Het rund functioneert zodoende tevens als model voor humane mycobacteriële ziekten en ontwikkeling van vaccins daarvoor. Tot op heden is er weinig kennis van CD1a, CD1b en CD1c gerestricteerde T cellen in vivo gedurende tuberculose. Echter, enkele studies over ex vivo lipide specifieke reactivieit van T cellen van mensen blootgesteld aan M. tuberculosis laten zien dat deze reageren op het totale lipide extract van de bacteriën en een aantal opgezuiverde lipiden (mycolic acid, GMM, mycoketide, diacylated sulfoglycolipid), hetgeen duidt op het ontstaan van een CD1 gerestricteerde immuunrespons. De studie beschreven in **hoofdstuk 2** liet zien dat mycobacteriële lipide antigenen een sterke T cel reactie oproepen in runderen die natuurlijk geïnfecteerd waren met MAP of M., maar ook dat er weinig kruisreactiviteit is van T cellen tegen lipide extracten van deze twee mycobacteriën. In conclusie, er moet rekening gehouden worden met T cel responsen specifiek voor lipiden van een mycobacteriële species en lipiden hebben potentiële waarde als vaccin kandidaten.

In hoofdstuk 3 wordt de immuunrespons van runderen na immunisatie met het glycolipid glucose monomycolaat (GMM) geëxtraheerd uit mycobacteriën beschreven. Van glycolipiden is bekend dat ze aan humane T cellen worden gepresenteerd door groep 1 CD1 moleculen, maar ze werden nog niet voor immunisatie gebruikt. Om te onderzoeken of GMM een adaptieve immuunrespons kan primen en T cel memory kan induceren, werden runderen geïmmuniseerd met gezuiverd GMM in een synthetisch adjuvans, dimethyl dioctadecyl ammonium bromide (DDA). Het eiwit "keyhole limpet haemocyanin" (KLH) werd gebruikt als controle immunogeen met hetzelfde adjuvans. In deze studie werd het duidelijk dat na immunisatie GMM specifieke T cel responsen, vergelijkbaar in sterkte met KLH specifieke te meten zijn in bloed, maar niet in lymfeklieren. Daarnaast, dat duidelijke KLH specifieke antilichaamtiters ontstaan, maar geen GMM specifieke. De responsen werden gevolgd en bleven aanwezig tot minstens drie maanden na laatste immunisatie. De resultaten suggereren dat eiwit en lipide antigenen immunogeen zijn, niet overlappende T cel populaties aansturen en zodoende dat lipiden potentieel hebben als een speciale klasse van subunit vaccins.

In de studie beschreven in **hoofdstuk 4** werd onderzocht of na immunisatie met een conjugaat van GMM en een eiwit antigeen, het eiwit in staat is helper T cellen te induceren ter ondersteuning van productie van GMM specifieke antilichamen door B cellen. De immunisatie met GMM/DDA, beschreven in **hoofdstuk 3**, resulteerde niet in productie van lipide specifieke antilichamen, in tegenstelling tot hetgeen werd waargenomen in natuurlijk geïnfecteerde dieren, waarin GMM specifieke antilichamen duidelijk aanwezig waren. Een mogelijke verklaring voor dit verschil is, dat gedurende natuurlijke infectie de fragmenten van de bacterie celwanden, die een variëteit aan eiwitten en lipiden bevatten, inclusief GMM, als een conjugaat vaccin functioneren. Om die reden werd onderzocht of lipide specifieke antilichamen werden geproduceerd in runderen geïmmuniseerd met GMM, non-covalent gebonden aan KLH of HSP70 als vaccin. Onverwacht induceerden de conjugaat vaccins geen antilichamen tegen GMM. Dit resultaat impliceert dat toevoeging van peptide epitopen voor T cel hulp aan GMM specifieke B cellen niet voldoende is voor inductie van lipide specifieke antilichamen. Toekomstig onderzoek zou gericht moeten zijn op het mechanisme van inductie van lipide specifieke antilichamen in natuurlijk geïnfecteerde, natuurlijke gastheren.

Kennis over CD1 moleculen en hun rol in de afweer van runderen is beperkt. In de context van dit proefschrift (**hoofdstuk 5 en 6**) werden bovine CD1 moleculen in detail bestudeerd en beschreven om een bijdrage te leveren aan het begrip van CD1 gerestricteerde immuniteit tegen mycobacteriële lipide antigenen. De aantallen CD1 genen verschillen sterk tussen species. In muizen komen uitsluitend CD1d moleculen tot expressie, terwijl in de mens 5 CD1 isovormen zijn geïdentificeerd: CD1 a, CD1b, CD1c, CD1d en CD1e. Expressie van bovine CD1 moleculen en hun identificatie met behulp van antilichamen is beschreven in hoofdstuk 5. De studies laten zien dat runderen tenminste twee CD1A en vijf CD1B genen bezitten, waarvan er twee, respectievelijk drie op eiwit niveau tot expressie komen. De moleculen van de CD1a en CD1b isovormen verschillen in hun antigeen bindende domeinen en cytoplasmatische staarten, hetgeen suggereert dat zij op verschillende wijzen door cellen migreren. In dit hoofdstuk worden ook de expressie patronen van bovine CD1 moleculen in B cellen en dendritische cellen beschreven, deze lijken sterk op die in humane antigeen presenterende cellen en voldoen aan de vereisten voor de presentatie van lipide antigenen aan bovine T cellen.

Van het CD1D gen in runderen werd in het verleden verondersteld dat het non-functioneel was, vanwege een mutatie in het start codon. Bovendien was het niet duidelijk of NKT cellen, waarvan, vanuit andere species, bekend is dat ze lipide antigeen gepresenteerd door CD1d eiwit herkennen, in runderen voorkomen. De ongewone gen structuur van CD1D werd opnieuw bestudeerd en in **hoofdstuk 6** werd voor het eerst de onverwachte expressie van het CD1d molecuul beschreven. Ondanks deze expressie van het bovine CD1d molecuul leidde in vivo toediening van a-galactosylceramide, een universeel antigeen voor NKT cellen, niet tot een toename in cytokines in serum, of temperatuursverhoging, de karakteristieke kenmerken van CD1d/NKT activatie in andere diersoorten. De aanwezigheid van NKT cellen in runderen blijft onzeker, maar, gezien de bevindingen is het duidelijk dat het CD1D gen van deze diersoort een ongewone intron/exon structuur en signaal peptide heeft en dat het niet waarschijnlijk is dat deze onderdeel uitmaken van een klassiek, functioneel geconserveerd CD1d/NKT systeem.

In **hoofdstuk 7** wordt CD1 expressie en immuniteit specifiek voor mycobacteriële lipiden in natuurlijk geïnfecteerde runderen samengevat en bediscussieerd, evenals de potentie van mycobacteriële lipiden als componenten van vaccins tegen mycobacteriële infecties.

De resultaten van de studies gepresenteerd in dit proefschrift, hebben de kennis omtrent lipide specifieke CD1 gerestricteerde T cellen en lipide specifieke antilichamen in runderen vergroot en daarmee het begrip van de afweer van het rund tegen mycobacteriën, en vormen een basis voor mogelijk toekomstig gebruik van bacteriële lipiden in vaccins tegen mycobacteriële ziekten in rund zowel als mens.

Tóm tắt

Tiêm chủng vắc-xin phòng ngừa các bệnh có nguyên nhân gây bệnh là mycobacteria, trong đó đặc biệt nhắc đến bệnh lao do các loài *M. tuberculosis* và *M. bovis* gây nên, đã và đang được coi là chiến lược để khống chế vi khuẩn lao hiện đang lây nhiễm trên khoảng 2 tỷ người, chiếm 1/3 dân số thế giới. Một căn bệnh khác gây thiệt hại nặng nề về kinh tế có tên gọi là phó lao xuất hiện trên trâu bò do vi khuẩn *M. avium subsp. paratuberculosis* gây ra cũng đang đòi hỏi cấp bách có vắc-xin phòng bệnh.

BCG hiện là vắc-xin duy nhất đang được sử dụng để phòng ngừa bệnh lao nhưng lại có hiệu quả rất hạn chế (hiệu quả của BCG chỉ khoảng 50%). Trong nhiều năm, đã có rất nhiều loại vắc-xin mới được nghiên cứu và đề xuất nhưng chưa có loại nào thực sự đạt hiệu quả cao. Ngoài ra, vắc-xin phòng ngừa bệnh phó lao trên bò cũng chưa đạt hiệu quả phòng bệnh tốt.

Những nghiên cứu của luận án này nêu ra tiềm năng sử dụng lipit tách chiết từ mycobacteria để chống lại bệnh do chính mycobacteria gây ra. Trong luận án nghiên cứu này, bò được sử dụng làm động vật thí nghiệm vì chúng là vật chủ tự nhiên của cả vi khuẩn gây bệnh lao *M. bovis* và bệnh phó lao *M. avium subsp paratuberculosis* (viết tắt là MAP). Do đó bò là loài động vật quan trọng cho mục tiêu nghiên cứu, đồng thời là động vật thí nghiệm trong mô hình nghiên cứu bệnh lao ở người.

Trong khi các kháng nguyên protein được trình diện tới các tế bào T qua các phân tử MHC, các kháng nguyên lipit được trình diện tới các tế bào T qua các phân tử CD1. Số lượng các gien CD1 và các đồng đẳng của mỗi gien là rất khác nhau giữa người và các loài động vật, cũng như giữa các loài động vật khác nhau. Ở người có 5 gen CD1 bao gồm CD1A, CD1B, CD1C, CD1D, CD1E mã hóa 4 phân tử protein được biểu hiện trên bề mặt tế bào bao gồm CD1a, CD1b, CD1c và CD1d. Riêng phân tử CD1e nằm trong các hạt cơ quan nội bào của các tế bào tua gai và không biểu hiện lên bề mặt tế bào. Dựa vào cấu trúc, sự biểu hiện trên bề mặt tế bào và chức năng chính, các phân tử CD1a, CD1b, CD1c và CD1e được xếp vào CD1 nhóm 1, còn CD1d được xếp vào CD1 nhóm 2. Do các phân tử CD1 đã được những nghiên cứu trước đây chỉ ra là các phân tử trình diện kháng nguyên lipit trên người nên chúng tôi tiến hành nghiên cứu sự tồn tại và biểu hiện của các gien CD1 trên bò, cũng như vai trò chức năng của những gien này trong các trường hợp nhiễm bệnh tự nhiên (Biểu hiện gien trong nghiên cứu này là quá trình liên quan đến việc chuyển đổi thông tin di truyền chứa trong gien thành protein). Đáp ứng của các tế bào lympho T đặc thù nhận diện lipit qua sự trình diện của phân tử CD1 cũng được nghiên cứu trên những con bò bị nhiễm bệnh tự nhiên, để từ đó nhận dạng thành phần glycolipid glucose monomycolate (viết tắt là GMM) là lipit có tác dụng gây đáp ứng miễn dịch chính của MAP trên chủ thể (động vật nhiễm bệnh). Thành phần lipit này sau đó được chúng tôi thử nghiệm trong hai loại vắc-xin để đánh giá các đáp ứng miễn dịch đặc hiệu.

Luận án này bao gồm 7 chương. Trong **chương 1**, những thông tin về mycobacteria (vi khuẩn gây bệnh), hoạt động miễn dịch của chủ thể chống lại mycobacteria (bao gồm hai hệ thống miễn dịch liên quan đến phân tử MHC và phân tử CD1), vắc-xin và sử dụng vắc-xin để chủng ngừa được giới thiệu tổng quan.

Nội dung trong **chương 2** mô tả đáp ứng miễn dịch đối với kháng nguyên lipit có nguồn gốc từ mycobacteria trên bò bị nhiễm bệnh tự nhiên. Hiểu biết cơ bản về những đáp ứng này sẽ

là nền tảng trong việc phát triển *subunit* vắc-xin sử dụng lipit tách chiết từ vi khuẩn. *Subunit* vắc-xin là vắc-xin được làm từ một phần cấu trúc của vi sinh vật có tính kháng nguyên đặc trưng của vi sinh vật đó, vì vậy ít có nguy cơ gây ra phản ứng bất lợi hơn so với những vắc-xin sử dụng nguyên vi sinh vật.

Cho đến nay nhiều nghiên cứu đã thử nghiệm *subunit* vắc-xin được làm từ các thành phần protein hay DNA của vi khuẩn thay vì sử dụng vi khuẩn (ở dạng sống, giảm độc lực, hay bất hoạt) như truyền thống. Tuy vậy, các thành phần lipit hiện vẫn chưa được sử dụng trong các *subunit* vắc-xin. Những đối tượng bò bị nhiễm bệnh tự nhiên còn được sử dụng làm động vật thí nghiệm cho mô hình nghiên cứu các bệnh do mycobacteria gây ra trên người và tìm kiếm vắc-xin phòng ngừa. Các hoạt động của phân tử CD1a, CD1b, and CD1c trên chủ thể nhiễm lao chưa được làm sáng tỏ. Trên thực tế, đã có một số nghiên *ex vivo* (là những nghiên cứu thực hiện trên các tổ chức mô cơ hoặc tế bào tách ra khỏi cơ thể sống) cho thấy các tế bào lympho T từ những người bị phơi nhiễm khuẩn lao *M. tuberculosis* có đáp ứng với chiết xuất lipit toàn phần cũng như một số loại lipit được tinh chế như mycolic acid, GMM, mycoketide, diacylated sulfoglycolipid. Những kết quả nghiên cứu đó cho thấy các kháng nguyên lipit được trình diện qua phân tử CD1 này gây ra đáp ứng miễn dịch nhận được đối với những loài mycobacteria kể trên. Những kết quả nghiên cứu trong khuôn khổ luận án này đã chứng tỏ rằng kháng nguyên lipit gây nên những đáp ứng miễn dịch tế bào lympho T rất mạnh mẽ ở những con bò bị nhiễm bệnh tự nhiên từ loài *M. avium subsp. paratuberculosis* cũng như loài *M. bovis*. Tuy nhiên, sự hoạt động chéo của đáp ứng miễn dịch tế bào lympho T giữa chiết xuất lipit từ hai loài trong cùng chi khuẩn mycobacteria này là rất yếu. Nghĩa là, lipit từ mỗi loài mang tính kháng nguyên gây ra đáp ứng miễn dịch tế bào T đặc thù cho chính loài đó. Tựu chung lại, do lipit có thể gây ra đáp ứng miễn dịch tế bào T đặc hiệu theo từng chủng mycobacteria nên chúng cần được nhìn nhận là một ứng cử viên có giá trị trong việc nghiên cứu vắc-xin.

Ở **chương 3**, các đáp ứng miễn dịch của bò sau khi gây miễn dịch bằng glycolipid glucose monomycolate (GMM) tách chiết từ mycobacteria được nghiên cứu. Glycolipids đã được biết đến là những kháng nguyên được các protein CD1 nhóm 1 trên người trình diện cho tế bào T. Dù vậy, chúng vẫn chưa được sử dụng để gây miễn dịch. Nhằm tìm hiểu xem GMM có thể khởi động hệ thống miễn dịch nhận được và gây ra sự hình thành dòng tế bào T ghi nhớ hay không, GMM sau khi tinh chế được dùng làm vắc-xin với chất phụ gia là dimethyl dioctadecyl ammonium bromide (DDA) để tiêm cho bò thí nghiệm. Nhóm đối chứng được tiêm protein keyhole limpet haemocyanin (KLH) được chế cùng phụ gia DDA làm vắc-xin. Thí nghiệm này cho thấy rõ GMM có thể gây nên đáp ứng miễn dịch tế bào T trong máu mạnh tương đương với kháng nguyên protein KLH, tuy nhiên tại các hạch bạch huyết (hay còn gọi là hạch lympho), chỉ có đáp ứng với KLH được tìm thấy. Thêm vào đó, kháng thể kháng lại kháng nguyên KLH rất mạnh, trong khi không có đáp ứng kháng thể nào đối với GMM được ghi nhận. Tất cả các đáp ứng miễn dịch tìm thấy xuất hiện và kéo dài trong mẫu thu thập suốt ba tháng sau khi động vật được tiêm gây miễn dịch. Những kết quả trong nghiên cứu này cho thấy mặc dù kháng nguyên protein và lipit đều có đặc tính gây miễn dịch, nhưng chúng kích hoạt các dòng tế bào T khác nhau. Do đó, glycolipid có thể là một nguồn kháng nguyên tiềm năng để chế tạo subunit vắc-xin.

Bởi vì thí nghiệm ở **chương 3** cho thấy động vật được tiêm vắc-xin GMM/DDA không gây ra đáp ứng kháng thể kháng lại kháng nguyên lipit, trong khi quan sát trên động vật nhiễm bệnh tự nhiên cho kết quả đáp ứng kháng thể đặc hiệu rất mạnh chống lại kháng nguyên GMM. Điều khác biệt này có thể được lý giải như sau: trong quá trình vi khuẩn xâm nhập tự nhiên vào động vật và gây bệnh, thành tế bào của vi khuẩn có chứa cả protein và rất nhiều loại lipit, trong đó có GMM, đóng vai trò như một vắc-xin lien kết protein-lipit. Từ suy luận đó ở **chương 4** chúng tôi tiến hành nghiên cứu xem liệu khi GMM được liên kết với protein KLH hoặc HSP70 trong một vắc-xin rồi tiêm cho động vật thí nghiệm thì có tạo ra kháng thể kháng lại GMM hay không. Không như mong đợi, kết quả thí nghiệm cho thấy kháng thể kháng GMM không được tạo ra. Kết quả này có mang ngụ ý nếu chỉ đơn giản bổ sung peptide epitopes thì vẫn chưa đủ điều kiện để hỗ trợ tế bào lympho B đặc hiệu với kháng nguyên lipit để sản sinh kháng thể chống lại kháng nguyên lipit. Trong tương lai cần phải tiến hành nghiên cứu sâu hơn để làm rõ các cơ chế tạo ra kháng thể chống lại kháng nguyên đặc trưng cho lipit, cụ thể hơn là glycolipit diễn ra thế nào trong các chủ thể nhiễm bệnh tự nhiên.

Các phân tử CD1 được nghiên cứu và miêu tả chi tiết trong **chương 5** và **chương 6**, cung cấp thêm kiến thức cơ bản về hệ miễn dịch liên quan đến phân tử CD1 chống lại các kháng nguyên lipit có nguồn gốc từ mycobacteria. Số lượng gien CD1 rất đa dạng giữa các loài động vật. Trong khi chuột chỉ có phân tử CD1d, người có 5 dạng CD1 khác nhau bao gồm CD1a, CD1b, CD1c, CD1d và CD1e. Sự biểu hiện của các phân tử CD1 trên bò cho thấy loài động vật này có các gien CD1A, CD1B, CD1D. Khác với loài người mỗi chỉ có một gien cho mỗi loại CD1 (từ A đến E), ở bò có ít nhất 2 gien CD1A và 5 gien CD1B, trong đó có 3 phân tử CD1b được biểu hiện thành protein. Trong các dạng CD1a và CD1b khác nhau, các phân tử khác biệt về những miền bám dính kháng nguyên và cả trong phần đuôi của tế bào chất, từ đó gợi ý chúng có thể di chuyển theo những con đường khác nhau bên trong tế bào. Ngoài ra trong chương này cách thức biểu hiện của các phân tử CD1 trong các tế bào trình diện kháng nguyên như tế bào B và tế bào tua gai của bò cũng được nghiên cứu. Kết quả cho thấy chúng khá tương đồng với cách thức biểu hiện của phân tử CD1 trên các tế bào trình diện kháng nguyên trên người và các phân tử CD1 của bò phù hợp với điều kiện cần thiết để trình diện các kháng nguyên lipit có nguồn gốc từ mycobacteria cho các tế bào lympho T của bò.

Trước đây gien quy định CD1D trên bò được cho là gien không hoạt động do sự xuất hiện của đột biến gien trong đơn vị mã khởi động (start codon). Hơn nữa, cho đến nay vẫn chưa có nghiên cứu nào chỉ ra sự tồn tại của tế bào NKT (natural killer T cells) trên loài bò. Tế bào NKT đã được biết đến trên nhiều loài động vật khác và trên người. NKT là dòng tế bào khác biệt với dòng tế bào lympho T. Vai trò của loại tế bào này là nhận biết các kháng nguyên lipit được trình diện qua phân tử protein CD1d. Trong chương 6 chúng tôi nghiên cứu lại cấu trúc bất thường của gien CD1D trên bò và đây là nghiên cứu đầu tiên phát hiện ra sự biểu hiện của phân tử protein CD1d từ gien này. Sự biểu hiện của phân tử CD1d được chúng tôi kiểm định lại trong thí nghiệm tiêm α-galactosylceramide trên bò để kiểm tra sự sản sinh của cytokine và sự gia tăng nhiệt độ cơ thể. Hàm lượng cytokine trong máu và nhiệt độ cơ thể tăng là hai chỉ số điển hình trong nghiên cứu hệ thống CD1d/NKT được áp dụng rộng rãi trên các loài động vật khác và trên người. Nhưng các chỉ số này không được tìm thấy trong thí nghiệm của chúng tôi trên loài bò. Vì thế có hay không tế bào NKT trên bò vẫn còn là một ẩn số, mặc dù gien CD1D rõ ràng đã tổng hợp nên protein CD1d cho dù gien có cấu trúc bất thường. Có vẻ như gien CD1D trên bò không có chức năng giống như các hệ thống CD1/NKT điển hình ở các động vật khác.

Tóm tắt và thảo luận chung về sự biểu hiện của các phân tử CD1 trên bò, miễn dịch đặc hiệu của chủ thể đối với kháng nguyên lipit có nguồn gốc từ mycobacteria trên những động vật nhiễm bệnh tự nhiên và tiềm năng sử dụng kháng nguyên lipit trong chủng ngừa các bệnh do mycobacteria gây ra được trình bày trong **chương 7**.

Những kết quả được trình bày trong luận án này nâng cao kiến thức cơ bản về các đáp ứng tế bào T đặc trưng và đáp ứng kháng thể chống lại kháng nguyên lipit trong hệ miễn dịch giới hạn cho CD trên bò. Hiểu biết về sự tương tác qua lại giữa hệ miễn dich của bò và vi khuẩn mycobacteria mang lại nền tảng cho tiềm năng sử dụng trong tương lai kháng nguyên lipit từ mycobacteria trong vắc-xin chống lại các bệnh truyền nhiễm do mycobacteria gây nên cả trên bò cũng như trên người.

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Curriculum vitae and publications

Curriculum vitae

Nguyễn Thị Kim Anh was born on June 28, 1974 in Hà Bắc, Vietnam. From 1988 to 1991 she studied at the Hanoi-Amsterdam gifted high school in Hanoi. She passed the national examination and was admitted to the Hanoi Agriculture University in 1991 and obtained the Doctor of Veterinary Medicine degree in 1996. Since 1997 she has been working for the National Institute of Animal Husbandry in Hanoi, Vietnam. In 2002 she enrolled in the Master program on Animal Pathology at the Department of Pathology, Faculty of Veterinary Medicine, Utrecht University, the Netherlands and graduated MSc in 2004. In 2007 she received a fellowship from the Vietnamese government to support her PhD studies in the Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, the Netherlands.

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