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Mechanism of the adjuvant activity of the synthetic mycobacterial cord factor analog Trehalose-6,6-dibehenate (TDB)

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Abbreviations

℃	Centigrade
µg	Microgram
µl	Microlitre
µM	Micromolar
Ag85B	Antigen 85B
AP-1	Activator protein 1
APC	Antigen-presenting cell
ASC	Apoptosis-associated speck-like protein containing a Card domain
ATF-2	Activating transcription factor 2
BCG	Bacillus Calmette-Guérin
BMDC	Bone-marrow-derived dendritic cell
BMDM	Bone-marrow-derived macrophage
BSA	Bovine serum albumine
CCl	CC motif chemokine ligand
CD	Cluster of development
CFA	Complete Freund's adjuvant
CLD	C-type lectin domain
Clec	C-type lectin like receptor
CLR	C-type lectin receptor
CpG	Cytosine-guanosine oligonucleotide
CRD	Carbohydrate recognition domain
CTL	Cytotoxic T lymphocyte
CXCl	CXC motif chemokine ligand
DC	Dendritic cell
DDA	Dimethyldioctadecylammonium bromide
ddH_2O	Double distilled water
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide tri-phosphate
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated protein kinase
ESAT6	Early secretory antigenic target 6

f.p.	Footpad
FcγR	Fc gamma receptor
h	Hour
hep A	Hepatitis A
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HPV	Human papilloma virus
Hsp65	Heat shock protein 65
ICAM	Intercellular adhesion molecule
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IKK	Inhibitor of κB kinase
IL	Interleukin
IL-1R	Interleukin 1 receptor
iNKT cell	Invariant natural killer T cells
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISCOM	Immunostimulating complexes
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JNK	C-JUN N-terminal kinase
kDa	Kilodalton
KO	Knock out
LPS	Lipopolysaccharide
LRR	Leucin rich repeats
LysM	Lysozm M
MAL	Myd88-adaptor-like
Man-LAM	Mannosylated lipoarabinomannan
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MBL	Mannose binding lectin
MCSF	Macrophage colony-stimulating factor

MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
min	Minute
Mincle	Macrophage-inducible C-type lectin
MPL	Monophosphoryl lipid A
MR	Mannose receptor
Mtb	*Mycobacterium tuberculosis*
MTT	Thiazolyl Blue Tetrazolium Bromide
Myd88	Myeloid differentiation primary response gene 88
NEMO	NF-κB essential modifier
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κB
ng	Nanogram
NK cell	Natural killer cell
NKT cell	Natural killer T cell
nm	Nanometre
NO	Nitric oxide
NOD	Nucleotide oligomerization domain
O/W	Oil in water
OD	Optical density
OSCAR	Osteoclast-associated receptor
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIM	Phosphatidylinositol monomannoside
PRR	Pattern recognition receptor
RANKL	Receptor-activator of nuclear-factor-κB ligand
RMA	Robust multi-array average algorithm
RNA	Ribonucleic acid
ROR	Receptor-related orphan nuclear receptor
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium
s	Second

SAP130	Splicosome-associated protein 130
SD	Standard deviation
SH2	Src homology 2 domain
SHIP	SH2 containing inositol phosphatase
TAK	Transforming growth factor β-activated kinase 1
TB	Tuberculosis
TDB	Trehalose-6,6-dibehenate
TDM	Trehalose-6,6-dimycolate
TGF	Transforming growth factor
Th cell	T helper cell
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor receptor associated factor
TRAM	Trif-related adaptor molecule
Trif	TIR domain-containing adaptor inducing interferonβ
UPL	Universal library probe
W/O	Water in oil
WT	Wild-type

1 Introduction

1.1 Vaccines and Vaccination

Every year, millions of people die of severe infections caused by bacterial pathogens or viruses. Tuberculosis (TB), malaria and HIV are the major microbial killers worldwide. Additionally the increasing threat of nosocomial infections of hospitalized patients, which are caused e.g. by staphylococci, enterococci, *Pseudomonas aeruginosa* and other bacterial pathogens must be taken into account. Protection against these existing and newly demanding infections therefore is a major goal in the field of vaccine development in the developing as well as in the industrialized world.

Human beings have benefited from vaccines for more than two centuries when the history of vaccination began with the story of Edward Jenner who performed the first vaccination against smallpox in 1796 (Baxby, 1996). In the 19th century, the work of Louis Pasteur continued the successful story of vaccination with the development of the first vaccines against cholera (1879) and rabies (1885). Further breakthroughs in the following years were the development of vaccines against tetanus (1927), typhus (1896) and diphtheria (1923).

To date, more than 20 vaccines against different bacterial pathogens and viruses are in use whereas a comprehensive understanding of the immune response is now needed for the rational design of vaccines that are directed at more complex pathogens. Such pathogens can often be controlled only if the vaccine induces a better immune response than the natural infection. So far, many strategies have been evolved for designing vaccines, exploiting the knowledge of the mechanisms and functions of the innate immune system to initiate a robust and protective immune response against different pathogens.

In recent years, the role of CD4$^+$ T-helper (Th) cells has been in the center of vaccine development. Th cells occupy a central role because they control the development of both the humoral immune response (characterized by production of antigen specific antibodies) and the cell mediated immune response (characterized by activation of antigen presenting cells, natural killer cells and cytotoxic T cells). In response to cytokines produced by antigen presenting cells, naïve T cells proliferate and differentiate into effector T cells in an antigen-specific fashion upon encounter with their cognate antigen (Glimcher and Murphy, 2000). The effector T cells then tailor their functions to the nature of the microbial threat, which means helping B cells to

produce antigen-specific antibodies, activating macrophages to kill intracellular pathogens and promoting the development of cytotoxic $CD8^+$ T cells, which can direct lyse infected target cells by means of perforins and granzymes. To date, three distinct $CD4^+$ effector T-cell lineages have been described: Th-1, Th-2 and Th-17 (Murphy and Reiner, 2002). In general, Th-1 cells confer immunity to infection by intracellular pathogens through production of the effector cytokines interferon-γ (IFN-γ) and Interleukin 2 (IL-2) while Th-2 cells play an important role in the clearance of multicellular helminthes and ectoparasites by the production of IL-4, IL-5 and IL-13 (Laurence and O'Shea, 2007). Th-17 $CD4^+$ T cells, which were recently described, confer protection against extracellular bacteria and fungi, particularly at epithelial surfaces (Bettelli et al., 2007; Weaver et al., 2006; Weaver and Murphy, 2007). Depending on the invading pathogen, mostly one T-cell subset predominates in an infection which influences the resulting adaptive immunity. The best success in immunization has been mediated through the induction of protective antibodies (Plotkin, 2001), whereas the major diseases like e.g. TB, HIV and malaria will require the induction of T cell immunity as well. Intracellular bacteria like *Mycobacterium tuberculosis* can survive in the phagosome of macrophages (Young and Dye, 2006) and even activated macrophages fail to eradicate this pathogen. In immunocompetent individuals, *M. tuberculosis* exhibits lifelong persistence without causing disease in 90 % of infected persons (Young and Dye, 2006). The bacteria are not eliminated, but are controlled by an active T-cell response. New vaccines have to be developed to target these intracellular pathogens (Kaufmann, 2007). Several strategies at this level are based on the knowledge about antigen targeting to the different MHC pathways, stimulation of APCs through pattern-recognition-receptors and the stimulation and development of the T cells triggered by the different pathogens. The major aim of such vaccines is the induction of a T-cell response, which is shifted towards a Th-1 direction and the induction of both $CD4^+$ and $CD8^+$ lymphocytes to guarantee an efficient elimination of the invader. However, induction of an immunological memory is central to all vaccination strategies, which comprises memory B cells, memory T cells (central memory T cells and effector memory T cells) as well as long living, antibody producing plasma cells.

To date, four types of traditional vaccines are in use or subjects of immunization strategies:

The first ones are vaccines containing chemical or heat-killed microorganisms. One example is the cholera vaccine, which contains a recombinant cholera toxin B (rCTB) in a liquid suspension of four strains of killed *Vibrio cholera* (The green book, 2006). Monovalent vaccines against hepatitis A also belong to this group. Here, different strains of the hep A virus are grown in cell culture followed by the adsorption of the virus particles onto aluminium hydroxide (The green book, 2006). The second group of classical vaccines contains live, attenuated pathogens. These are live microorganisms that have been cultivated under conditions that disable their virulent properties or which use closely-related but less dangerous organisms to induce a broad immune response. One example is the live BCG vaccine for immunization against TB, which contains an attenuated strain derived from *Mycobacterium bovis* (The green book, 2006). Other examples include vaccines against measles, rubella and yellow fever.

The use of toxoids represents the third group of vaccines. These are inactivated toxic products of microorganisms, for which it is known that they cause illness. Toxoid-based vaccines are in use for immunization against tetanus or diphtheria. E.g. the tetanus vaccine is made from a cell-free purified toxin extracted from a strain of *Clostridium tetani*. Treatment with formaldehyde then converts the toxin into the tetanus toxoid, which is adsorbed to aluminium hydroxide (The green book, 2006).

In recent years, the development of so called subunit vaccines is in the center of research. Instead of “whole agent” vaccines, subunit vaccines only contain fragments of pathogens. Normally, those subunits are peptides, capsular polysaccharides, DNA or recombinant proteins. E.g., the vaccine against hepatitis B contains only the surface protein of the virus (HBsAg) which is prepared from yeast cells using recombinant DNA technology (The green book, 2006). Another example is the vaccine against human papillomavirus (HPV), which contains only the major capsid protein of HPV (The green book, 2006). The advantage of subunit vaccines is the greater safety compared to conventional vaccines, but because subunit antigens alone are less immunogenic, the use of peptides or recombinant proteins as vaccines needs strong adjuvants to induce a robust immune response.

1.2 Adjuvants

Adjuvants are defined as compounds that can increase and/or modulate the intrinsic immunogenicity of an antigen. The term adjuvant itself derives from the latin word *adjuvare*, meaning to help. New vaccines, based on recombinant viruses, recombinant proteins, DNA, purified subunits, peptides etc., have a more defined composition that is often linked to a lower immunogenicity compared with “traditional” live vaccines or killed/attenuated whole-cell based vaccines. Adjuvants are therefore required to assist new vaccines to induce potent and persistent immune responses. There is also now a major interest in developing adjuvants which are capable of eliciting strong cellular immune responses of the Th-1 type, which is highly desirable for vaccines targeting either chronical viral diseases (e.g. HIV), infections linked to intracellular pathogens (e.g. TB) or cancer (known as therapeutic vaccines). Adjuvants differ in their mode of action. For example, certain adjuvants are able to convey long-term presentation of the antigen (also known as depot effect), others help to target immune cells (e.g. by delivering the antigen to APCs) or exhibit a capacity to elicit the production of different cytokine patterns by activating different pattern-recognition-receptors (PRRs), thereby shifting the immune response towards Th-1 or Th-2. Another possibility is that the adjuvant enhances the levels of co-stimulatory molecules on APCs (Cox and Coulter, 1997; Guy, 2007).

The “ideal” adjuvant is non-toxic, does not induce autoimmunity and is effective for infants and adults. Furthermore, it has to be stable and biodegradable and is non-immunogenic by itself.

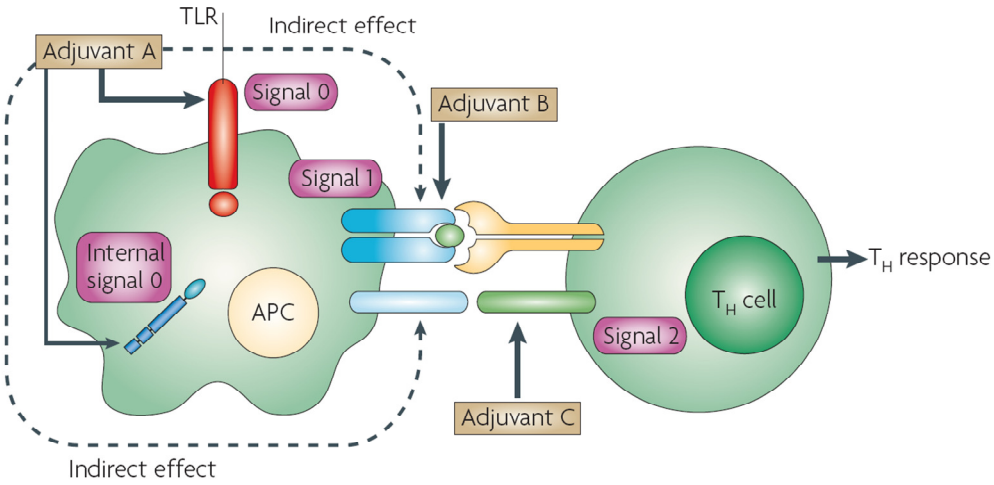


Fig 1: Where do adjuvants act?
The initiation of T helper (Th)-cell responses requires three signals, referred to as signal 0 (recognition of PAMPS by PRRs), signal 1 (specific peptide presentation) and signal 2 (co-stimulatory signals). Adjuvants can act — alone or in combination — on each of these three signals. Type A adjuvants act on signal 0 and indirectly on signal 2 by activating antigen presenting cells (APCs) and triggering the secretion of pro-inflammatory cytokines. Type B adjuvants (e.g. TLR agonists) can act on signal 1 by favouring efficient presentation of the co-administered antigen. PAMPS, pathogen-associated molecular pattern; PRR, pattern-recognition receptor; TLR, Toll-like receptor (from Guy, 2007)

The most widely used adjuvants in humans so far are mineral salts like aluminium hydroxide ($AlOH_3$) or aluminium phosphate ($AlPO_4$), in general called alum. The main mechanism of alum adjuvant is that it delays clearing of the antigen from the injection site, which leads to a prolonged exposure of the antigen to antigen presenting cells. E.g., adsorption of tetanus toxoid to alum enhances the uptake of the antigen by APCs compared to the soluble form of the toxoid alone (Tritto et al., 2009). Recent publications reported that macrophages are activated by alum to present the antigen (Rimaniol et al., 2004) and a previously unknown population of IL-4 producing cells was shown to be required for alum-induced *in vivo* priming and expansion of antigen-specific B cells (Jordan et al., 2004). Alum induces a Th-2 biased immune response, which is not likely to protect against diseases for which Th-1 immunity and MHCII restricted CTLs are essential; furthermore $AlOH_3$ is not the best adjuvant for raising high antibody titers against small size peptides.

After aluminium compounds, emulsions are among the most frequently used adjuvants in humans and animals. Emulsions can be divided in two prototypes: oil-in-water (O/W) and water-in-oil (W/O). One example for a water-in-oil emulsion is Freund's complete adjuvant (CFA), which contains killed mycobacteria. CFA induces a strong and long lasting inflammatory reaction which appears at the site of injection

and in draining lymph nodes and leads to granuloma formation. Furthermore it induces tubercle-like lesions in lymph nodes and non lymphoid tissues (Billiau and Matthys, 2001). Although CFA triggers the development of a Th-1 response, it is too toxic for the use in humans and, if possible, should be replaced by other adjuvants for animal vaccination.

An alternative to CFA is the Ribi adjuvant system. Ribi adjuvants are oil-in-water emulsions where antigens are mixed with small volumes of sqalene (metabolizable oil) which are then emulsified with saline containing the surfactant Tween 80. This system also contains refined mycobacterial products (Trehalose-6,6-dimycolate, cell wall skeleton) as immunostimulators and bacterial monophosphoryl lipid A, which can be recognized by Toll-like receptor 4 (Mata-Haro et al., 2007). This adjuvant system is much less toxic and less potent than CFA but generally induces satisfactory amounts of high avidity antibodies against protein antigens and is used in veterinary medicine. Another emulsion is incomplete Freund's adjuvant (IFA), which is composed of paraffin oil containing mannide mono oleate as surfactant. The mode of action of IFA lies in the depot formation at the site of injection, which guarantees a slow release of the antigen resulting in an efficient stimulation of APCs. Both CFA and IFA are known to enhance phagocytosis of the antigen by APCs (Billiau and Matthys, 2001).

Besides these "classical" adjuvants, recent progress in adjuvant research focused on the use of compounds, which are known to be recognized by Toll-like receptors (TLRs). One example is the use of monophosphoryl lipid A (MPL), the membrane anchor of LPS. Like LPS, MPL is recognized by TLR4, but it can also activate TLR2, suggesting that TLR2 might be involved in activation of APCs by MPL (Martin et al., 2003). LPS was also tested as a potent adjuvant, but because of its high toxicity it can not be used in humans. Both LPS and MPL convert B cells, macrophages and DCs into potent APCs, resulting in an increased capacity to prime antigen-specific T cells *in vivo* (De Becker et al., 2000; Okemoto et al., 2006). Furthermore, MPL can induce both Th-1 and Th-2 type immune responses in the systemic and mucosal compartments of the immune system (Baldridge et al., 2000) and could enhance the production of antigen-specific $CD8^+$ CTLs (Richards et al., 1998). The use of MPL as adjuvant was tested in the prevention of several diseases, e.g. in a vaccination model against malaria (Alonso et al., 2005; Garcon et al., 2003).

Recent publications have identified the immunotherapeutic uses of CpG oligonucleotides (CpG ODNs) (Klinman, 2004), containing CpG motifs similar to those found in bacterial DNA. Recognized by TLR9, CpG ODNs induce the maturation/activation of professional APCs and boost the humoral and cell-mediated response to proteins (Zhang et al., 2007). Furthermore, CpG DNA promotes a robust Th-1 response and the induction of pro-inflammatory cytokines. It is a potent enhancer of systemic and mucosal immune responses against the hepatitis B surface antigen in mice (McCluskie and Davis, 2000).

Besides TLR agonists, the use of pathogen-associated-molecular-pattern (PAMPs) which are recognized by other PRRs, is subject of several studies. One example is zymosan, a yeast cell wall component, which is recognized by TLR2 and the C-type lectin Dectin-1 (Dillon et al., 2006). Zymosan was shown to enhance the immune response to a DNA vaccine for HIV type 1 through the activation of the complement system (Ara et al., 2001). Another one is muramyl dipeptide (MDP), a cell wall component which signals via intracellular NOD proteins (Tanabe et al., 2004).

A further popular method in the adjuvant development field is the use of liposomes, biodegradable polymer microspheres or cage-like structures (ISCOMs), which can serve as vehicles or delivery systems for vaccine antigens and/or immunostimulators such as LPS, MPL, MDP or Cytokines.

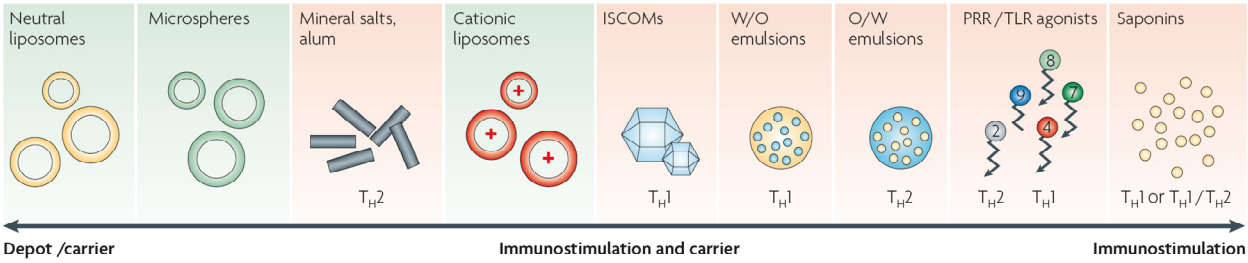


Fig 2: Properties of adjuvants.
The main type of adjuvants with respect to their depot/carrier and immunostimulatory properties is shown. Some compounds can possess both characteristics whereas others possess only one. Shown in red are adjuvants which have immunomodulatory properties to direct the T cell response specifically towards Th-1 or Th-2. ISCOMs, immunostimulating complexes; O/W, oil-in-water emulsion; PRR, pattern-recognition receptor; TLR, Toll-like receptor; W/O, water-in-oil emulsion. (from Guy, 2007)

One example for cationic liposomes, which have been shown to act as a potent carrier for antigens and are subject of the present PhD thesis, is the quaternary ammonium compound dimethyldioctadecylammonium (DDA). DDA was reported as

an adjuvant by Gall in the mid 1960s (Gall, 1966) and has been tested in combination with a number of different viral and bacterial antigens in different animal species. DDA is a synthetic amphiphilic lipid compound comprising a hydrophilic positively charged dimethylammonium head group attached to two hydrophobic 18-carbon alkyl chains. In an aqueous environment, DDA self-assemble into closed vesicular bilayers similar to liposomes made from natural phospholipids (Carmona-Ribeiro and Chaimovich, 1986; Ribeiro and Chaimovich, 1983). In 1992, it was shown by Hilgers *et al*, that DDA induces cell-mediated immunity and delayed-type hypersensitivity (Hilgers and Snippe, 1992) and it is suggested, that DDA liposomes can form a depot (Holten-Andersen et al., 2004). Furthermore DDA vesicles interact rapidly with different biomolecules with very high affinity (Carmona-Ribeiro, 2000) and are taken up by active endocytosis (Friend et al., 1996; Wrobel and Collins, 1995) via an actin-dependent pathway. In 2007, Korsholm *et al* focused on the adjuvant mechanism of the cationic DDA liposomes by using a vaccination model with ovalbumine (OVA) as the specific antigen (Korsholm et al., 2007). DDA liposomes predominantly target the antigen to splenic DCs, macrophages and B cells, but not to T cells and the liposomes enhanced the antigen-presentation by murine bone-marrow-derived DCs (BMDC) and, to a lesser extend, by B cells. Furthermore DDA liposomes rapidly adsorb OVA and bind avidly to BMDCs, thereby mediating enhanced cellular adsorption and uptake of the antigen.

1.3 Combination of a synthetic cord factor analog with DDA liposomes as a powerful new adjuvant system (CAF01)

1.3.1 Biological effects of the mycobacterial cord factor Trehalose-6,6-dimycolate (TDM)

The ability of *Mycobacterium tuberculosis* (Mtb) to manipulate macrophage biology is critical to the pathogen's persistence. Mtb mediates macrophage activation by modulating the cytokine production and the phagosomal maturation during infection. Phagosomes that contain mycobacteria do not fuse with lysosomes (Armstrong and Hart, 1971), thereby enabling the pathogen to survive in the infected host cell. The dynamic interactions between Mtb and the host macrophages are influenced by lipid components, which represent more than 60% of the mycobacterial cell wall (Kolattukudy et al., 1997). Thus, due to the high amount of lipids, MtB cells have an extremely low permeability barrier which is responsible for the resistance of the

pathogen to most common therapeutic agents (Jarlier and Nikaido, 1994) as well as for the ability of this microorganism to survive within the hostile environment of the macrophage (Spargo et al., 1991). The major mycolic-acid containing cell wall molecule in mycobacteria is Trehalose-6,6-dimycolate (TDM), also known as the cord factor (Fig 1).

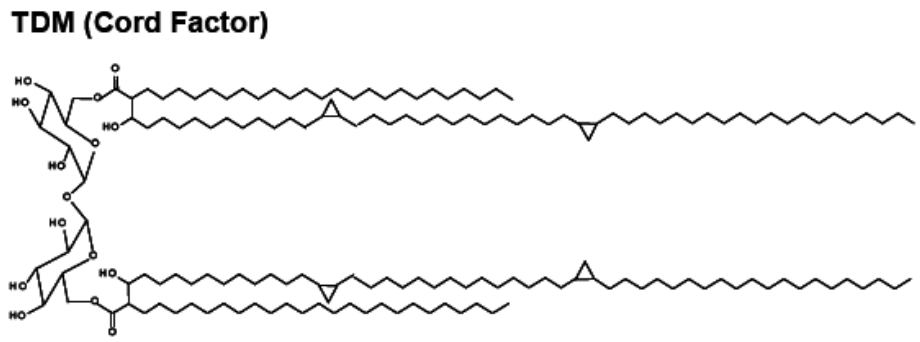


Figure 3: Chemical structure of Trehalose-6,6-dimycolate (TDM, cord factor)

In 1985, Silva *et al* found by HPLC analysis, that petroleum ether extracts of mycobacteria contain primarily TDM (>95% of the total extract) (Silva et al., 1985). Recent studies implicated this glycolipid as the major immunomodulatory component of the mycobacterial cell wall, because macrophages which were infected with de-lipidated BCG showed impaired production of pro-inflammatory cytokines, which could be “rescued” by reconstitution of TDM to the cells (Indrigo et al., 2002). Immune responses to purified TDM mimic certain aspects of a natural Mtb infection, including production of pro-inflammatory cytokines like TNF-α, IL1-β and IL-6, development of granulomas, increased pro-coagulant activity and production of antibodies (Behling et al., 1993; Perez et al., 2000; Perez et al., 1994). *In vitro*, Perez *et al* showed that macrophages treated with TDM-coated beads produced high amounts of pro-inflammatory cytokines (Perez et al., 2000). Furthermore TDM leads to up-regulation of MHCII expression on macrophages (Ryll et al., 2001a) and causes expansion of Th-1 cells by inducing the production of IL-12 and IFN-γ, cytokines that are known to trigger a Th-1 response (Oswald et al., 1997). Also it mediates trafficking events during a mycobacterial infection of murine macrophages (Indrigo et al., 2003). Due to its strong immunstimulatory and Th-1 promoting effect, several studies have used TDM as a possible new adjuvant in different vaccination models. First experiments were done by Saito *et al* in 1976, who compared the adjuvant effect

of IFA-incorporated TDM with the adjuvant effect of IFA alone in combination with different antigens (Saito et al., 1976). It turned out, that TDM caused a significantly stronger footpad swelling and higher antigen-specific antibody titers in mice than IFA alone and it appeared to exert a stronger adjuvant effect than tubercle bacilli. In 2003, Lima *et al* found out, that when TDM was co-encapsulated with a hsp65-encoding DNA plasmid into microspheres, vaccinated mice were protected against a virulent strain of *Mycobacterium tuberculosis* (Lima et al., 2003). Furthermore this vaccine induced protection in mice against *Leishmania major* infection (Coelho et al., 2006). Although TDM has been identified as a potent adjuvant driving Th-1 polarized immune reactions, the problem for its use lies in the relatively high toxicity of the mycolic acids and in possible contaminations of the TDM during the preparation process. A synthetic analog of the cord factor, namely Trehalose-6,6-dibehenate (TDB), appears to be an effective and safe alternative. TDB is a glycolipid comprising a 6, 6'-diester of α, α'-trehalose with two 22-carbon acyl chains instead of the mycolic acid chains of TDM (Fig 2). According to the shorter fatty acid chains, TDB has been associated with a lower toxicity than the natural cord factor. Furthermore it is easier to produce, making it a potent candidate in the field of vaccine development.

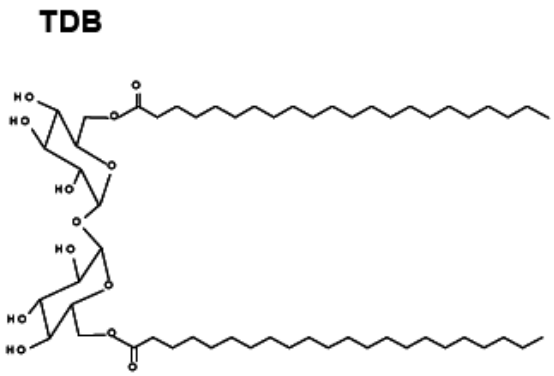


Figure 4: Chemical structure of Trehalose-6,6-dibehenate (TDB)

1.3.2 Immunological analysis of the adjuvant system CAF01

In 2001, Olsen *et al* could show that a subunit vaccine based on a fusion protein of two mycobacterial antigens protected mice against a TB infection (Weinrich Olsen et al., 2001). These two antigens were the 6 kDa early secretory antigenic target (ESAT-6) and the antigen 85B (Ag85B), which is the major secretory protein in actively replicating *Mycobacterium tuberculosis* (Mtb) (Belisle et al., 1997). Both

antigens have been identified to represent very promising vaccine candidates, because they are strongly recognized by T cells in the first phase of infection (Ravn et al., 1999; Ulrichs et al., 1998), they have demonstrated protective efficacy in different animal models (Brandt et al., 2000; Horwitz et al., 1995) and they contain numerous well-characterized epitopes recognized in TB-patients (Ravn et al., 1999; Ulrichs et al., 1998). The fusion protein of these two molecules (Ag85B-ESAT6, also called H1 antigen) together with DDA-coupled monophosphoryl lipid A (MPL) as adjuvant induced protective immunity similar to BCG in the mouse model of TB infection. Similar results were found in an aerosol guinea pig model (Olsen et al., 2004).

In 2004, it was shown that a vaccine containing H1 as the antigen and DDA fused BCG lipids as the adjuvant induced a strong Th-1 response characterized by high amounts of IFN-γ and the production of antigen-specific IgG2a antibodies, whereas the major IFN-γ producing cells could be identified as $CD4^+$ helper T cells (Rosenkrands et al., 2005). Based on these results, the synthetic analogon of the cord factor, Trehalose-6,6-dibehenate (TDB) was next tested in combination with DDA liposomes and used as an adjuvant in a vaccine containing the H1 antigen. Incorporation of the TDB into the DDA liposome bilayers not only stabilized the physically unstable DDA structure but furthermore induced a very substantial cell-mediated immunity as well as a humoral response in mice with a strong Th-1 bias, indicated by high titers of IgG2b and high levels of IFN-γ release from $CD4^+$ T cells that were primed by the vaccine (Davidsen et al., 2005). Similar results were found in a TB vaccination model with maquaques (Langermans et al., 2005). Because of its high potential to polarize the immune response towards Th-1, this adjuvant formulation, designated the CAF01 system, is now tested in vaccines against different diseases. It was shown by Agger *et al* that CAF01, in combination with different antigens, led to high levels of protection against blood-stage malaria and chlamydia infection (Agger et al., 2008).

Following the positive preclinical testing, this adjuvant system is now due to enter phase I clinical trials.

1.4 Activation of antigen-presenting cells

1.4.1 Toll-like receptors and Toll-like receptor signaling

The innate immune system is the first line of the defensive mechanisms that protect hosts from invading microbial pathogens. Antigen presenting cells like macrophages and dendritic cells (DCs) are key players of innate immunity, because they can discriminate between pathogens and self and, upon activation, induce a defensive immune response including the production of antimicrobial peptides and cytokines. APCs recognize microbial components via various pattern recognition receptors (PRRs). PRRs sense diverse pathogen associated molecular patterns (PAMPs) ranging from lipids, lipoproteins, proteins and nucleic acids. This activates intracellular signaling pathways that trigger the induction of inflammatory cytokines, chemokines, interferons and up-regulation of co-stimulatory molecules. A prominent and well-studied family of PRRs is the Toll-like receptor (TLR)-family. TLRs serve as key PRRs with central roles in induction of innate immune responses as well as subsequent development of adaptive immune responses. Toll, the founding member of the TLRs was initially identified as a gene product essential for the development of embryonic dorsoventral polarity in *Drosophila* (Lemaitre et al., 1996). Later, studies revealed its additional role for an effective immune response against fungal infection in flies (Lemaitre, 2004). So far, thirteen Toll-like receptors in mice and eleven TLRs in humans were identified (Beutler, 2004) whereas only TLRs 1-9 are conserved between both species. TLR10 can be found exclusively in humans and TLR11 is only functionally active in mice (Chuang and Ulevitch, 2001; Zhang et al., 2004a). To date, the biological role of TLRs 10, 12, and 13 is not clear.

The main immune cells expressing TLRs are DCs, macrophages and B cells, but also specific types of T cells and non-immune cells such as endothelial and epithelial cells (Hornung et al., 2002; Pasare and Medzhitov, 2005; Zarember and Godowski, 2002). Based on their subcellular localization, TLRs can be classified into two groups. The first group includes TLR1, 2, 4, 5 and 6, all of which are present at the cell surface. The second group includes TLR3, 7, 8 and 9, which are localized intracellular within endosomal compartments. Common to all TLRs is the characteristic ectodomain composed of leucine rich repeats (LRR) that are responsible for PAMP recognition (Akira and Takeda, 2004) and a cytoplasmic domain homologous to the cytoplasmic region of the IL-1 receptor, which is known as the Toll/ Interleukin-1 receptor (TIR) domain. TIR domains are conserved in all TLRs

and they are also components of the IL-18 receptor and of several adaptor molecules. TLRs can also be divided in several groups based on their ligand specificity. Bacterial lipids are recognized by TLR1, 2 and 6 whereas TLR4 is the main receptor for bacterial cell-wall derived lipopolysaccharides. The intracellular receptors TLR3, 7, 8 and 9 are known to recognize nucleic acid like structures, including single stranded (TLR7/8) and double stranded RNA (TLR3). TLR9 is the receptor for bacterial DNA which contains CpG-rich motifs. TLR5 is activated by flagellin (Akira and Takeda, 2004). Beside these microbial ligands, TLRs are also capable to sense some endogenous non-pathogenic molecules, which can be derived from dead or apoptotic cells (Guillot et al., 2002; Termeer et al., 2002; Vabulas et al., 2002).

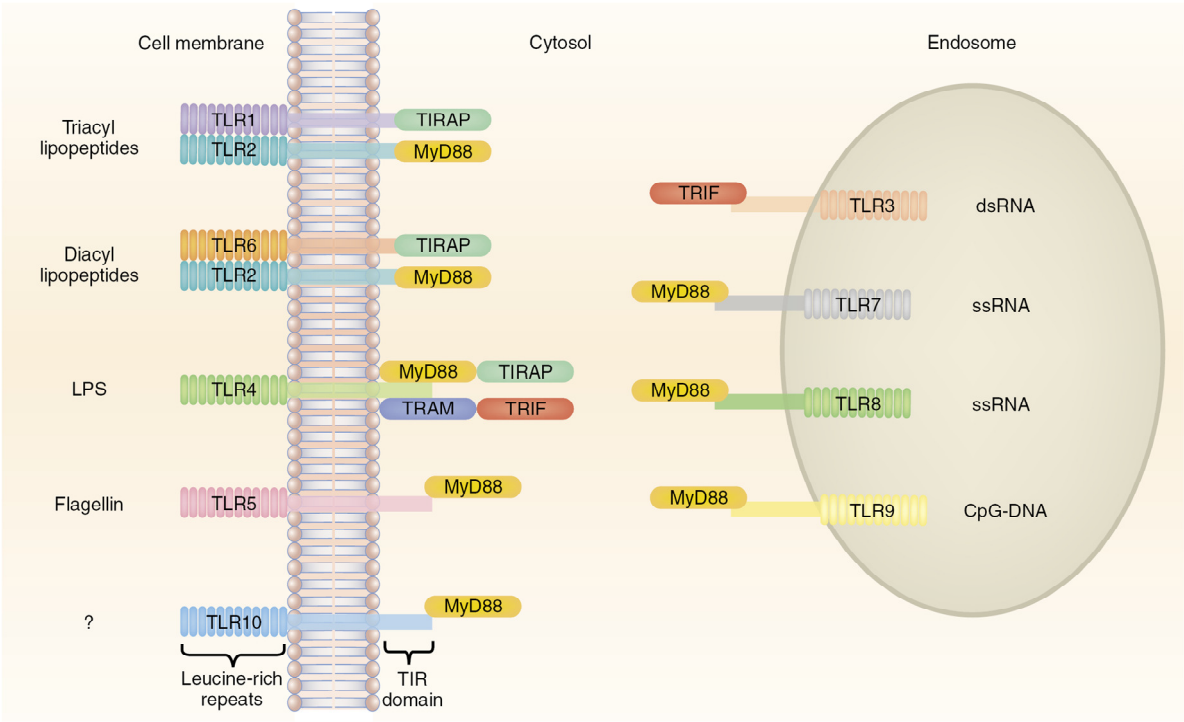


Fig 5: Toll-like receptors.
Schematic diagram of Toll-like receptors showing adaptors, cellular orientation and examples of ligands. TLR10 is only found in humans but not in mice. (from Kanzler et al., 2007)

After ligand binding, TLRs dimerize and undergo conformational changes which are required for the recruitment of downstream signaling molecules, thereby initiating a signaling cascade resulting in the activation of transcription factors like nuclear factor κB (NF-κB) and interferon regulatory factors (IRFs). These transcription factors regulate the expression of several pro-inflammatory cytokines and chemokines as

well as type I Interferons, which mediate innate immune responses and induce adaptive immunity. Most of the TLRs are known to form homodimers upon activation, which enables the cytosolic TIR domains in the receptor tails to associate with the downstream adaptor molecules (Takeda and Akira, 2005). However, TLR2 can also form heterodimers with TLR1 or TLR6 and each dimer responds to different ligands (Ozinsky et al., 2000). To date, four TIR-domain-containing adaptor molecules are known, including myeloid differentiation primary-response protein 88 (Myd88), Myd88 adaptor-like protein (MAL, also known as TIRAP), TIR-domain-containing adaptor protein inducing IFNs (Trif, also known as TICAM1) and Trif-related adaptor protein TRAM (also known as TICAM2). Different TLRs use different adaptor molecules, resulting in different expression profiles upon activation, whereas the initiated signaling cascades induced by TLRs can be divided in Myd88-dependent and independent pathways (Takeda and Akira, 2005; West et al., 2006).

1.4.1.1 Myd88-dependent pathway

Myd88 was originally isolated as a myeloid differentiation primary response gene that is rapidly induced upon IL-6 stimulated differentiation of M1 myeloleukaemic cells into macrophages (Lord et al., 1990). It represents the most prominent TIR-domain-containing adaptor protein because it is recruited by all TLRs except for TLR3. In 1994, Hultmark *et al* found out, that the C-terminal domain of Myd88 is highly homologous with the cytoplasmic regions of the IL-1 receptor family, which led to the hypothesis that Myd88 might have an important role in immunity (Hultmark, 1994). However, unlike the IL-1 receptor family, Myd88 does not contain a transmembrane portion and contains a death domain (DD) in its N-terminus.

It was shown by Adachi *et al*, that mice deficient in Myd88 are completely defective in their responses to IL-1 and the IL-1 related cytokine IL-18, indicating that Myd88 is a critical component in the signaling cascades of both cytokines (Adachi et al., 1998). Furthermore, Myd88-deficient mice were also unresponsive to other immunostimulatory bacterial components including peptidoglycan, lipoproteins and CpG DNA, demonstrating an essential role for Myd88 in the response to most pathogen-derived immunostimulatory molecules (Kawai et al., 1999).

Upon TLR activation, Myd88 binds and recruits IRAK4 to this complex through interaction between the death domains of both molecules. IRAK4 is a member of the IL-1 receptor-associated kinases, which includes IRAK1, IRAK2, IRAK4 and IRAK-M

(Akira and Takeda, 2004; West et al., 2006). IRAK4 is initially activated which in turn phosphorylates and activates IRAK1.The IRAK4/ IRAK1 complex then dissociates from Myd88 and interacts with tumor necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 is a RING-domain E3 ubiquitin ligase, which promotes Lys63-linked polyubiquitination of target proteins in a complex with the two ubiquitin-conjugating enzymes Ubc13 and Uev1a. This polyubiquitination includes TRAF6 itself and also NEMO (Adhikari et al., 2007; Chen, 2005). Ubiquitinated NEMO and TRAF6 subsequently recruit another protein kinase complex involving transforming growth factor-β-activated-kinase-1 (TAK1) and TAK1 binding proteins (TAB1, TAB2 and TAB3), which then activates two distinct pathways involving the IKK complex and the mitogen-activated protein kinase (MAPK) pathway. In the first pathway, active TAK1 phosphorylates components of the inhibitor of κB kinase (IKK) complex, resulting in phosphorylation of IκB, which is associated with NF-κB in the cytosol. Upon this, IκB is degraded and Nf-κB is translocated into the nucleus, where the gene expression program is started. In the second pathway, TAK1 phosphorylates mitogen-activated protein kinase kinases (MAPKKs), which in turn phosphorylate the mitogen-activated protein kinases (MAPKs) p38, C-Jun terminal kinase (JNK) and extracellular-signal regulated kinase (ERK), leading to activation of transcription factors like activator protein 1 (AP-1) and activating transcription factor 2 (ATF-2) (Wang et al., 2001). Activation of NF-κB, AP-1 and ATF-2 ultimately results in the expression of pro-inflammatory cytokines and chemokines, including tumor necrosis factor α (TNF-α), IL-6, IL-12, IL-1β and CXCL8 (Hayden and Ghosh, 2004; Takeda and Akira, 2005).

Myd88 is also capable to induce the expression of interferons (IFNs) by activation of interferon regulatory factors (IRFs) in macrophages and DCs. Stimulation of the intracellular receptors TLRs 7,8 and 9 led to activation of IRF1 and IRF7, which are known to be the main inducers of type I IFNs. TLR-mediated activation of plasmacytoic DCs, the main producers of type I IFNs during viral infections, led to complex formation of Myd88, IRAK4, IRAK1, TRAF6 and IRF7 in the cytoplasm, whereas TRAF6 activates IRF7 through its ubiquitin E3 ligase activity. IRF7 is then translocated into the nucleus to induce the expression of IFN-α and IFN-β (Honda et al., 2004; Honda et al., 2005; Kawai et al., 2004). In myeloid DCs, Myd88 interacts directly with IRF1 and this complex is translocated into the nucleus to initiate transcription of IFN-β (Negishi et al., 2006).

1.4.1.2 Myd88-independent pathway

In 1999, Kawai *et al* have shown, that cells deficient in Myd88 still responded to the TLR4 ligand LPS, whereas with different kinetics (Kawai et al., 1999). This observation suggested the existence of a Myd88-independent pathway using different adaptor molecules.

TIRAP could be identified as an alternative adaptor protein specific for TLR2 and TLR4 signaling but this molecule acts rather in combination with than independent of Myd88 (Yamamoto et al., 2002). However, the adaptor molecule TRIF for an alternative TLR4 and also TLR3 signaling could be identified by database screening for TIR-domain-containing protein. Its important role was shown in TRIF-deficient mice, which were unable to initiate TLR3 and TLR4-induced IFN-β production and activation of IRF3. Furthermore inflammatory cytokine production was impaired in response to TLR4, but not TLR2, 7 or 9 activation (Oshiumi et al., 2003b; Yamamoto et al., 2003). Upon stimulation of TLR3 or 4, TRIF is recruited to the TIR domains of the receptor, where it interacts directly with TRAF6 via its TRAF6-binding motifs in the N-terminal region (Jiang et al., 2004; Sato et al., 2003). TRIF activation also leads to the recruitment and activation of TRAF3, resulting in activation of IRF3 via an IKK-like kinase termed TBK-1 (Fitzgerald et al., 2003). IRF3 is translocated into the nucleus where it initiates the expression of IFN-β. IFN-β in turn can induce several other IFN-inducible genes like CXCL10, GARG-16 or IRG-1, which are important mediators for an effective antiviral response (Kawai et al., 2001; Oshiumi et al., 2003a; Toshchakov et al., 2002; Yamamoto et al., 2002).

1.4.2 C-type lectin receptors and signaling

Besides TLRs, other receptors can also bind pathogens or PAMPs, thereby regulating the expression of innate response genes. Those receptors include members of the TREM (triggering receptors expressed on myeloid cells), the Siglec and the C-type lectin family, which are all classified as a non-TLR class of PRRs. C-type lectin receptors (CLRs) encompass a large superfamily of proteins, including members which appear to have central roles in antifungal immunity. In general, CLRs are defined as proteins, that contain one or more so called C-type lectin domains (CLDs), which are characterized by a structural motif originally identified as a protein fold in the carbohydrate recognition domain of mannose-binding lectin (MBL). The CLD structure has a characteristic double-loop (loop-in-a-loop) stabilized by two

highly conserved disulfide bridges located at the bases of the loops, as well as a set of conserved hydrophobic and polar interactions (Zelensky and Gready, 2005). The second loop, called the long loop region, is structurally and evolutionarily flexible, and is involved in Ca^{2+}-dependent carbohydrate binding and interaction with other ligands (Zelensky and Gready, 2005). This loop is completely absent in a subset of CLDs, indicating calcium independency of several receptors. Furthermore, many CLRs do not bind carbohydrates (Zelensky and Gready, 2005).

Myeloid-expressed CLRs, in particular, have become a focus of intense interest, because they have been linked to many aspects of immunity ranging from pathogen recognition to the maintenance of immune homeostasis (Robinson et al., 2006). However, the ligands and physiological functions of many of these CLRs are still unknown. According to their cytoplasmic motifs or associations with adaptor molecules, these receptors can be divided in three major categories. Members of the first group, including the CLRs Dectin-1 and Clec-2, contain an atypical immunoreceptor-tyrosine-based (ITAM) motif in their cytoplasmic tail (hemi-ITAM). ITAM motifs are conserved sequence motifs and can be found in a variety of proteins that participate in receptor signaling initiated by ligand binding. Upon activation, these motifs become phosphorylated and in turn bind to SH2 (src homology) domains of recruited downstream adaptor molecules. In contrast to common ITAMs, hemi-ITAMs only contain one instead of two ITAM motifs, but this still allows binding of the kinase Syk. Although Syk contains two SH2 domains, it has been shown for Dectin-1, that it is still recruited and bound to the phosphorylated receptor tail (Rogers et al., 2005; Underhill et al., 2005).

The second group of CLRs completely lack ITAM motifs but contain basic residues in their transmembrane domain, which allows them to associate non-covalently with ITAM-containing adaptor proteins, including DAP10, DAP12 and Fc receptor γ-chain (FcRγ), thereby initiating a downstream signaling cascade similar to the hemi-ITAM group. The last group of CLRs contains ITIM instead of ITAM motifs. These are also conserved amino acid sequences that are found in many inhibitory receptors. Upon receptor activation, ITIM motifs become phosphorylated by members of the Src family of kinases, allowing them to recruit other enzymes such as the phosphotyrosine phosphatases SHP-1 and SHP-2 or the inositol-phosphatase SHIP.

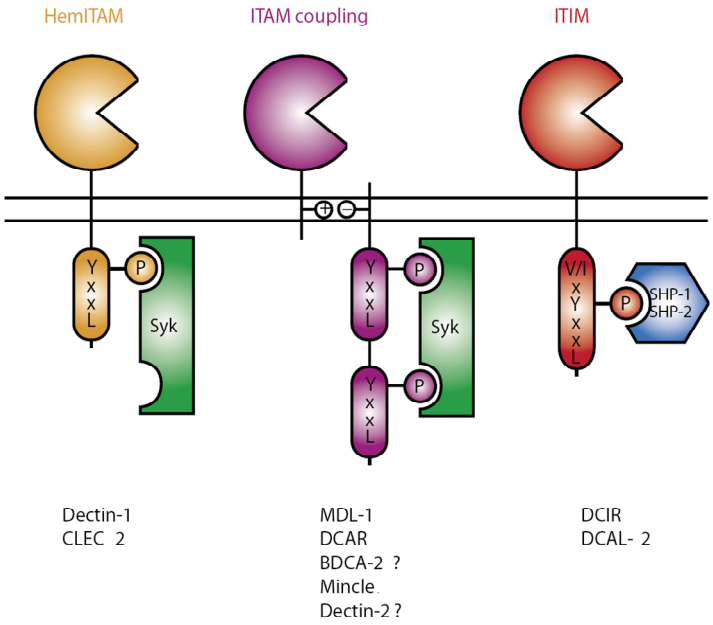


Fig 6: Distinct myeloid C-type lectin receptors (CLRs) use distinct proximal signaling mechanisms.
Myeloid CLRs may activate Syk directly through a hemITAM or indirectly by means of ITAM-bearing adaptor proteins (such as FcRγ chain or DAP12). ITIM-containing CLRs may inhibit myeloid cell activation by coupling to SHP-1 or SHP-1 phosphatases. (from Robinson et al., 2006)

Besides this classification, CLRs can additionally be divided into two major groups, including soluble and membrane-bound forms of the proteins. E.g. the soluble collectins (CLR group III), found in serum, can bind microbes and activate complement. The prototypic collectin thereby is the mannose-binding lectin (MBL), which binds to various sugar moieties presented on viruses, bacteria, fungi and protozoa. MBL provides protection against infections with *Staphylococcus aureus* or *Streptococcus pneumonia* (Takahashi et al., 2006), whereas in contrast to other CLRs, it does not directly signal to induce gene transcription. Prominent CLRs that can act as activating signaling receptors on NK cells are represented by NKG2, NKR-P and Ly49, whereas these receptors respond mainly to self rather than to microbe-derived ligands. Therefore they are not generally thought as PRRs (Lanier, 2005). Many of the membrane-bound CLRs belong to the groups II, V and VI (Zelensky and Gready, 2005) and are expressed mainly by myeloid cells. Group VI e.g. is called the MR family and includes the mannose receptor (MR, CD206) and the DC-specific DEC-205 (CD205). Both receptors have a carbohydrate recognition domain (CRD) and act in a calcium-dependent way. The MR is widely present on tissue macrophages and on some DCs and has been found to induce NF-κB activation as

well as the production of several pro-inflammatory cytokines in response to fungi (Pietrella et al., 2005; Tachado et al., 2007; Taylor et al., 2005; Zhang et al., 2004b). DEC205 is involved in mediating antigen uptake and delivering antigens to late endosomes or lysosomes, where they are degraded (Figdor et al., 2002), resulting in the generation of efficient peptide/ MHC complexes for presentation to T cells.

Group II of the CLRs also contain a CRD domain and are calcium-dependent. Examples are DC-SIGN, Dectin-2 and macrophage-inducible C-type lectin (Mincle). DC-SIGN (CD209) was originally defined as an intercellular adhesion molecule-3 (ICAM-3) receptor on DCs supporting DC-mediated T-cell proliferation (Geijtenbeek et al., 2000). It also functions as receptor for several viruses such as HIV and hepatitis C (Geijtenbeek et al., 2000; Lozach et al., 2007) and recent studies have shown that DC-SIGN can also initiate innate immunity by modulating TLRs by a yet unknown mechanism (den Dunnen et al., 2008). Furthermore it has been proposed to mediate fungal uptake (Cambi et al., 2003; Serrano-Gomez et al., 2004), although the role of this receptor in response to fungi has not been studied extensively.

Dectin-2 is a group II CRD that is present widely on tissue macrophages, Langerhans cells and DCs and its expression is up-regulated on maturing monocytes during inflammation (Taylor et al., 2005). Like many CLRs, it has a high specificity for mannose structures and recognizes hyphal forms of fungi preferentially, including *Candida albicans*, *Microsporum audouinii* and *Trichophyton rubrum* (McGreal et al., 2006). Sato *et al* have shown 2006 that the cytoplasmic tail of Dectin-2 appears to associate through a novel interaction with FcRγ chain, and that Dectin-2 can induce TNF and IL-1 receptor antagonist in response to hyphal forms of *C. albicans* (Sato et al., 2006). Although this is the first receptor shown to recognize and respond to fungal hyphae, the role of Dectin-2 in antifungal immunity has still to be determined.

Mincle (Clec4e) was originally identified as a lipopolysaccharide inducible protein in macrophages (Matsumoto et al., 1999) and has been shown to stimulate inflammatory responses to several fungi, including *C. albicans* (Wells et al., 2008) and the pathogenic fungus Malassezia (Yamasaki et al., 2009). Like Dectin-2, Mincle has a positively charged residue in its transmembrane domain, which mediates association with the FcRγ chain (Yamasaki et al., 2008). Hara *et al* reported 2007, that upon antibody crosslinking of Mincle on macrophages, the tyrosine activation motif of FcRγ triggers intracellular signaling through the tyrosine kinase Syk and the caspase recruitment domain protein CARD9, resulting in the production of pro-

inflammatory cytokines and chemokines like IL-6, TNF, CXCL1 (KC) and CXCL2 (MIP-2) (Hara et al., 2007). These responses were absolutely dependent on FcRγ and CARD9 but independent of Myd88, suggesting that TLR signaling is not involved in this pathway. The authors were also capable to identify the splicosome-associated protein 130 (SAP130), a soluble factor released by necrotic cells, as a possible ligand for Mincle. Although several other CLRs have been linked to the recognition and clearance of apoptotic cells, Mincle is the first example that recognizes an endogenous nuclear ligand associated with necrotic cells. There is evidence that Mincle can induce inflammatory responses even in non-macrophage cell types and it probably induces a variety of other responses in these cells, such as the respiratory burst (Underhill et al., 2005).

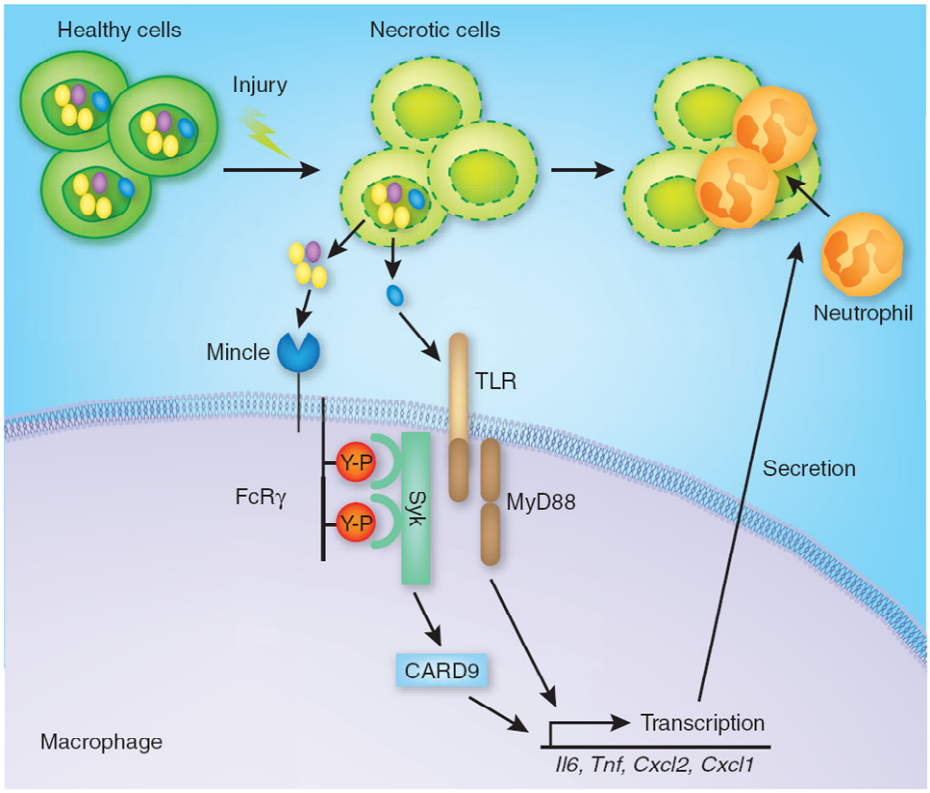


Fig 7: The C-type lectin receptor Mincle triggers inflammatory responses to necrotic cells. The induction of excessive cell death leads to cellular necrosis and disruption of cytoplasmic and nuclear membranes. This causes the release of cellular components such as SAP130, which is recognized by Mincle expressed on myeloid cells. The recognition of SAP130 by Mincle triggers intracellular signaling through the associated FcRγ adaptor and the Syk-Card9 pathway, which induces the production of proinflammatory cytokines and chemokines and recruitment of neutrophils to the site of inflammation. (from Brown, 2008)

The best studied C-type lectin so far is Dectin-1, which belongs to group V of membrane bound CLRs expressed in myeloid cells. Members of this group are also called “NK-cell-receptor-like C-type lectins” because many of them are exclusively

expressed on NK cell and T-cell subsets. Dectin-1 was first identified by Brown and Gordon in an expression cloning scheme for receptors that could mediate phagocytosis of zymosan, a β-glucan rich cell wall particle prepared from *Saccharomyces cerevisiae* (Brown and Gordon, 2001). In addition to zymosan, Dectin-1 can specifically recognize other soluble and particulate β-1,3 linked glucans like e.g. Curdlan, a high molecular weight polymer of glucose. All these compounds are carbohydrate polymers with immunomodulatory activity that are found mainly in the cell wall of fungi (Gantner et al., 2005; Steele et al., 2003), but also in some plants and bacteria. Several studies indicated, that Dectin-1 recognizes several fungi species, including *Candida* spp., *Pneumocystis* spp., *Saccharomyces* spp. *Coccidoides* spp. and *Aspergillus* spp. (Brown et al., 2003; Steele et al., 2003; Viriyakosol et al., 2005) and that in macrophages, Dectin-1 mediates the uptake and killing of live fungal particles by the induction of the respiratory burst and the production of pro-inflammatory cytokines and chemokines. In addition to single carbohydrate ligands and whole fungi, Dectin-1 is also thought to recognize an endogenous, yet unknown T cell ligand. Dectin-1 can bind to $CD4^+$ and $CD8^+$ T cells *in vitro*, thereby increasing their proliferation. According to these observations, Dectin-1 has been proposed to act as a co-stimulatory molecule for T cells (Ariizumi et al., 2000; Grunebach et al., 2002).

In contrast to other C-type lectin receptors, Dectin-1 lacks cysteine residues in its stalk region, indicating that it probably does not dimerize upon activation. However, it is the first example of a signaling non-TLR PRR. Underhill *et al* have shown in macrophages, that Dectin-1 activates the tyrosine kinase Syk through its hemi-ITAM motif in its cytoplasmic tail resulting in the production of reactive oxygen species (ROS) like e.g. nitric oxide (NO) (Underhill et al., 2005). After binding of particulate β-glucans, Dectin-1 induces the production of various cytokines and chemokines, including TNF, CXC-chemokine ligand 2 (CXCL2, also known as MIP-2), IL-2, IL-10 and IL-12 from macrophages and DCs (Brown et al., 2003; Gantner et al., 2003; Rogers et al., 2005). In 2006, Gross *et al* identified the adaptor proteins Card9, Bcl10 and Malt1 as essential components of the signaling cascade induced by Dectin-1 in DCs (Gross et al., 2006). Card9-deficient DCs showed impaired production of TNF-α, IL-6 and IL-2 in response to zymosan as well as to *Candida albicans* infection, whereas the response to LPS was unaffected. Similar results were determined in Bcl10 and Malt1-deficient DCs. Furthermore Card9 relays Dectin-1/Syk signals to

NF-*k*B, whereas Goodrigde *et al* reported that activation of Dectin-1 also triggers NFAT activation in macrophages and DCs (Goodridge et al., 2007). In 2007, LeibundGut-Landmann additionally reported, that Dectin-1 stimulation with the β-glucan Curdlan activates DCs by enhancing the expression of the two surface markers CD40 and CD86 (LeibundGut-Landmann et al., 2007). Curdlan stimulation also induced the expression of TNF-α, IL-6, IL-2 and IL-12p40 in DCs in a Syk-Card9-dependent pathway, whereas this was independent of Myd88 and Trif-signaling. Furthermore, Dectin-1 activated DCs were able to induce the priming of Th-1 as well as Th-17 T cells *in vitro*. *In vivo*, infection with *C. albicans* also triggered the development of a Th-17 response in a strictly Card9-dependent way (LeibundGut-Landmann et al., 2007).

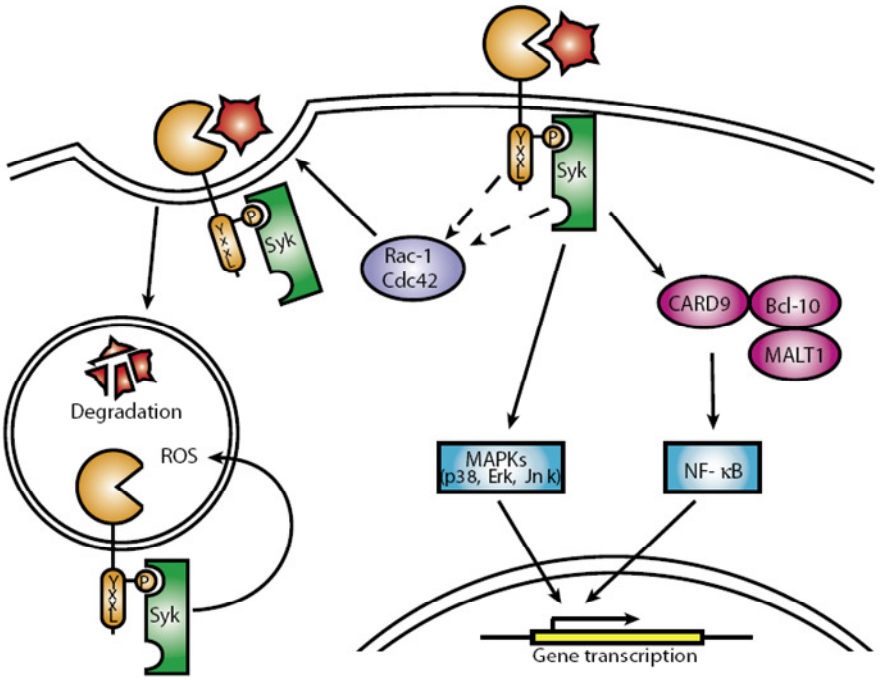


Fig 8: Intracellular consequences of dectin-1 stimulation.
Stimulation of Dectin-1 by zymosan or fungal components triggers activation of mitogen-activated protein kinases (MAPKs) and NF-κB (the latter via a complex containing Card9, Malt1 and Bcl-10) and ultimately results in the transcription of innate immune response genes. Zymosan phagocytosis by DCs is partially dependent on Syk, whereas in macrophages it is Syk-independent but the production of reactive oxygen species (ROS) depends on Syk activity. (from Robinson et al., 2006)

2 Aims of the study

The development of adjuvants that elicit strong cellular immune responses of the Th-1 type has recently become a focus of vaccine research. The mycobacterial cord factor Trehalose-6,6-dimycolate (TDM) has been identified to be a potent Th-1-promoting immunostimulatory component in several vaccination models. In the adjuvant system CAF01, the lesser toxic synthetic cord factor analog Trehalose-6,6-dibehenate (TDB) was incorporated into DDA liposomes bilayers (DDA/TDB) and tested as an adjuvant in combination with the mycobacterial fusion protein Ag85B-ESAT6 (H1 antigen). CAF01 induced robust and protective cell-mediated and humoral immunity in mice, indicated by high titers of antigen-specific IgG2b antibodies and IFN-γ produced by $CD4^+$ T cells. Thus, TDB represents an attractive adjuvant for potential use in humans. However, the mechanism behind the Th-1 promoting adjuvant activity of the glycolipids TDB and TDM has remained unknown.

To address this question, in the present study, both glycolipids should first be tested in terms of their capacity to activate antigen-presenting cells *in vitro* by using different biochemical and molecularbiological methods. TDB/TDM induced pro-inflammatory mediators associated with the development of a Th-1 response should be analyzed and compared to the responses induced by classical TLR and non-TLR agonists.

Second, by using bone-marrow-derived macrophages and DCs from different knock out mice, the intention was to analyze the role of Toll-like receptors and other pattern-recognition receptor families in the recognition of TDB/TDM and to possibly identify receptors(s) and molecules involved in the TDB/TDM-initiated downstream signaling.

Third, it was planned to study the type of the TDB/TDM induced immune response *in vivo* by immunization of mice with the adjuvant system CAF01 in combination with the H1 antigen and fourth, based on the *in vitro* findings, it should be analyzed by immunization of specific knock-out mice with the CAF01/H1 vaccine, which receptors and signaling molecules are involved in controlling the TDB/TDM-induced immune response *in vivo.*

3 Material and Methods

3.1 Material

3.1.1 Equipment

Analytical balance	Scaltec, Göttingen
Balance	Ohaus, USA
Centrifuge Biofuge Pico/ Fresco	Heraeus, Hanau
Centrifuge Multifuge 3	Heraeus, Hanau
Electrophoresis Apparatus	Peqlab, Erlangen
Electrophoresis power supply 301	Amersham/ GE Healthcare, Munich
ELISA reader sunrise	Tecan, Switzerland
Experion Bioanalyzer	BioRad, Munich
Freezer -20°C	Siemens, Munich
Freezer -80°C	Thermo Scientific, USA
Fridge	Liebherr, Switzerland
Gene Chip® Fluidics station 450	Affymetrix, USA
Gene Chip® Scanner 3000	Affymetrix, USA
Incubator BBD 6220	Heraeus, Hanau
Incubator Hera-Cell 240	Heraeus, Hanau
Lightcycler LC 480	Roche, Mannheim
Microcentrifuge	Roth, Karlsruhe
Microscope Zeiss Axiovert 40 C	Zeiss, Jena
Microwave	Privileg, Munich
Multichannel pipettes	ThermoLabsystems, USA
Multipipette plus	Eppendorf, Hamburg
Nanodrop®ND-1000 Spectrophotometer	Peqlab, Erlangen
Neubauer counting chamber	Roth, Karlsruhe
pH-meter Multical	WTW, Weilheim
Pipettes	Gilson, USA
Pipetboy Acu	Integra Biosciences, Fernwald
Sealing apparatus Folio	Severin, Sundern
Shaker	Peqlab, Erlangen
Sterile bench	Heraeus, Hanau
Thermocycler Trio Thermoblock	Biometra, Göttingen

Thermomixer Comfort	Eppendorf, Hamburg
UV water bath	Elma, Singen
Vortexer Genie 2	Scientific Industries, USA
Waterbath	Memmert, Schwabach

3.1.2 Consumable items, kits and enzymes

Cell culture plates and petri dishes	Falcon, USA Greiner, Austria
Cell scraper	TPP, Switzerland
Cell strainer, 100 µM	Falcon, USA
Combitips	Eppendorf, Hamburg
Cryotubes, 1 ml	Corning, USA
ELISA kits	R&D Systems, USA
Filter tips	Kisker, Steinfurt
Gene Chip® WT Kit	Affymetrix, USA
Gene Chip® Mouse 1.0 ST	Affymetrix, USA
HotStar Taq PCR Kit	Qiagen, Hilden
LC480 PCR plates 96-well	Roche, Mannheim
M-MulV reverse Transcription kit	Peqlab, Erlangen
MaxiSorp 96-well ELISA plate	Nunc, Wiesbaden
Microtest™ 96-well ELISA plate	Becton Dickinson, Heidelberg
Microcentrifuge tubes 1,5 ml	Eppendorf, Hamburg
Microsentrifuge tubes 2 ml	Eppendorf, Hamburg
Parafilm®	Roth, Karlsruhe
RNeasy mini isolation kit	Qiagen, Hilden
Serological pipettes	TPP, Switzerland
Sterile injection needles Microlance™3	Becton Dickinson, Heidelberg
Syringe Discardit™ 10 ml and 5 ml	Becton Dickinson, Heidelberg

3.1.3 Reagents

Accutase	PAA, Cölbe
Agarose	Invitrogen, Karlsruhe
Ag85B-ESAT6 fusion protein (H1)	provided by Statens Serum Institute, Denmark
BD Opteia™ Substrate Reagent	Becton Dickinson, Heidelberg
β-Mercaptoethanol	Gibco, Karlsruhe
Bovine Serum Albumin	Sigma-Aldrich, Taufkirchen
Concanavalin A	Sigma-Aldrich, Taufkirchen
CpG 1826	Coley Pharmaceutical GmbH, Düsseldorf
Curdlan	Wako Chemicals,
Dimethyldioctadecylammonium (DDA) bromide	Avanti Polar lipids, USA
DEPC-H_2O	Ambion, USA
DMSO	Sigma-Aldrich, Taufkirchen
DNA-Ladder 1 Kb	Invitrogen, Karlsruhe
dNTP	Invitrogen, Karlsruhe
Dulbecco's MEM	Biochrom, Berlin
Ethidiumbromide	Roth, Karlsruhe
Fetale Bovine Serum	Biochrom, Berlin
Freund's Adjuvant, incomplete	Sigma-Aldrich, Taufkirchen
L-cell-conditioned medium	Own production
LC480 Mastermix	Roche, Mannheim
LPS from *E.coli* O55:B5	Sigma-Aldrich,Taufkirchen
MTT (Thiazolyl Blue Tetrazolium Bromide)	Sigma-Aldrich, Taufkirchen
Murine IFNγ recombinant	tebu-bio, Offenbach
Oligo(dT) primer	Applied Biosystems, Darmstadt
	Amersham/ GE Healthcare, Munich
Orange G	Sigma-Aldrich, Taufkirchen
PBS	Biochrom, Berlin
Penicillin/ Streptomycin	Biochrom, Berlin
Piceatannol	Merck/ Calbiochem, Darmstadt

Polybead® Polystyrene beads 90 µM	Polysciences, Eppelheim
Proteinase K	Roche, Mannheim
Random Hexamer	Applied Biosystems, Darmstadt
	Amersham/ GE Healthcare, Munich
RPMI	Biochrom, Berlin
SYBR-green	Molecular Probes, Karlsruhe
Streptavidin-HRP	R&D Systems, USA
Trehalose-6,6-dibehenate (TDB)	Avanti Polar lipids, USA
	Sigma-Aldrich, Taufkirchen
Trehalose-6,6-dimycolate (TDM)	Gerbu, Gaiberg
	Sigma-Aldrich, Taufkirchen

3.1.4 Primers and probes

Table 1: Oligonucleotides for real-time PCR

Gene name	Primer forward	Primer reverse	Associated UPL probe	Company
IL-6	RL409 gctaccaaactggatataatcagga	RL410 ccaggtagctatggtactccagaa	6	Metabion
Mincle	RL569 gcctccatcctgtttctcag	RL570 tgagagctgcgatatgttacg	9	Metabion
iNOS	RL453 ctttgccacggacgagac	RL454 tcattgtactctgagggctgac	13	Metabion
IL-1β	RL522 ttgacggaccccaaaagat	RL523 agctggatgctctcatcagg	38	Metabion
HPRT	RL415 tcctcctcagaccgctttt	RL416 cctggttcatcatcgctaatc	95	Metabion

Table 2: Universal library probes for real-time PCR

UPL probe number	Sequence	Cat. No	Company
6	ttcctctg	4685032001	Roche library probe
9	catcacca	4685075001	Roche library probe
13	aggcagag	4685121001	Roche library probe
38	ctgcttcc	4687965001	Roche library probe
95	agtcccag	4692128001	Roche library probe

3.1.5 Antibodies

Table 3: Detection antibodies for ELISA

Isotype	Conjugate	Dilution	Source	Company
IgG_{2a} [b]	Biotin	1:1000	Rabbit anti-mouse	BD Pharmingen™
IgG_1	Biotin	1:1000	Rabbit anti-mouse	BD Pharmingen™

3.2 Methods

3.2.1 Cell biology

3.2.1.1 Media used for eukaryotic cell culture

cRPMI: 500 ml RPMI 1640
50 ml heat inactivated FBS
5 ml Penicillin/ Streptomycin
500 µl β-Mercaptoethanol 50 mM

cDMEM: 500 ml DMEM
50 ml heat inactivated FBS
5 ml Penicillin/ Streptomycin
500 µl β-Mercaptoethanol 50 mM

3.2.1.2 Generation of murine bone-marrow-derived macrophages

After sacrificing the mice, the hind legs were removed and the bones were separated. The bone marrow was washed out with cDMEM under sterile conditions by using a 27G syringe and the cell suspension was collected in a 50 ml Falcon tube.
After 5 minutes of Erythrocyte lysis cells were taken up in cDMEM supplemented with 10 % of L-cell-conditioned medium (LCCM) as a source of M-CSF and incubated over night at 37°C in 10-cm bacteriological plastic dishes. Non-adherent cells were counted and re-plated at a density of $0.5x10^6$ cells/ml in cDMEM with 10 % LCCM in 10-cm plastic dishes. At day four, 5 ml of fresh cDMEM with 10 % LCCM were added and the cells were incubated for another two days. After 6-7 days of differentiation, non-adherent cells were removed and the remaining cells were incubated with 5 ml of Accutase for 15 minutes at 37°C. The Accutase was then inhibited by the addition of 5 ml cDMEM and the cells were counted. Afterwards the cells were re-plated at a density of $1x10^6$ cells/ml and rested overnight at 37°C before they were stimulated.

3.2.1.3 Coating of cell culture dishes with TDB and TDM

Lyophilized powder of either TDB or TDM was solved in Isopropanol by heating the solution in a water bath for 1 hour at 55°C. The solution was mixed by vortexing every 10 minutes during the whole incubation time. Afterwards, the required amounts of TDB and TDM were coated directly to the bottom of sterile cell culture plates by incubation of the plates overnight at 37°C until the Isopropanol had evaporated. The plates were then stored at 4°C. The original procedure was described by Ozeki *et al.* 2005.

3.2.1.4 Coating of Polystyrene beads with TDB and TDM

1 ml of the Polystyrene bead solution was centrifuged and the sedimented beads were washed twice with 1ml of sterile PBS. The supernatant was aspirated and the beads were mixed with 200 µg of TDB. For coating the lipids on the bead surface, the solution was sonicated three times in a UV-water bath for 5 minutes at 60°C.

In case of TDM, the required amounts of TDM (200 µg) were first given into a 1,5 ml Eppendorf-tube followed by vacuum-centrifugation at 60°C until the Isopropanol had completely evaporated. Afterwards the washed beads were given to the dried TDM and the suspension was incubated 30 minutes at 55°C with 1000 rpm of agitation. This step was followed by the coating procedure as described for TDB (Geisel et al., 2005).

3.2.2 Mice

All mice were kept under pathogen-free conditions at the animal facility of the Institute of Medical Microbiology, Immunology and Hygiene of the Technical University Munich. Animal experiments were approved and authorized by the local government. Prior to the experiments, all transgenic mice were genotyped according to standard laboratory protocols. C57BL6 mice, ASC-/- mice, IL-18-/- mice, TLR2/3/4/7-/- mice and TLR2/3/4/7/9-/- mice were bred at our own animal facility or were ordered from Harlan-Winkelmann. Breeding pairs for mice deficient in Myd88 were provided by S. Akira (Research Institute for Microbial Diseases, Osaka, Japan). Card9-/-, Bcl10-/- and Malt1-/- mice have been previously described (Gross et al., 2006; Ruland and Mak, 2003), and were kindly provided by J. Ruland. Dectin-1 deficient mice have been previously described (Taylor et al., 2007), and were kindly provided by G. Brown. IL1R-deficient mice have been previously described (Glaccum

et al., 1997), and were kindly provided by Esther von Stebut (Department of Dermatology, Johannes Gutenberg-University, Mainz). Syk-/- and control mice were provided by V. Tybulewicz (National Institute for Medical Research, London, England, UK). Dap12 -/- and Fcer1g -/- mice were used with permission from T. Takai (Kaifu et al., 2003; Takai et al., 1994), and kindly provided by Falk Nimmerjahn (Department of Molecular Immunology, Friedrich-Alexander University Erlangen-Nürnberg). All knockout mice used for immunization experiments were backcrossed to C57BL6 for at least six generations.

3.2.3 Animal Experiments

3.2.3.1 Preparation of adjuvant formulas

The preparation oft the adjuvant formulations was kindly accomplished by the group of Peter Andersen at the Staten Serum Institute in Denmark.

DDA liposomes were suspended in sterile distilled water at 2.5 mg/ml, heated to 80°C for 20 minutes with continuous stirring in order for liposomes to form and subsequently left to cool at room temperature. A stable formulation of DDA/TDB was prepared by the lipid film hydration method. TDM was suspended in sterile distilled water containing 2 % dimethylsulfoxide to a concentration of 5 mg/ml by repeated passaging through a fine-tipped pipette followed by vortexing. This step was repeated three times before freezing the solution at -20°C until use. Before immunization, TDB was finally mixed with DDA.

3.2.3.2 Immunization of mice with the fusion protein Ag85B-ESAT6 (H1)

Mice were immunized subcutaneously by footpad injection two or three times with a two-week interval between injections. All mice were immunized with 2 µg of the vaccine antigen H1 emulsified in 100 µl of each adjuvant (50 µl per foot), comprising either 250 µg DDA liposomes alone, 250 µg DDA liposomes plus 50 µg TDB or 250 µg DDA liposomes plus 50 µg TDM. For CpG as adjuvant, H1 was mixed in PBS with the oligonucleotide such that one dose (50µl) contained 2 µg H1 antigen and 5 nmol CpG. The footpad thickness was determined with a caliper before and at several timepoints after injection. Baseline values were subtracted individually for both feet of each mouse.

3.2.3.3 Removal of lymph nodes and heart blood

Four weeks after the first immunization, the mice were sacrificed and the draining lymph nodes were removed and passed through a 100 µm cell strainer to separate the cells. After 5 minutes of Erythrocyte lysis the cells were washed twice with cRPMI and counted. For re-stimulation, $3x10^6$ cells (in a total volume of 250 µl cRPMI) were plated in triplicates in 96-well round-bottom cell culture dishes.

Heart-blood was taken out immediately with a Pasteur-pipette and collected in heparin-coated capillary tubes. The blood samples were centrifuged for 5 minutes at 13000 rpm and the sera were frozen at -20°C.

3.2.3.4 Measurement of antigen-specific IFN-γ and IL-17 production

For re-stimulation of the extracted LN cells, the H1 Antigen was used at a final concentration of 1 µg/ml and 10 µg/ml. As a negative or positive control, wells containing only medium or 1 µg/ml Concanavalin A were used. After 96 hours of incubation the supernatants were harvested and the amount of IFNγ and IL-17 was determined by ELISA.

3.2.3.5 Determination of antigen-specific antibody response

To evaluate the presence of H1-specific antibodies, mouse sera of immunized mice were analyzed in triplicates in 3-fold dilutions (beginning at a 1:1000 dilution in Reagent Diluent) in 96-well ELISA reaction plates. The procedure followed a standard sandwich ELISA protocol, except that in every step the total reaction volume per well was 50 µl instead of 100 µl. For determining specific antibody titers, biotinylated rabbit anti-mouse IgG_1 and $IgG_{2a}{}^b$ were used as detection antibodies. The absorbance was measured on an ELISA reader at 450 nm.

3.2.4 Molecular Biology

3.2.4.1 Buffers and Solutions

50x TAE	242	g	Tris Base
	57.1	ml	Glacial acetic acid
	100	ml	0.5 M Na_2EDTA pH 8
	Ad 1000 ml ddH_2O		

4X DNA running buffer	50	mg	Orange G
	15	ml	Glycerol
	0.5	ml	1 M Tris-HCl
	Ad 50 ml ddH_2O		

3.2.4.2 Agarosegel electrophoresis

Based on the expected DNA fragment sizes, 1-2 % Agarose gels were used for the separation. The Agarose was first heated in 1x TAE buffer until the solution became clear. After cooling of the solution, Ethidiumbromide was added to a final concentration of 100 ng/ml. The dried gel was overlayed with 1x TAE buffer and the DNA fragments were separated at 80 V and 120 mA. 5 µl of a 1kb DNA ladder served as control to determinate the fragment size of the amplificates. The fluorescence of the DNA bands was visualized with UV light at a wave length of 254 nm.

3.2.4.3 Standard PCR

All PCRs were performed in a total volume of 20 µl using the *Pfx* Platinum Hot start Taq Polymerase Kit (Invitrogen). Preperation of the PCR mix followed the manufacturer's protocol. With modifications for annealing temperature (dependent on used primer combination) and extension time (dependent on the size of the amplified fragment), amplification occurred using the following protocol:

5	min	95C°	
30	s	95C°	} 35-40x
45	s	65C°	
4	min	72C°	
10	min	72C°	

3.2.4.4 Total RNA extraction

Total RNA from monolayer cells was extracted by using the RNeasy mini kit (Quiagen, Hilden). The preparation followed the standard protocol except that 50 µl instead of 20 µl were used for the elution step. The RNA concentration was measured on a Nanodrop spectrophotometer. For microarray analysis the RNA quality was additionally controlled by using Biorad Experion Standard Sensitivity Chips (Biorad, Munich).

3.2.4.5 First strand cDNA synthesis

The synthesis of first strand cDNA from total RNA was carried out by using the peqGOLD M-MulV Reverse Transcriptase. 500 ng of total RNA per sample in a total reaction volume of 15 µl was given into a 0.2 ml reaction tube and 1 µl of a prepared primer mix (100 ng random hexamers and 250 ng Oligo(dT) primer) was added to each sample. To denature the RNA the mixture was incubated at 70°C for 5 minutes and then cooled on ice. Afterwards the following Mastermix was added to each sample:

6	µl	5x buffer for M-MulV RT complete
2	µl	dNTP Mix
0.25	µl	M-MulV RT (50 U)
6.75	µl	DEPC-H_2O

The reaction mix was incubated at 42°C for 1 hour followed by the addition of 30 µl DEPC-H_2O to each sample. The cDNA was stored at -80°C for further usage.
All reaction steps were prepared in a Thermocycler block.

3.2.4.6 Quantitative Real-time PCR

To determine the expression levels of different genes, primer-probe combinations designed and selected from the Roche Universal Probe Library were used.
All PCRs were performed in 96-well reaction plates in a total volume of 20 µl on the LC480 light cycler (Roche, Mannheim).

PCR mix for each sample:	3	µl	cDNA
	10	µl	Roche LC480 Mastermix
	0.2	µl	UPL probe
	0.2	µl	forward primer (20 µM stock)
	0.2	µl	reverse primer (20 µM stock)
	6.4	µl	DEPC-H_2O

The thermal cycling conditions were based on the manufacturer's instructions for the usage of UPL probes (Roche).

PCR cycling program:	5	min	95°C	
	10	s	95°C	} 45x
	30	s	60°C	
	1	s	72°C	
	10	s	45°C	

Expression of target genes (fold changes) was normalized to the house-keeping gene HPRT and calculated with the ΔΔCT method using the untreated sample as a calibrator.

3.2.4.7 Microarray analysis of gene expression

Affymetrix GeneChips were used for genome-wide transcriptome analysis of 1 µg total RNA per sample, following the manufacturer's instruction for labeling and hybridization of the samples.

After a 48h stimulation of C57BL6 and Card9-/- macrophages with CpG, Curdlan and TDB the total RNA was extracted as previously described and the samples were processed with the Whole Transcript Assay Kit and hybridized to Mouse Gene 1.0 ST GeneChips, as detailed by the manufacturer. CEL files were processed for global normalization and generation of expression values using the robust multi-array average algorithm (RMA) (Bolstad et al., 2003). To select regulated genes, the data were filtered by excluding all probe sets that over all conditions showed less than 5-fold change and an absolute difference between maximum and minimum expression values of less than 100. The probe sets passing these criteria were z-score normalized and subjected to hierarchical clustering using Spotfire DecisionSite

Functional Genomics software. The CEL files were submitted to the public repository Gene Expression Omnibus (Accession numbers GSE10532).

3.2.5 Immunology

3.2.5.1 Enzyme-linked immunosorbent assay (ELISA)

Buffers and Solutions

Blocking buffer/ Reagent Diluent	9.55	g	PBS
	10	g	BSA
	Ad 1000 ml		ddH_2O
Washing buffer	9.55	g	PBS
	0.5	ml	Tween 20
	Ad 1000 ml		ddH_2O
Stop Solution	2 N		H_2SO_4

Determination of cytokine levels

Cytokines were detected by using the DuoSet ELISA Development System (R&D Systems). The procedure followed the manufacturer's protocol.

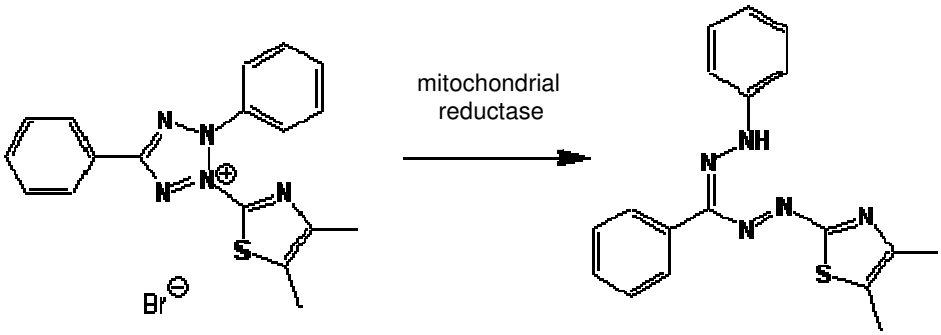
Briefly, 96-well ELISA reaction plates were coated with 100 µl/well of the capture antibody (final concentration 720 ng/ml, diluted in PBS) and incubated overnight at 4°C. On the next day the plates were washed twice, dried and 200 µl blocking buffer/well was added. After 1 hour incubation at room temperature the plates were washed and dried again. Afterwards, 100 µl of the samples and the standard (all diluted in Reagent Diluent) was added to the plates followed by an overnight incubation step at 4°C. On day three, the plates were washed and dried again and 100 µl/well of the detection antibody (final concentration 36 µg/ml, diluted in Reagent diluent) was added for 2 hours at room temperature. This was followed again by two washing steps and addition of 100 µl/ Streptavidin (diluted 1:200 in Reagent Diluent) to each sample. After 30 minutes, the plates were washed and dried again and 100 µl/well of freshly prepared substrate solution was added to the plates. Afterwards the plates were incubated in the dark dependent on the light sensitivity of the substrate reagent. The incubation time ranged from 10-40 minutes. To stop the reaction 50 µl/well stop solution was added and the plate was analyzed in an ELISA reader at a

wave length of 450 nm (reference wavelength at 570 nm). For the calculation of the final protein concentration the dilution factor of the samples was included.

3.2.5.2 MTT assay

The MTT assay is a standard colorimetric assay for measuring the activity of enzymes that reduce the yellow MTT to formazan, which gives a purple color. Addition of a solubilization solution (acidic Isopropanol) dissolves the insoluble purple formazan product into a colored solution and the absorbance of this solution can be quantified by measuring at a wavelength between 500 and 600 nm by a spectrophotometer.

The reduction takes place only when the mitochondrial reductase enzymes are active and therefore this assay can be used as a measure of viable (living) cells.



Buffers and solutions

MTT stock solution	5 mg/ml Thiazolyl Blue Tetrazolium Bromide in	
ddH_2O	Solution has to be filtered through a 0.2 µM filter and stored at 2-8°C	
Acidic Isopropanol	0.04 M	HCl in absolute Isopropanol

Detection of the cell viability

The supernatants of cultured monolayer cells (in 96-well cell culture plates) were aspirated and 50 µl of fresh medium was added to each well together with 20 µl of a MTT stock solution. The plate was incubated for 2 hours at 37°C followed by the addition of 150 µl/well acidic Isopropanol. The solution was pipetted up and down

several times to solubilize the formazan crystals. All plates were analyzed in an ELISA reader at a wave length of 570 nm.

3.2.5.3 NO assay

Buffers and solutions

Grieß Reagent	0.5	g	Sulfanilamid
	0.05	g	N-(1-Naphtyl)-Ethylendiamine
	1.17	ml	phosphoric acid
	Ad 50 ml		ddH_2O
$NaNO_2$ stock solution	10 mM		$NaNO_2$ in ddH_2O

The concentration of nitric oxide in the supernatant of cultured monolayer cells was measured using the Grieß-Reagent (Green *et al*, 1982). The assay was prepared in 96-well cell culture plates. Respectively 50 µl of cell culture supernatant and standard concentrations of $NaNO_2$ (in duplicates) in a range from 400 µM to 0.4 µM were mixed with 50 µl Grieß reagent solution and incubated 10 minutes at room temperature. Afterwards the plate was measured in an ELISA reader at a wave length of 540 nm. The NO concentration of every sample was calculated by comparing their absorbance to that of the standard concentrations.

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4 Results

4.1 Differential induction of Th-1 and Th-17 responses by CpG ODN and TDB adjuvants

According to the findings of Rosenkrands *et al* 2005, who showed that Trehalose-6,6-dibehenate (TDB) as a component of the novel adjuvant system CAF01 induces a strong Th1-response in combination with various antigens, the adjuvant activity of TDB *in vivo* was tested and compared to the adjuvant activity of the TLR9 ligand CpG. Therefore, C57BL6 mice were immunized in the footpad with one of the two adjuvants TDB (embedded in Dimethyldioctadecylammonium (DDA) liposomes) or CpG ODN 1826 in combination with the mycobacterial fusion protein Ag85B-ESAT6 (H1 Antigen, 2 µg) and received a second immunization 14 days later (Fig 1). On day 30 post vaccination, lymph nodes were removed and prepared and the cellular suspension was re-stimulated for 96 hours with H1 antigen to investigate the priming of the T-cell response. Unvaccinated mice were used as controls.

Both TDB and CpG induced a robust and comparable Th-1 response characterized by high amounts of antigen-specific IFN-γ production (Fig 2), which confirmed the findings of the Danish group. Additionally, TDB was also shown to elicit a strong H1-specific Th-17 response (Fig 2). Thus, this indicates that this glycolipid has an adjuvant activity which differs from that of a classical TLR agonist.

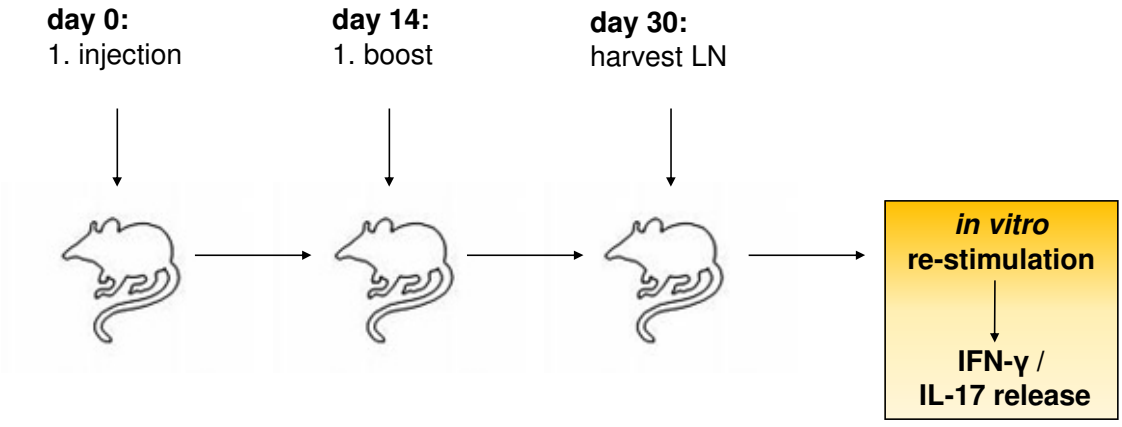

Figure 1: Immunization scheme
Mice were immunized in the footpad and received a second immunization 14 days later. On day 30 post vaccination, lymph nodes were removed and prepared and the cellular suspension was re-stimulated for 96 hours with H1 antigen followed by detection of antigen-specific production of IFN-γ and IL-17 by ELISA.

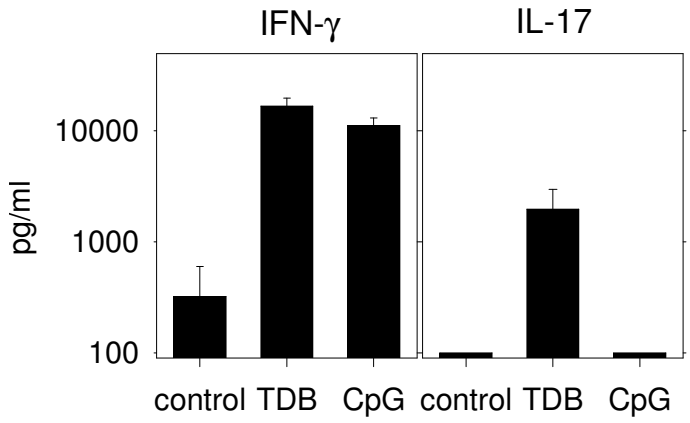

Figure 2: TDB induces a Th1/Th17 response
C57BL6 mice were s.c. immunized twice with H1 antigen in combination with CpG or liposome-embedded TDB (DDA/TDB) or left untreated (control). Two weeks after the last immunization, draining lymph node cells were re-stimulated with 10 µg/ml H1 for 96 h, followed by detection of IFN-γ and IL-17 by ELISA. Mean and SD (n= 3-4 mice) from representative experiment out of three are shown.

4.2 TDB and TDM are potent activators of APCs

Activation of T cells by antigen-presenting cells (APCs) like macrophages or dendritic cells (DCs) is essential for the development of the following immune response to the according antigen. Given that TDB triggers a strong IFN-γ and IL-17 response from primed T cells, it was analyzed whether TDB and the mycobacterial cord factor Trehalose-6,6-dimycolate (TDM) activate macrophages and DCs *in vitro*.

Bone-marrow-derived macrophages (BMDMs) from C57BL6 mice were generated and stimulated with different concentrations of TDB and TDM. The glycolipids were either directly coated to the bottom of the cell culture dishes (in a range from 4 to 0.25 µg/ml) or given as a suspension with a final concentration of 40 µg/ml. To compare the effects of TDB and TDM with the effects of classical TLR activation, the cells were also stimulated with LPS (TLR4 ligand) and CpG (TLR9 ligand). As a control for a TLR-independent activation of APCs, cells were additionally stimulated with Curdlan, a β-glucan ligand of the C-type lectin receptor Dectin-1.

To investigate the effect of macrophage priming in terms of the TDB response, cells were additionally pre-incubated with or without IFN-γ 3 hours prior to stimulation.

After 60 hours, cells treated with the plate-coated TDB or TDM showed a very high cell viability which was comparable to that of CpG and LPS stimulated cells (Fig 3). In contrast, when the glycolipids were given in suspension, only TDB, but not TDM, induced a strong activation of the cells. However, in some experiments TDM in suspension also activated BMDMs, although less efficiently compared to the plate-coated form.

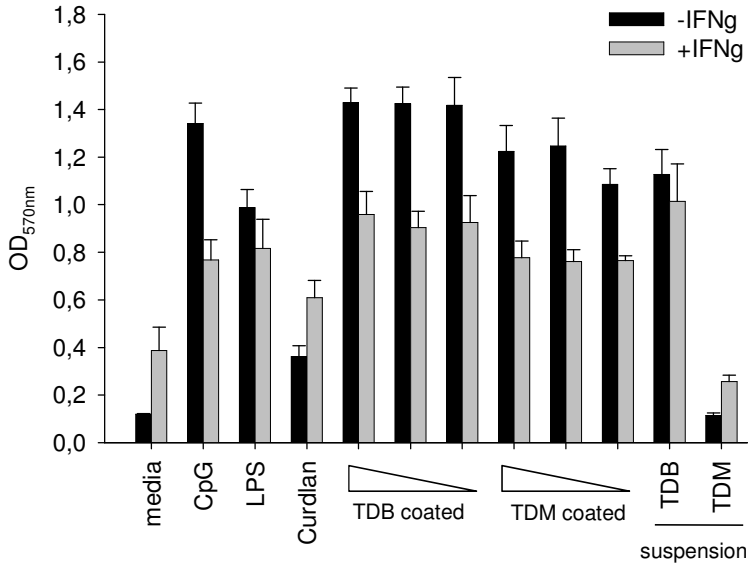

Figure 3: TDB and TDM are potent activators of macrophages
IFN-γ primed C57BL6 BMDMs were stimulated for 60 h in triplicates with 1µM CpG ODN, 100 ng/ml LPS, 100 µg/ml Curdlan and TDB and TDM either plate-coated (4 to 0.25 µg/ml range) or in suspension (40 µg/ml). Cell viability was measured as OD at a wave length of 570 nm by MTT assay. Mean and SD of triplicate wells, representative of three experiments.

Possibly, due to the poor solubility of TDM as a consequence of the fatty mycolic acid chains, TDM given in suspension cannot reach the surface of the cells ideally. This would explain the loss of cell activation although the final concentration was higher than when TDM was coated directly to the plates. Surprisingly, additional IFN-γ in the cultures led to a decrease in macrophage cell viability. Given that the coated glycolipids led to a strong activation of the cells even at low concentrations, it is therefore possible, that IFN-γ priming caused an “over-stimulation” of the cells, resulting in an earlier cell death. However, different batches of IFN-γ were used during the experimental phase of the present PhD thesis which differed in their intensity to activate BMDMs. Overall, priming of the macrophages increased the response of the cells to the glycolipids in comparison to naive cells, therefore all stimulation assays were performed with IFN-γ primed cells, whereas the stimulation time points differed to prevent early cell death.

NO production of macrophages is an important anti-microbial effector and NO release by activated APCs can be measured easily. However, measurable levels of NO produced by activated macrophages as a result of the induced respiratory burst can be measured above 48 hours of stimulation. Therefore IFN-γ primed and

unprimed C57BL6 BMDMs were stimulated for 60 hours and the NO levels were detected by Griess assay (Fig 4). Both TDB and TDM induced substantial amounts of NO similar to CpG whereas, as expected, the strongest NO response occurred after LPS stimulation. In contrast, the NO response was boosted when the cells were primed with IFN-γ with the highest NO production after TDM stimulation. TDB also lead to a threefold higher NO production compared to the unprimed cells whereas the values were similar to that of LPS and CpG. As for the cell viability, this strong response to TDM and TDB was equal for the three used concentrations. TDM given as suspension had no activating effect on macrophages. In the case of Curdlan, NO release by BMDMs was induced when the cells were primed with IFN-γ, whereas NO production in response to this stimulus was not detectable in unprimed cells.

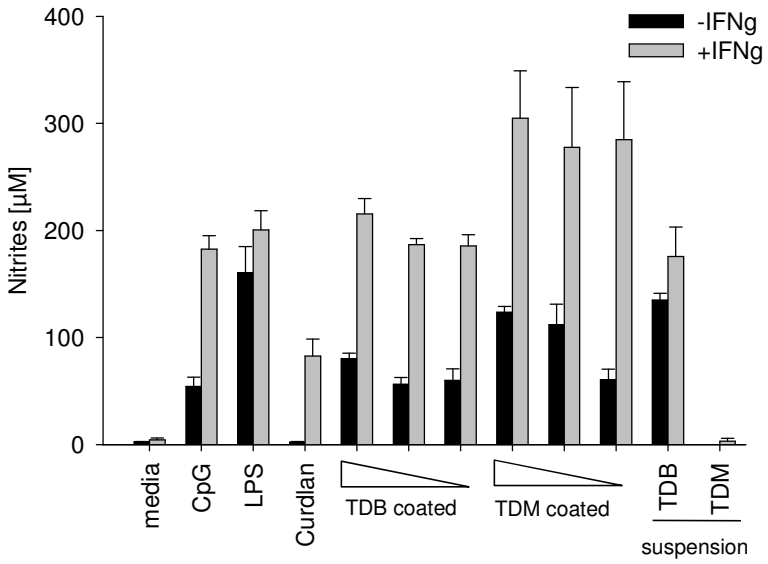

Figure 4: TDB and TDM are potent inducers of NO production by macrophages
IFN-γ primed C57BL6 BMDMs were stimulated for 60 h in triplicates with 1µM CpG ODN, 100 ng/ml LPS, 100 µg/ml Curdlan and TDB and TDM either plate-coated (4 to 0.25 µg/ml range) or in suspension (40 µg/ml). NO production was measured as nitrites by Griess assay. Mean and SD of triplicate wells, representative for three experiments.

Prior experiments have shown that the kinetics of TDB-mediated NO production was delayed when compared to CpG-stimulated cells but that the released amounts of NO were similar after 96 hours of stimulation (Werninghaus et al., 2009). Possibly, the recognition of TDB is delayed, because receptor molecules for TDB may be poorly expressed by resting BMDMs. This would require TDB-triggered expression of

the receptor, which in turn would accelerate the response to TDB. Another hypothesis is that the interaction between TDB and its possible receptor occurs more slowly than the interaction between CpG and TLR9, which also would explain the delayed response of the macrophages to TDB.

To investigate differences in the effects of TDB or CpG on macrophage activation more generally, changes in gene expression over time were measured by microarray analysis. Both stimuli induced substantial transcriptional reprogramming with again an overall slower response to TDB but with qualitative differences in gene expression. Hierarchical gene clustering indicated CpG-selective as well as TDB-selective gene induction and genes induced early by CpG and late by TDB. Of interest, the microarray data indicated that the expression of cytokines that can direct T-cell differentiation was differentially induced by CpG and TDB.

The pro-inflammatory cytokine IL-12 is necessary to stimulate IFN-γ production by T and natural killer (NK)-cells and therefore triggers a Th-1 response (Schmitt et al., 1997). Sources of IL-12 are activated phagocytes and DCs, which produce this cytokine in response to different microbial stimuli and bacterial or viral infections. Classically, CpG and LPS are known to elicit strong IL-12 production from macrophages and DCs.

To confirm whether TDB also induces the release of IL-12 by APCs, BMDMs as well as BMDCs were stimulated with TDB or, as positive controls, with CpG or LPS. Interestingly, TDB failed to induce IL-12 release from macrophages and induced only a weak response in DCs, indicating that the mechanisms of TDB-induced T-cell priming towards a Th1 response might differ from that of a classical TLR agonist. However, in macrophages, TDB as well as TDM strongly induced the production of the two pro-inflammatory cytokines IL-6 (Fig 5A) and IL-1ß (Fig 5B). As these two cytokines have been identified to play an essential role in the development of a Th-17 response, the results may help to explain the *in vivo* findings (Fig 2). Similar to the preceding findings, priming of the macrophages with IFN-γ led to a decrease in the amounts of IL-6 and IL-1ß in almost all samples.

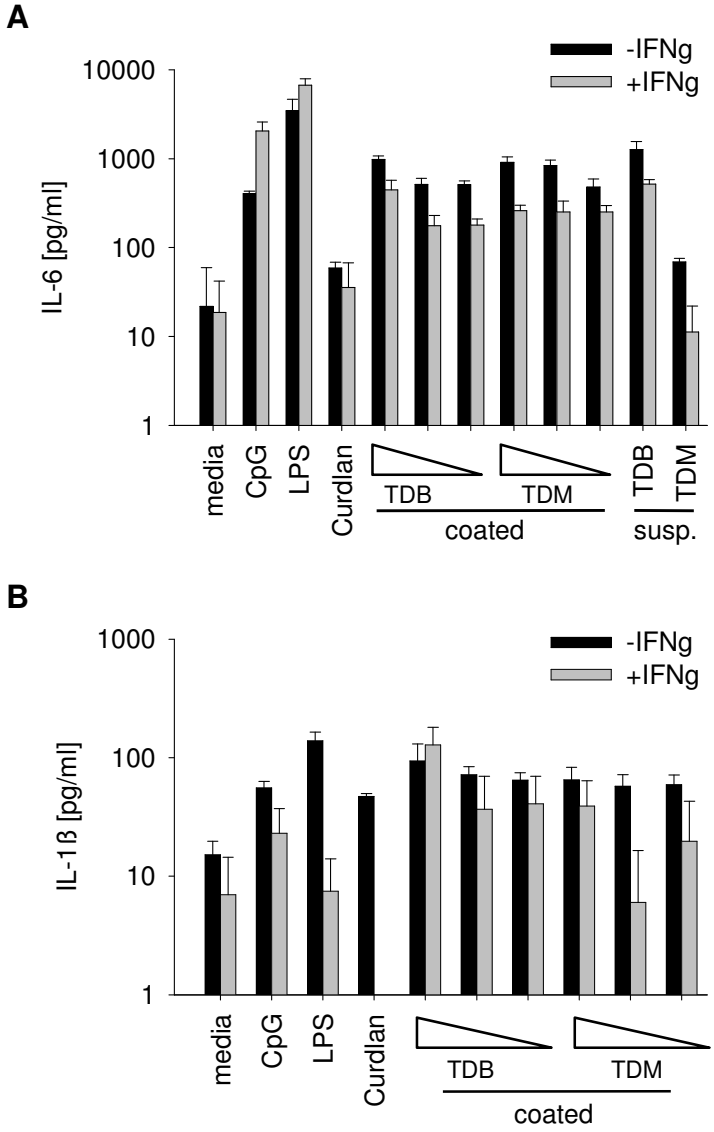

Figure 5: TDB and TDM are potent inducers of IL-6 and IL-1β production by macrophages
IFN-γ primed C57BL6 BMDMs were stimulated for 60 h in triplicates with 1µM CpG ODN, 100 ng/ml LPS, 100 µg/ml Curdlan and TDB and TDM either plate-coated (4 to 0.25 µg/ml range) or in suspension (40 µg/ml). IL-6 (A) and IL-1β production (B) was measured by ELISA. Mean and SD of triplicate wells, representative of two (B) to three (A) experiments. susp, suspension

Further parallel studies with DCs indicated that TDB caused up-regulation of MHC-II and of the co-stimulatory molecules CD40, CD80 and CD86 on the cell surface and, in addition, TDB triggered cytokine and NO release in DCs similar as in BMDMs (Werninghaus et al., 2009).

In summary, TDB and the cord factor TDM have been identified to be potent activators of APCs which trigger a gene expression program that drives inflammation and helper T-cell differentiation whereas this activation program is apparently distinct from classical TLR activation.

4.3 *in vitro*, TDB and TDM induced activation of APCs is independent of TLR signaling

Diverse pathogen-associated-molecular-patterns (PAMPS) use different pattern recognition receptors (PRRs) to mediate their biological effect. The family of Toll-like receptors (TLRs) represents the most prominent group of PRRs and for CpG and LPS it is known, that they signal via TLR 9 and TLR 4. As it was not clear, how TDB and TDM could be recognized by myeloid cells, it was studied whether any of these receptors was required for TDB and TDM-mediated cell activation *in vitro*. Almost all TLRs signal via the adaptor protein Myd88, therefore the effect of TDB and TDM was first analyzed in Myd88-deficient macrophages.

IFN-γ primed cells were stimulated with CpG and LPS as classical TLR agonists and with different concentrations of TDB and TDM (4 to 0.25 µg/ml range). Curdlan was used as control stimulation for a TLR-independent activation of APCs.

Following 60 hours of stimulation, NO production was measured and, as expected, the NO production of Myd88-deficient BMDMs was reduced or abrogated in response to LPS or CpG (Fig 6). In contrast, TDB and TDM both induced stronger NO production in the Myd88-/- cells (Fig 6).

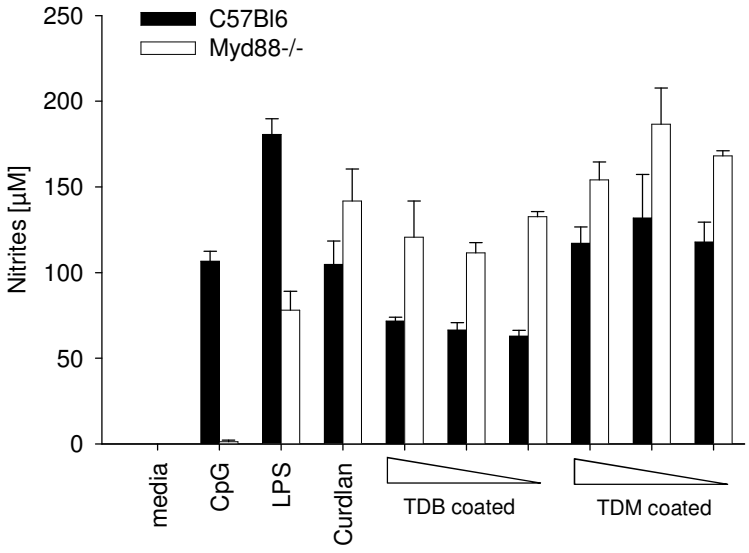

Figure 6: TDB and TDM induced NO production by macrophages is independent of Myd88
IFN-γ primed C57BL6 and Myd88-/- BMDMs were stimulated for 60 h in triplicates with 1µM CpG ODN, 100 ng/ml LPS, 100 µg/ml Curdlan and plate-coated TDB and TDM (4 to 0.25 µg/ml range). NO production was measured as nitrites by Griess assay. Mean and SD of triplicate wells, representative for two experiments.

Although Myd88-deficiency did not alter the TDB and TDM-induced activation of BMDMs, the possible role of TLRs in the TDB and TDM-mediated effect was studied in more detail using TLR2/3/4/7-/- and TLR2/3/4/7/9-/- macrophages. BMDMs from both mouse lines were generated and the stimulation scheme followed the same conditions as for the Myd88-deficient cells. The NO response from IFN-γ primed macrophages from TLR2/3/4/7-/- (Fig 7A) and TLR2/3/4/7/9-/- (Fig 7C) mice stimulated with TDB and TDM was not significantly different to wild-type cells. However, in the absence of IFN-γ priming, BMDMs of both TLR2/3/4/7-/- and TLR2/3/4/7/9-/- mice responded even stronger to both glycolipids, indicated by higher levels of NO compared to the wild-type controls (Fig 7B and 7D). Possibly, the deficiency of the different TLRs resulted in a higher sensitivity of the according BMDMs to external stimulation with IFN-γ in combination with the coated glycolipids, resulting in an earlier cell death of the TLR-deficient cells compared to the control cells. However, as expected, the NO production of TLR2/3/4/7-/- and TLR2/3/4/7/9-/- BMDMs was reduced or abrogated after stimulation with CpG (TLR2/3/4/7/9-/-) and LPS (TLR2/3/4/7-/- and TLR2/3/4/7/9-/-) (Fig 7 A-D).

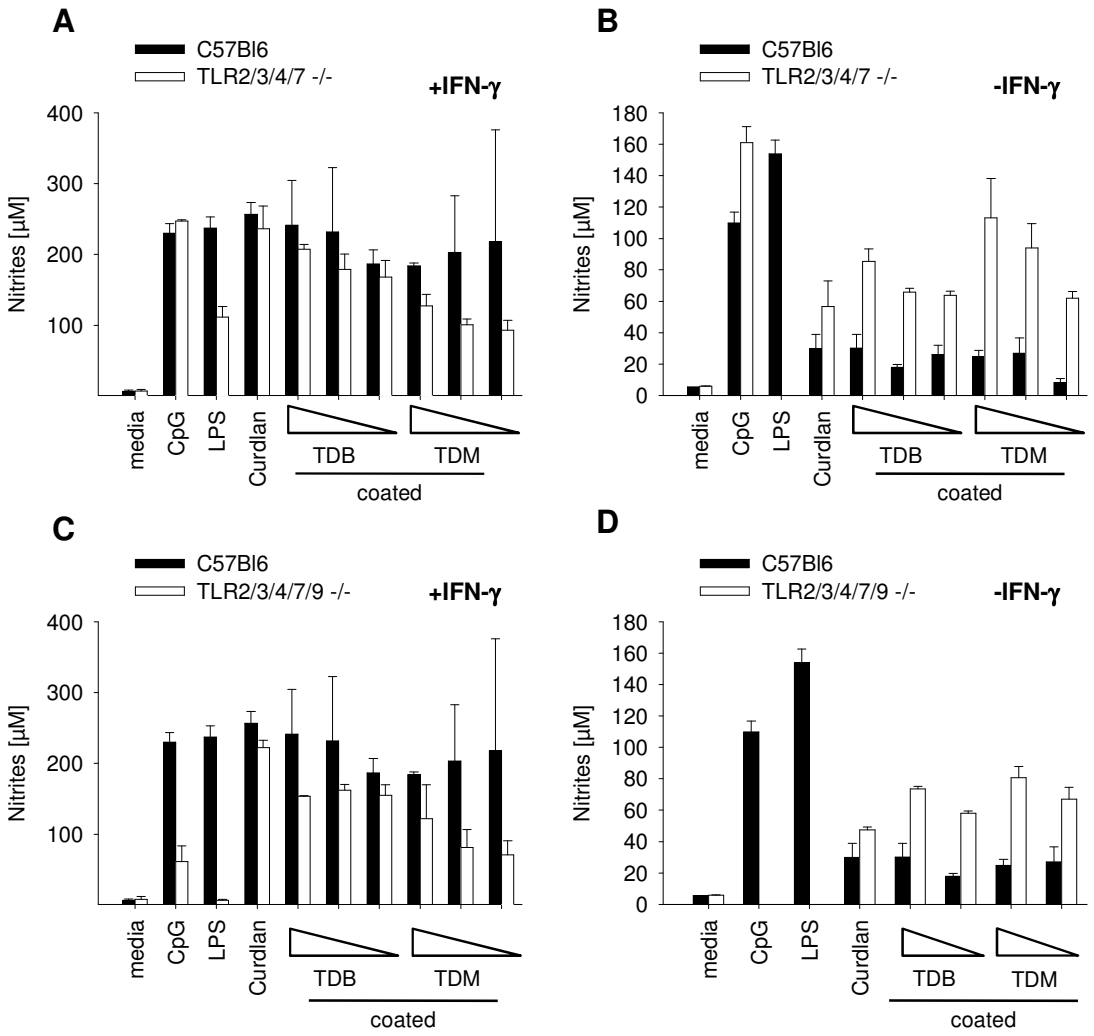

Figure 7: TDB and TDM induced NO production by macrophages is independent of TLR 2,3,4,7 and 9

C57BL6 and TLR2/3/4/7-/- (A,B) or TLR2/3/4/7/9-/- (C,D) BMDMs were stimulated for 60 h in triplicates with 1µM CpG ODN, 100 ng/ml LPS, 100 µg/ml Curdlan and plate-coated TDB and TDM (4 to 0.25 µg/ml range) with (A,C) or without pre-stimulation (B,D) with 10 ng/ml IFN-γ. NO production was measured as nitrites by Griess assay. Mean and SD of triplicate wells.

Focusing on the expression levels of the pro-inflammatory cytokines IL-6 (Fig 8, left) and IL-1β (Fig 8, right) in Myd88-deficient BMDMs after 48 hours of stimulation, it turned out that similar to the data of the NO production, Myd88-deficient macrophages showed higher expression levels for both cytokines in response to TDB and TDM compared to the wild-type cells. As expected, because of the blocked TLR-signaling the response to CpG was weak or absent and the response to LPS was strongly reduced (Fig 8, left and right).

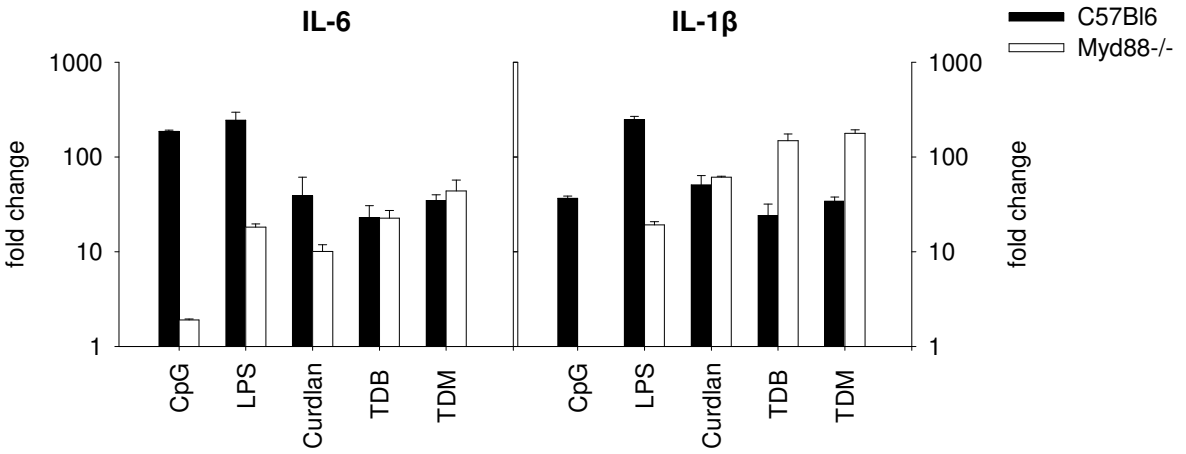

Figure 8: TDB and TDM induced IL-6 and IL-1β expression by macrophages is independent of Myd88
IFN-γ primed C57BL6 and Myd88-/- BMDMs were stimulated for 48 h in duplicates with 1µM CpG ODN, 100 ng/ml LPS, 100 µg/ml Curdlan, TDB (40 µg/ml) and TDM (40 µg/ml). Expression levels of IL-6 (left) and IL-1β (right) were analyzed by real-time PCR. Fold change is relative to medium control. Mean and SD of quadruplicate determinations.

Analysis of IL-6 production by TLR2/3/4/7-/- (Fig 9A) and TLR2/3/4/7/9-/- (Fig 9B) BMDMs after 48 hours of stimulation showed that the IL-6 release in response to the different TDB and TDM concentrations was stronger in both knock out cell lines compared to the wild-type controls. Surprisingly, the TLR fivefold deficient BMDMs still responded to CpG, which was unexpected, as CpG is recognized by TLR9 (Fig 9B). Possibly this was due to a contamination of the used CpG. However, the amount of IL-6 in response to CpG was significantly reduced in the TLR2/3/4/7/9-/- macrophages in comparison to the wild-type cells.

Together, the data suggested that recognition of TDB and TDM was independent of TLR signaling.

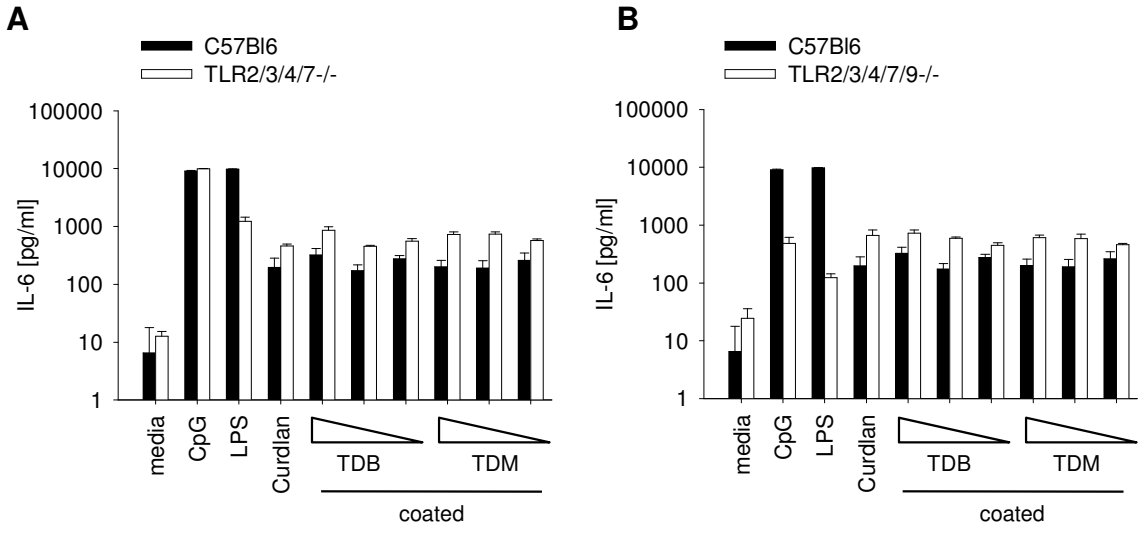

Figure 9: TDB and TDM induced IL-6 expression by macrophages is independent of TLR 2,3,4,7 and 9
IFN-γ primed C57BL6 and TLR2/3/4/7-/- (A) or TLR2/3/4/7/9-/- (B) BMDMs were stimulated for 60 h in duplicates with 1µM CpG ODN, 100 ng/ml LPS, 100 µg/ml Curdlan and plate-coated TDB and TDM (4 to 0.25 µg/ml range). Expression levels of IL-6 were analyzed by real-time PCR. Fold change is relative to medium control. Mean and SD of quadruplicate determinations.

4.4 TDB and TDM activate APCs via a Syk-Card9-Malt1-dependent signaling pathway

Besides the Toll like receptor family, several other groups of PRRs have been identified to play a major role in innate immunity, including members of the C-type lectin-, the Siglec- and the TREM family. Recent work established members of the C-type lectin family as important for innate immune recognition against fungal infections and for some it is known, that initiated downstream signaling includes activation of Syk kinases, which bind to the phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) in the tails of the receptors and in turn recruit and activate further downstream molecules.

Gross *et al* have shown, that activation of the β-glucan receptor Dectin-1, which is the major mammalian pattern recognition receptor for the fungal component zymosan, triggers a pathway that depends on the adaptor proteins Card9, Malt1 and Bcl10 and leads to activation of NF-κB and mitogen activated protein kinases (MAPKs) resulting in myeloid cell activation, cytokine production and innate anti-fungal immunity (Gross et al., 2006). Based on these findings, the following experiments focused on the role of this novel signaling pathway in mediating the effect of TDB and TDM on macrophages and DCs, since the data described in

section 3.3 strongly indicated that recognition of both glycolipids was independent of TLR signaling.

According to the Syk-dependency of some C-type lectin receptors, it was tested, whether Syk-deficiency affected the response of BMDMs to TDB and TDM. In a first experiment, C57BL6 BMDMs were treated with different concentrations of the Syk-kinase inhibitor Piceatannol (in a range from 3 to 20 µM) followed by stimulation of the cells with LPS (100 ng/ml) or two different concentrations of TDB (5 and 20 µg/ml).

Treatment with Piceatannol blocked the NO production in response to TDB in a dose-dependent manner whereas the LPS induced NO release was only slightly inhibited (Fig 10A). In the case of LPS, this can be explained by the fact, that the Piceatannol was prepared in sterile DMSO, which itself has an inhibiting effect on the cells at higher concentrations. However, similar effects could be observed using Syk-deficient BMDMs. NO production was blocked in Syk-/- macrophages in response to TDB and TDM whereas the response to CpG and LPS was Syk-independent (Fig 10B). Furthermore Syk-deficient macrophages failed to produce IL-6 in response to TDB, but not to LPS or CpG (Fig 10C), indicating that activation of macrophages by TDB as well as TDM is strictly dependent on Syk activation.

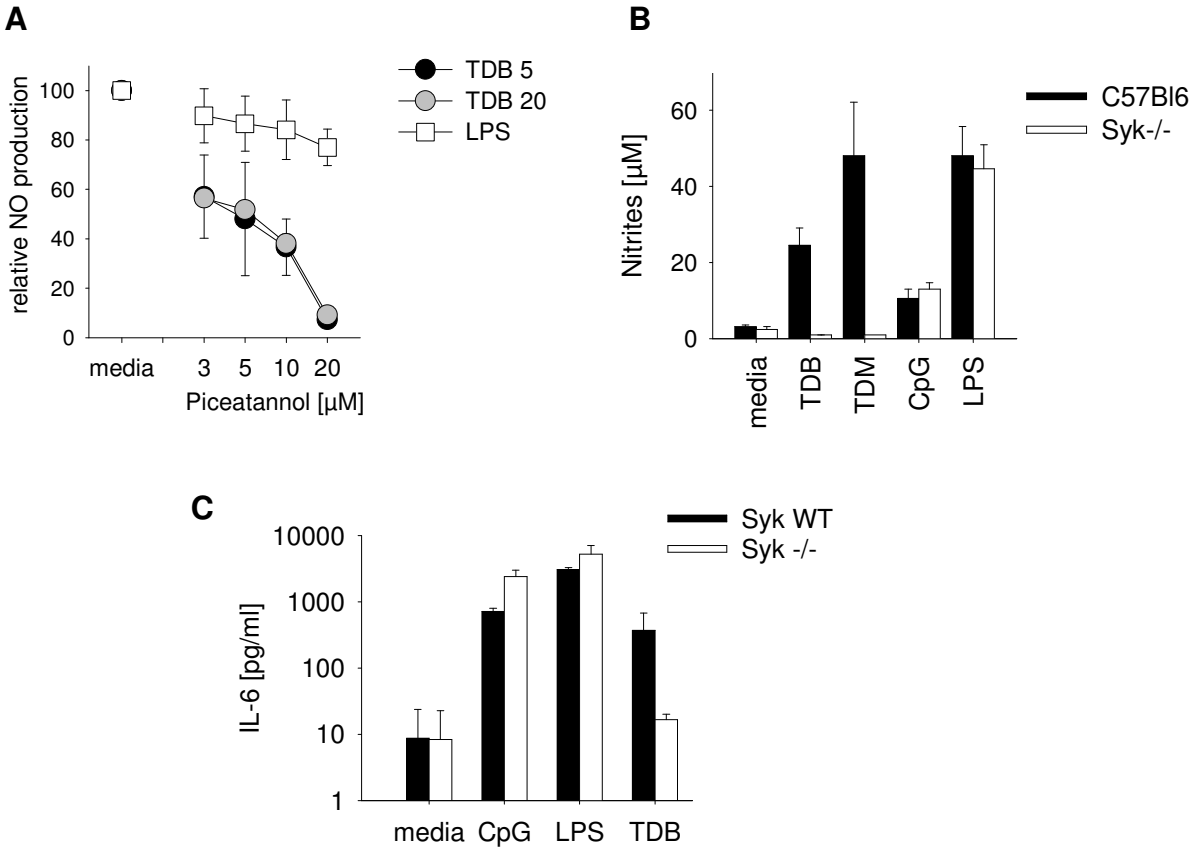

Figure 10: TDB/ TDM-induced macrophage activation requires Syk activity
(A) TDB-induced macrophage activation is sensitive to Piceatannol. IFN-γ-primed C57BL6 BMDMs were pretreated for 30 min with increasing doses of Piceatannol before stimulation with TDB (5 or 20 µg/ml) or LPS (100 ng/ml). Mean and SD of triplicates wells, representative of two experiments. **(B)** TDB and TDM-induced NO production depends on Syk activity. IFN-γ primed C57BL6 and Syk-/- BMDMs were stimulated for 96 h with TDB (20µg/ml), TDM (20 µg/ml), Cpg (1 µM) or LPS (100 ng/ml). NO production was measured as nitrites by Griess assay. Mean and SD of triplicate wells, representative for two experiments. **(C)** TDB-induced IL-6 production depends on Syk activity. IFN-γ primed Syk wild-type and Syk-/- BMDMs were stimulated for 72 hours with CpG (1 µM), LPS (100 ng/ml) or TDB (40 µg/ml). IL-6 production was measured by ELISA. Mean and SD of triplicate wells, representative of two experiments.

Syk activation results in the recruitment of several downstream adaptor molecules, including Card9, which plays a major role in signaling pathways induced by different members of C-type lectins. Thus, to test whether this molecule is involved in the TDB and TDM-mediated effects on macrophages, *in vitro* generated BMDMs from Card9-deficient mice were tested for NO production after 72 hours of stimulation. Card9-deficiency resulted in a completely abrogated NO production in response to TDB and TDM, whereas the response to CpG and LPS was unaffected and similar to the wild-type controls. As expected, Card9-/- cells also lack NO production in response to the Card9-dependent ligand Curdlan (Fig 11).

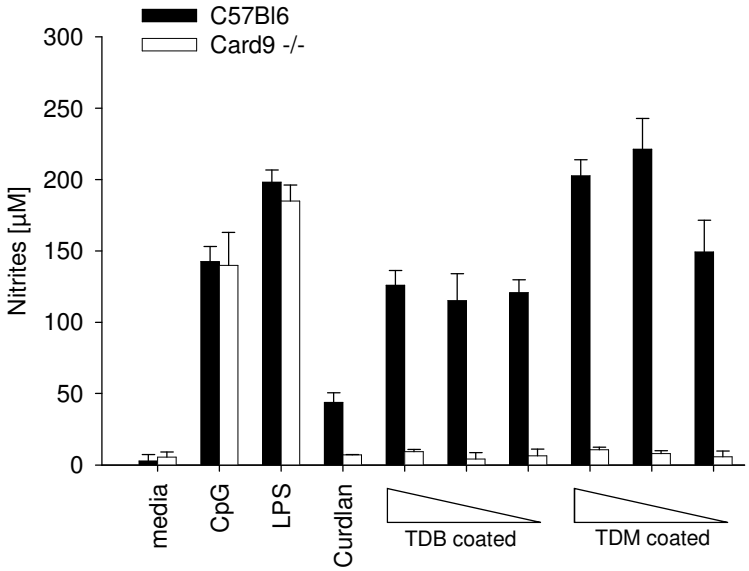

Figure 11: TDB/ TDM-induced NO production by macrophages depends onCard9
IFN-γ primed C57BL6 and Card9-/- BMDMs were stimulated for 72 h with CpG (1 µM), LPS (100 ng/ml), Curdlan (100 µg/ml) or plate coated TDB and TDM (4 to 0.25 µg/ml range). NO production was measured as nitrites by Griess assay. Mean and SD of triplicates wells, representative of three experiments.

Similar results were found for the expression levels of IL-6 (Fig 12, left) and IL-1β (Fig 12, right) after 48 hours of stimulation. Expression of both genes was abrogated (TDM) or significantly reduced (TDB) in Card9-/- BMDM when compared to the wild-type cells whereas Card9-deficient cells responded normally to CpG and LPS.

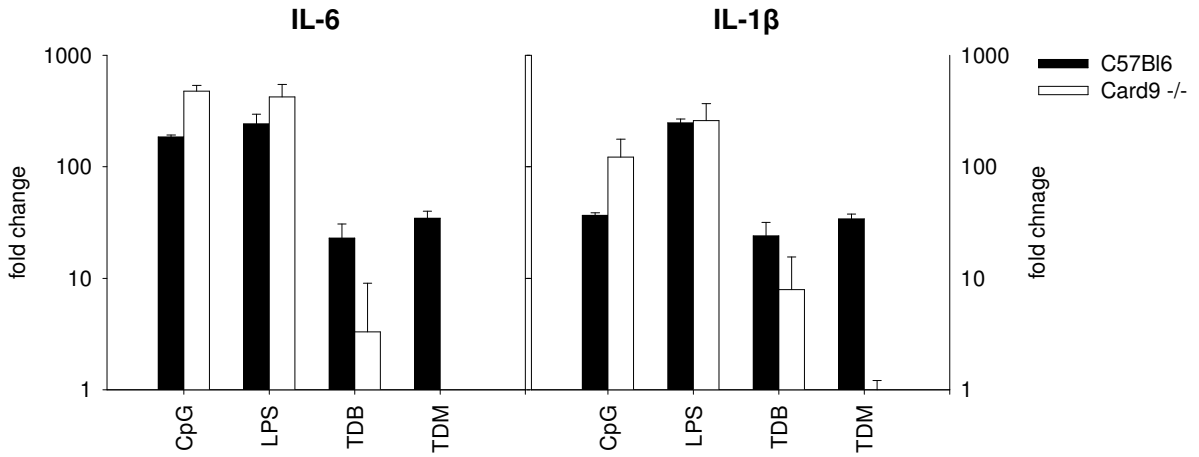

Figure 12: TDB/ TDM-induced IL-6 and IL-1β expression by macrophages depends on Card9
IFN-γ primed C57BL6 and Card9-/- BMDMs were stimulated in duplicates for 48 h with CpG (1 µM), LPS (100 ng/ml) or plate coated TDB and TDM (1 µg/ml). Expression levels of IL-6 (left) and IL-1β (right) were analyzed by real-time PCR. Fold change is relative to medium control. Mean and SD of quadruplicate determinations, representative of two experiments.

It is known for several signaling pathways that the adaptor molecule Malt1 acts downstream of Syk kinase by forming a complex with Card9. Thus, as the TDB and TDM effects on macrophages were identified to be strictly dependent on a pathway involving Syk-Card9 activity, Malt-1-deficient BMDMs were tested for expression of IL-1β and IL-6 after 48 hours of stimulation with TDB and CpG (Fig 13).

Similar to the results for Syk and Card9-deficient BMDMs, TDB, but not CpG, failed to induce IL-6 (Fig 13, left) and IL-1β expression (Fig 13, right) in Malt1-/- cells, indicating an essential role for Malt1 in the TDB-mediated effect on macrophage activation.

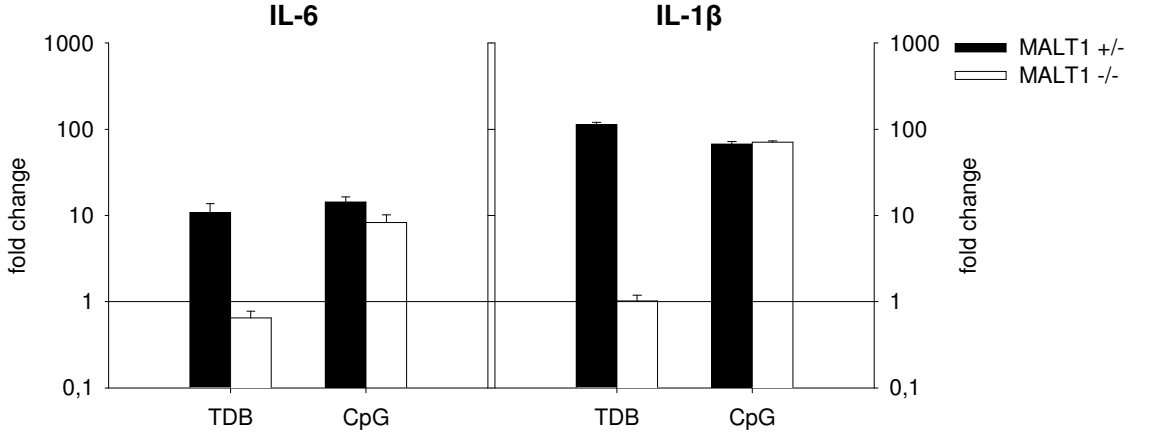

Figure 13: TDB-induced IL-6 and IL-1β expression by macrophages depends on Malt1
Malt1+/- and Malt1-/- BMDMs were stimulated in duplicates for 48 h with CpG (1 µM) or TDB (40 µg/ml). Expression levels of IL-6 (left) and IL-1β (right) were analyzed by real-time PCR. Fold change is relative to medium control. Mean and SD of quadruplicate determinations.

The TDB effect was also analyzed in BMDCs *in vitro*. Card9-deficiency resulted in an impaired production of IL-1ß, IL-6 and TNF-α by BMDCs in response to TDB, TDM and Curdlan, indicating that activation of DCs by both glycolipids followed the same mechanisms that were identified for macrophages (Werninghaus et al., 2009).
In addition, it was shown that activation of BMDCs by TDB resulted in the activation of the transcription factor NF-κB, whereas, in contrast to LPS-induced activation, TDB-induced activation followed a slower kinetic and was completely dependent on Card9 (Werninghaus et al., 2009).

To study the role of Card9 in terms of the TDB-induced gene expression profile in macrophages in more detail, BMDMs from wild-type and Card9-deficient animals were generated and stimulated for 48 hours with CpG, TDB or Curdlan. Thereafter, RNA was prepared from all samples. Affymetrix Mouse Gene 1.0 ST GeneChips were then used for genome-wide transcriptome analysis of 1 µg total RNA per sample, following the manufacturer's instruction for labeling and hybridization. The CEL files were processed for global normalization and generation of expression values using the robust multi-array average algorithm (RMA) (Bolstad et al., 2003). All regulated genes were then z-score normalized and subjected to hierarchical clustering using the Spotfire DecisionSite Functional Genomics software.
Curdlan and TDB induced very similar genome-wide transcriptional responses, whereas some gene clusters were induced stronger by TDB than by Curdlan (Fig

14). However, the TDB and Curdlan-induced responses were distinct from the pattern of CpG-induced activation. In the absence of Card9, the responses to TDB and to Curdlan were almost completely abrogated, whereas CpG-triggered gene expression was unaffected (Fig 14).

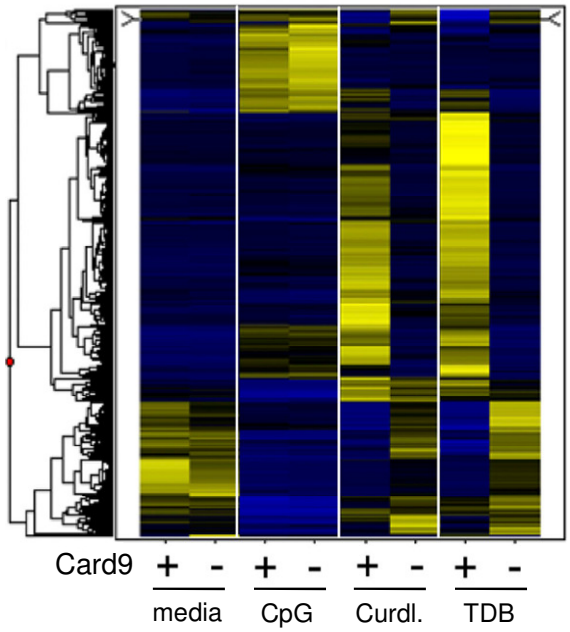

Figure 14: TDB-induced expression profile in macrophages depends on Card9
Expression profiling of IFN-γ primed C57BL6 and Card9-/- BMDMs 48 h after stimulation with CpG (1 µM), Curdlan (100µg/ml) or TDB (40 µg/ml). Hierarchical clustering of 606 probe sets with regulated expression (Max/Min > 5, Max-Min > 100). Yellow indicates high expression, blue indicates low relative expression. The genotype of BMDMs is indicated with + (C57BL6) and – (Card9-/-). Curdl, Curdlan

Further analysis of the TDB-induced gene expression profile identified several members of the C-type lectin family as prominent target genes of TDB, including Clec4e (Mincle), Clec4n (Dectin-2), Clec5a (DAP12 associating lectin-1), Clec4d (macrophage endocytic receptor) and Clec7a (Dectin-1) as the major induced genes (table 1). With a fold change of 268.15 and 58.44, Clec4e (Mincle) and Clec4n (Dectin2) showed the strongest induction by TDB, indicating that these two genes might play an important role in the adjuvant mechanisms of TDB. Furthermore expression of Mincle and Dectin-2 as well as expression of Clec5a, Clec4d and Dectin-1 in response to TDB was strictly dependent on Card9 (table 1). Interestingly, TDB also enhanced the expression of the scavenger receptor MARCO (fold change 16.03), which was recently identified to play a role in the recognition of the mycobacterial cord factor (Bowdish et al., 2009), in a Card9-dependent way (table 1).

Besides up-regulation of different members of the C-type lectin family, TDB induced a strong expression of the pro-inflammatory cytokines IL-1β and IL-6 with fold changes of 196.70 (IL-1β) and 62.88 (IL-6) in a Card9-dependent manner, which confirms the results of the detected protein and expression levels of both cytokines in the earlier sections (table 1). As mentioned, both proteins are known to be important factors for the development of a Th-17 response. However, effective T-cell priming towards the Th-17 direction also depends on the availability of the cytokines TGF-β and IL-23 (Mangan PR et al 2006). Indeed, expression of the α-subunit of IL-23 was fivefold up-regulated by TDB in a Card9-dependent manner whereas, in contrast, expression of the two isoforms of TGF-β was not detectable, indicating that production of this cytokine in response to TDB might not be related to macrophages (table 1).
Besides the induction of pro-inflammatory cytokines, TDB led to enhanced expression levels of several chemokines, which play an important role in recruiting effector cells to sites of infection, especially of CCl17, which is known to induce chemotaxis of T cells (table 1) (Campbell et al., 2007). As expected, according to the high levels of NO production by TDB-stimulated BMDMs *in vitro*, expression of inducible NO synthase (iNOS) was also strongly up-regulated by TDB in a Card9-dependent way (table 1).

The obtained data were compared with microarray data of a prior experiment, where only wild-type macrophages were stimulated with TDB or CpG for 48 hours. Instead of Affymetrix Mouse Gene 1.0 ST GeneChips, MOE430 2.0 GeneChips were used for genome-wide transcriptome analysis of 1 µg total RNA per sample (Werninghaus et al., 2009). The data confirmed the previous results as TDB strongly induced the expression of twelve genes encoding different C-type lectins and the fold change data between both experiments were comparable with very high values for Clec4n (Dectin-2), Clec4e (Mincle), Clec1b and Clec5a (DAP12 associated lectin) (table 2). Furthermore the expression levels for IL-1β, IL-6, iNOS, Tgf-β1 and IL-23α were elevated after TDB stimulation (table 2).

Affymetrix probe set ID	*gene symbol*	*other name*	*WT ctrl*	*WT TDB*	*ko ctrl*	*ko TDB*	*WT TDB fold change*	*ko TDB fold change*
10547664	Clec4e	Mincle	20	5473	13	28	268.15	2.12
10541605	Clec4n	Dectin-2	73	4255	58	79	58.44	1.36
10544273	Clec5a	DAP12 associating lectin-1	83	1327	81	183	15.95	2.26
10541614	Clec4d	Macrophage endocytic receptor	1631	4293	1194	1395	2.63	1.17
10548375	Clec7a	Dectin-1	364	857	278	377	2.35	1.35
10541587	Clec4a2		55	106	49	65	1.92	1.32
10590558	Clec3b		14	20	18	19	1.42	1.05
10433536	Clec16a		64	81	68	7	1.27	1.13
10541575	Clec4a4		4	5	5	5	1.26	1.09
10541555	Clec4a1		402	496	303	371	1.24	1.23
10542156	Clec2d		192	226	138	176	1.18	1.28
10542156	Clec2d		192	226	138	176	1.18	1.28
10542181	Clec9a	DNGr-1	9	10	11	9	1.16	0.82
10575702	Clec3a		20	21	19	20	1.04	1.09
10548359	Clec12b		10	10	12	9	1.02	0.73
10487597	IL1b	Interleukin-1β	21	4188	30	23	196.70	0.76
10520452	IL6	Interleukin-6	4	225	3	4	62.88	1.09
10373358	IL23a	Interleukin-23 α	15	76	20	17	5.19	0.83
10345032	Il17a	Interleukin-17 A	15	24	18	17	1.62	0.94
10551185	Tgfb1	transforming growth factor β, 1	1714	2047	1691	1766	1.19	1.04
10360920	Tgfb2	transforming growth factor β, 2	17	17	17	13	1.00	0.76
10353415	Il17f	Interleukin-17 F	30	31	39	29	1.03	0.73
10456136	Il17b	Interleukin-17 B	28	28	29	26	0.98	0.89
10415636	Il17d	Interleukin-17 D	16	14	16	15	0.86	0.95
10576115	Il17c	Interleukin-17 C	44	25	52	29	0.55	0.56

Table 1: Expression levels of TDB- induced genes in bone-marrow-derived macrophages of Card9-deficient and wild-type mice.
Genes induced after 48 h by TDB in bone-marrow-derived macrophages of Card9 KO and wild-type mice. Depicted are the expression levels of the most important target genes of TDB, including the most important inflammatory mediators and members of the C-type lectin receptor family.
Data Shown are from one representative experiment.

Affymetrix probe set ID	*gene symbol*	*other name*	*WT ctrl*	*WT TDB*	*ko ctrl*	*ko TDB*	*WT TDB fold change*	*ko TDB fold change*
10545154	Il23r	Interleukin-23 receptor	9	42	9	7	4.53	0.79
10541246	Il17ra	Interleukin-17 receptor A	68	283	89	105	4.16	1.18
10413398	Il17rd	Interleukin-17 receptor D	22	24	23	20	1.07	0.85
10540705	Il17rc	Interleukin-17 receptor C	40	33	40	35	0.81	0.89
10418341	Il17rb	Interleukin-17 receptor B	35	26	36	25	0.75	0.71
10540679	Il17re	Interleukin-17 receptor E	36	24	32	29	0.65	0.90
10574226	Ccl17	Chemokine ligand 17	19	401	22	13	21.40	0.59
10379721	Ccl4	Chemokine ligand 4	107	1082	98	133	10.13	1.36
10389207	Ccl5	Chemokine ligand 5	175	1241	206	244	7.08	1.18
10574213	Ccl22	Chemokine ligand 22	27	161	27	21	5.98	0.76
10389222	Ccl6	Chemokine ligand 6	643	3198	523	948	4.97	1.81
10389231	Ccl3	Chemokine ligand 3	1273	6304	1035	1996	4.95	1.93
10379511	Ccl2	Chemokine ligand 2	69	209	96	307	3.04	3.19
10379518	Ccl7	Chemokine ligand 7	94	285	125	304	3.03	2.44
10389214	Ccl9	Chemokine ligand 9	1411	3462	1085	1796	2.45	1.66
10534493	Ccl24	Chemokine ligand 24	18	40	25	23	2.16	0.94
10379228	NOS2	inducible NO synthase (iNOS)	20	517	23	15	25.92	0.66
10357261	Marco	MARCO	22	350	82	28	16.03	0.34
10499160	CD1d1	CD1d	31	150	42	42	4.83	0.99

Table 1 (continued): Expression levels of TDB- induced genes in bone-marrow-derived macrophages of Card9-deficient and wild-type mice.
Genes induced after 48 h by TDB in bone-marrow-derived macrophages of Card9 KO and wild-type mice. Depicted are the expression levels of the most important target genes of TDB, including the most important inflammatory mediators and members of the C-type lectin receptor family.
Data Shown are from one representative experiment.

Affymetrix probe set ID	*gene symbol*	*other name*	*WT ctrl*	*WT CpG*	*WT TDB*	*TDB fold change*	*CpG fold change*
1419627_s_at	Clec4n	Dectin-2	147	2313	7439	50.72	15.77
1420330_at	Clec4e	Mincle	306	2939	8653	28.32	9.62
1421182_at	Clec1b		17	11	443	25.36	0.62
1421366_at	Clec5a	DAP12 associated lectin	56	39	919	16.42	0.69
1420804_s_at	Clec4d	Macrophage endocytic receptor	1403	5678	7678	5.47	4.05
1420699_at	Clec7a	Dectin-1	2829	387	4171	1.47	0.14
1449466_at	Clec3b		23	23	25	1.08	0.98
1418796_at	Clec11a		18	18	18	1.03	1.01
1431575_at	Clec4g	LSECtin	17	21	16	0.95	1.26
1451438_s_at	Clec2h	C-type lectin related protein	28	29	27	0.93	1.03
1419467_at	Clec14a	DCAR	12	9	11	0.86	0.75
1450297_at	IL6	Interleukin-6	6	140	432	67.23	21.74
1449399_a_at	IL1b	Interleukin-1β	310	7799	12264	39.56	25.16
1420393_at	NOS2	inducible NO synthase (iNOS)	21	102	203	9.78	4.93
1419529_at	IL23a	Interleukin-23, α-subunit	10	11	16	1.60	1.11
1420653_at	TGFb1	TGF-β1	661	566	896	1.35	0.86

Table 2: Expression levels of TDB- and CpG-induced genes in bone-marrow-derived macrophages of wild-type mice.
Genes induced after 48 h by TDB and CpG in bone-marrow-derived macrophages of wild-type mice. Depicted are the expression levels of the most important target genes of TDB, including the most important inflammatory mediators and members of the C-type lectin receptor family.
Data Shown are from one representative experiment.

The obtained data out of both microarray experiments identified Mincle and Dectin-2 as the most prominent targets of TDB in macrophages and the expression of both genes was strictly dependent on Card9.
To validate the data and to also analyze the involvement of Syk as well as Malt1 in the TDB-induced expression of both genes, *in vitro* generated BMDMs from Syk-/-, Card9-/- and Malt1-/- mice were stimulated with TDB and CpG for different time points. Thereafter RNA was prepared for real time PCR.
In correlation with the microarray data, expression of Mincle and Dectin-2 was strongly induced by TDB and strictly dependent on Card9 (Fig 15C). Furthermore deficiency of Malt1 (Fig 15B) and Syk (Fig 15A) resulted in a reduced or absent expression of both genes in response to TDB when compared with the wild-type controls. Surprisingly, CpG induced the expression of Mincle but not of Dectin-2. This was in contrast to the obtained microarray data which indicated higher fold change levels for Dectin-2 than for Mincle expression in response to Cpg (table 2). However, as expected, the induction of both genes by CpG was independent of Syk-Card9-Malt1 signaling (Fig 15A-C).

All together, by using different read out systems it could be clearly shown that TDB and TDM both trigger a Syk-Card9-Malt1-dependent signaling pathway, which induces an innate immune response in macrophages and DCs, characterized by the production of several pro-inflammatory cytokines and chemokines and release of NO. Furthermore TDB led to the up-regulation of many genes encoding different C-type lectins in macrophages with Clec4e (Mincle) and Clec4n (Dectin-2) as the major targets. How TDB and TDM are recognized by APCs is not known, but according to the TDB-induced high expression values of members of the C-type lectin family and to the fact, that the effects of TDB and TDM seemed to be TLR-independent, it is suggested that the receptor for both glycolipids may belong to this family of PRRs.

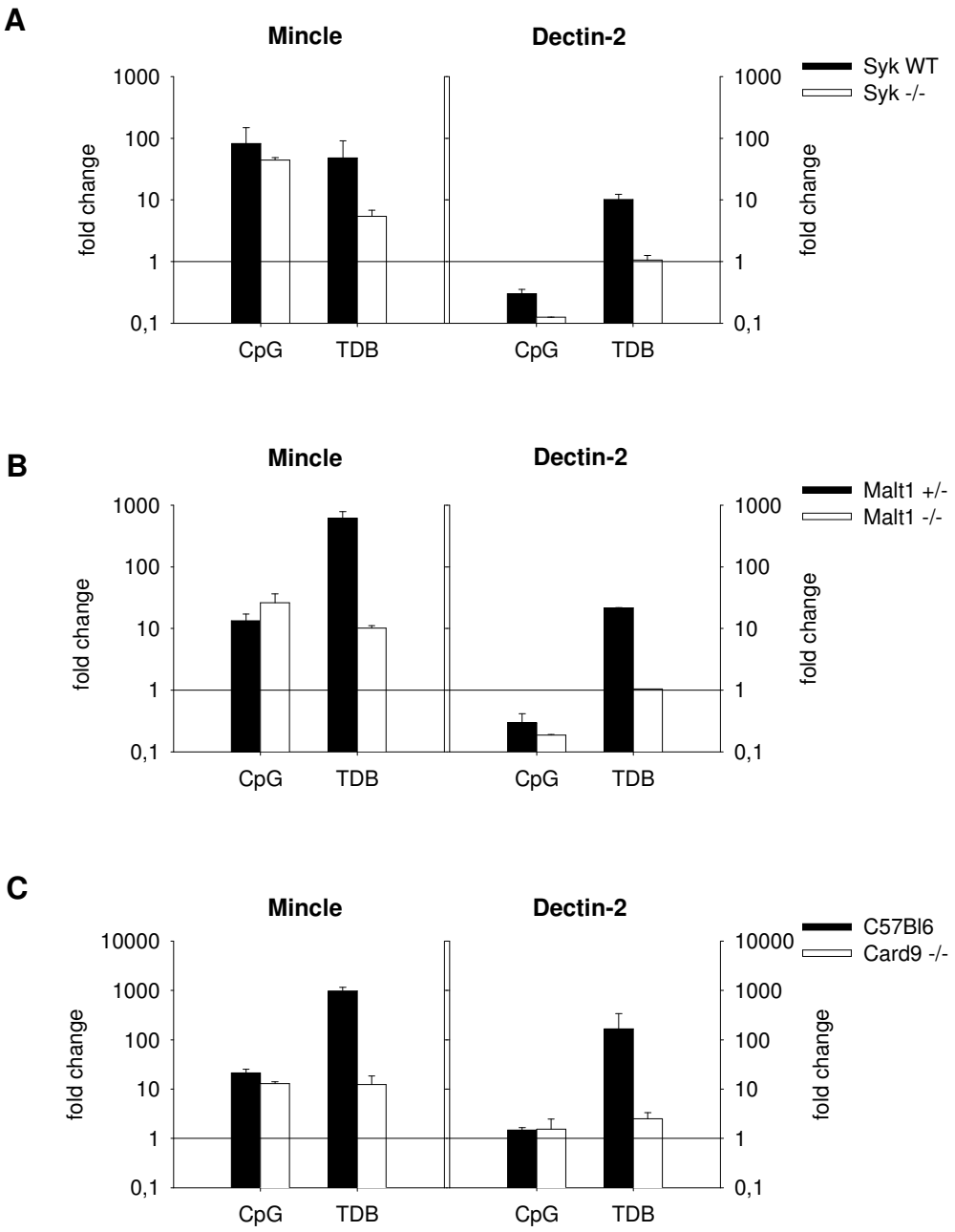

Figure 15: TDB-induced expression of Mincle and Dectin-2 depends on Syk-Card9-Malt1 activation

IFN-γ primed BMDMs from Syk wild-type and Syk-/- mice (A), Malt1+/- and Malt1-/- mice (B) and C57BL6 and Card9-/- mice (C) were stimulated in duplicates for 72 h (A and C) and 48 h (B) with CpG (1µM) or TDB (40 µg/ml). Expression levels of Mincle (A,B,C left) and Dectin-2 (A,B,C right) were analyzed by real-time PCR. Fold change is relative to medium control. Mean and SD of quadruplicate determinations, (A, C) representative of four experiments.

4.5 The TDB/TDM effect is independent of the C-type lectin Dectin-1

The previous results identified the TDB and TDM effect on APCs to be strictly dependent on a signaling pathway including Syk kinase and the adaptor proteins Card9 and Malt1. Activation of this pathway in turn triggers the expression and up-regulation of several cytokines, chemokines and surface receptors and the production of nitric oxide by APCs whereas the possible specific receptor for both glycolipids is unknown.

However, the induction of a Syk-Card9-Malt1 dependent signaling pathway was also found for the C-type lectin Dectin-1 (Gross et al., 2006), which has been identified to play an essential role in APC activation in response to mycobacterial infection (Rothfuchs et al., 2007). As TDB was shown to induce the expression of Dectin-1 in macrophages in a Card9-dependent way (table 1), the following question was, whether this receptor might be responsible for the recognition of TDB and TDM *in vitro*. Therefore Dectin-1 deficient BMDMs were generated and analyzed in terms of their response to both glycolipids whereas CpG, LPS and the Dectin-1 specific ligand Curdlan were used as control stimulations.

Neither the cell viability (Fig 16A) nor the NO production (Fig 16B) was affected in the Dectin-1-deficient macrophages in response to different concentrations of TDB and TDM after 60 hours of stimulation, whereas, as expected, Dectin-1-deficiency resulted in impaired cell viability and NO production in response Curdlan (Fig 16A/B).

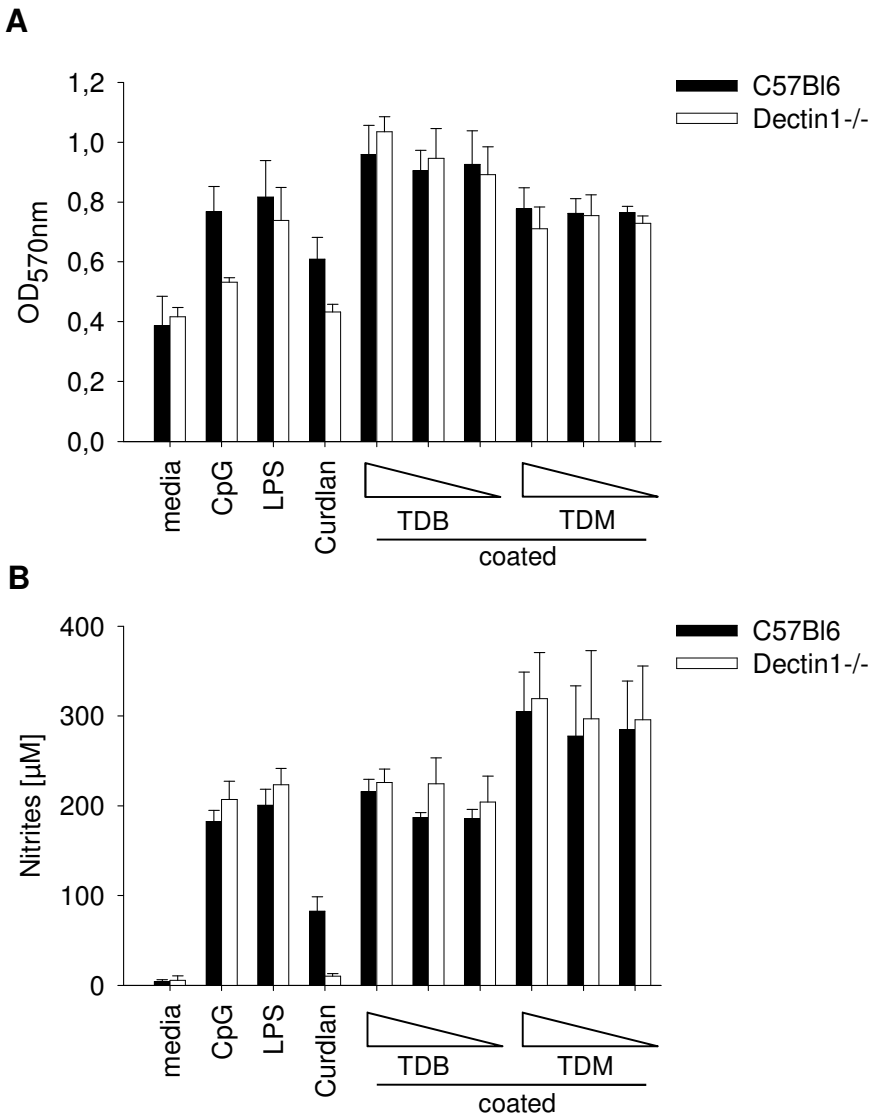

Figure 16: TDB-induced activation of macrophages is independent of Dectin-1
IFN-γ primed BMDMs from C57BL6 and Dectin-1-/- mice were stimulated for 60 h with CpG (1µM), LPS (100 ng/ml), Curdlan (100 µg/ml) and plate-coated TDB and TDM (4 to 0.25 µg/ml range). (A) Cell viability was measured as OD at a wave length of 570 nm by MTT assay. (B) NO production was measured as nitrites by Griess assay. Mean and SD of triplicate wells, representative for two experiments.

Analysis of IL-6 (Fig 17, left) and IL-1ß expression (Fig 17, right) after 48 hours of stimulation led to similar results with no detectable differences between Dectin-1-/- and wild-type BMDMs. Thus, the results excluded Dectin-1 as the specific receptor for TDB and TDM *in vitro*, which led to the hypothesis that its contribution to the activation of APCs by whole mycobacteria is likely due to a mycobacterial ligand distinct from TDM.

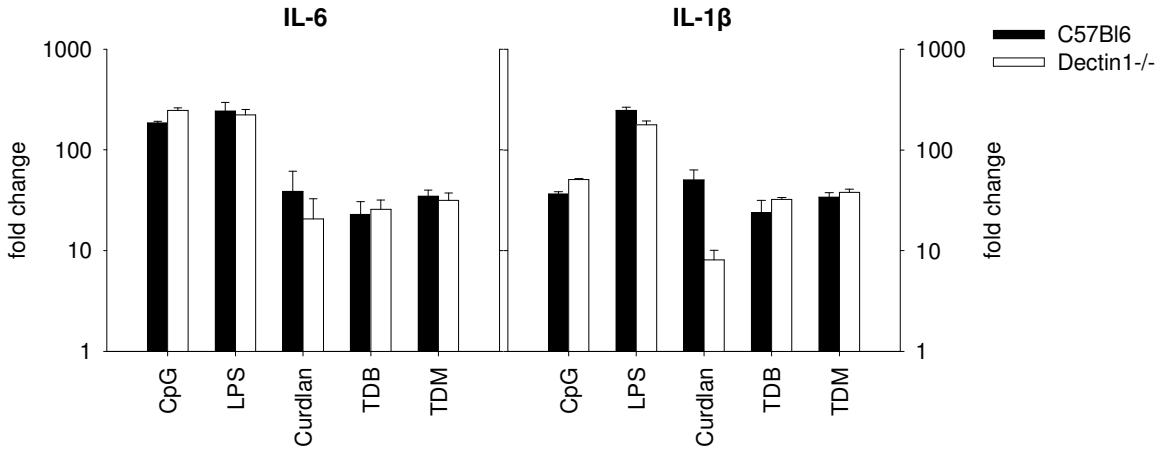

Figure 17: TDB-induced expression of IL-6 and IL-1β in macrophages is independent of Dectin-1
IFN-γ primed BMDMs from C57BL6 and Dectin-1-/- mice were stimulated in duplicates for 48 h with CpG (1µM), LPS (100 ng/ml), Curdlan (100 µg/ml) and plate-coated TDB and TDM (1 µg/ml). Expression levels of IL-6 (left) and IL-1β (right) were analyzed by real-time PCR. Fold change is relative to medium control. Mean and SD of quadruplicate determinations.

4.6 Recognition of TDB and TDM requires FcRγ

In contrast to Dectin-1, that possesses an ITAM-like motif in its cytoplasmic region, a large number of Syk-activating myeloid cell receptors are associated with one of the ITAM-bearing adaptor proteins DAP12 or Fc-receptor gamma (FcRγ) chain. According to the Syk-dependency of the TDB and TDM-induced effects on APCs and to gain further insight into the signaling requirements of APC activation by TDB and TDM, DAP12 and FcRγ-deficient BMDMs were generated and analyzed in terms of their ability to respond to both glycolipids.

After 48 hours of stimulation, no differences could be detected in the expression levels of IL-6 (Fig 18A, left) and iNOS (Fig 18A, right) in DAP12-deficient BMDMs in comparison to the wild-type controls. In contrast, FcRγ-deficiency impeded the expression of IL-6 (Fig 18B, left) and iNOS (Fig 18B, right) in response to TDB, but not to CpG.

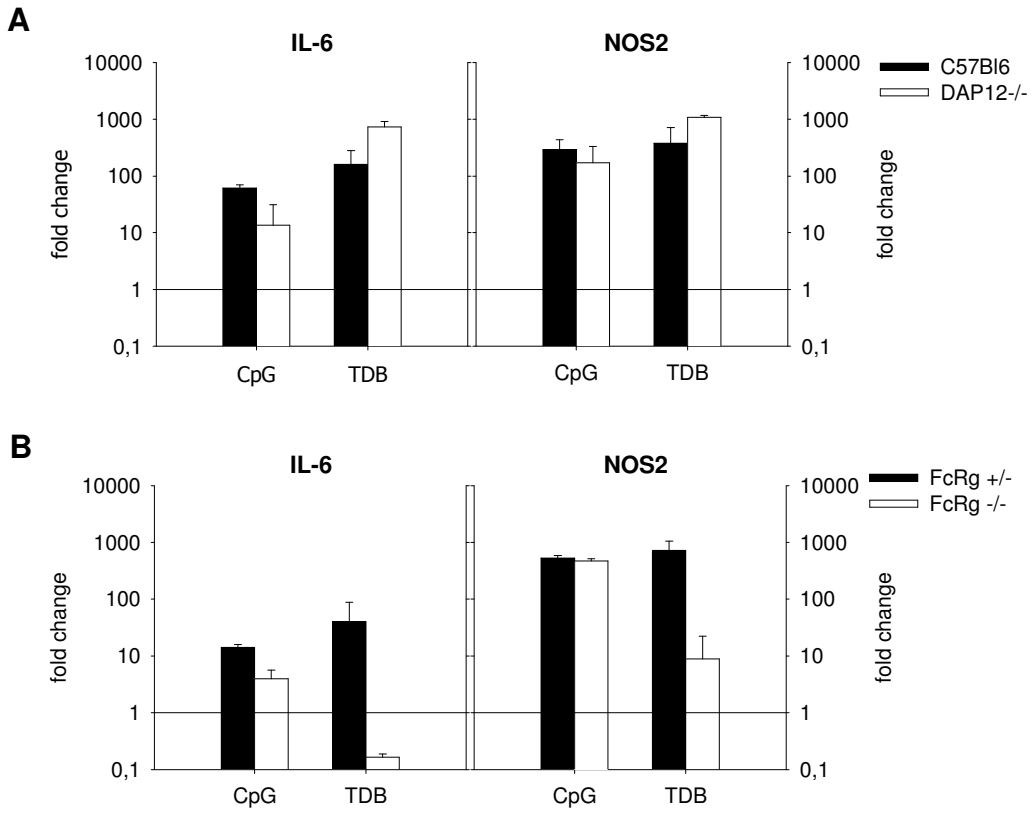

Figure 18: TDB-induced expression of IL-6 and iNOS is independent of DAP12 but requires the adaptor protein FcRγ
IFN-γ primed BMDMs from C57BL6 and DAP12-/- mice (A) and FcRγ+/- and FcRγ-/- mice (B) were stimulated in duplicates for 48 h with CpG (1µM) and TDB (40 µg/ml). Expression levels of IL-6 (A,B left) and iNOS (A,B right) were analyzed by real-time PCR. Fold change is relative to medium control. Mean and SD of quadruplicate determinations, representative of two experiments.

In addition, TDB and TDM-induced consequential NO production was completely dependent of FcRγ (Werninghaus et al., 2009). The specific requirement for FcRγ was also found in terms of the cell viability of TDB and TDM-stimulated cells (Fig 19). However, the response to Curdlan and the TLR ligands was normal in all cell types.
In summary, these results identified the ITAM-bearing adaptor protein FcRγ, but not DAP12, as a critical component for linking TDB and TDM recognition to the Syk-Card9 pathway.

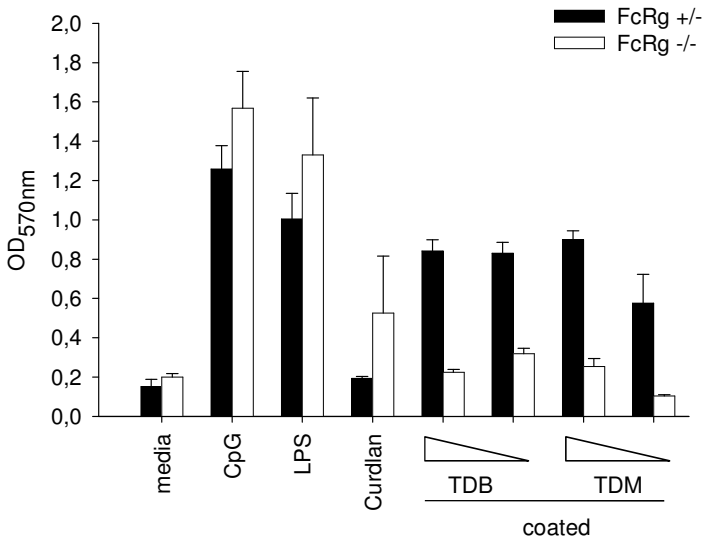

Figure 19: TDB/TDM induced activation of macrophages requires the adaptor protein FcRγ
Cell viability in response to TDB/TDM depends on FcRγ. IFN-γ primed BMDMs from FcRγ+/- and FcRγ-/- mice were stimulated for 48 h with CpG (1 µM), LPS (100 ng/ml), Curdlan (100 µg/ml) and plate-coated TDB and TDM (4 and 1 µg/ml). Cell viability was measured as OD at a wave length of 570 nm by MTT assay. Mean and SD of triplicate wells, representative of three experiments.

4.7 Adjuvanticity of TDB *in vivo* requires Myd88, but is largely independent of TLR2, 3, 4, 7and 9

The previous *in vitro* studies have shown, that both TDB and TDM are capable of activating myeloid cells independent of classical TLR-signaling but that this activation requires a pattern recognition pathway that triggers a FcRγ-Syk-Card9-Malt1-dependent signaling cascade.

To investigate whether this TLR-independency is also given in the adjuvant effect of TDB *in vivo*, Myd88-/-, Myd88/Trif-/-, TLR2/3/4/7-/- and TLR2/3/4/7/9-/- mice were immunized in the footpad with H1 antigen (2 µg) in combination with DDA liposomes alone or DDA-liposome-embedded TDB or incomplete Freund's (IFA) as adjuvant. IFA was used as a control adjuvant because of its Myd88-independent mode of action. Thus, it should lead to similar reactions between immunized Myd88-deficient and C57BL6 mice, which were used as controls in all experiments.

As a first read out to analyze the knock out effects in terms of the adjuvant activity of TDB, the change in footpad thickness over time was determined. Myd88-/- mice had a reduced swelling of the feet in response to H1/DDA/TDB in comparison to the wild-type animals, which showed a strong increase in the swelling of the feet after the first immunization, with a sharp rise after the second injection (Fig 20 A). Comparable to

the reaction of Myd88-/- mice, Myd88/Trif-deficient mice also showed reduced footpad swelling in response to H1/DDA/TDB vaccination, although the differences in footpad swelling between the genotypes were detectable only after the second immunization (Fig 20 B). However, Myd88/Trif-/- mice were only tested once whereas Myd88-/- mice were used in two independent experiments with similar results. Thus, the reproducibility of the data in Fig 20A confirmed the Myd88-dependence of the footpad swelling in response to H1/DDA/TDB immunization.

In contrast, the change in footpad thickness between wild-type and TLR2/3/4/7-/- (Fig 20 C) or wild-type and TLR2/3/4/7/9-/- (Fig 20 D) mice in response to H1/DDA/TDB vaccination was equal with similar characteristics. Vaccination with H1/DDA alone induced only a slight swelling of the feet in comparison to vaccination with H1/DDA/TDB with similar responses of all mouse genotypes (Fig 20 A-D).

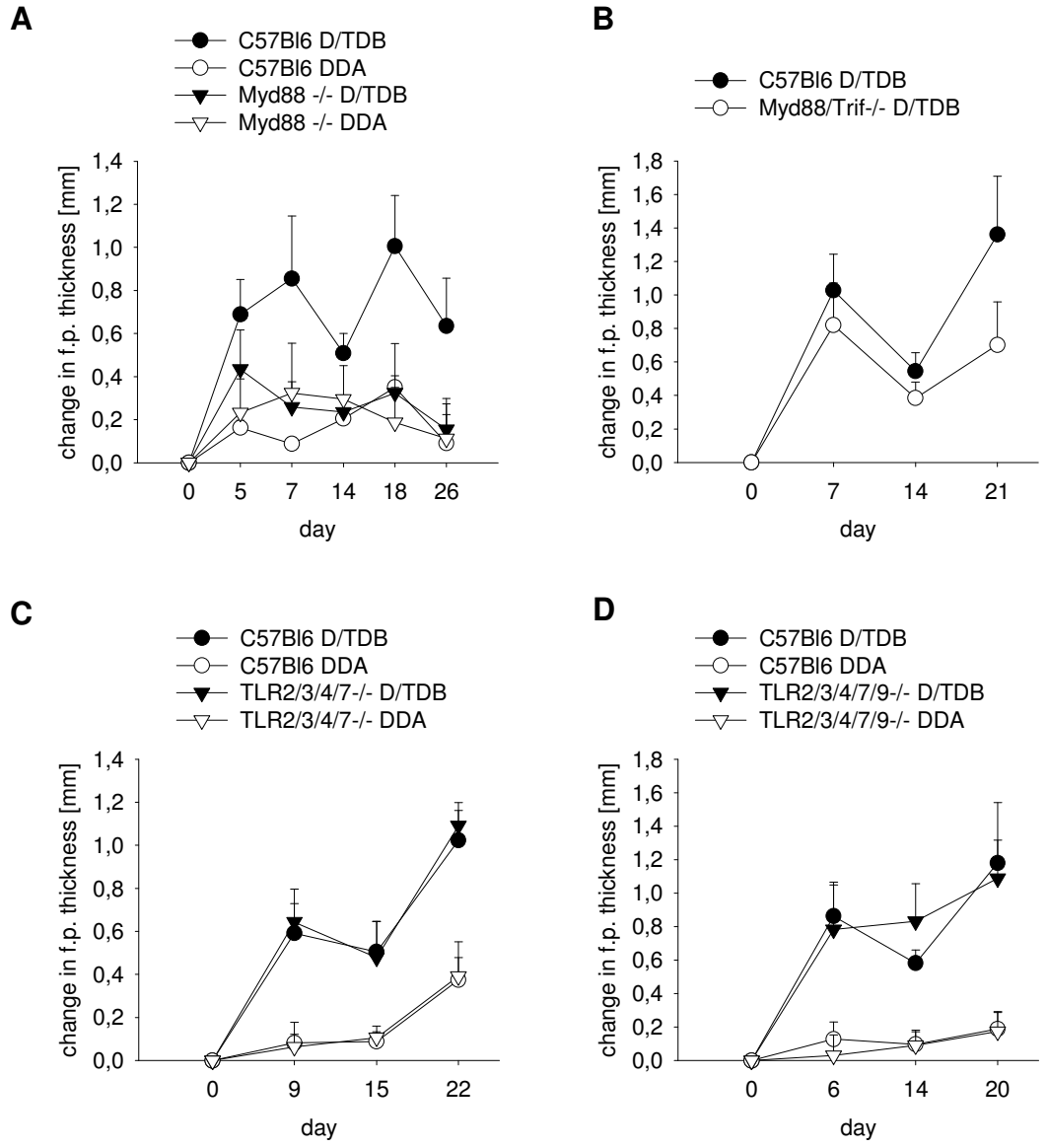

Figure 20: Deficiency of Myd88, but not of TLR2, 3, 4, 7 and 9 results in a reduced footpad swelling after TDB-immunization
C57BL6 and Myd88-/- mice (A) and Myd88/Trif-/- mice (B) and TLR2/3/4/7-/- mice (C) and TLR2/3/4/7/9-/- mice (D) were immunized via hind footpad injection on day 0 and 14/15 with H1 antigen in DDA or DDA/TDB liposomes. Mean and SD of the increase in footpad thickness is shown. Footpad number per genotype n = 6-8 (A-D), (A, D) representative of two independent experiments. D, DDA; D/TDB, DDA/TDB

When the cellularity of the draining lymph nodes (LNs) was examined, the increase in the cell numbers after immunization with H1/DDA/TDB was strongly Myd88-dependent (Fig 21 A/B). In contrast to Myd88-deficient mice ($P<0.009$, Fig 21 A) the reduction in LN cellularity of Myd88/Trif-/- mice after H1/DDA/TDB immunization was not significant compared to the control animals ($P<0.08$, Fig 21 B). However, the findings of significantly reduced LN cell numbers were confirmed under equal conditions in a third experiment with Myd88-deficient and C57BL6 mice. Vaccination

with H1/DDA alone resulted in only small cell numbers in the LNs of Myd88-/- and control mice, with no differences between the different genotypes (Fig 21A). In addition, immunization of Myd88/Trif-/- and control animals with IFA resulted in equal amounts of cells in draining LNs of both genotypes (Fig 21 B).

In contrast, cell numbers in LNs of TLR2/3/4/7-deficient and wild-type animals were comparable in response to both vaccines with a higher cellularity after H1/DDA/TDB immunization (Fig 21C). TLR2/3/4/7/9-/- knock out mice had reduced cell numbers after H1/DDA/TDB as well as after H1/DDA immunization (Fig 21D), although only in case of H1/DDA vaccination with significant effects ($P<0.009$, Fig 21D). However, the amounts of cells in the LNs of TLR2/3/4/7/9-/- animals were higher after immunization with H1/DDA/TDB than with H1/DDA alone (Fig 21D), which indicated, that the immune response was not completely abrogated. Furthermore, based on the fact that with DDA alone the migration of immune cells into the LN was also impaired (Fig 21 D), it seems that TLR9-deficiency is associated with a general impairment of cell recruitment after DDA liposome injection. Possibly, the basal level of the cell number in the LNs of TLR2/3/4/7/9-/- animals is lower than in wild-type mice, which has to be analyzed in further experiments. If so, it has to be calculated if the ratio between the cell numbers after H1/DDA/TDB and H1/DDA immunization is similar to the ratio between the vaccinated wild-type controls, which would indicate a similar response of both genotypes to the immunization.

Together, using the local inflammatory response and regional LN cell number as readout, it could be shown that the response to DDA/TDB *in vivo* was strictly Myd88-dependent, but largely independent of TLR2,3,4,7 and 9.

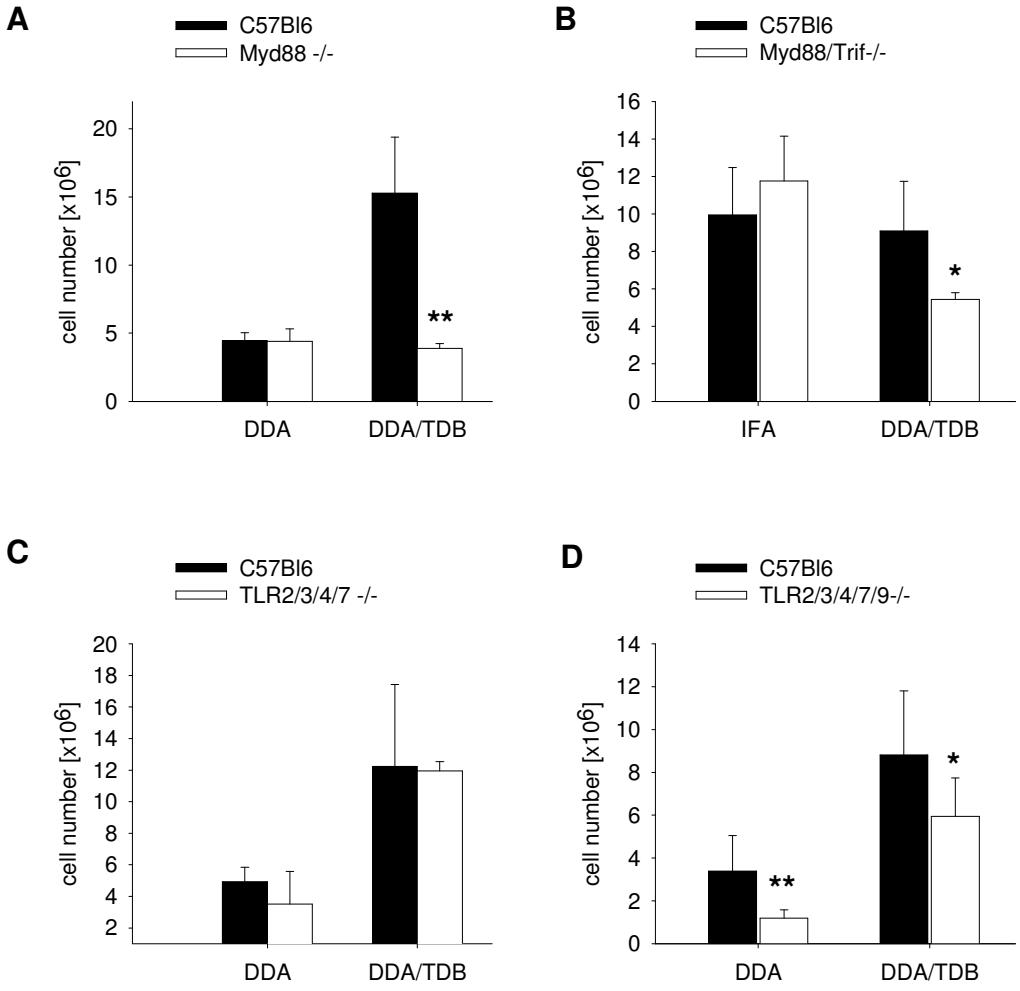

Figure 21: Deficiency of Myd88 and TLR9 results in reduced cell numbers in draining LNs after TDB-immunization.
Numbers of cells in draining LNs of C57BL6 and Myd88-/- mice (A) and Myd88/Trif-/- mice (B) and TLR2/3/4/7-/- mice (C) and TLR2/3/4/7/9-/- mice (D) two weeks after the second immunization with H1 in IFA or in DDA or DDA/TDB liposomes. Mice per genotype n = 3-4 (A-D). (A) Representative of two independent experiments. (D) Cell numbers were pooled from two independent experiments.
*p value <0.08; **p value <0.009

To test the efficiency of the adjuvant effect of DDA and DDA/TDB in the different mouse lines in terms of T-cell priming, the extracted draining LN cells of all animals were re-stimulated with H1 antigen (1 µg/ml) for 96 hours. The amounts of antigen-specific produced IFN-γ and IL-17 were determined by ELISA.

Immunization with H1/DDA/TDB failed to induce a Th-1 response in Myd88-/- as well as in Myd88/Trif-/- mice, indicated by significantly reduced IFN-γ production by re-stimulated LN cells compared to wild-type controls ($P<0.007$, Fig 22A, left). Of note, IFN-γ production induced by H1/DDA alone was also dependent on Myd88 (Fig 22A, middle). As expected, immunization with H1/IFA failed to induce a Th-1 response in wild-type as well as in Myd88-/- mice (Fig 22A, right).

Deficiency of TLR2, 3, 4 and 7 did not affect the development of a H1-specific immune response, indicated by similar levels of IFN-γ produced by re-stimulated LNs of H1/DDA/TDB and H1/DDA immunized TLR2/3/4/7-/- and control animals (Fig 22 B, left and right). DDA alone also induced a strong Th-1 response in both mouse genotypes (Fig 22 B, right), albeit to a lower extent than in combination with TDB.
Comparable to the results of H1/DDA/TDB immunized Myd88-/- and Myd88/Trif-/- mice, re-stimulated LN cells from H1/DDA/TDB immunized TLR2/3/4/7/9-/- showed significantly less IFN-γ production after re-stimulation with the antigen compared to the wild-type controls ($P<0.02$; Fig 22 C, left) while immunization with H1/DDA alone failed to induce a Th-1 response in TLR2/3/4/7/9-deficient mice (Fig 22C, right).

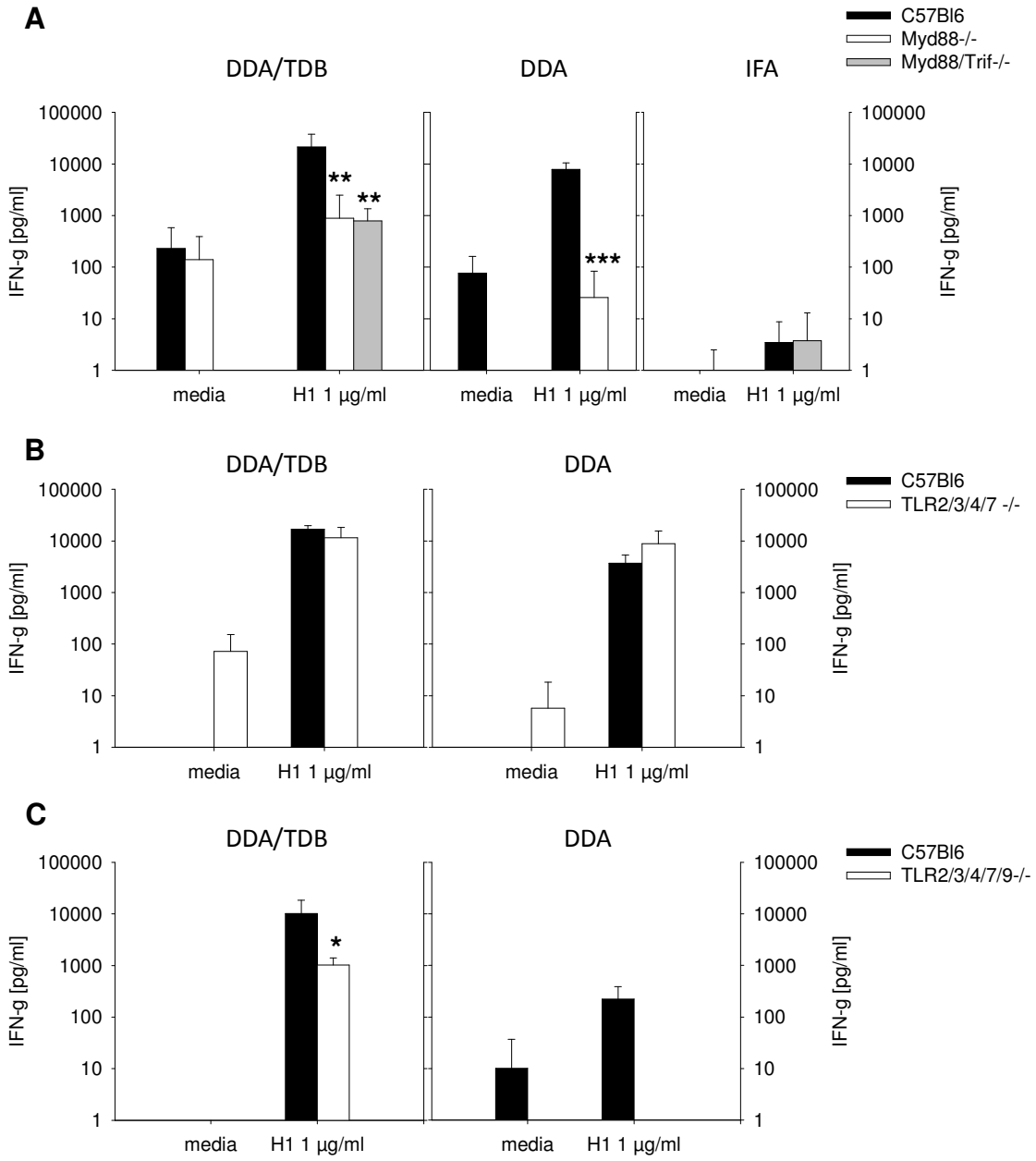

Figure 22: Development of a Th-1 response depends on Myd88 and partial on TLR9, but is independent of TLR2, 3, 4 and 7

Production of IFN-γ by draining LNs of immunized C57BL6 and Myd88-/- and Myd88/Trif-/- mice (A) and TLR2/3/4/7-/- mice (B) and TLR2/3/4/7/9-/- mice (C) two weeks after the second immunization with H1 in IFA or DDA or DDA/TDB liposomes. Cells were re-stimulated for 96 h with 1 µg/ml H1 or left untreated. Production of IFN-γ was measured by ELISA. Mean and SD of duplicate wells. Mice per genotype n = 3-4 (A-C). (A) Data were pooled from three independent experiments. (C) Representative of two independent experiments. ***p value<0.00025, **p value<0.007, *p value<0.02

Next, the requirement for Myd88 and TLRs in the Th-17 induction was tested. IL-17 production was absent in re-stimulated LN cells of H1/DDA/TDB and H1/DDA immunized Myd88-/- and Myd88/Trif-/- mice ($P<0.002$) whereas wild-type controls showed a strong Th-17 response as a consequence of H1/DDA/TDB vaccination and, to a lesser extent, also to H1/DDA vaccination (Fig 23A, left and middle). Immunization with H1/IFA failed to induce a Th-17 response in Myd88-/- and Myd88/Trif-/- mice, whereas control animals responded to the immunization in a way comparable to immunization with H1/DDA (Fig 23A, right).

Like for the Th-1 response, induction of a Th-17 response by DDA/TDB was independent of TLR 2, 3, 4 and 7 and even stronger in the re-stimulated LN cells of H1/DDA immunized TLR2/3/4/7-deficient mice compared to the wild-type controls (Fig 23B left and right).

Although deficiency of TLR9 seemed to be responsible for the development of a Th-1 response as a consequence of H1/DDA/TDB immunization, the development of the adjuvant-induced Th-17 response in mice deficient in TLR2, 3, 4, 7 and 9 was not affected (Fig 23C, left). However, immunization with H1/DDA alone induced a Th-17 response in wild-type, but not TLR2/3/4/7/9-deficient mice (Fig 23C, right).

All together, the data indicated that the induction of a robust Th-1 and Th-17 response by TDB is strongly Myd88-dependent but independent of TLR 2, 3, 4 and 7. In contrast, TLR9-deficiency led to an impaired Th-1 response whereas the induction of a Th-17 response is independent of TLR9.

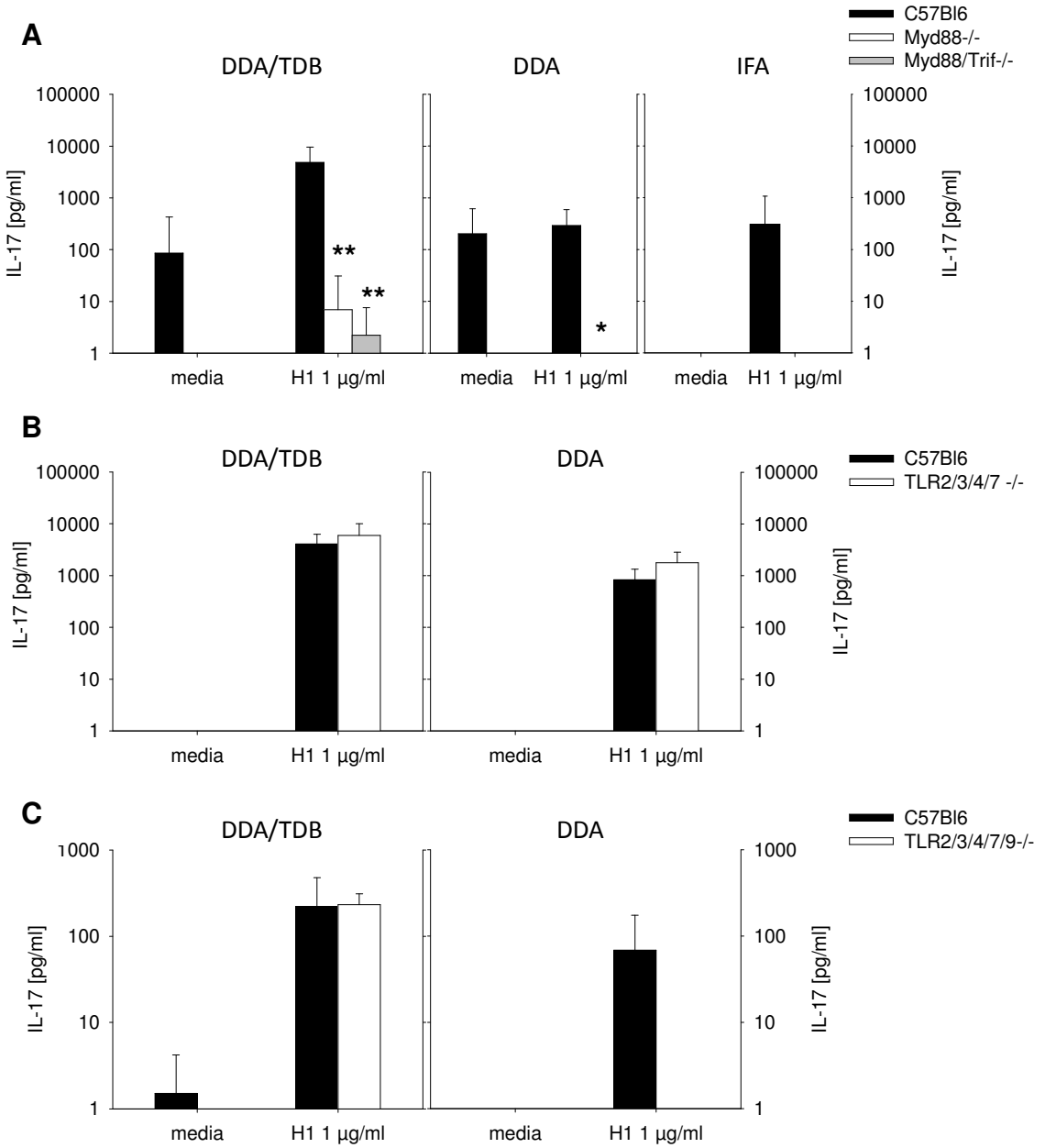

Figure 23: Development of a Th-17 response depends on Myd88, but is independent of TLR2, 3, 4, 7 and 9

Production of IL-17 by draining LNs of immunized C57BL6 and Myd88-/- and Myd88/Trif-/- mice (A) and TLR2/3/4/7-/- mice (B) and TLR2/3/4/7/9-/- mice (C) two weeks after the second immunization with H1 in IFA or DDA or DDA/TDB liposomes. Cells were re-stimulated for 96 h with 1 µg/ml H1 or left untreated. Production of IL-17 was measured by ELISA. Mean and SD of duplicate wells. Mice per genotype n = 3-4 (A-C). (A) Data were pooled from three independent experiments. (C) Representative of two independent experiments. *p value<0.04, **p value<0.002

As an additional read out to investigate the Th-1-directing adjuvant effect of TDB *in vivo*, the amounts of antigen-specific IgG2a antibody titers in the sera of all immunized animals were measured.

Myd88-/- and Myd88/Trif-/- mice failed to produce H1-specific IgG2a antibodies in response H1/DDA/TDB or H1/DDA immunization (Fig 24A and B). DDA alone induced production of IgG2a in wild-type mice to a lower extent than in combination with TDB, which is in agreement to the data of the H1/DDA induced Th-1 response in control animals (Fig 22A-C).

H1/DDA/TDB immunized TLR2/3/4/7-/- mice had comparable levels of IgG2a compared to the wild-type controls (Fig 24C), which is consistent with the data of the determined IFN-γ levels produced by re-stimulated LN cells (Fig 22 B).

Mice deficient in TLR2, 3, 4, 7 and 9 had comparable levels of IgG2a to the wild-type animals in response to H1/DDA/TDB immunization (Fig 24D), therefore, although deficiency of TLR9 resulted in an impaired IFN-γ response of re-stimulated LN cells to the antigen (Fig 22C, left), the development of the Th-1 response in the TLR2/3/4/7/9-/- mice seemed to be strong enough to trigger production of high levels of IgG2a antibodies in a way similar to the wild-type controls.

In summary, the TDB-triggered antibody response was independent of TLR 2, 3, 4, 7 and 9, but strictly dependent on Myd88.

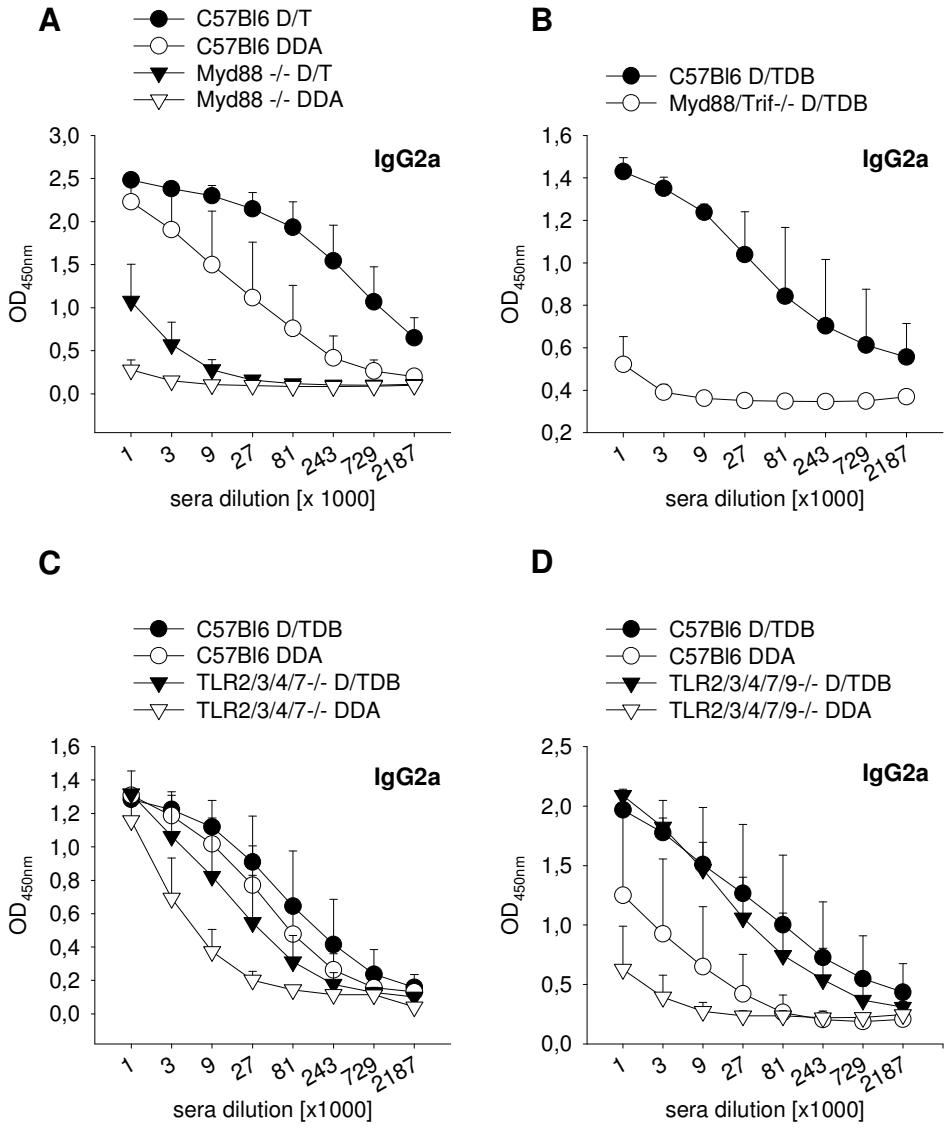

Figure 24: Production of IgG2a antibodies is strictly dependent on Myd88, but largely independent of TLR2, 3, 4, 7 and 9
IgG2a levels in sera of C57BL6 and Myd88-/- mice (A) and Myd88/Trif-/- mice (B) and TLR2/3/4/7-/- mice (C) and TLR2/3/4/7/9-/- mice (D) two weeks after the second immunization with H1 in DDA or DDA/TDB liposomes. Mice per genotype n = 3-4 (A-D). (A, D) Representative of two independent experiments. D/TDB, DDA/TDB

4.8 Adjuvanticity of TDB *in vivo* requires inflammasome formation, but is independent of IL-1R signaling

The previous results have shown that the adjuvant effect of TDB *in vivo* is strictly dependent on Myd88 whereas Myd88 is not required for TDB-induced activation of APCs *in vitro*. However, besides its essential role in TLR signaling, Myd88 is also the important downstream adaptor molecule in the signaling pathways induced by IL-1β and IL-18 (Adachi et al., 1998). Both cytokines are produced in response to different PAMPs in an inactive precursor-form (pro-IL-1β, pro-IL-18), which need further cleavage by active caspase-1 for the generation of the biologically active form of the cytokines (Dinarello, 2006). Activation of Caspase-1 requires the formation of a cytosolic protein complex, called the inflammasome, in which the intracellular adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD domain) has a central role.

IL-1β was identified to be induced in macrophages in response to TDB *in vitro* (table 1) and to further study the role of Myd88 in the *in vivo* adjuvant effect of TDB in more detail, mice deficient either in the IL-1 receptor (IL-1R) or in ASC were immunized with H1/DDA/TDB or H1/DDA alone to investigate the consequences of blocked IL-1 signaling and inflammasome formation.

In terms of the footpad swelling, IL-1R-deficient mice responded similar to the wild-type controls. Immunization with H1/DDA/TDB resulted in a strong increase in the swelling of the feet after the first immunization with a sharp rise after the second injection whereas H1/DDA only induced a slight swelling of the feet independent of the mouse genotype (Fig 25A).

In contrast, ASC-/- mice showed reduced footpad swelling in response to H1/DDA/TDB compared to the wild-type animals, indicating an essential role of inflammasome formation for the adjuvant effect of TDB *in vivo* (Fig 25B). The swelling was significantly reduced on day seven after the first injection whereas this difference was less striking after the second injection (Fig 25B). Like for the previous experiments, the response to H1/DDA was similar between wild-type and ASC-/- mice with only a slight footpad swelling (Fig 25B).

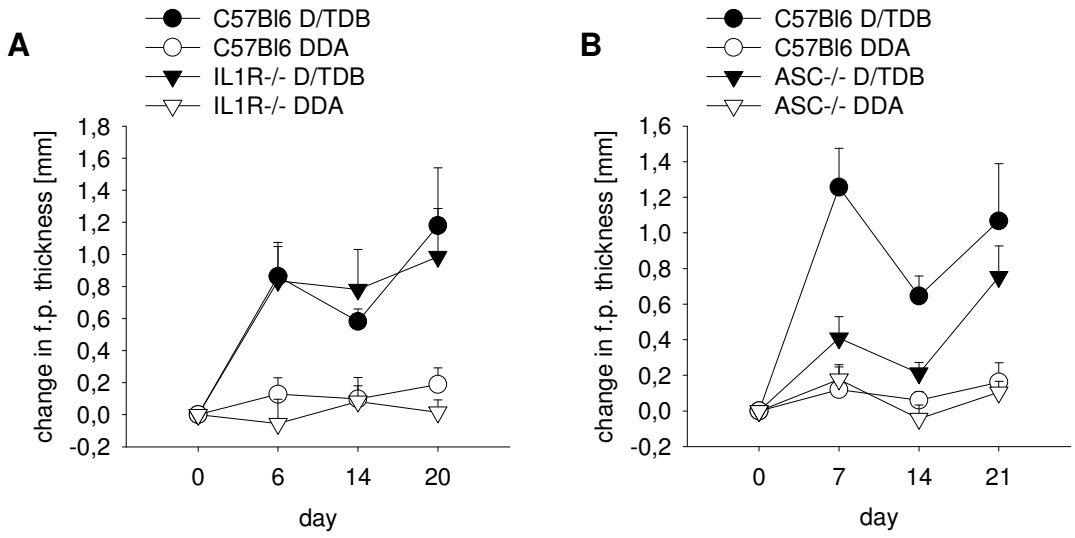

Figure 25: Deficiency of ASC, but not of IL1R results in a reduced footpad swelling after TDB-immunization
C57BL6 and IL1R-/- (A) and ASC-/- mice (B) were immunized via hind footpad injection on day 0 and 14 with H1 antigen in DDA or DDA/TDB liposomes. Mean and SD of the increase in footpad thickness is shown. Footpad number per genotype (A) n = 8 (B) n = 6. (B) Representative of two independent experiments. D/TDB, DDA/TDB

Analysis of the cell numbers in the draining LNs indicated that mice deficient in the IL-1 receptor had comparable cell numbers to the LNs of wild-type animals after H1/DDA/TDB vaccination (Fig 26A).

In contrast to the results of IL-1R-deficient mice, LNs of ASC-/- mice had significantly lower cell numbers after H1/DDA/TDB immunization ($P<0.035$, Fig 26B) whereas the response to H1/DDA was similar to the cell numbers in LNs of wild-type animals, which is in agreement with the data of the footpad swelling analysis (Fig 25B).

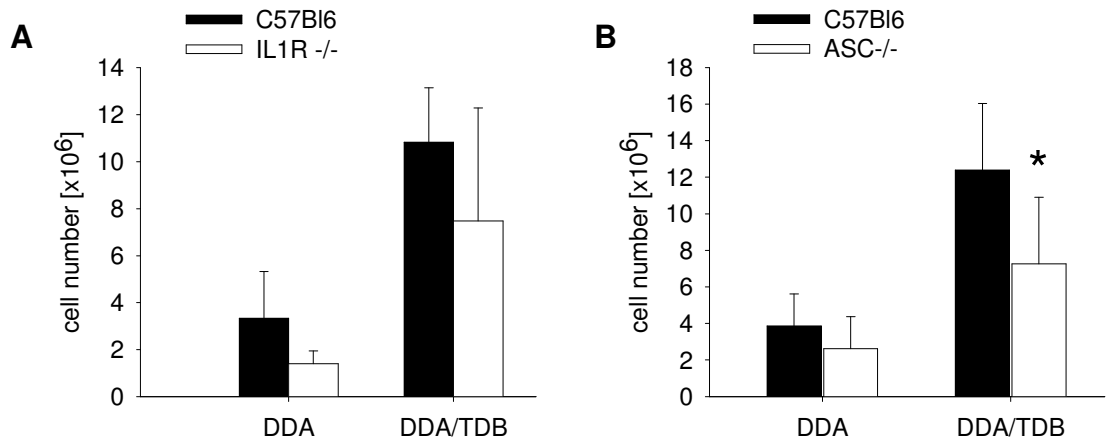

Figure 26: Deficiency of ASC, but not of IL1R results in reduced cell numbers in draining LNs after TDB-immunization.
Numbers of cells in draining LNs of C57BL6 and IL1R-/- mice (A) and ASC-/- mice (B) two weeks after the second immunization with H1 in DDA or DDA/TDB liposomes. Mice per genotype (A) n = 4 (B) n = 3-5. (B) Data were pooled from two independent experiments.*p value<0.04

Analysis of the induced T-cell priming in response to TDB indicated elevated levels in the amounts of released IFN-γ in re-stimulated LN cells of H1/DDA/TDB vaccinated IL-1R-/- in comparison to wild-type controls (Fig 27A, left). Surprisingly, although the cell numbers in LNs of H1/DDA immunized IL-1R-/- mice were lower than in LNs of the wild-type animals, similar effects were seen in the Th-1 response of the re-stimulated LN cells of H1/DDA immunized mice (Fig 27A, right). Comparable to the results from IL-1R-deficient cells, the IFN-γ response of re-stimulated LN cells of H1/DDA/TDB immunized ASC-/- mice was similar to the wild-type controls, indicating that the TDB-induced development of a Th-1 response is independent of inflammasome formation (Fig 27B, left).

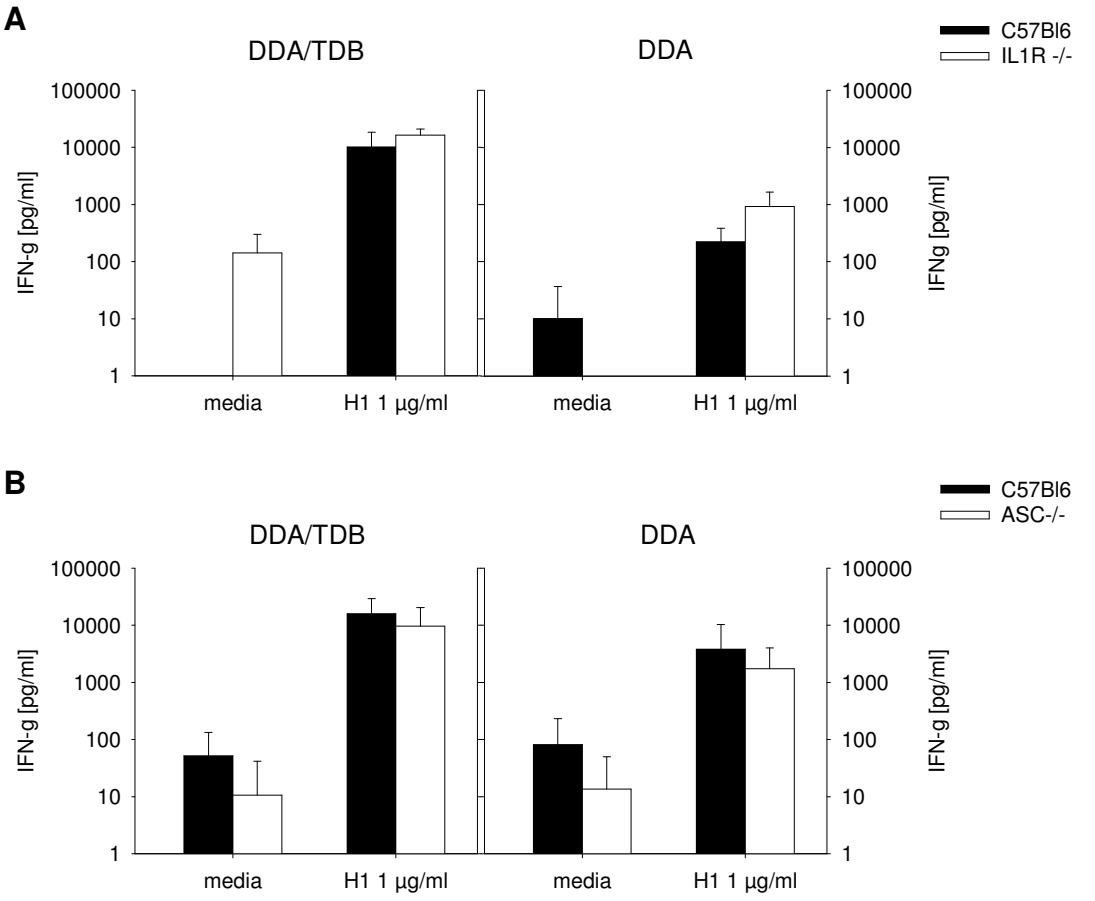

Figure 27: Induction of a Th-1 response by TDB is independent of IL1R signaling and inflammasome formation
Production of IFN-γ by draining lymph node cells of C57BL6 and IL1R-/- mice (A) and ASC-/- mice (B) two weeks after the second immunization with H1 in DDA (A, B right) or DDA/TDB liposomes (A, B left). Cells were re-stimulated for 96 h with 1 µg/ml H1 or left untreated. Production of IFN-γ was measured by ELISA. Mean and SD of duplicate wells. Mice per genotype (A) n = 4 (B) n = 3-5. (B) Data were pooled from two independent experiments.

Analysis of the Th-17 response in the different mouse genotypes indicated that deficiency of IL-1 signaling did not impair the T-cell priming in response to H1/DDA/TDB (Fig 28A, left), whereas blocking of inflammasome formation limited the Th-17 response to H1/DDA/TDB immunization significantly ($P < 0.00005$, Fig 28B, left).

Immunization with H1/DDA alone resulted in a reduced Th-17 response of re-stimulated LN cells of control animals in comparison to the effects induced by the DDA/TDB combination (Fig 28A/B, right). In contrast, DDA alone failed to induce a Th-17 response in IL-1R as well as in ASC-deficient mice, indicated by unresponsiveness of the according LN cells to re-stimulation (Fig 28A/B, right).

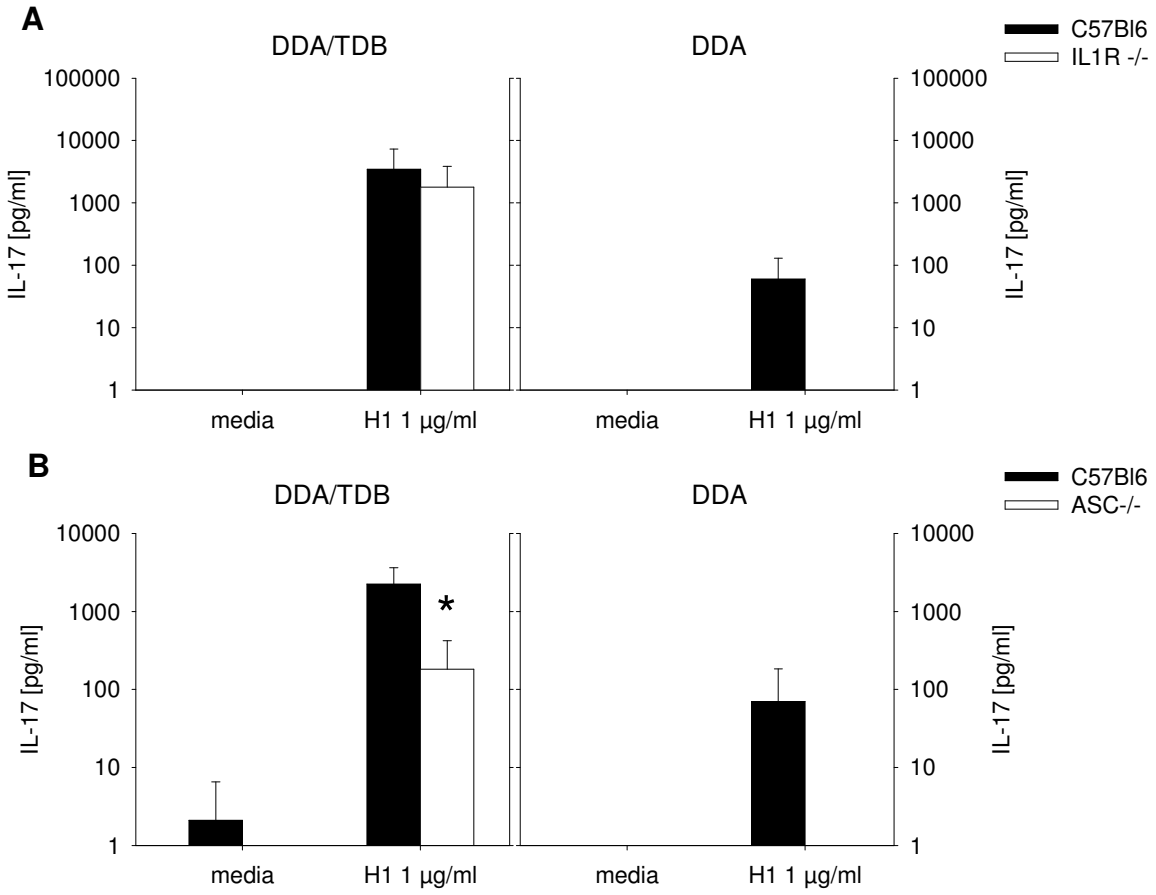

Figure 28: Induction of a Th-17 response by TDB is independent of IL1R signaling but requires inflammasome formation
Production of IL-17 by draining lymph node cells of C57BL6 and IL1R-/- mice (A) and ASC-/- mice (B) two weeks after the second immunization with H1 in DDA (A, B right) or DDA/TDB liposomes (A, B left). Cells were re-stimulated for 96 h with 1 µg/ml H1 or left untreated. Production of IL-17 was measured by ELISA. Mean and SD of duplicate wells. Mice per genotype (A) n = 4 (B) n = 3-5. (B) Data were pooled out of two independent experiments.*p value<0,00005

Analysis oft the IgG2a titers in the sera of all vaccinated mice indicated, that Th-1 specific antibody production in response to H1/DDA/TDB immunization was independent of IL-1R- (Fig 29A) and ASC-deficiency (Fig 29B), indicated by similar IgG2a antibody levels in sera of both genotypes in comparison to the antibody levels of wild-type controls. This is consistent with the IFN-γ data in re-stimulated LN cells of the different genotypes (Fig 27). DDA alone also induced the production of IgG2a, but with lower levels than in combination with TDB as adjuvant and independent of IL1R (Fig 29A) and ASC (Fig 29B) deficiency.

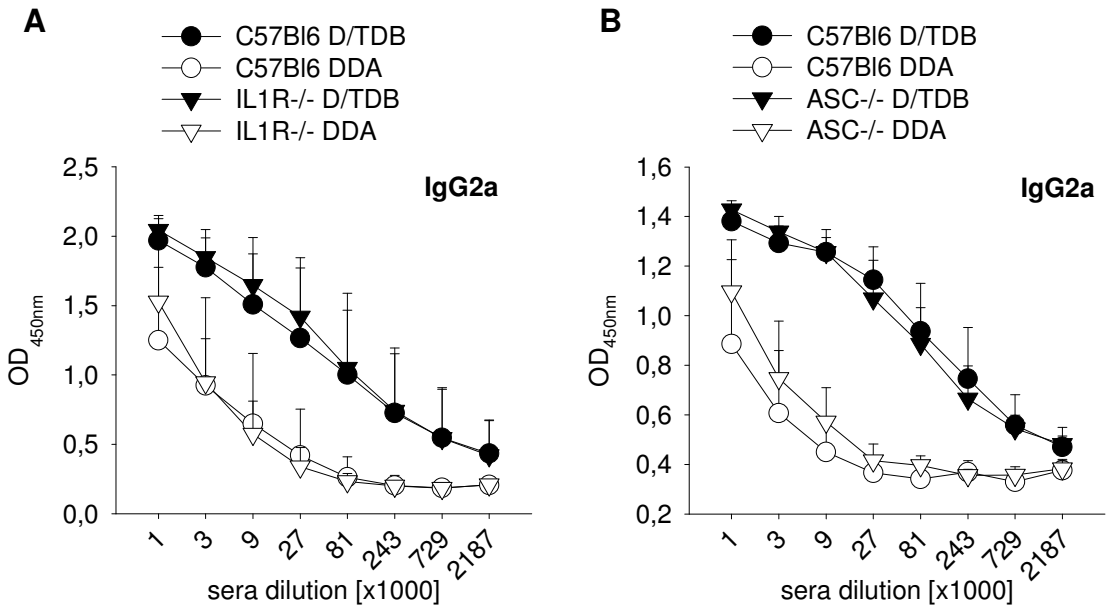

Figure 29: TDB induces production of Th-1 specific IgG2a antibodies independent of IL1R signaling and inflammasome formation
IgG2a levels in sera of C57BL6 and IL1R-/- mice (A) and ASC-/- mice (B) two weeks after the second immunization with H1 in DDA or DDA/TDB liposomes. Mean and SD of triplicate wells. Mice per genotype (A) n = 4 (B) n = 3. (B) Representative of two independent experiments.
D/TDB, DDA/TDB

All together, the previous results indicated, that the Myd88-dependency of the TDB adjuvant effect is not due to blocked IL-1β signaling, because the development of the Th-1 and Th-17 response was unaffected in IL-1R-/- animals. However, for the induction of a stable Th-17 response, inflammasome formation seemed to be essential. The role of other cytokines signaling via Myd88 was not analyzed here. As IL-18 production requires ASC-dependent inflammasome activation, and IL-18 signals via Myd88, this cytokine may explain at least in part the Myd88-dependence of the TDB-adjuvant effect *in vivo*.

4.9 The Myd88 dependency of TDB/TDM *in vivo* is restricted to myeloid cells

To investigate whether the Myd88-dependence of the TDB adjuvant effect is restricted to myeloid or lymphoid cells of the immune system, $Myd^{Stop/Stop}LysM^{Cre/Cre}$ mice were analyzed in terms of their response to H1/DDA/TDB immunization. These transgenic mice have a loxP-flanked stop cassette in the Myd88 gene locus and backcrossing $Myd88^{Stop/Stop}$ animals with $LysM^{Cre/Cre}$ mice leads to excision of this stop cassette only in Lysozym M expressing cells. Therefore, these animals express Myd88 only in the myeloid fraction of immune cells whereas the expression is blocked in the lymphoid cell fraction. The characteristics of the footpad swelling over time (Fig 30A) as well as the amount of cell numbers in the draining LNs (Fig 30B) were similar between $Myd^{Stop/Stop}LysM^{Cre/Cre}$ and wild-type animals. Furthermore no differences could be observed in the amounts of released IFN-γ (Fig 30C) and IL-17 (Fig 30D) between both genotypes, when the LN cells were re-stimulated with H1 antigen, indicating a normal Th-1 and Th-17 response of $Myd^{Stop/Stop}LysM^{Cre/Cre}$ and wild-type mice to the adjuvant. Also the titers of the Th-1 specific antibody IgG2a in the sera of all immunized animals were comparable between $Myd^{Stop/Stop}LysM^{Cre/Cre}$ mice and wild-type controls (Fig 30E). Together, the results indicated that the Myd88-expression in myeloid cells is sufficient to engender the TDB adjuvant responsiveness.

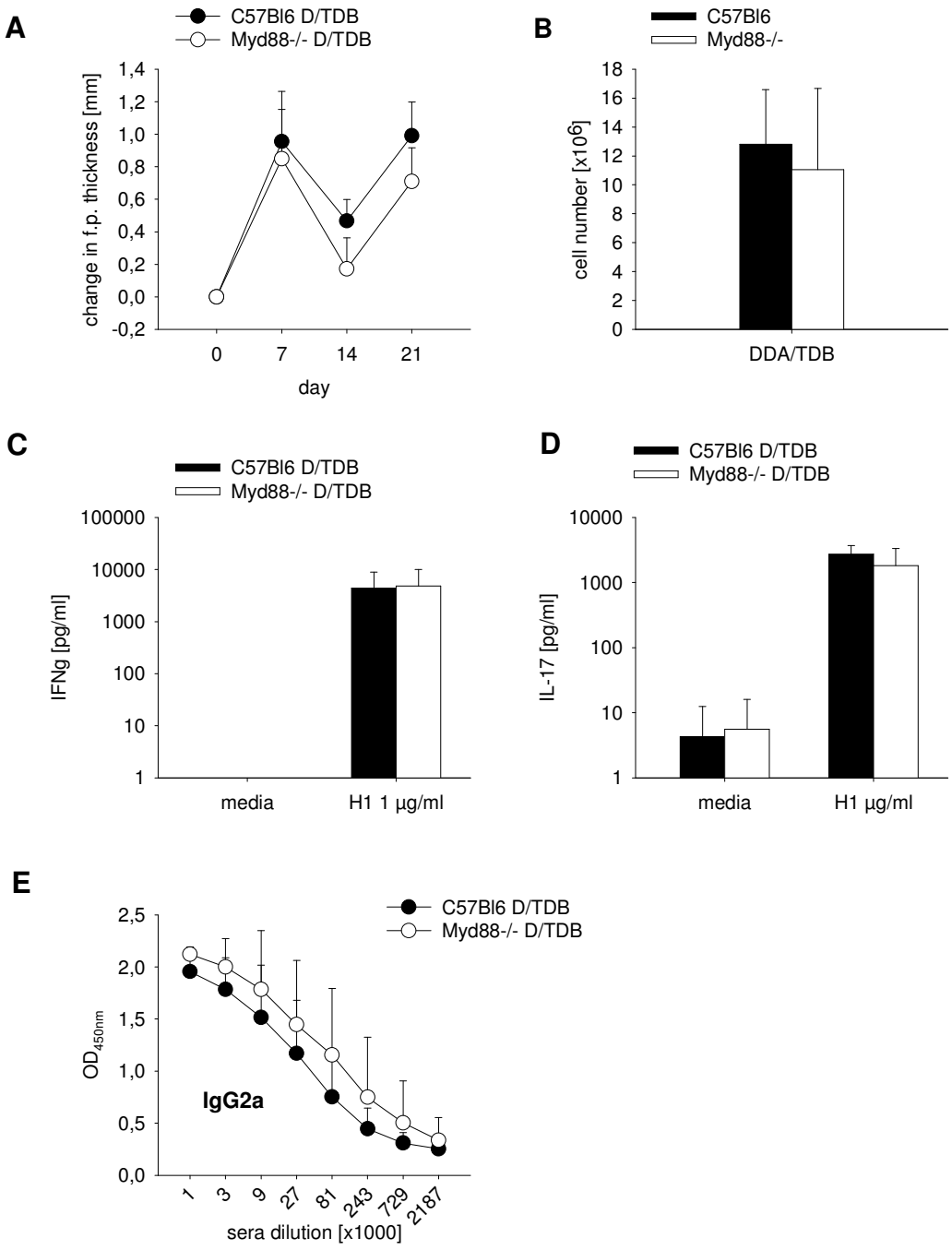

Figure 30: The Myd88-dependence of the TDB-adjuvant effect is independent of lymphoid cells
C57BL6 and Myd88$^{Stop/Stop}$LysM$^{Cre/Cre}$ mice were immunized via hind footpad injection on day 0 and 14 with H1 antigen in DDA/TDB liposomes. (A) Mean and SD of the increase in footpad thickness is shown. Footpad number per genotype n = 6 (B) Numbers of cells in draining LNs of C57BL6 and Myd88$^{Stop/Stop}$LysM$^{Cre/Cre}$ mice two weeks after the second immunization with H1 in DDA/TDB liposomes. Mice per genotype n = 3. (C) Production of IFN-γ and IL-17 (D) by draining lymph node cells of C57BL6 and Myd88$^{Stop/Stop}$LysM$^{Cre/Cre}$ mice two weeks after the second immunization with H1 in DDA/TDB liposomes. Cells were re-stimulated for 96 h with 1 µg/ml H1 or left untreated. Production of IFN-γ and IL-17 was measured by ELISA. Mean and SD of duplicate wells. Mice per genotype n = 3. (E) IgG2a levels in sera of C57BL6 and Myd88$^{Stop/Stop}$LysM$^{Cre/Cre}$ mice two weeks after the second immunization with H1 in DDA/TDB liposomes. Mean and SD of triplicate wells. Mice per genotype n = 3.

4.10 Induction of protective Immunity to challenge with TB requires Card9

It has been shown that the Syk-Card9 pathway is a pattern recognition pathway that can couple to adaptive immunity (LeibundGut-Landmann et al., 2007). To investigate whether this innate pathway is also responsible for the adjuvant activity of TDB *in vivo*, Card9-/- and control animals were immunized three times with H1/DDA, H1/DDA/TDB or H1/DDA/TDM. In contrast to Syk-/- and Malt1-/- mice, Card9-/- mice have defects in myeloid cells but intact lymphocyte activation (Gross et al., 2006; Ruland and Mak, 2003). As a first readout, changes in the footpad swelling over time were measured of all immunized animals. Swelling of the feet was only slightly induced in response to H1/DDA and comparable between wild-type and Card9-deficient mice, whereas, in sharp contrast, the increase in the footpad thickness was completely abrogated in Card9-/- animals after immunization with H1/DDA/TDB. However, the wild-type animals responded normally to the immunization with transient swelling of the feet after the injections (Fig 31).

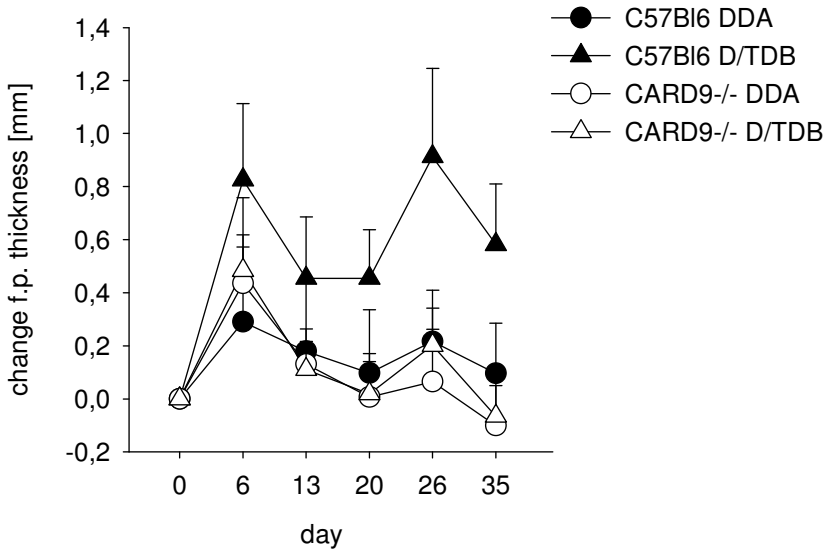

Figure 31: Deficiency of Card9 results in reduced footpad swelling after TDB-immunization
C57BL6 and Card9-/- mice were immunized via hind footpad injection on day 0 and 20 with H1 in DDA or DDA/TDB liposomes. Mean and SD of the increase in footpad thickness is shown. Footpad number per genotype n = 12, representative of two independent experiments. D/TDB, DDA/TDB

Moreover, the injection of TDB and most notably TDM led to a strong increase in the amount of cells in the draining lymph nodes of wild-type, but not Card9-deficient mice, whereas the response to CpG was relatively comparable between both genotypes (Fig 32).

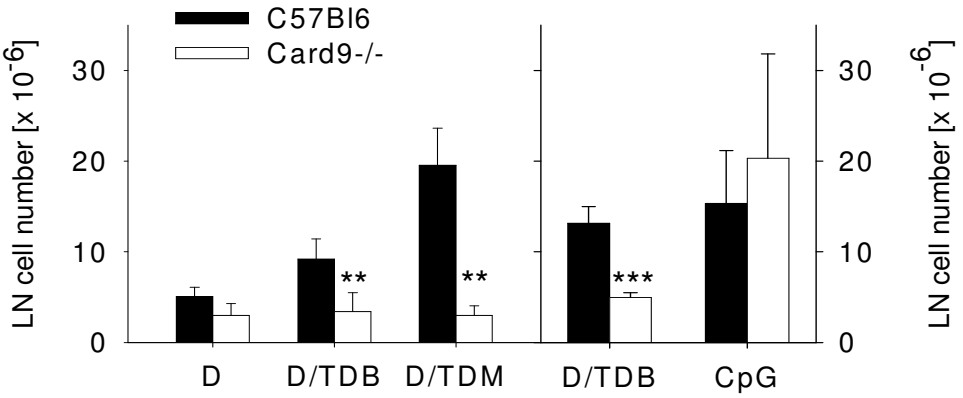

Figure 32: Deficiency of Card9 results in reduced cell numbers in LNs after TDB, but not after CpG-immunization
C57BL6 and Card9-/- mice were immunized via hind footpad injection on day 0 and 20 with H1 in DDA or DDA/TDB liposomes or in combination with CpG. Number of cells in draining LNs two weeks after the second injection are shown. Mice per genotype n = 3-5. Data are from two independent experiments. **p value <0.01; ***p value <0.001

To study the TDM and TDB-induced capacity of the extracted lymph node cells for IFN-γ and IL-17 production, the cells were re-stimulated with two different concentrations of the H1 antigen. It turned out, that Card9-deficient animals had a reduced Th-1 response compared to the wild-type controls, which indicated, that the Th-1 differentiation directed by these two glycolipids depends on Card9. The development of a H1-specific IL-17 production conferred by TDB and TDM was completely abrogated in Card9-/- mice whereas the wild-type animals responded normal, which suggested a crucial role of the Syk-Card9 pathway for the TDB-/TDM-induced Th-17 differentiation (Fig 33).

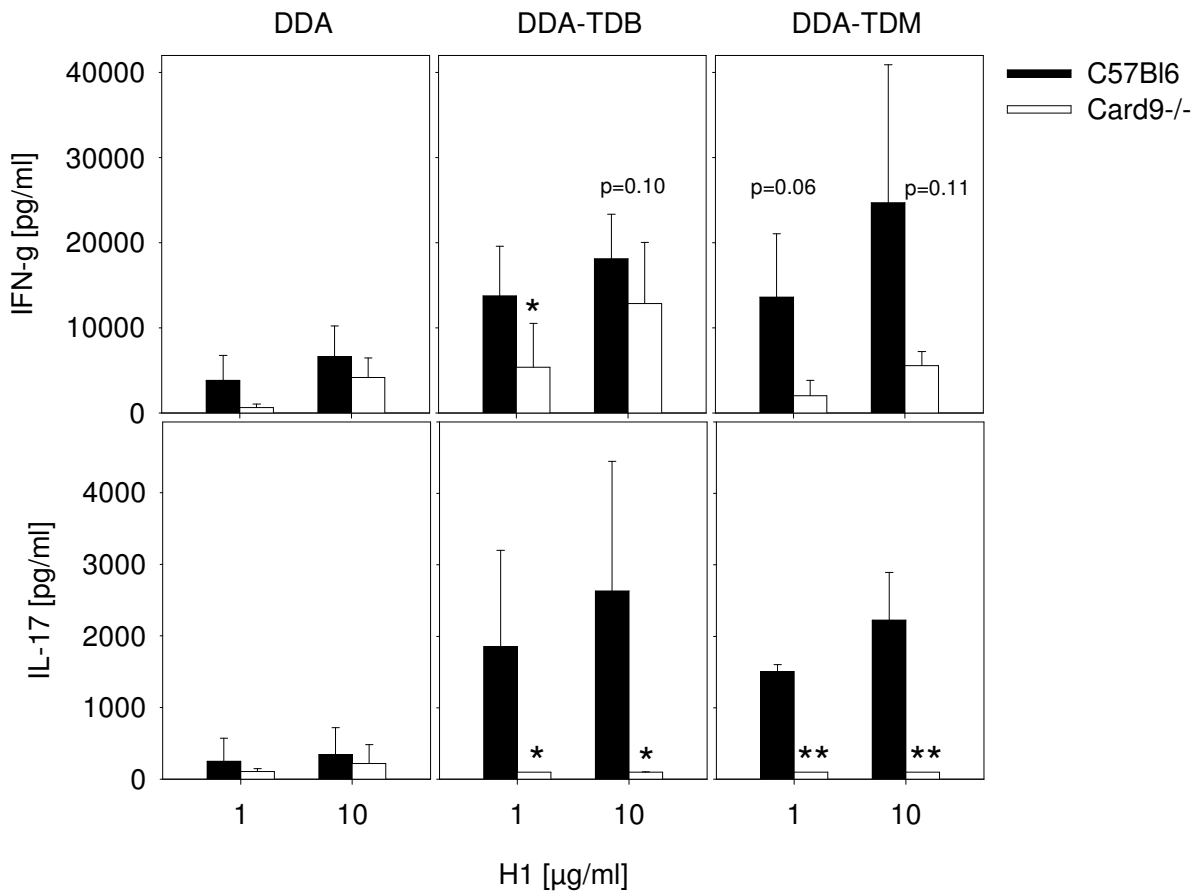

Figure 33: Deficiency of Card9 results in a reduced Th1/Th17 response after TDB/TDM immunization

Production of IFN-γ and IL-17 by draining lymph node cells of immunized C57BL6 and Card9-/- mice after 96 h re-stimulation with H1 antigen (1 µg/ml). Mean and SD of duplicate wells. Levels of IFN-γ and IL-17 were measured by ELISA. Mice per genotype n = 3-5, representative of two experiments.
*p value <0.05; **p value <0.01

As a further readout for the TDB and TDM-induced Th-1 response, IgG2a antibody titers in the sera of all vaccinated mice were measured. Only wild-type animals showed high antibody levels, whereas Card9-deficiency decreases the induction of this antibody response to a level comparable to H1/DDA immunized wild-type and Card9-/- animals (Fig 34). Thus, by using different readout systems for Th-1, Th-17 and antibody responses, Card9 was identified to be essential for mediating the adjuvanticity of TDB and TDM *in vivo*.

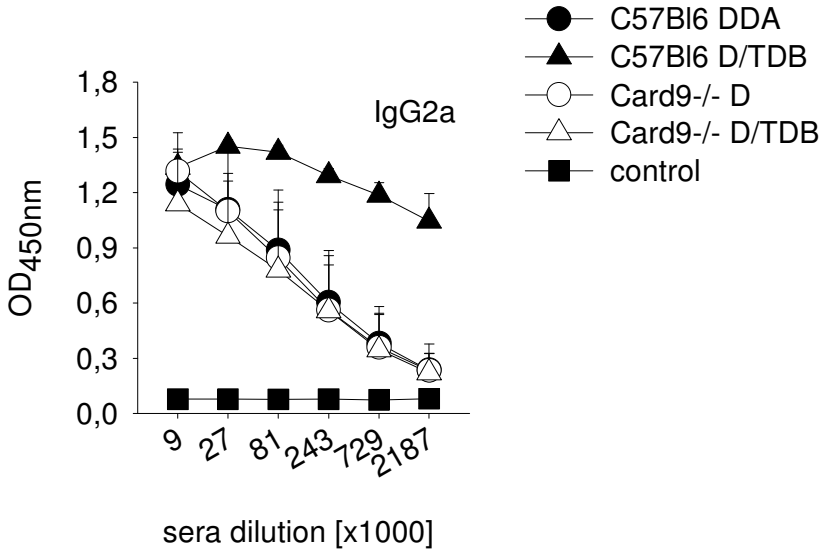

Figure 34: Deficiency of Card9 results in reduced Th1-specific antibody titers after TDB immunization
IgG2a levels in sera of immunized C57BL6 and Card9-/- mice injected three times with H1 in DDA or DDA/TDB liposomes. Mean and SD of triplicate wells, representative of two experiments. Mice per genotype n = 6. Control, untreated mice; D/TDB, DDA/TDB.

To test whether Card9 signaling is also responsible for the immune protection after subunit vaccination with H1/DDA/TDB, which confers a similar reduction in pulmonary mycobacterial load as the live BCG vaccine (Holten-Andersen et al., 2004), H1/DDA and H1/DDA/TDB immunized wild-type and Card9-/- mice were challenged with low-dose aerosol *Mycobacterium tuberculosis* (MTB) infection. Six weeks later, the amount of colony-forming units (CFUs) in the lungs of the infected animals were analyzed. As expected, compared to non-vaccinated mice, immunization with H1/DDA/TDB caused a significant reduction of the mycobacterial load in the lungs of wild-type mice whereas H1/DDA immunization had no significant effect (Werninghaus et al., 2009). In contrast, vaccination of Card9-deficient mice with H1/DDA/TDB failed to reduce the number of mycobacterial CFUs in the lungs of these animals. Like for the wild-type controls, immunization with only H1/DDA had no protective effect (Werninghaus et al., 2009).

5 Discussion

5.1 TDB/TDM potently activate macrophages and DCs

The major goal of this study was to analyze the mechanistic effects underlying the adjuvanticity of the mycobacterial cord factor TDM and its synthetic analogue TDB. In the first set of experiments both glycolipids were tested for their ability to activate antigen presenting cells (APCs) *in vitro* and compared with classical TLR ligands or non-TLR pattern-recognition receptor (PRR) ligands like the specific Dectin-1 ligand Curdlan. Both glycolipids potently activated macrophages with comparable effects to CpG and LPS, and these effects were even stronger, when the cells were primed with IFN-γ. Priming of macrophages with IFN-γ augments cell activation by inducing anti-tumor and direct anti-microbial mechanisms as well as by up-regulating antigen processing and presentation pathways, resulting in a stronger and quicker response of macrophages to different PAMPs (Schroder et al., 2004). Thus, it is conceivable that IFN-γ priming might also increase the expression of yet unknown TDB/TDM-specific surface molecules or enhance the phagocytic activity of the macrophage for efficient TDB/TDM internalization. This could explain the elevated responsiveness of primed BMDMs to TDB and TDM. Stimulation with bot glycolipids induced a strong and robust NO response as well as the production of the pro-inflammatory cytokines IL-6 and IL-1ß. These results were consistent with the data from Lima *et al* 2001, who showed that *in vitro,* TDM induced the production of IL-1β, IL-6, TNF-α and IL-12 in peritoneal macrophages. Furthermore this group detected enhanced levels of NO, IL-6, IL-12 and IL-10 in the homogenate and supernatant of bulk lung cells from mice treated with TDM-PGLA microspheres or with encapsulated microspheres containing TDB (Lima et al., 2001).

By measuring changes in global gene expression of TDB, CpG and Curdlan stimulated macrophages, it was shown here that TDB and Curdlan induced very similar genome-wide transcriptional responses that were distinct from the pattern of CpG-induced activation. Of note, TDB and CpG-induced transcriptional re-programming followed different kinetics, with an overall slower response to TDB. The TDB response was only partially overlapping with TLR activation and hierarchical clustering revealed TDB-selective and CpG-selective gene induction in addition to common down- and up-regulated target genes. Interestingly, IL-12p40 was strongly induced by CpG, but not by TDB, indicating different mechanisms behind the TDB

adjuvanticity in triggering a Th-1 response. On the other hand, TDB induces some genes not activated by CpG, e.g. the MHC-like molecule CD1d.

In comparison with the results of the *in vitro* studies, IL-6 and IL-1ß were identified as the main TDB-induced pro-inflammatory cytokines, whereas TDB also up-regulates the expression of several chemokines which are associated to induce migration of T cells and eosinophils in inflammatory reactions (Pinho et al., 2003; Quandt and Dorovini-Zis, 2004). In bone-marrow-derived DCs (BMDCs), TDB caused an up-regulation of MHC class II molecules and of the co-stimulatory molecules CD40, CD80 and CD86, essential components for the APC/ T-cell interaction resulting in the development of effector T cells (Chambers, 2001; Mueller, 2000). Furthermore TDB triggered cytokine and NO release in DCs in a manner similar to bone-marrow-derived macrophages. In addition, it could be shown that recognition of TDB by DCs induced the activation of the transcription factor NF-κB (Werninghaus et al., 2009), which is known to play a key role in regulating the immune response to infection (Ghosh et al., 1998). Thus, this study demonstrates that the adjuvants TDB and TDM activate APCs, in which they triggered a different expression program compared with TLR stimulation resulting in immunostimulatory effects that can be linked to the mechanisms behind the adjuvant activity of both glycolipids to trigger the development of a T cell response.

Although the TDB- and TDM-induced release of NO and IL-6 by bone-marrow-derived macrophages (BMDMs) was comparable to that of classical TLR activation, additional experiments indicated that activation of macrophages with TDB and CpG followed different kinetics, thereby confirming the results of the microarray data. In terms of NO production, the TDB response was delayed, whereas the amounts of NO after treatment with CpG or TDB were equal after 96 hours (Werninghaus et al., 2009). The reason for this difference is not known at present. Possibly, assuming that the TDB/TDM receptor is located on the cell surface, receptor molecules may be only poorly expressed on the surface of resting BMDMs but up-regulated in response to TDB, representing a positive feedback loop. An alternative explanation may be that the interaction between TDB and its possible receptor occurs slower than the interaction between CpG and TLR9 or that the possible TDB receptor, like TLR9, is localized intracellularly. This would require previous internalization and/or processing of the glycolipids and, dependent on the time-flow of internalization could result in a delayed response of the cells compared to classical TLR ligands. Identification of the

possible TDB receptor could clarify these hypotheses, as this would give the opportunity to analyze the time-flow of receptor-ligand interactions.

5.2 Macrophages and DCs recognize TDB/TDM via a FcRγ-Syk-Card9 pathway

The signaling requirements for macrophage activation by TDB/TDM were investigated mainly by using macrophages and DCs from mice genetically deficient in defined components of TLR and non-TLR pathways. Toll-like receptors represent a very prominent group in the recognition of several microbial components and except for TLR3 all known TLRs signal via the adaptor protein Myd88 (Akira and Takeda, 2004). Several mycobacterial pathogen-associated molecular patterns (PAMPs) such as phosphatidylinositol monomannoside (PIM) or Man-LAM, stimulate TLR2 to produce a pro-inflammatory response in macrophages which can promote mycobacterial killing (Underhill et al., 1999) or induce apoptosis in infected cells (Aliprantis et al., 1999; Lopez et al., 2003). Thus, to study if TLR signaling is involved in the recognition of TDB and TDM, Myd88-deficient as well as TLR2/3/4/7-/- and TLR2/3/4/7/9-/- BMDMs were analyzed. No evidence for an important role of TLR 2, 3, 4, 7, 9 or for Myd88 was found in macrophages, indicating that the mechanism behind the adjuvant effect of both glycolipids is independent of TLR signaling.

Besides TLRs, other receptors can also bind pathogens or pathogen-associated molecular patterns (PAMPs), thereby regulating the expression of innate response genes. Several publications reported that the C-type lectin Dectin-1 functions together with TLR2 as mediator for macrophage activation upon infection with different strains of mycobacteria (Shin et al., 2008; Yadav et al., 2006). Certain ITAM receptors or ITAM-coupled receptors like C-type lectins use the kinase Syk for downstream signaling (Tohyama and Yamamura, 2009), thus it was studied whether Syk-coupled pathways are required for TDB-mediated cell activation. Syk-deficiency resulted in an impaired immune response of BMDMs to TDB and TDM, indicating its essential role in the signaling mechanism induced by both glycolipids. Syk was also identified to use the myeloid-specific adaptor protein Card9 for the activation of macrophages and DCs in response to various signals (Gross et al., 2006; Hara et al., 2007; LeibundGut-Landmann et al., 2007). Therefore, Card9-deficient antigen-presenting cells (APCs) were analyzed in terms of their response to TDB and TDM. In a manner similar to Syk-/- macrophages, Card9-deficiency resulted in an

abrogated immune response of the APCs, indicating that Syk activity in response to TDB/TDM is also coupled to this downstream molecule. Gross *et al* have also shown in 2006 that Card9 can furthermore couple to the downstream adaptor molecules Bcl10 and Malt1 and analysis of Malt1- and Bcl10-deficient APCs resulted in a loss of the immune response after stimulation with TDB. Analysis of the gene expression profile in Card9-/- BMDMs indicated that TDB induced genome-wide transcriptional responses very similar to the Dectin-1 ligand Curdlan and, in contrast to CpG, these responses were completely Card9-dependent for both ligands. Dectin-1 is the best studied C-type lectin receptor so far and has been identified as a receptor that specifically recognizes β-1,3 glucans which mediates the uptake and killing of live fungal particles in macrophages by inducing the respiratory burst and the production of pro-inflammatory cytokines and chemokines (Brown, 2006). Because of its ability to recognize sugar-containing components and to initiate a downstream signaling cascade similar to that found for TDB and TDM, Dectin-1 represented a first receptor candidate for the recognition of TDB. Analysis of the expression profiling in wild-type and Card9-deficient BMDMs furthermore showed that, in a Card9-dependent manner, Dectin-1 expression was indeed up-regulated in response to TDB, whereas with a relatively low level compared to the untreated control. However, Dectin-1 deficiency did not affect the response of BMDMs to TDB and TDM, indicating that this C-type lectin is not required for the recognition of both glycolipids. To exclude a role for Dectin-1 in the adjuvanticity of TDB/TDM, it has to be tested, if Dectin-1 deficiency influences the adjuvant effect of TDB and TDM *in vivo*.

Syk has been reported to play a crucial role in signal transduction through the classical immunoreceptors, including the B-cell receptor (Kurosaki et al., 1995; Takata et al., 1994), Fc receptors (Crowley et al., 1997; Kiefer et al., 1998) and activating receptors of NK cells (Lanier, 2005). Furthermore, the ITAM-containing adaptor molecules DAP12 and Fc receptor γ (FcRγ) chain have been found to associate with Syk and mediate β2-integrin signaling in neutrophils and macrophages (Mocsai et al., 2006). It is unknown whether the mycobacterial cord factor can bind to and activate integrins. Majeed *et al* reported 2001 that Syk kinase is required for FcγR-induced phagocytosis by macrophages, thus, it is conceivable that Syk-signaling is also essential for the internalization of TDB and TDM. Syk-deficiency could block the TDB/TDM induced activation of APCs in two different ways, either by preventing downstream signaling resulting in an abrogated immune response of the

APCs or by inhibiting the incorporation of the glycolipids, thereby preventing binding to a potential intracellular receptor.

DAP12 and FcRγ are adaptor proteins associated with several C-type lectin and other cell membrane receptors and couples them to activation of Syk kinase (Robinson et al., 2006). To reduce the large number of possible candidates for the TDB/TDM specific receptor, the responsiveness of DAP12-/- and FcRγ-/- BMDMs to both glycolipids was analyzed. Whereas DAP12-deficiency did not affect the immunostimulatory effects of TDB and TDM on macrophages, deficiency of FcRγ resulted in an abrogated immune response of BMDMs to both glycolipids. Based on these findings, further analysis of the mechanism behind the recognition of TDB/TDM by APCs should focus on FcRγ-coupled receptors. Together, the present study identified TDB and TDM as potential activators of APCs that trigger a pathway which requires the adaptor molecule FcRγ as well as Syk kinase activity and activation of the downstream adaptors Card, Bcl10 and Malt1. To our knowledge, the cord factor and its synthetic analog represent the first mycobacteria-derived compounds that induce this TLR-independent pathway of APC activation.

5.3 TDB/TDM induce a Th-1 and Th-17 response

Our data confirm that TDB as a component of the adjuvant system CAF01 induces a strong Th-1 response. Importantly, we describe here the additionally strong and distinct Th-17-inducing property of TDB/TDM adjuvants. Furthermore, this study identifies Card9 as a crucial player for the Th-1/Th-17 adjuvanticity of TDB/TDM *in vivo*, suggesting that the FcRγ-Syk-Card9-dependent activation of APC directs the development of Th-1 and Th-17 responses.

Agger *et al* reported, that the adjuvant system CAF01 in combination with different antigens induces a strong Th1-response indicated by high titers of IFN-γ and IgG2a (Agger et al., 2008). LeibundGut-Landmann *et al* identified the Syk-Card9 pathway as the initiator for the development of a Th17-response directed by the Dectin-1 ligand Curdlan or during infection with *Candida albicans* (LeibundGut-Landmann et al., 2007). Our analysis of TDB/TDM-induced T-cell responses showed that the induction of a Th-1 as well as of a Th-17-response was impaired in Card9-deficient animals, indicating an essential role for Card9 in mediating the adjuvanticity of TDB/TDM *in vivo*.

In contrast to Syk-/- mice, Card9-/- mice have a normal development of T , B , and regulatory T cells and no differences in the basal immunoglobulin levels compared to wild-type animals. Therefore the data indicate that the Card9-dependence of the TDB/TDM adjuvanticity is related to the myeloid and not the lymphoid cell fraction. This is consistent with the findings that TDB/TDM failed to activate Card9-deficient BMDMs as well as BMDCs *in vitro*. A key role of Card9-signaling in the TDB-induced protective immunity to *Mycobacterium tuberculosis* (Mtb) *in vivo* could furthermore be demonstrated by Mtb challenge of TDB-immunized mice, as immunization with TDB failed to reduce the number of bacterial CFUs in the lungs of Card9-/- mice in sharp contrast to the significantly reduced mycobacterial load in the lungs of wild-type animals. This protective effect correlated with the Card9-dependent induction of antigen-specific Th17 cells in the draining LNs after vaccination, but not with the frequency of H1-specific IFN-γ producing $CD4^+$ T cells. Interestingly, the lung mycobacterial load in non-immunized Card9-deficient mice was higher than in the wild-type controls, which suggests an additional role for the Card9 pathway in the natural resistance to Mtb infection that deserves further detailed investigation. The higher bacterial load in Card9-/- mice may also explain the substantially elevated numbers of IFN-γ-producing T cells.

The mechanisms behind the adjuvanticity of TDB/TDM to induce a Th-1/Th-17 response can be linked at least in part to the TDB-induced gene expression profile in BMDMs. According to the current paradigm, the development of a Th-17 response in mice is dependent on the cytokines TGF-β and IL-6, which synergistically initiate the differentiation of T cells into the Th-17-lineage by inducing the expression of a transcription factor called retinoic acid receptor-related orphan nuclear receptor (ROR)-γt (Bettelli et al., 2006; Ivanov et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006; Zhou et al., 2007). In contrast, it has been shown that in humans IL-1β and IL-6, but not TGF-β are essential for the development of Th-17 cells (Acosta-Rodriguez et al., 2007) while the cytokine IL-23 was identified to be essential to further support the differentiation and survival of the Th-17 lineage in humans and mice (Bettelli et al., 2006). However, according to three recently published paper reporting TGF-β and IL-23 to be also essential cytokines in the differentiation and survival of human Th-17 cells, these results were controversial (Manel et al., 2008; Volpe et al., 2008; Yang et al., 2008). Interestingly, Khader *et al* reported that the presence of IL-23 is necessary for an efficient generation of Th-17 cells during the

initial priming upon mycobacterial infection. Furthermore it was shown that IL-23 can compensate for IL12p70 to induce a Th-1 response in Mtb-infected IL-12p35-deficient mice (Khader et al., 2005) and that IL-23 co-delivered with Ag85B induces a strong, Ag85B-specific Th-1 response in IL12p40-deficient mice (Wozniak et al., 2006). Gene expression data together with the *in vitro* results of the present study indicated that in BMDMs, TDB strongly enhanced the expression of IL-1β and IL-6 and, though with relatively low levels, also of IL-23α and TGF-β1 in a Card9-dependent manner. However, to definitely assess the effects of TDB/TDM on the TGF-β pathway, mRNA data are not sufficient. Future work should therefore determine whether active TGF-β is released by APCs upon TDB/TDM stimulation.

According to the findings of LeibundGut-Landmann 2007, who showed that activation of the Syk-Card9 pathway led to an enhanced production of IL-23 by BMDCs it is speculated that DCs, and not macrophages, are the main producers of this cytokine in response to TDB/TDM. This suggestion is furthermore strengthened by the findings that DCs are induced to produce IL12p70 as well as IL-23 upon Mtb infection (Khader et al., 2005; Wozniak et al., 2006). As TDB/TDM activates IL-23 production in DCs in a Card9-dependent manner, this may explain the loss of a Th-17 response in immunized Card9-deficient animals, as IL-23-deficiency together with the absent IL-6 production by Card9-deficient macrophages would inhibit the differentiation and survival of this T cell lineage. Therefore the TDB/TDM-induced IL-23 response of *ex vivo* DCs needs further investigation. In addition, it has to be determined, if DCs are also the main source of TGF-β production, as the macrophage response to TDB/TDM in terms of TGF-β expression was only weak. However, analysis of the TGF-β response was determined only in BMDMs, thus it is conceivable that the TDB/TDM-induced cytokine-profile in macrophages differs *in vivo*. Another possibility is that production of TGF-β in response to TDB/TDM is restricted to a cell population different from macrophages and DCs or that another (yet unknown) cytokine can compensate for TGF-β in terms of T cell priming towards Th-17. Acosta-Rodriguez *et al* reported IL-1β instead of TGF-β as the essential cytokine that acts synergistically with IL-6 in triggering the development of the Th-17 lineage in humans (Acosta-Rodriguez et al., 2007). Even so, IL-1β is unlikely to be involved in the induction of a Th-17 response by TDB/TDM, as the H1-specific IL-17 production by re-stimulated T cells of immunized IL-1R-deficient mice was comparable to wild-type animals. However, *in vivo* analysis of ASC-deficient mice showed that inflammasome

formation is essential for the induction of a Th17-response by TDB/TDM, indicating a possible role for the two additional inflammasome-dependent cytokines IL-18 and/or IL-33 in triggering T-cell priming towards the Th-17 lineage, although to date, involvement of these cytokines in triggering a Th-17 response has not been shown.

Interestingly, it was reported by several publications that in Mtb infection the most abundant IL-17 producing cells were $CD4^{-}CD8^{-}\gamma\delta^{+}$- rather than $CD4^{+}\alpha\beta^{+}$ T cells (Lockhart et al., 2006; Umemura et al., 2007) and that absence of γδ-cells resulted in an altered granuloma response (D'Souza et al., 1997). Moreover, Roark *et al* 2008 speculated that γδ- T cells are the first IL-17-producing cells upon an immune response and that antigen-specific $CD4^{+}\alpha\beta^{+}$ Th-17-cells develop later, thereby contributing to the response (Roark et al., 2008). Although LeibundGut-Landmann reported 2006 that activation of the Syk-Card9 pathway via the C-type lectin Dectin-1 resulted in the production of IL-17 and IFN-γ by $CD4^{+}$ T cells, it would be interesting to analyze the role of IL-17 producing γδ-T cells in promoting the adjuvanticity of TDB and TDM.

Besides the induction of a Th-17 response, we and others have shown the potential of TDB/TDM to induce an efficient and robust Th-1 response in different vaccination models. Given that IL-12 is a potent and essential inducer of Th-1-responses (Hsieh et al., 1993; Manetti et al., 1993) and mainly produced by phagocytes and DCs (D'Andrea et al., 1992; Macatonia et al., 1995) in response to microbial stimulation, the question is how TDB directs the development of a Th-1-response. In contrast to macrophages, DCs did produce measurable quantities of IL-12 upon stimulation with TDB in a Card9-dependent manner (Werninghaus et al., 2009). Interestingly, Oswald *et al* reported that TDM induces IL-12 synthesis at the mRNA level of peritoneal cells and that IL-12 production is a required step in TDM-induced activation of mouse peritoneal macrophages (Oswald et al., 1997). In the present study only bone-marrow derived cells were used for *in vitro* analysis of the TDB/TDM effect on APCs. According to the findings of Lima *et al* 2001, who showed that peritoneal macrophages do produce IL-12 in response to TDM, it is possible that the "maturation route" of macrophages and DCs influences the cytokine response to TDB/TDM. Therefore it is necessary to also analyze *ex-vivo* derived cells to investigate whether these APCs produce more IL-12 than *in vitro* generated BMDMs or BMDCs.

On the other hand, IL-23 can compensate for IL12p70 to induce a Th-1 response in Mtb-infected IL-12p35-deficient mice (Khader et al., 2005). Thus, it is also conceivable that TDB/TDM induced production of IL-23 by DCs is sufficient enough for triggering the T cell development towards the Th-1 lineage.

Flesch *et al* reported that early IL-12 production by macrophages in response to mycobacterial infection depends on IFN-γ and TNF-α, suggesting that production of the latter two cytokines must precede IL-12 production (Flesch et al., 1995). In addition, several publications have shown that TDM induces the expansion of NK-cells in several mouse strains (Ryll et al., 2001a; Tabata et al., 1996). Indeed, early in infection, NK cells are the main source of IFN-γ, whereas the NK-cell IFN-γ response to most pathogens is mediated by IL-12, indicating that IL-12 production by APCs precedes IFN-γ secretion by NK cells and not vice versa. However, two recent studies have proposed that type I IFNs, in combination with IL-18 or TNF-α, can induce the secretion of IFN-γ by NK cells independently of IL-12 signaling (Freudenberg et al., 2002; Marshall et al., 2006), although the *in vivo* relevance of this finding is not clear. Analysis of the TDB-induced gene expression profile in macrophages indicated that TDB led to a strong up-regulation in the gene expression of TNF-α, whereas probe sets for type I IFNs were not represented on the array. Furthermore stimulation of BMDM with TDB up-regulated the expression level of IL-18 only slightly (fold change 1.5). Thus, it is speculative that the induction of a Th1-response by TDB could be due to an IL-12 independent activation of NK cells, which would result in the production of IFN-γ and in turn to the secretion of high amounts of IL-12 by macrophages *in vivo*, resulting in the development of a Th1-response. Besides NK cells, natural killer T cells (NKT cells) have been identified as a potent source of large quantities of cytokines, including IFN-γ and IL-4 and this cell subset is hypothesized to be important in the initiation and regulation of various immune responses (Taniguchi et al., 2003; Wilson and Delovitch, 2003). NKT cells represent an unusual population of T cells which express certain receptors typical for NK cells, e.g. NK1.1 and Ly49 (Bendelac and Medzhitov, 2002) and, in addition, they have a restricted T cell receptor (TCR) set, which includes one invariant α-chain and a limited repertoire of β-chains. Therefore these cells are also called invariant NKT (iNKT) cells. Characteristic for NKT cells is their restriction to the MHCI-like molecule CD1d, a glycoprotein which is a member of the CD1 family of antigen-presenting molecules.

In humans, five members of CD molecules have been identified so far, which are divided in group 1 (CD1a, CD1b, CD1c) and group 2 (CD1d, CD1e) whereas in mice only two CD1 molecules exist (CD1d1, CD1d2) (De Libero and Mori, 2005). Besides several other ligands, human CD1b has been shown to present mycobacterial lipoarabinomannan (Sieling et al., 1995) and mycolic acid (Beckman et al., 1994) whereas mouse CD1d presents phosphatidylinositol tetramannosides (PIMs), which are compounds of the mycobacterial cell wall (Brutkiewicz, 2006). We and others have found that TDB as well as TDM causes an up-regulation of the CD1d molecule on macrophages, indicating that this molecule might play a role in the presentation of TDB to NKT cells (Ryll et al., 2001b). Interestingly, Emoto *et al* reported an IL-12 dependent depletion of an IL-4 producing NKT subset (normal-density NKT cells) and the appearance of IFN-γ-secreting cells (low-density NKT cells) (Emoto et al., 1997) after infection of mice with *Mycobacterium bovis* BCG, which is consistent with the results of Ryll *et al* that mycobacterial cord factor causes depletion of NKT cells, but that this depletion lasts long enough to allow the establishment of a Th1-response (Ryll et al., 2001b). Furthermore it was shown that NKT cells recognize *M. tuberculosis*-infected macrophages in a CD1d-dependent way, resulting in the production of IFN-γ and killing of intracellular bacteria (Sada-Ovalle et al., 2008). Thus, one could speculate that TDB and TDM might be presented by the CD1d molecule to NKT cells, which in turn are activated and rapidly produce high amounts of IFN-γ and IL-4, thereby inducing DC and macrophage maturation and IL-12 production, which would trigger a “forward loop” towards further IFN-γ production by developing Th-1 cells. The subsequent depletion of the IL-4 producing NKT-cell subset then would guarantee the perpetuation of the T- cell priming towards a Th1-response, because IL-4 is known to promote a Th-2-response. However, besides recognition of the CD1d/ glycolipid complex, IL-12 is also essential for the activation of NKT cells (Brigl et al., 2003; Schaible and Kaufmann, 2000). In the present study, only DCs could be identified to produce small amounts of IL-12 in response to TDB *in vitro*. However, these low levels of IL-12 may be sufficient to trigger NKT cell activation. Thus, to study the mechanisms of the TDB-induced Th-1 development in more detail, one interesting approach would be to analyze CD1d-deficient mice in terms of the adjuvant effect of TDB and to determine the influence of CD1d-deficiency and NKT cells on the T- cell response.

IFN-γ negatively regulates the induction and expansion of Th-17 cells (Cruz et al., 2006). Therefore, it is assumed that both cytokines may counter-regulate each other during chronic mycobacterial infection.

Regarding the hypothesis, that NKT cells might play a role in the TDB effect by supporting the development of a Th-1-response, and according to a recent publication where NKT cells were identified as an inhibitory cell subset for the development of the Th-17 lineage (Mars et al., 2008), one could speculate that the kinetics in the establishment of both T-cell responses to TDB differ. Khader *et al* reported that in primary Mtb infection, the induction of a Th-1 and Th-17 response followed the same kinetics, but that there are 5-10 fold more Th-1 than Th-17 cells (Khader et al., 2007; Khader et al., 2005). Interestingly, memory Th-17 cells responded more rapidly and populated the lungs more quickly than Th-1 cells after Mtb aerosol challenge and the Th-1 memory response to Mtb seen in the lungs of vaccinated mice was dependent on IL-23 and IL-17 (Khader et al., 2007). Furthermore IL-17 induced the up-regulation of CXCL9, CXCL10 and CXCL11 in the lung and depletion of IL-17 during Mtb challenge prevented the recruitment and accumulation of Th-1 cells, indicating a possible role for the Th-17 lineage in protection against mycobacterial infections. Moreover, Sergejeva *et al* could identify IL-17 as a recruitment and survival factor of macrophages in allergic airway inflammation and Dragon et al could show that IL-17 together with IL-23 alters the functional profile of neutrophils, resulting in reduced tissue damage and inflammation (Dragon et al., 2008; Khader and Cooper, 2008; Sergejeva et al., 2005). Thus, the possible function of IL-17 and IL-23 in the late stage of Mtb induced inflammation could lie in maintaining the integrity of the granuloma by inhibiting neutrophil cell death. According to these findings it is conceivable that the induced Th-17 response upon TDB/TDM immunization results in the generation of IL-17 producing surveillance cells which can recognize the invading pathogen rapidly in the tissue and promote the recruitment of protective cells, including neutrophils, monocytes and Th-1 cells. Furthermore cross regulation of IL-12 and IL-23 by each other and by IL-17 and IFN-γ may be critical to the inflammatory outcome of any mycobacterial infection. Therefore, it will be important to determine the effects of IFN-γ as well as of IL-17-deficiency on the protective effect of TDB/TDM vaccination against Mtb infection and whether the development of both lineages occurs with altered kinetics in the absence of IFN-γ or IL-17.

5.4 Myd88 is essentially required for TDB adjuvanticity *in vivo*, but dispensable for APC activation *in vitro*

By using different read-out systems to analyze the effect of TDB and TDM on APC activation *in vitro*, it turned out that both glycolipids acted in a Myd88-independent and therefore TLR-independent way. However, immunization of Myd88-/- and Myd88/Trif-deficient mice with TDB in combination with H1 antigen resulted in an impaired footpad swelling of the animals as well as in a strongly reduced Th-1 and an abrogated Th-17 response of the re-stimulated lymph node cells compared to the wild-type controls. Furthermore, Myd88-deficient animals had significantly reduced cell numbers and Th-1 specific antibody titers. Interestingly, Geisel *et al* reported, that Myd88 is required for cord factor induced inflammation *in vivo* (Geisel et al., 2005), including cell recruitment and production of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α whereas the precise role of this molecule in the mycobacterial-induced effects is not clear. Furthermore, Su *et al* found an essential role of the Myd88 pathway, but nonessential roles of TLRs 2, 4 and 9 in the adjuvant effect of complete Freund's adjuvant promoting Th-1 mediated autoimmunity (Su et al., 2005).

Bafica *et al* reported that TLR9 regulates Th1-responses and cooperates with TLR2 in mediating optimal resistance to *M. tuberculosis* (Bafica et al., 2005). However, when TLR2/3/4/7-/- mice were analyzed in terms of the adjuvant effect of TDB *in vivo*, no differences could be detected between both genotypes, indicating that the Myd88-dependence of the TDB effect *in vivo* is not due to an involvement of these TLRs. In contrast, immunization of TLR2/3/4/7/9-/- mice resulted in normal footpad swelling as well as in normal IgG2a titers compared to control mice whereas the IFN-γ response of re-stimulated LN cells was strongly reduced in the knock out animals. The results fit well to data of earlier experiments, were TLR9-deficient animals were analyzed. Similar to the T-cell response of immunized TLR2/3/4/7/9-/- mice, TLR9-deficiency alone resulted in reduced IFN-γ levels of lymph node cells upon re-stimulation with the H1 antigen, indicating a possible role for TLR9 in the development of a Th-1 response upon DDA/TDB immunization. In contrast, deficiency of TLRs 2, 3, 4, 7 and 9 did not affect the Th-17 response (Fig 23). Therefore, and because of the weaker attenuation of IFN-γ production in the absence of TLR9 compared to Myd88, deficiency of TLR9 alone can not account for the Myd88-dependent adjuvant effect of TDB *in vivo*. Possibly, the impaired effects in TLR2/3/4/7/9-/- and TLR9-/- mice are due to TLR9-dependent changes in the

lymphoid compartment, resulting in a general reduction of the vaccine response. Another option is that TLR9 recognizes TDB *in vivo* and synergizes with a Syk-coupled receptor, although deficiency of TLR9 did not impair the response of BMDMs to TDB/TDM *in vitro.* Haas *et al* have shown, that the sugar backbone 2' deoxyribose represents a prime determinant for ssDNA-TLR9 interactions and that base-free 2' deoxyribose homopolymers act as basal TLR9 agonists (Haas et al., 2008). Possibly, TLR9 is also capable to recognize the trehalose backbone of TDB and TDM upon internalization of the glycolipids and thus "boosts" the immune response of the APCs to TDB and TDM, whereas the major activation of the APCs is provided by a yet unknown TDB-specific receptor that triggers the induction of a Syk-Card9-dependent signaling pathway. However, the TLR-dependence of the TDB/TDM effect on APC activation was not analyzed in BMDCs, thus it is conceivable that the recognition of TDB/TDM by *in vitro* generated DCs in terms of TLR9-deficiency is different from macrophages, although the TDB/TDM induced signaling cascades were similar in both cell types.

By immunization of $Myd^{Stop/Stop}LysM^{Cre/Cre}$ mice, it was shown here that Myd88 expression in myeloid cells is sufficient to confer TDB-adjuvanticity, thereby ruling out the possibility that Myd88-signaling in T cells or B cells contributes significantly to the Th-1/ Th-17 induction or IgG production. As deficiency of Myd88 did not impair the response of BMDMs to TDB/TDM *in vitro*, the role of other myeloid subtypes (including monocytes, mast cells, eosinophils and neutrophils) in the Myd88-dependent adjuvanticity of TDB has to be analyzed. Neutrophils have previously been described as the first cells recruited to tissues following infection with various mycobacterial species (Appelberg and Silva, 1989; Fulton et al., 2002; Seiler et al., 2000; Silva et al., 1989). Furthermore they play an essential role in the early granulomatous response in the lung after TB infection in mice (Seiler et al., 2003). Interestingly, a recent publication reported that neutrophils can act as shuttle cells for live bacilli to the draining lymph nodes. Intradermal vaccination of mice with *M. bovis* BCG resulted in massive infiltration of neutrophils at the site of injection and to migration of infected neutrophils from the periphery to the secondary lymphoid organs via afferent lymphatics. Furthermore contacts between neutrophils and T cells were observed in the draining lymph nodes, which suggests that neutrophils, together with resident DCs, may play an important role in the mycobacterial antigen presentation process (Abadie et al., 2005). Therefore, neutrophils may promote the

adjuvant effect of TDB and TDM *in vivo*, because they may also play an important role in presenting the H1 antigen as well as the according glycolipid to T cells of the draining LNs. One could speculate that recognition of the adjuvant by neutrophils, in contrast to DCs and macrophages, requires Myd88. Thus, it would be interesting to see by FACS analysis, which cells are recruited to injection sites of TDB and TDM and if the amount and profile of the recruited cells differ between wild-type and Myd88-deficient mice.

Besides its role in TLR signaling, Myd88 is also a critical component in the signaling cascade mediated by the IL-1 receptor as well as the IL-18 receptor (Akira, 2000; Akira et al., 2000). Sugawara *et al* reported that in mice, deficiency of the IL-1 type 1 receptor resulted in larger granuloma formation as well as in a low IFN-γ production by splenic cells in response to a mycobacterial infection (Sugawara et al., 2001; Sugawara et al., 1999). To gain further inside into the role of Myd88 in the TDB mediated effect, it was analyzed, whether inhibited IL-1 signaling would influence the adjuvant effect of TDB *in vivo* with similar effects to Myd88-deficient animals. Deficiency of IL-1R did not alter the TDB-induced immune response. Thus, the Myd88-dependency of the adjuvant effect of TDB *in vivo* is not due to an abrogated IL-1R signaling.

In contrast to the results of the IL-1R -deficient mice, analysis of the TDB adjuvant effect in ASC-deficient mice, which have defects in inflammasome activation, indicated that the development of a Th-17 response was impaired whereas the Th-1-response was unaffected. Interestingly, Gross *et al* reported that Syk signaling induces the activation of the Nlrp3 inflammasome in response to fungal infections, resulting in the production and release of IL-1β (Gross et al., 2009). As our results demonstrate that the development of a Th-17 response seemed to be independent of IL-1β but dependent on inflammasome activation, this indicates a possible participation of IL-18 and/or IL-33, two further inflammasome-dependent cytokines of the IL-1 superfamily. IL-18 was first identified by Okamura *et al* as a novel cytokine that induces IFN-γ production by Th-1 cells (Okamura et al., 1998) and Takeda *et al* could show that IL-12 and IL-18 synergistically induce the development of a Th-1 response (Takeda et al., 1998). Furthermore Sugawara *et al* reported that mice deficient in IL-18 have reduced levels of IFN-γ but intact IL-12 production upon mycobacterial infection (Sugawara et al., 1999). However, to date, an involvement of IL-18 in triggering T-cell priming towards Th-17 has not been reported, but as IL-18 is

known to induce a signaling pathway identical to IL-1β (Akira, 2000), it is necessary to analyze IL-18-deficient mice, as this might clarify the role of Myd88 in the TDB/TDM adjuvant effect in more detail. IL-33, the third inflammasome-dependent cytokine of the IL-1 superfamily was identified to induce T helper cells, mast cells, eosinophils and basophils to produce type 2 cytokines in a Myd88-dependent way (Kroeger et al., 2009; Schmitz et al., 2005). As a possible role of IL-33 in mycobacterial infections or an involvement of this cytokine in triggering the development of a Th-17 response has not been investigated, it would be interesting to also analyze the consequences of IL-33 deficiency in terms of the TDB/TDM-induced Th-1/Th-17 response. However, according to the Th-2 directed activity of IL-33 it is assumed that adjuvanticity of both glycolipids is independent of this cytokine.

5.5 Outlook: Possible receptors for TDB and TDM

Several studies have identified TDM as the major immunostimulatory component of the mycobacterial cell wall but the receptor(s) responsible for recognition of this glycolipid have not been defined. In the present study, Toll-like receptors, which represent a well studied and prominent group of PRRs, could be excluded as possible receptors for TDM and its synthetic analogue TDB *in vitro*, thus, other PRRs have to be involved in the recognition of both glycolipids.

The role of C-type lectins in mycobacterial infections has been subject of several studies in the past and is still under investigation, whereas to date, detailed information about C-type lectin/ Mycobacteria interactions are given for the membrane-bound myeloid-cell-associated DC-SIGN, Dectin-1 and the soluble surfactant-associated proteins SP-A and SP-D (Torrelles et al., 2008). In addition, Mannose-binding lectin (MBL) and the mannose receptor were identified to recognize mycobacterial surface molecules (Dobos et al., 1996; Ortalo-Magne et al., 1995; Schlesinger et al., 1994) and a recently published paper furthermore identified the class A scavenger receptor MARCO to cooperate with TLR2 and CD14 for TDM-induced signaling in macrophages (Bowdish et al., 2009). Marco-/- macrophages were shown to be defective in activation of MAP kinases and production of pro-inflammatory cytokines in response to TDM and infection with *Mycobacterium tuberculosis* (Mtb). Analysis of the TDB-induced gene expression profile showed that TDB indeed strongly induced an up-regulation in MARCO expression in a Card9-

dependent way. Thus, this scavenger receptor represents a potent receptor candidate for TDB/TDM recognition that needs further investigation.

Further detailed analysis of the TDB-induced gene expression profiling in macrophages indicated that besides induction of Dectin-1 expression, TDB additionally led to an enhanced expression of fourteen different members of the C-type lectin family in macrophages with Clec4e (Mincle) and Clec4n (Dectin-2) as the major induced genes. Furthermore, increased expression of these TDB-induced genes was shown to be strictly Card9-dependent. Analysis of the TDB-induced expression levels of both genes in wild-type macrophages validated the microarray data, whereas deficiency of Syk, Card9 and Malt1 resulted in an abrogated induction of both genes in response to TDB. Mincle and Dectin-2 both belong to the group of membrane-bound, ITAM coupled C-type lectin receptors that lack ITAM motifs in their cytoplasmic tails but are associated with ITAM containing adaptor molecules. These adaptor molecules include DAP10, DAP12 and FcRγ which are associated with different C-type lectins but also associate with several other membrane receptors including Fc-receptors, activating and inhibitory NK-receptors and members of the TREM family (Lanier, 2009; Mocsai et al., 2004). In the present work, an involvement of DAP12 in the adjuvant effect of TDB and TDM could be excluded, as DAP12-deficient macrophages responded normally to both glycolipids. In contrast, deficiency of the Fc receptor γ chain resulted in an abrogated NO response as well as in an impaired expression of IL-6 and iNOS. Consequently, the repertoire of possible receptors for TDB and TDM can now be reduced to those transmembrane proteins which are associated with the FcRγ chain. In recent publications, Mincle as well as Dectin-2 were identified to be associated with the FcRγ adaptor protein (Sato et al., 2006; Yamasaki et al., 2008). Interestingly, Hara *et al* could also show that activation of Mincle induced a signaling pathway that depends on Syk as well as on the adaptor protein Card9 but was independent of Myd88 (Hara et al., 2007), suggesting that TLRs do not co-operate with Mincle to induce downstream signaling. According to these findings, Mincle and Dectin-2 are receptor candidates for TDB and also for the natural cord factor TDM. Therefore combination of biochemical and molecular genetic methods is needed to determine the possible involvement of Mincle as well as of Dectin-2 in the recognition of TDB and TDM *in vitro*. Furthermore it has to be tested if deficiency of one of both candidates will affect the adjuvant activity of both glycolipids *in vivo* in terms of T cell priming and protection against mycobacterial infection.

Besides its association with some C-type lectins, the FcRγ chain is also found in several other membrane-bound receptors, including members of the Fc receptor (FcR) family. To date, four different classes of IgG-specific Fcγ receptors (FcγRs), known as FcγRI, FcγRIIB, FcγRIII and FcγRIV have been recognized in mice of which only FcγRI, FcγRIII and FcγRIV have activating functions. Monocytes and macrophages express all activating and inhibitory FcγRs, whereas the expression of FcγRI, FcγRIIB and FcγRIII dominates on DCs (Nimmerjahn and Ravetch, 2008; Ravetch, 2002). The group of FcγRs has a well-defined role in triggering activation of innate effector cells, because they function in antigen presentation and immune-complex-mediated maturation of DCs, regulation of B-cell activation and plasma-cell survival (Nimmerjahn and Ravetch, 2006). Moreover, by regulating DC activity, FcγRs control whether an immunogenic or tolerogenic response is initiated after the recognition of antigenic peptides that are presented on the surface of DCs to cytotoxic T cells, T-helper cells and regulatory T cells. In general, an activating FcR usually consists of a ligand-binding α-chain, which has a high affinity for the Fc fragment of the different IgG antibody isotypes, and a signal transducing, ITAM containing adaptor molecule. In mice, this adaptor molecule is common for all activating FcγRs and could be identified as the FcRγ chain. Cross linking of FcRs by immune complexes starts with phosphorylation of the ITAM motifs by kinases of the Src family (Ghazizadeh et al., 1994; Wang et al., 1994), resulting in the recruitment of Syk-family kinases. The Syk-dependency as well as the FcRγ chain containing architecture of FcRs makes this class of receptors possible candidates for the recognition of TDB and TDM. It is not known if Fc receptors can bind other components besides the Fc fragments of antibodies. Analysis of the microarray data indicated, that the basal expression level of FcRγ chain (encoded by the FceR1g gene), and FcγRIII is very high in macrophages and slightly up-regulated by TDB. In addition, also the expression of FcγRI is high at the basal level, but down-regulated in response to TDB. It will be important to analyze Fc-receptor-deficient macrophages and mice to test the possible involvement of Fc receptors in the adjuvant effect of TDB and TDM *in vitro* and *in vivo*.

Two additional molecules are known that are also associated with the FcRγ chain, including the osteoclast-associated receptor (OSCAR) and the paired Ig-like receptor A (Pir-A) (Ishikawa et al., 2004; Maeda et al., 1998). OSCAR was identified as a molecule expressed mainly on osteoclasts, which are large multinucleate cells that

resorb bone. Monocytes are thought to be precursors of osteoclasts, which, under the influence of macrophage colony-stimulating factor (M-CSF) and receptor-activator-of-nuclear-factor-κB ligand (RANKL) differentiate to mature osteoclasts on the bone surface (Gordon and Taylor, 2005). It could be shown by Ishikawa *et al* that FcRγ mediates signal transduction by OSCAR, but it remains unclear how OSCAR transduces an activation signal that is required for osteoclast differentiation.

Pir-A is a transmembrane protein which possesses similar ectodomains with six Ig-like loops, but has different transmembrane and cytoplasmic regions. The protein was first identified by Kubagawa *et al* 1997, who showed that Pir-A expression was restricted to cells of B and myeloid lineages, including granulocytes, macrophages and mast cells (Kubagawa et al., 1997). Maeda *et al* identified PIR-A as an activating receptor on mast cells as a consequence of its association with the ITAM bearing FcRγ chain (Maeda et al., 1998). However, the receptor structure and ligand(s) of PIR-A together with the downstream signaling events induced by this protein are presently unknown. As PIR-A was not represented on the microarray used there is no evidence for a possible role of this protein in the TDB or TDM-mediated effects. In contrast, the expression of OSCAR could be detected, but the expression level did not change in response to TDB. Therefore OSCAR as well as PIR-A can be put on the list of possible receptor candidates for TDB and TDM, but their involvement in the adjuvant effect of both glycolipids is rather unlikely.

Gene symbol	Protein name	Adaptor protein	Expression in macrophages
Clec4e	Mincle	FcRγ	Strongly induced by TDB
Clec4n	Dectin-2	FcRγ	Strongly induced by TDB
Fcgr1	CD64	FcRγ	Highly expressed
Fcgr3	CD16	FcRγ	Highly expressed
Oscar	OSCAR	FcRγ	Low expression
Pira1	Pir-A	FcRγ	Not represented on array
Marco	MARCO	?	Strongly induced by TDB

Table 1: Receptor candidates for TDB/TDM recognition

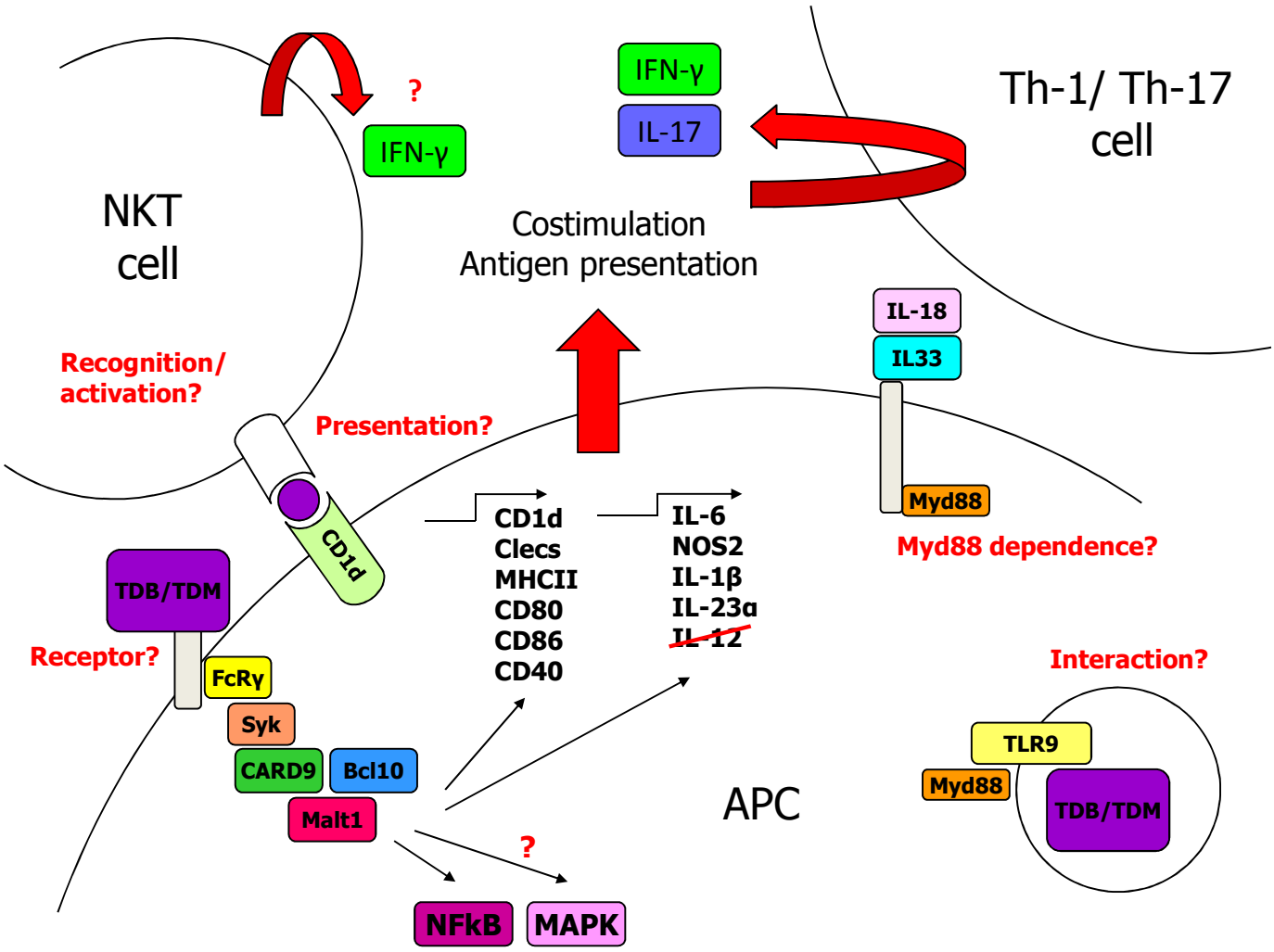

Figure 1: Schematic overview of the TDB/TDM induced effects on APC activation and T cell priming

Macrophages and DCs recognize TDB/TDM via a FcRγ-Syk-Card9-Bcl10-Malt1 pathway and are activated independent of TLR signaling. Downstream signaling includes activation of the transcription factor NF-κB and enhanced gene expression of pro-inflammatory cytokines, co-stimulatory molecules, inducible NO synthase, members of the C-type lectin family and MHC class II molecules. The induced effect results in efficient priming and activation of Th-cells and in the development of a Th-1/Th-17 response. TDB does not induce production of IL-12 but induces gene expression of the MHC-like molecule CD1d, indicating a possible role for this molecule in presentation of the glycolipids to NKT cells. If NKT cells do produce IFN-γ as a consequence of CD1d restricted recognition of TDB/TDM is not clear. Furthermore the TDB/TDM mediated effect on MAPK activation in APCs is unknown. The Myd88-dependency *in vivo* is to date unexplained and might be related to the signaling pathways induced by the cytokines IL-18 and IL-33. Involvement of TLR9 in the Th-1 induction by TDB/TDM *in vivo* contrasts with intact activation of TLR9-/- macrophages. APC, antigen-presenting cell; NKT, natural killer T cell; Clecs, C-type lectins

6 Summary

In the present study, the mechanism behind the adjuvant activity of the mycobacterial cord factor TDM and its synthetic analog TDB as a component of the new adjuvant system CAF01 was investigated. *In vitro*, TDB and TDM were both found to be potent activators of APCs. Stimulation of bone-marrow-derived macrophages and DCs with both glycolipids resulted in a strong production of nitric oxide (NO) and pro-inflammatory cytokines and the response was elevated upon IFN-γ priming. Compared to the TLR ligands LPS and CpG, the glycolipids TDB and TDM induced overlapping but distinct gene activation programs. In contrast to CpG and LPS, recognition of TDB and TDM by *in vitro* generated macrophages and DCs was independent of Myd88 and of TLR2, 3, 4, 7 and 9, but strictly dependent on a signaling pathway including Syk kinase and the adaptor proteins Card9, Malt1 and Bcl10, indicating that APC activation by TDB/TDM follows a TLR-independent pathway. Microarray analysis of the TDB-induced gene expression profile in Card9-deficient and wild-type BMDMs indicated that TDB induced very similar genome-wide transcriptional responses to the specific Dectin-1 ligand Curdlan, whereas some gene clusters were induced stronger by TDB than by Curdlan. However, the TDB and Curdlan-induced responses were distinct from the pattern of CpG-induced activation and completely Card9-dependent, whereas Card9-deficiency did not affect CpG-triggered gene expression. Further *in vitro* studies excluded the C-type lectin Dectin-1 as the specific TDB/TDM receptor and identified the adaptor protein FcRγ to be essential for TDB/TDM-induced APC activation. Surprisingly, TDB did not induce the expression of the Th-1 promoting cytokine IL-12, but strongly enhances the expression of the cytokines IL-1β, IL-6 and IL-23α and furthermore of several chemokines, which are associated with the development of a Th-17 response and the recruitment of T cells.

In vivo, similar to CpG, TDB induces a strong Th-1 response, indicated by high titers of H1-specific IgG2a antibodies and strong levels of IFN-γ produced by re-stimulated draining lymphnode cells of immunized mice. Furthermore and in contrast to CpG, TDB additionally triggers the development of an antigen-specific Th-17 response. Interestingly, Myd88 was dispensable for APC activation *in vitro*, but essentially required for the TDB adjuvant effect *in vivo*. However, TDB adjuvanticity was independent of TLR2, 3, 4 and 7 and largely independent of TLR9, as deficiency of TLR9 indeed affected the production of antigen-specific IFN-γ by re-stimulated T cells

of draining LNs, but did not block the production of H1-specific IgG2a antibodies or the development of a Th-17 response. Analysis of IL-1R-/- and ASC-/- animals indicated that adjuvanticity of TDB is independent of IL-1R signaling but requires inflammasome formation for efficient T cell priming towards the Th-17 lineage. Myd88 is an essential molecule in the downstream signaling of the two additional inflammasome-dependent cytokines IL-18 and IL-33, thus, an involvement of both cytokines in the TDB mediated adjuvant effect should be further studied to clarify the role of Myd88. However, by using specific knock out mice, it was shown that the Myd88-dependence of TDB *in vivo* is restricted to myeloid cells.

The essential importance of the Syk-Card9 pathway for the TDB/TDM adjuvant effect *in vivo* became evident in studies with Card9-deficient mice. Absence of this signaling molecule abrogated all immunization response in vaccinated mice, including the development of a Th-1 as well as of a Th-17 response. Furthermore, it was shown by a TB challenge model that Card9 is essential for the induction of protective immunity to TB.

The major open question in our understanding of the adjuvant effect of TDB and TDM is the identification of the receptor(s) binding these glycolipids. Interestingly, TDB strongly enhances the expression of several members of the C-type lectin family in a Card9-dependent manner with Clec4e (Mincle) and Clec4n (Dectin-2) as the most prominent targets. Recent studies identified Mincle as a FcRγ-chain coupled membrane receptor that initiates a Myd88-independent downstream signaling cascade including Syk kinase and Card9 upon activation. Thus, this C-type lectin represents a potent receptor candidate for TDB and TDM and further *in vitro* and *in vivo* studies using Mincle-deficient mice might clarify the role of this C type lectin in the TDB/TDM mediated effect in more detail. According to the FcRγ chain dependency of the TDB/TDM induced effect, further studies will also focus on the role of other FcRγ-coupled receptors to possibly identify the TDB/TDM-specific receptor.

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