Role of TLR-MyD88 signalling in B-cells during *Salmonella* infection

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Abbreviations

Ab	Antibody
Ag	Antigen
AP	Alkaline phosphatase
APC	Antigen-presenting cell
BCG	Bacille Calmette-Guérin
BCR	B cell receptor
BM	Bone marrow
BSA	Bovine serum albumin
CD#	Cluster of differentiation
CFA	Complete Freund’s adjuvant
CIA	Collagen-induced arthritis
CNS	Central nervous system
CpG	Oligodeoxynucleotides carrying unmethylated CpG motifs
CTL	Cytotoxic (cytolytic) T lymphocyte
Cy5	Cy-Chrome 5, fluorescent dye
CLR	C-type lectin receptor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmatic reticulum
FACS	Fluorescent-activated cell sorting
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box protein 3
FSC	Forward scatter
GC	Germinal centre
HKS	Heat-killed *Salmonella*
HLA	Histocompatibility leukocyte antigen
HSC	Hematopoietic stem cell
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
iTregs	Induced regulatory T cells
i.v.	Intravenous
LPS	Lipopolysaccharide
LT	Lymphotoxin
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
min.	Minute
MHC	Major histocompatibility complex
ml	Milliliter
mg	Milligram
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response gene 88
MZ	Marginal zone
NK	Natural killer
NKT	Natural killer T cells
NO	Nitric oxide
NOD	Non-obese diabetic
nTregs	Natural regulatory T cells
OD	Optical density
PBS	Phosphate buffered saline
PBMCs	Peripheral-blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PI	Propidium iodide
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RT	Room temperature
SA	Streptavidin
SCID	Severe Combined Innune Deficiency
SEM	Standard errors of mean
SLE	Systemic Lupus Erythematosus
SSC	Sideward scatter
T1D	Type 1 Diabetes Mellitus
TCR	T cell receptor

TGF	Transforming growth factor
T_H1	T helper cell type 1
T_H2	T helper cell type 2
T_H17	T helper cell type 17
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UC	Ulcerative colitis
WT	Wild-type

1 Introduction

The immune system provides defences against pathogens (e.g. viruses, bacteria, and parasites) and tumors. An important process of the immune system is the recognition of the microorganism by receptors on the cell surface that lead to the activation of the cell and subsequently the induction of responses. In mammals the immune system consists in two parts: the innate and the adaptive immunity. In the innate immune response the antigen recognition is done by evolutionary conserved receptors that recognize conserved microbial structures common to many pathogens. The binding of specific components of pathogens by these receptors gives rise to very rapid responses. One of the important responses is the ingestion of the pathogens after recognition by phagocytic cells, such as neutrophils, macrophages and dendritic cells. Other responses that are associated to the recognition of the innate receptors are the induction of soluble components. These components include cytokines that affect the behaviour of other cells, chemokines that attract cells, growth factors that control growth, division and maturation of cells, and small soluble proteins that make part of the complement system. Many of these soluble components have an important role in the subsequent activation of the adaptive immunity.
In the adaptive immunity the antigen recognition is done by receptors, which are generated by the random recombination of a variable gene segments creating a highly diverse repertoire of lymphocyte antigen receptors. This immunity takes longer to develop and involves antigen specificity of lymphocytes. After antigen-specific recognition, B and T cells clonally expand and differentiate into effector cells that effectively target the pathogen for elimination. Another important characteristic of the adaptive immunity is the development of immunological memory, in which each pathogen is “remembered” by an antigen-specific antibody and antigen-specific T cells.
These memory cells can be called upon to quickly eliminate the same pathogen on subsequent infections.

The lymphocytes beside the expression of highly selective antigen-receptors also have innate receptors, such as Toll-like receptors (TLRs). Particularly, TLR-pathway of inflammation depends on the signalling protein MyD88 (myeloid differentiation primary response gene 88). How MyD88-signalling in B cells influences the host response to intracellular bacteria is the subject of this study. The many known functions and the involvement of MyD88 in many immune responses by macrophages and dendritic cells as

well the importance of TLR/MyD88 mediated signalling in B cells during auto-immune diseases lead us to the analysis of MyD88 during *Salmonella typhimurium* infection in mice with MyD88-restricted deficiency on B cells.

1.1 The innate immune system

The innate immune system constitutes the first line of defence against pathogen invasion. It is initiated immediately after contact to foreign antigens. The recognition of pathogens relies on germline-encoded receptors termed pattern recognition receptors (PRR). After pathogen recognition by these receptors on innate immune cells, the inflammation process starts, which leads to the local recruitment and activation of different subsets of phagocytes that limit the multiplication and spread of the microbe very early after infection.

1.1.1 Innate immune receptors

The recognition of microbial threats by PRRs, such as TLRs (Toll-like receptors), NLRs [NOD (nucleotide-binding oligomerization domain)-like receptors], CLRs (C-type lectin receptors), and RLRs (RIG-I-like receptors) (Figure 1) is often the first step of the inflammatory cascade. These receptors are able to recognize a high range of microbial products. The TLRs recognize a variety of pathogen-associated molecular patterns (PAMPs) derived from different microbes. The NLRs sense bacteria, RLRs sense viruses and CLRs sense fungi. These PRRs are localized in different cells of the immune system, such as neutrophils, macrophages, dendritic cells, endothelial cells, epithelial cells and lymphocytes.

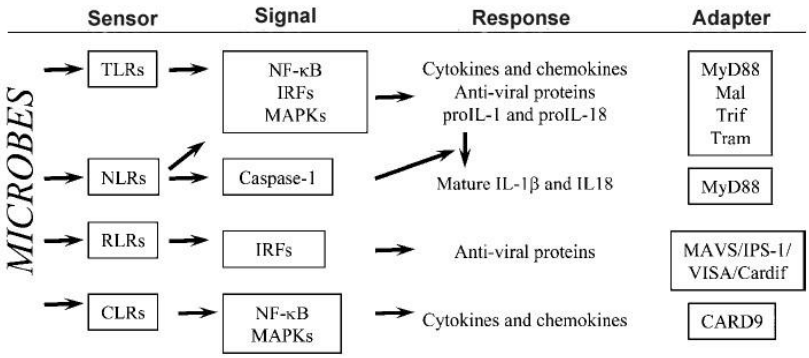

Figure 1: Schematic representation of four types of PRRs and their gene expression in response to invasive microbes. The TLRs and CLRs activate the transcription factor NF-κB and also MAPKs. Some TLRs and the RLRs activate IRFs, which are required for expression of some antiviral genes. Certain NLRs (e.g. Nalp3) activate caspase 1, which processes the pro-forms of IL-1β and IL-18. Signalling is activated via a receptor-specific subset of adaptors, with MyD88, Mal, TRIF and TRAM mediating the signalling. MyD88 is also used by IL-1R and IL-18R. CLRs signal via the adaptor CARD9. RLRs signal via the adaptor IPS-1; Cardif, CARD adaptor inducing IFNβ; MAVS, mitochondrial antiviral signalling protein; VISA, virus-induced signalling adaptor. The figure is adapted from [1].

1.1.1.1 Toll-like receptors

Toll-like receptor family are the best characterized class of PRRs in mammalian species. The first studies identifying the roles of Toll receptors in innate immunity were performed in *Drosophila melanogaster.* These studies demonstrated that the gene toll is absolutely required for activation of antifungal innate immunity [2]. The first ortholog of Drosophila Toll was found in mammalians in 1996 [3]. Since then ten TLRs (TLR1-TLR10) have been described for human and twelve TLRs are known in the mouse (TLR10 is not present in mouse). TLRs are type I transmembrane receptors, which are characterized by an extracellular Leucine-rich repeat (LLR) domain for ligand binding, a single transmembrane domain, and an intracellular Toll/IL-1 receptor (TIR) domain involved in signalling [4]. TLRs can be localized on the cell surface, such as TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 or can be localized intracellularly, such as TLR7, TLR8 and TLR9, where their natural ligands might only be found within acid compartments, such as phagolysomes. The TLR3 could locate at the cell surface or intracellularly [5].

TLRs detect a broad range of pathogens including viruses [6-10], bacteria [11-14], fungi [15] and parasites [16]. The recognition of molecular patterns by TLRs includes: lipoproteins from gram-negative bacteria, Mycoplasma and spirochetes [17-21], lipoteichoic acids and peptidoglycans from gram-positive bacteria, are recognized by TLR2; TLR9 recognizes unmethylated CpG motifs from bacterial DNA; flagellin from bacterial flagella is recognized by TLR5 [11]; double-stranded RNA produced by most viruses during the infection cycle is recognized by TLR3; and lipopolysaccharide (LPS) of gram-negative bacteria is recognized by TLR4.

The mechanism of LPS recognition by TLR4 requires several membrane-linked and soluble molecules, including CD14 and MD-2. CD14, an LRR-containing, GPI-linked molecule binds LPS binding protein/LPS complexes and is thought to transfer LPS to the TLR4 complex [22, 23]. MD-2 is another protein that interacts with TLR4, and is required for LPS responsiveness [24, 25].

Signal transduction from TLRs requires adaptor molecules. The protein MyD88 is the major adaptor molecule in the TLR signalling cascade [26] except for TLR3, and it is also essential for signalling via interleukin 1 (IL-1) and IL-18 receptors [27]. Other adaptor proteins contribute to TLR signalling, such as TIRAP (TIR domain-containing adapter protein) also known as MAL, TRIF (TIR domain-containing adapter-inducing interferon-β), TRAM (TRIF-related adapter molecule), and SARM (sterile α-and armadillo-motif-containing protein) [28].

The usage of these adaptor proteins varies between TLRs. For example TLR2 activation on macrophages triggers via TIRAP and MyD88, a signalling pathway ending in activation of NF-κB and production of cytokines such as tumour necrosis factor-α (TNFα). TLR4 leads to the activation of NF-κB via TIRAP or MyD88 but additionally activates the transcription factor interferon (IFN) regulatory factor-3 (IRF3), leading to the production of type I IFNs in addition to TNF-α [29, 30].

TLRs can form heterodimers or even associate with non-TLR membrane clusters, to further diversify their recognition potential [31-33]. The co-activation of TLR2 and TLR4 leads to higher production of TNF-α, IL-6, and macrophage inflammatory protein 1α (MIP-1α) by mouse macrophages and human monocytes than either receptor alone elicits [34]. TLR2 and TLR4 synergize for production of nitric oxide (NO) by macrophages [35]. Activation of TLR4 together with TLR7 increases the production of IL-12p70 by 10 to 100-fold compared to triggering of either receptor alone [34]. Therefore, TLRs are key receptors in the identification of pathogens by the innate immune system. Figure 2 summarizes some of the components in intracellular signalling cascade of the TLRs.

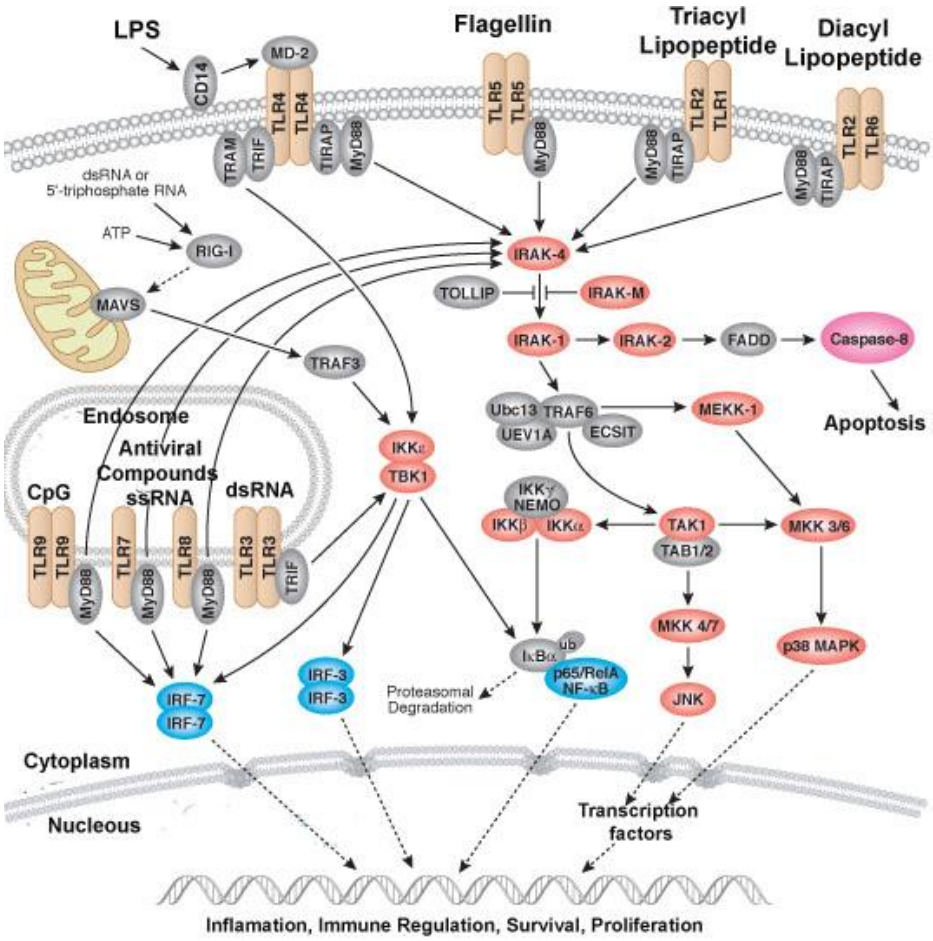

Figure 2: TLR pathway. TLR1, 2, 4, 5 and 6 are located on the cell surface and TLR3, 7, 8 and 9 are localized to the endosomal/lysosomal compartment. The activation of the TLR signalling pathway originates from the cytoplasmic Toll/IL-1 receptor (TIR) domain that associates with a TIR domain-containing adaptor, MyD88. Upon stimulation with ligands, MyD88 recruits IL-1 receptor-associated kinase (IRAK) to TLRs through interaction of the death domains of both molecules. IRAK activated by phosphorylation then associates with TRAF6, finally leading to activation of JNK and NF-κB. Tollip and IRAK-M interact with IRAK-1 and negatively regulate the TLR-mediated signalling pathways. MyD88-independent pathways induce activation of IRF3 and expression of interferon-β. TIR-domain containing adaptors such as TIRAP, TRIF and TRAM regulate TLR-mediated signaling pathways by providing specificity for individual TLR signalling cascades. This figure was adapted from www.cellsignal.com

1.1.1.2 Other receptors: NLRs, CRLs and RLRs

NLRs such as NODs (Nucleotide-binding oligomerization domain), NALPs (NACHT-LRR-PYD-containing protein), NAIP (Neuronal apoptosis inhibitor protein) and IPAF (ICE protease-activating factor) are cytoplasmic receptors, which recognize microbial products and/or other danger signals derived from the host [36]. These receptors either sense organisms that enter the cytoplasm or sense components that may be released or transported into the cytoplasm by processes such as phagocytosis and degradation of microbes [34]. Activation of NLRs by bacterial products can stimulate two major signalling pathways: the nuclear transcription factor (NF-κB) pathway initiated by Nod1 (expressed ubiquitously) and Nod2 (expressed by monocytes, macrophages, dendritic cells and

intestinal epithelial cells), or the activation of caspase-1 triggered by IPAF and Nalp3 through the formation of a multiprotein complex called inflammasome [37]. NF-κB is a key regulator of the pro-inflammatory response, activating genes that encode cytokines and costimulatory factors [38]. Caspase-1 leads to the secretion of inflammatory mediators IL-1β and IL-18 and it can also result in rapid host cell death termed pyroptosis [39-41]. Several studies reported NAIP5 [42] and IPAF-mediated intracellular recognition of *Salmonella* flagellin in a TLR5-independent manner [43-45]. The detection of flagellin by IPAF activates caspase-1 resulting in the secretion of IL-1β and IL-18 [44, 45].

Other families of different receptors are also important for the innate immune system. This includes the RIG-I-like receptor (RLR) family and the C-type lectin receptor (CLR) family.
The RLRs are cytosolic domains, such as RIG-1 and MDA-5, that play an important role in the detection of RNA species derived from viruses in the cytoplasm [46].
CLR family comprises a large family of receptors that binds carbohydrate structures in calcium-dependent manner but the family also includes proteins that do not bind calcium or have any carbohydrate specificity. The majority of CLRs mediate endocytosis and/or phagocytosis, play a role in antigen presentation and keep endogenous glycoprotein levels constant. Another small subset of CLRs gives rise to altered gene expression in response to various PAMPs. CLRs are expressed by most cell types including neutrophils, macrophages, dendritic cells, platelets and B cells [47]. One important group of CLRs is represented by DC-SIGN. The SIGN (specific ICAM-3-grabbing non-integrin) family of CLRs consists of DC-SIGN, L-SIGN and LSECtin in humans, and the murine homologues mDC-SIGN and mSIGNR1 to mSIGR4. For example mSIGNR1 is present on the splenic marginal zone macrophages and peritoneal macrophages [48]. SIGNR1 is able to capture pathogens from the blood, such as *Escherichia coli* and *Salmonella typhimurium* [49]. It is thought that pathogen degradation products from MZ macrophages, which do not express MHC class II molecules, are shed from the cell surface and are taken up by MZ B cells after opsonisation by complement [48]. Murine Dectin-1 is expressed on dendritic cells, monocytes, macrophages, neutrophils, and some splenic T cells. In humans, it is also found on mast cells, eosinophils, and B cells. Dectin-1 is restricted to recognize fungal components [50] and upon ligand recognition initiates the activation of NK-κB resulting in an increased expression of pro-inflammatory cytokines [50]. The mannose receptor found in macrophages and DCs recognizes some sugar molecules found on the surface of many bacteria and some viruses, including the human immunodeficiency virus (HIV) [47].

In response to a given pathogen, several PRRs can be sequentially or simultaneously activated on the same cell, and on different cell types. For example, TLR4 and TLR2 are

sequentially involved in macrophages responses to *Salmonella typhimurium*. [51]. PRRs are involved within a system, in which the cross-talk between or even within groups of PRRs is crucial in balancing immune responses through collaborative induction or negative feedback mechanisms [52]. For example, to control the over-activation or dysregulation of the TLR-signalling that can lead to severe diseases, many TLR signal-induced or TLR signal-associated negative regulators have evolved. For instance, SOCS1 (suppressor of cytokine signalling 1) is negative regulator of TLR4 through the inhibition of type I IFN signalling pathway following IFN-β upregulation by TLRs [53]. Also, activation of the same TLR in different cell types can lead to different functions in the immune response. One example is that TLR-activated DCs and B cells by LPS provides cytokine environments with opposite effects on T cell activation [54].

1.1.2 Cells from the innate immune system

Phagocytic cells, such as neutrophils, monocytes, macrophages, DCs, and NK cells are essential for the innate immune response. Neutrophils are the first cells recruited at the injured tissue where they contribute to the early defence by eliminating infected cells and by restricting growth of the microbe [55]. Resident tissue macrophages are also responsible for the secretion of neutrophil chemoattractants CXCL1 and CXCL2 [56, 57]. If the pathogen persists, more macrophages are recruited to the site of infection. Macrophages and neutrophils express receptors for antibody and complement which increases phagocytosis of antibody- or complement-coated antibodies [58, 59]. NK cells are able to defend the organism in the early phases of infection against intracellular pathogens or viruses. These cells are able to kill other cells upon activation by cytokines or upon encountering the target cells, such as pathogen infected cells and tumours. Dendritic cells are the last ones to be described since they have an extraordinary capacity to activate naive T cells and elicit the adaptive immunity. The cascade of cellular events taking place after an infection are described in more detailed in the following paragraphs.

1.1.2.1 Granulocytes – neutrophils

Granulocytes also called polymorphonuclear leucocytes (PMN) are a group of white blood cells characterized by the presence of granules in the cytoplasm. The neutrophils belong to this group and are considered the second major group of phagocytic cells, after macrophages.

Upon activation neutrophils leave the blood stream and move in the direction of the infection site as a result of chemotaxis. At sites of infection, neutrophils rapidly phagocytose pathogens, destroying them with antimicrobial proteins and proteases stored in the

cytoplasmic granules and with the production of high concentrations of superoxide. Neutrophils can also generate chemotactic signals to recruit monocytes and dendritic cells, such as chemerin that is responsible for the recruitment of immature DCs and plasmacytoid dendritic cells [60]. Neutrophils also produce TNF and other cytokines that differentiate and activate DCs and macrophages [61-63].

Neutrophils are short lived cells that undergo constitutive apoptosis and are specifically recognized and phagocytosed by macrophages. Neutrophil apoptosis and clearance is also very important for the cessation of the acute phase of inflammation. [64].

1.1.2.2 Macrophages

Macrophages are present in almost all tissues. They differentiate from monocytes that leave the circulation and then migrate into tissue in the steady state or in response to inflammation [65].

Tissue macrophages have a broad role in the maintenance of tissue homeostasis, through the clearance of senescent cells and the remodelling and restoration of tissues after inflammation [66, 67]. These cells show a high degree of heterogeneity according to their specific function in different organs, such as lungs, brain, in the submucosal layer of the gastrointestinal tract, spleen and liver. In the liver the resident macrophages are known as kupffer cells.

Inflammatory macrophages derive from monocytes that leave rapidly the bloodstream upon injury or infection. This population is subdivided into two other main populations: classically activated macrophages and alternatively activated macrophages [68].

Classically activated macrophages arise in response to TLR agonists, IFN-γ, which can be produced during innate immune response by natural killer (NK) T cells or during adaptive immune response by T helper 1 (T_H1) cells or $CD8^+$ T cells, and tumor necrosis factor (TNF), which is produced by several cells, such as neutrophils, antigen presenting cells, T_H1 T cells [68]. This population have microbicidal activity and secrete high levels of pro-inflammatory cytokines, such as TNF-α, interleukin 6 (IL-6), IL-1β, and IL-12 [68, 69]. In addition, this population produces reactive oxygen species and have higher expression of co-stimulatory molecules [68]. Classically activated macrophages are crucial in host defence against intracellular pathogens, for example, mice lacking this population are more susceptible to *Salmonella* typhimurium infection [70]. However, the activation of classically activated macrophages must be tightly controlled because cytokines as IL-1, IL-6 and IL-23 give rise to T_H17 cells, which can contribute to autoimmune responses [68].

Alternatively activated macrophages arise in response to IL-4, IL-10 and/or IL-13, cytokines that are generally produced in T_H2 responses, particularly in allergic, cellular and humoral

responses to parasites and extracelullar pathogens [68, 71]. This population promotes tissue repair and suppresses inflammation by the secretion of high levels of IL-10 [65, 68].

1.1.2.3 NK cells

Natural killer (NK) cells are large granular lymphocytes that can lyse infected cells with a variety of pathogens by releasing proteins into the target cell. Their activation is controlled by NK cell receptors that are encoded in the germline and do not undergo somatic recombination. They can respond within hours upon infection. The mechanism, by which NK cells are triggered, depends on a balance between two opposing signals: activating receptors that recognize target cell surface structures, including viral products, and give a ''kill'' signal, and inhibitory receptors that recognize MHC class I proteins present in all healthy cells, and may suppress NK cell responses. When target cells express ligands for both inhibitory and activating receptors the outcome is determined by the outline of the strength of signals [72]. NK cells can kill target cells through perforin and granzymes, which are proteins, pre-stored in cytoplasmic granules and can be exocytosed directly in the target cells. This killing mechanism is very similar to the $CD8^+$ cytotoxic T lymphocytes (CTL).

NK cells upon activation secrete inflammatory cytokines such as, IFN-γ and TNF-α and chemokines [72, 73], which have a direct role in the activation and function of other cells. For instance, NK cells facilitate the capacity of DCs to trigger adaptive T cell response [74]. DCs also secrete IL-12, IFN-α and IFN-β, which activates IFN-γ production by NK cells [74]. Moreover, the IFN-γ produced by NK cells is critical also for the activation of macrophages [75, 76]. NK cells enhance the response of $CD8^+$ T cells and $CD4^+$ T cells [77] and also IFN-γ producing NK cells promote a T helper type 1 (T_H1) cell polarization.

1.1.2.4 Dendritic Cells

Dendritic cells (DCs) are the most potent antigen presenting cell (APC) of the immune system, as illustrated by their ability to trigger activation of naïve $CD4^+$ and $CD8^+$ cells compared to other APCs such as macrophages and B cells [78-80]. Dendritic cells are present in lymphoid organs, in the epithelia of the skin and gastrointestinal and respiratory tracts, and in the interstitium of most parenchymal organs.

A large variety of DC subsets has been described. Mouse DCs have been classified into six subpopulations according to their phenotype and properties [81]. The main populations are characterized according to the T cell markers CD8 or/and CD4 [81]. There are also the plasmacytoid DCs (PDC), which also can express CD8 but their principal characteristic is the expression of B220 [81]. Upon viral activation these DCs are principal producers of type I

interferons (IFN-α/β) [82]. Additionally, there are also the dermal DCs and the langerhans cells, both present in the skin [81].

Dendritic cells recognize and ingest pathogens through PRRs that recognize features common to microbial surfaces, and they are very active in taking antigens by phagocytosis using receptors such as TLRs. The recognition of pathogens via TLRs activates DC, causing an increase of expression of class II molecules [83, 84], co-stimulatory molecules (CD80 and CD86) [85-87] and production of several cytokines such as IL-12 [88]. Remarkably, ligation of distinct TLRs can trigger differential cytokine production in a single DC type or result in different cytokines in distinct DC sub-types. Previous studies have shown that $CD8^-$ DCs mainly induce T_H2 cell responses, whereas $CD8^+$ DCs elicit strong T_H1 cell responses due to their high capacity to produce IL-12 [89, 90]. However certain characteristics of the microbe also play an important role in tuning the immune response. TLR4-dependent *E. Coli* LPS induces a T_H1 response [91], but LPS from the oral bacterium *Porphorymonas gingivalis*, which signals through TLR4 independent way [92], induces a T_H2 response. Consistent with this, *E. Coli* LPS, but not P. *gingivalis*, LPS induces IL-12 in splenic $CD8\alpha^+$ DCs [91]. Cytokines secreted by other cells can also modulate DC function. For example, T_H1-inducing DCs, when exposed to IL-10 or TGF-β, induce T_H2-like responses [93]. On the other hand, IFN-γ can induce DCs to acquire some T_H1-inducing capacity [93].
The DC subsets also comprises different repertoire of TLRs. For example, splenic $CD8^+$ DC do not express TLR7 mRNA and do not respond to the ligand R-848 in vitro [94]. Other study claims that $CD8^+$ DCs upregulate co-stimulatoty molecules in response to a different TLR7 ligand in vivo [95]. This subset also expresses lower level of TLR5 mRNA compare to other splenic DC subsets, but in contrast have the higher expression of TLR3 mRNA, which appears to be absent in PDCs [94].

1.2 The adaptive immune system

Adaptive immunity also called acquired immunity is characterized by two important properties: specificity and memory. B and T cells are able to recognize all pathogens specifically and to provide enhanced protection against reinfection.

1.2.1 Cells from the adaptive immune system

B and T cells are the main components of adaptive immunity and one of their principal properties is the specificity, which resides in the antigen receptors. During their development, every individual pro-B or pro-T cell acquires a unique antigen-specific receptor on its surface. The broad range of antigen specificities in the antigen receptor repertoire is due to variation in the amino-acid sequence at the antigen-binding site, which is made up from the variable (V) regions of the receptor proteins chains. In each chain the V region is linked to an invariant constant (C) region, which provides effector or signalling functions.
The variable (V) region is encoded by more than one gene segments. The first segment is termed a V gene segment because it encodes most of V domain. The second segment is termed diversity (D) gene segment. The third is termed joining (J) gene segment which is located close to the C region. Each gene segment has multiple different copies, and different combinations of gene segments can be used in different rearrangement events [96, 97]. This generates an enormous repertoire of antigen receptors with different specificities.

Upon activation, when the lymphocyte receptor recognizes the specific antigen this leads to clonal expansion of this specific lymphocyte. The antigen recognition molecules of B cells are immunoglobulins (Ig), which are membrane-bound receptor for antigen, the B cell receptor (BCR). Upon antigen recognition, B cells differentiate into cells producing antibody molecules of the same antigen specificity as the BCR.
T cells recognize antigen through cell-surface receptors called T cell receptor (TCR), which recognizes a peptide bound to a MHC molecule, which is displayed on the surface of APCs. T cells are subdivided into two distinct classes based on their TCR. The majority of T cells express receptors made up of α and β chains. A small group of T cells express receptors made up of γ and δ chains. Among the α/β T cells are two important subgroups: those that express the co-receptor molecule CD4 ($CD4^+$ T cells) and those that express CD8 ($CD8^+$ T cells). These cells differ fundamentally in how they recognize antigen but they mediate different types of regulatory and effector functions.

1.2.2 T lymphocytes

1.2.2.1 CD4 T cells

The $CD4^+$ T cells are important orchestrators of the immune system. Their functions depend on the cytokines that they secrete when activated and also on the cell surface molecules that are induced upon activation. The $CD4^+$ T cells can differentiate into various T helper subsets,

such as T helper 1 (T_H1), T helper 2 (T_H2) cells, T follicular helper (T_{FH}) and T helper 17 (T_H17) cells, distinguished by the arrays of cytokines they produce.
The T_H1 cells produce IL-2, IFN-γ and lymphotoxin (LT). These cytokines are strong inducers of cellular immune responses. They improve the microbicidal activity of monocytes and macrophages resulting in an increased efficiency in lysing microorganisms in intracellular vesicular compartments. In addition these cytokines induce the proliferation and activation of CD8 T cells. T_H1 also provide some B-cell help, but higher T_H1-cell numbers can inhibit B cell activation [98]. There is strong evidence that T_H1 T cells are required in bacterial infections. For instance, mice deficient in IFN-γ receptor are highly susceptible to attenuated *Salmonella typhimurium* infection [99] and CD4 T cell production of IFN-γ is required for protection against *Listeria monocytogenes* [100].

T_H2 cells produce IL-4, IL-5, IL-6, IL-10, TGF-β and IL-13 cytokines [101]. These cytokines are excellent promoters for antibody production and also enhance eosinophil proliferation and function. T_H2 cytokines are commonly associated with strong antibody and allergic responses [102].

Follicular helper T cells (T_{FH}) have a distinct phenotype and cytokine profile than other $CD4^+$ T cells. These cells are localized in B-cell areas of lymphoid organs and their main function is to provide specialized help to germinal centre (GC) B cells, which are involved in the generation of memory B cells or long-lived plasma cells (explained in detail in section 1.2.3). T_{FH} cells are distinguished from T_H1 and T_H2 by chemokine receptors, such as CXC-chemokine receptor 5 (CXCR5) and transcription factors. Upon activation of T_{FH} cells, the expression of CXCR5 is up-regulated and binds to CXC-chemokine ligand 13 (CXCL13), which promotes follicular homing by B and T cells [103]. Several features, not all unique to T_{FH} cells, help to characterize and define this subset. This includes CD40L, which is crucial for B cell proliferation, immunoglobulin class-switching, and GC B cell survival [104-106], ICOS and IL-10, which support GC B-cell growth and differentiation [104, 107], and IL-21, which also helps B cell activation [108, 109]. T_{FH} can also produce IL-4 upon infection. For instance, mice infected with a helminth have a strong IL-4 production by T_{FH} cells. Additionally, T_{FH}-cell derived IL-4 is required for the generation of high IgG1 affinity antibodies [110].

T_H17 T cells are a recently identified $CD4^+$ T cell subset that secretes not only IL-17, but also IL-22 and IL-21 [111] and exhibit effector functions distinct from T_H1 and T_H2 cells. T_H17 T cells have been implicated in the pathogenesis of many experimental autoimmune diseases and human inflammatory conditions [111]. However, the primary function of these cells may

be the clearance of extracellular pathogens during infections [111]. It seems that T_H17 T cells are required in infections by gram negative bacteria to induce an appropriate host immune response and for abscess formation [112]. They induce a strong neutrophil response and also control the migration of various other cell types, such as macrophages and probably also T cells. For example in mycobacterial infection, T_H17 cells trigger the induction of chemokines that attract T_H1 cells, which eventually control the infection [113]. T_H17 cells also play a role in protection against *Salmonella*. For instance, IL-17 deficient mice have a mild effect in bacterial clearance during *Salmonella* infection [114].

Several factors are affecting the differentiation of T helper cells, for example, IL-12 production by DCs and macrophages induces T_H1 differentiation, IFN-γ produced by NK cells and CD8 T cells also induce T_H1 differentiation, whereas IL-4 stimulates differentiation of T_H2 cells, which may be contributed by mast cells, and basophils [115]. IL-6 production by activated DCs promotes T_H17 differentiation [116].

The characteristic cytokine products of T_H1 and T_H2 are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype. Thus, IFN-γ producing T_H1 cells are able to block the growth of T_H2 cells [117]. The IL-10 acts on macrophages to inhibit T_H1 activation and TGF-β inhibits the growth of T_H1 cells [118, 119]. If a subset is activated first or preferentially in a response, it can suppress the development of the other subset [119]. In general, infection are associated with dominance of either a T_H2 or a T_H1 response [102] The cross-inhibition may partly explain the strong biases towards T_H1 or T_H2 responses during many infections in mice and humans [102].

1.2.2.2 CD8 T cells

$CD8^+$ T cells or cytotoxic T cells (CTL) are important for the host defense against intracellular pathogens as viruses and bacteria like *Listeria* and *Salmonella* [120, 121].
Naive $CD8^+$ T cells differentiate into cytotoxic T cells after recognition of antigen peptide-MHC class I complexes on the surface of dentritic cells, which express high levels of co-stimulatory molecules, or they recognize the antigen in weakly co-stimulatory cells requiring the presence of CD4 T cells bound to the same APC [122]. Both types of activation induce the autocrine IL-2 production by $CD8^+$ T cells leading to their proliferation [123, 124].

Cytotoxic T cells have several ways of destroying infected cells. One is through the delivery of cytotoxins (perforins, granzymes and granulysins), contained in specialized lysosomes called lytic granules that are released and lyse the infected cells upon antigen recognition on the surface of the infected cell [125]. Another way is through the interaction of Fas (CD95)

with Fas-ligand on the surface of the cytotoxic cells leading to the apoptosis of the infected cell [125]. In addition cytotoxic CD8 T cells also secrete cytokines such as IFN-γ, TNF-α and TNF-β, which contribute to the defense. IFN-γ inhibits viral replication, increases expression of MHC class I, and activates macrophages. TNF-α and TNF-β can synergize with IFN-γ in macrophage activation and in killing some target cells through their interaction with TNFR-I.

1.2.2.3 NKT cells

Natural Killer T (NKT) cells are T lymphocytes that express both αβ T cell receptors (TCRs) and NK cell receptors [126, 127]. NKT cells can respond very fast to their cognate antigens, which is a characteristic of innate immunity,-and they can also promote T_H1 and T_H2 adaptive responses [128]. In mice, most NKT cells express an invariant chain Vα14Jα18 TCR with predominant coexpression of Vβ8.2, Vβ2 and Vβ7 [129]. Human NKT cells express a Vα24Jα18 rearranged TCR α chain usually coexpressed with Vβ11. NKT cells recognize an MHC class IB molecule, CD1, which presents endogenous or bacterial lipid antigens.

The most precise description of NKT cells is their expression of an invariant TCRα antigen receptor and their reactivity to αGalCer, which is an unusual α-glycosylatedsphingolipid initially derived from marine sponge. NKT cells upon activation release large amounts of T_H1 cytokines such as IFN-γ and TNF but also T_H2 cytokines like IL-4, IL-10 and IL-13 [130-132]. Deficits in NKT cells are involved with autoimmune diseases, such as diabetes, indicating that these cells are also important for suppressing autoimmunity and graft rejection. NKT Cells also facilitate immunity against infections and tumors. For instance, NKT cells participate in protection of mice from a variety of bacterial, viral, and protozoan parasites [133].

1.2.2.4 Regulatory T cells

Regulatory T cells (Tregs) are an important subset of T cells that mediate peripheral tolerance. These cells are classified in two subsets: one is called natural regulatory T cells (nTregs), which develop in the thymus and the other is called inducible regulatory T cells (iTregs), which derive from naive T cells in the periphery. The natural regulatory T cells are $CD4^+$ T cells characterized by high expression of CD25 and the transcriptional factor Foxp3 (forkhead box P3). In humans not all the Foxp3-expressing cells are Tregs. The nTregs utilize the cytokines IL-10, IL-35 and TGF-β to exert their suppressive effects upon conventional T cells. Beside the secretion of inhibitory cytokines, nTregs use other suppression mechanisms, such as cytolysis through granzyme A or B for humans or mice respectively, perforin, which kills the target cell, metabolic disruption, in which Tregs provoke death of effector T cells by cytokine deprivation, and modulation of APC function [134-137].

The iTregs are originally coming from $CD4^+$ $CD25^-$ or $CD8^+$ $CD25^-$ T cells. The iTregs population includes distinct subtypes of $CD4^+$ T cell: T regulatory 1 (Tr1), which secrete high levels of IL-10, TGF-β, no IL-4 and no or low levels of IFN-γ [138]; and T helper 3 (T_H3) cells, which secretes high levels of TGF-β. Although $CD8^+$ T cells are associated with cytotoxic T-lymphocyte function and IFN-γ production, a subtype of these cells can secrete IL-10 and TGF-β [139, 140] and have been called $CD8^+$ regulatory T cells.

Regulatory T cells are involved in the control of various immunological processes, including protective immune responses in autoimmunity, for example nTregs are functionally impaired or have deficits in their maturation or in their thymic emigration in multiple sclerosis (MS) [141-144], iTregs and n Tregs are involved in the prevention of type 1 diabetes (T1D) [145, 146], iTregs are also implicated in protection against inflammatory bowel disease (IBD) [147]. The nTregs play also a significant role in the immune response to infections. For instance, they control an excess of effector immune responses against a pathogen preventing immunopathology [148], for example, during infection of mice with *Candida albicans*, a reduction in nTregs numbers induces better control of the infection but on the other hand enhances inflammatory gastrointestinal pathology [149]. There are also some pathogens that favor nTregs induction, promoting their survival inside of the host [148].

1.2.2.5 Memory T cells

Memory T cells can develop directly from effector precursors after the primary response or are can be generated in parallel with effector populations continuing on to their predetermined fate after the primary immune response [150, 151].

Memory T cells can generate a rapid recall response to secondary pathogen challenge [152-154]. CD4 and CD8 memory T cells derived from human blood and mouse spleen can be distinguished based on the expression of the lymph node homing receptors CD62L and CCR7.

There are two kinds of memory T cells, the effector memory T cells (T_{EM}), which are $CD62L^{low}$ and $CCR7^-$ and the central memory T cells (T_{CM}), which are $CD62L^{high}$ and $CCR7^+$. The T_{EM} cells are localized in the peripheral tissues and display immediate effector function, whereas central memory T cells home to the T cell areas of secondary lymphoid organs, have little or no effector function, but readily proliferate and differentiate to effector cells in response to antigenic stimulation. Both subsets produce large amounts of effector cytokines such as IFN-γ and TNF-α after antigen stimulation, central memory T cells produce predominantly IL-2 which could increase their ability to proliferate in response to antigen [155]. T_{EM} cells have more lytic activity *ex vivo*, probably because of increase expression of perforin [156]. Furthermore a small fraction of these cells express the low-affinity Fc receptor

for IgG IIIa (FcγRIIIa), which allows them to directly mediate antibody-dependent cell mediated cytotoxicity [157].
Some studies have shown that memory $CD8^+$ T cells after their generation in secondary lymphoid organs, preferentially locate to BM for homeostatic maintenance [158] and recently some memory $CD4^+$ T cells have been shown to reside also in the BM [159].

1.2.3 B lymphocytes

There are two main groups of B cells, the B-1 cells, which develop from fetal liver-derived HSCs and B-2 cells, which derive from bone marrow HSCs.
Most of all studies done with B-1 cells are based on rodent studies, in humans this population is still not clear [160]. B-1 cells reside mainly in peritoneal and pleural cavities and are subdivided in B-1a and B-1b B cells. B-1a cells are characterized by the $CD5^+$ $CD11b^+$ $B220^{low}$ IgM^{high} IgD^{low} phenotype, while the B-1b B cells do not express the CD5 [161]. B-1a cells are the source of natural antibodies, which are predominantly of IgM class and provide protection during the early stages of infection, whereas induced antibodies derived from B-1b cells are produced subsequently and are key to the clearance of, and long term protection, from pathogens [162, 163].

B-2 cell population is heterogeneous, consisting of follicular B cells (FO), in LN and splenic lymphoid follicles, and a subset residing in the marginal zone (MZ) of the spleen [164]. MZ and FO B cells are distinguished by differential expression of several cell surface markers: MZ B cells are $IgD^{low}CD21^{high}CD23^{low/-}$, whereas FO B cells are $IgD^{high}CD21^{inter}CD23^{high}$ [165, 166]. Marginal zone B cells are located at the margin between the white pulp and red pulp. In rodents, MZ B cells primarily appear to mediate T-independent responses to antigens from blood-borne pathogens. During these pathogen-driven responses, LPS and possibly other microbial products may drive the migration of activated B cells out of the MZ into the red pulp where they may differentiate into short-lived plasma cells secreting mainly IgM and IgG3 antibody [164, 167].

The follicular B cells can occupy different niches. Once they mature, they recirculate in the spleen and lymph nodes (or in the BM). Naive follicular B cells reside in the ''follicular niche'' and may present T-dependent antigens to activated T cells. Differentiation of activated follicular B cells into antibody-secreting cells is enhanced by TLR activation on follicular B cells, but only prior to BCR and CD40 activation. It has been previously proposed that for an effective activation of lymphocyte it is necessary a second signal in addition to the specific antigen recognition by the BCR or TCR. [168]. Ruprecht and Lanzavecchia [169]

demonstrated that CpG, provides a strong signal that in synergy with cognate T-cell help and BCR cross linking helps to sustain optimal proliferation and differentiation of human B cells. Furthermore, it has been demonstrated by Fillatreau et al. that for experimental autoimmune encephalomyelitis (EAE), which is an animal model for Multiple Sclerosis (autoimmune disease that affects the central nervous system (CNS)) CD40 participates in the production of IL-10 by B cells which stimulates disease resolution [170].
The binding of CD40 by CD40L also induces the expression of co-stimulatory molecules such as B7-family molecules (CD80/CD86) [171]. In addition, cytokine secretion by T cells, such as IL-4, IL-5 and IL-6 stimulate B cells to proliferate, to undergo isotype switching and differentiate into antibody-secreting plasma cells.

B cells, which have recognized antigen trough BCR and received T cell help can also form germinal centres. Germinal centres are specialized areas in the follicle where B cells proliferate rapidly and undergo a process of somatic mutation so that a single initial B cell can give rise to variant progeny [172, 173]. The germinal centre B cells that have high affinity BCRs for the antigen are selected and differentiate into memory or into plasma cells secreting antibody of high affinity [173-177]. MZ B cells also participate in T-dependent (TD) immune responses to protein antigen as well as in responses to lipid antigens. For instance, Song and colleagues have demonstrated that MZ B cells upon stimulation with TD antigen can differentiate very rapidly into antibody forming cells (AFC), which have a distinct clonotypic repertoire and also they can give rise to GCs with somatic hypermutation and generate immunological memory [178].

The first antibodies to be produced in humoral immune response are always IgM, because IgM can be expressed without isotype switching. In addition to IgM, there are three antibody isotypes, such as IgG, IgA and IgE. Among these, IgG are by far the most abundant immunoglobulin and contain several subclasses. In mouse there are IgG1, IgG2b, IgG2c and IgG3. In humans there are IgG1, IgG2, IgG3 and IgG4.
It is known that in mice cytokines can induce isotype switching. For example, IFN-γ secretion by CD4 T cells induce the isotype switching to IgG2c or to IgG3 and the IL-4 switches to IgG1 or IgE.

The antibodies play different roles in the immune defence. Apart of direct neutralization of pathogens, antibodies can also activate complement and opsonise microorganisms by phagocytic cells or killing by NK cells.

1.2.3.1 Memory B cells and long lived plasma cells

B cell memory develops in GC reactions after primary immune response to thymus-dependent (TD) antigens [179-182]. Populations of affinity-matured memory B cells persist for long periods of time in multiple cellular forms [183-185]. These post-germinal center B cells can be categorized into long-lived plasma cells, which will not respond to antigen recall and the precursors for the memory response [181, 186, 187].

Memory B cells do not secrete antibodies until expansion and differentiation into plasma cells after rechallenge with antigen. These memory B cells contain high affinity BCRs, enabling them to respond *in vivo to* little amounts of antigen. These cells recirculate in the periphery, where they can encounter easily the antigen leading to an efficient secondary immune response [188]. Many of them are localized in the marginal zone of the spleen [189, 190]

Long-lived plasma cells are terminally differentiated, affinity-matured and home to the bone-marrow [191, 192]. Although there is evidence that some long-lived plasma cells are present in the spleen [193]. It has been shown in vitro that the survival of these cells is promoted by IL-6, CXCL12, and ligands for CD44 [194, 195]. The continued production of antibodies by the long-lived plasma cells is crucial for immunity, because such antibodies provide immediate protection without the requirement for clonal expansion and differentiation of B cells [196].

1.2.3.2 Regulatory functions of B cells

B cells positively regulate immune responses through antibody production and $CD4^+$ T cell activation. However, B cells can also negatively regulate immune responses in mouse autoimmunity and inflammation models through the production of auto-antibodies. For instance, it was shown that transfer of serum from patients suffering from idiopathic thrombocytopenia purpura (ITP), an autoimmune disease associated with low platelet counts and mucocutaneous bleedings, could transfer disease to healthy patients [197]; in hyperthyroidism, the increased production of thyroid hormone is sustained through the stimulation of thyroid-stimulating hormone receptor by auto-antibodies [198].

Despite the role of pathogenic auto-antibodies, recent studies indicate that B cells can have a regulatory function and suppress autoimmune diseases [170, 199, 200]. The first observation was provided by Wolf and Janeway who observed that mice lacking B cells fail to control EAE, while wild-type mice recover after a short episode of paralysis [201].

A characteristic feature of B cell-induced regulation is the involvement of IL-10 in the regulatory process. B cells are able to produce large amounts of IL-6 and IL-10 that play instrumental roles in T cell mediated immunity [54]. It has been shown that IL-10 deficient mice suffer a chronic EAE, a more severe CIA and also develop a spontaneous inflammatory

bowel disease (IBD) [170, 199, 202]. In fact, it has been revealed that B cells regulate these autoimmune diseases through the provision of IL-10 [170, 199, 203]. Thus, mice in which only B cells lack IL-10 develop a chronic EAE while mice with wild type B cells recover after a short time of paralysis [170]. In addition, B cells isolated from recovered mice secrete IL-10 upon *in vitro* stimulation and transfer of these cells confers protection to recipient mice [170]. The importance of B cell-derived IL-10 in the regulation of autoimmune diseases has been extended in numerous studies. IL-10 production by B cells are involved in the suppression of T_H1 and T_H17 mediated pathologies such as EAE [204] and T1D [205]. They are also involved in the suppression of diseases driven by T_H1 cells and/or auto-antibodies such as CIA [199, 206], SLE [207] and act as regulators in T_H2 mediated diseases such as UC [202, 203]. The regulatory role of B cells is not limited to autoimmune situations. For instance, infection with *Schistosoma mansoni* induces IL-10 production by B cells, which can suppress anaphylaxis [208]. Similarly, some viruses have built up mechanisms to stimulate IL-10 producing B cells and therefore subvert the immune system [209, 210].

The mechanism how IL-10 production by B cells regulates the immune response is not completely clear, it has been shown to strongly inhibit macrophages and dendritic cells [211]. IL-10 from B cells also inhibits the capacity of activated CpG-dendritic cells to stimulate T cell proliferation *in vitro*. The production of IL-6 and IL-12 by DCs is also repressed by IL-10 from B cells, which then inhibits differentiation of T_H17 and T_H1 cells respectively [54]. IL-10 production by B cells elicited by infection with *Brugia pahangi* attenuates their efficiency as APCs to $CD4^+$ T cells restricting their expansion [212].

1.2.3.3 TLR-activated B cells

The signalling through B-cell antigen receptor (BCR) is very important in shaping the development and functions of peripheral follicular B cells; however it has also been described that, innate TLRs, which recognize specific microbial products, also help to sculpt humoral immune responses. For instance, MyD88 signalling in B cells can amplify several aspects of the humoral response, including antibody production, germinal centre formation, and accumulation of long-lived plasma cells in the bone marrow [213-216]. Beside the fact that B cells respond to TLR stimulation by proliferating and secreting antibodies, they are also able to secrete cytokines [54, 217]. Previously it has been demonstrated that LPS or CpG are potent inducers of IL-10 production by naive B cells [54]. Mice with B cell deficiencies in MyD88 or both TLR2 and TLR4 do not recover from EAE [54]. In contrary mice with TLR9 deficient B cells are able to recover [54]. In EAE, components from *Mycobacterium tuberculosis* are present in the complete freund's adjuvant used to induce disease. This suggests that *Mycobacterium tuberculosis* provides the TLR agonists that induce the

regulatory function in B cells. It seems that only particular TLR agonists can trigger a regulatory function in B cells. This can be explained by the fact that B cells and cells from the innate immune system, such as DCs or macrophages use TLRs in different ways to sense their environment. Additionally, MyD88 signalling in B cells controls disease resolution while MyD88 signalling in other cells is required for disease induction [218]. For example, B cells activated via TLR can secrete a milieu of cytokines that inhibits T cell activation in an IL-10 dependent manner, whereas activation of DCs in the same way produced a milieu that contained very little IL-10 and was able to enhance T cell proliferation [54]. These observations suggest that in EAE model MyD88-signalling in distinct cell types can modulate immunity in opposite ways.

1.3 *Salmonella enterica* infection

Salmonella enterica belong to the family of *enterobacteriaceae* and are facultative intracellular gram-negative bacteria that infect humans and animals causing a spectrum of disease ranging from systemic infection to gastroenteritis, depending on the bacterial serovar and the host species infected. More than 2400 serotypes have been described and all of them are considered potentially pathogenic. Some serotypes are host specific but the majority can affect different hosts. The *Salmonella* serotypes are classified according to the Kauffman-White classification scheme. The identification is based on the ‘‘O’’ antigens, which consist of the lipopolysaccharide-protein chains exposed on the cell surface, and ‘‘H’’ antigens, which consist on the flagellar antigens. Typhoid fever is a systemic disease caused by *Salmonella enterica, serovar typhi,* a highly invasive enteric pathogen that infects only humans. *Salmonella enteric serovar typhimurium* (Figure 3) referred usually as *S. typhimurium* infects animals and humans. In particular, *S. typhimurium* causes gastroenteritis and occasionally septicemia in humans, and systemic disease in susceptible mouse strains that is similar to the human typhoid fever. This model is widely accepted as the best experimental system for studying human typhoid fever and has proved extremely valuable in uncovering mechanisms of innate and adaptive immune resistance to intracellular pathogens.

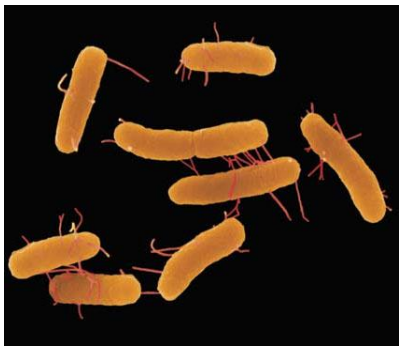

Figure 3: *S. typhimurium*. This figure was adapted from www.britannica.com/EBchecked/topic-art/478531/121515

1.3.1 The mouse typhoid model

Salmonella is a food- and water-born pathogen. Therefore, after oral ingestion and colonization of the small intestine, *S. typhimurium* penetrates the intestinal epithelium and enters the Peyer's patches through the M cells or through the enterocytes in the microvilli [219, 220].

After bacteria have penetrated the mucosal barrier, the bacteria moves in the tissues and many reach the mesenteric lymph nodes. At this stage antibodies, complement and tissue macrophages exert their protective function, resulting in a reduction of the numbers of bacteria. *Salmonella* move with the efferent lymph to the circulation, and are carried by it to all parts of the body which results in a transient bacteremia [221]. Bacteria are rapidly removed from the blood by phagocytic cells in the spleen and liver. During this stage a big part of the *Salmonella* are killed [219]. The *Salmonella* that survived this stage starts to replicate intracellularly in the spleen and liver. This process can take several days, during which nonspecific host defence mechanisms such as IFN-γ and TNF-α modify the rate of bacterial growth. Depending on this rate of bacterial multiplication and the initial numbers of *Salmonella* in the liver and spleen, the infection can either progress to an overwhelming disease or be restricted. If the bacteria reach a number of 10^8 in the organs the host seems unable to control the infection [219]. As a consequence, secondary bacteremia, in which bacteria invades hepatocytes, these are lysed and release large numbers of bacteria leading to an endotoxic shock and soon death [219]. If the growth rate of the bacteria is slower or the inoculum was smaller, the bacterial multiplication can be suppressed with the recruitment of more defences. This phase is called plateau phase and is characterized by splenomegaly, recruitment of bone marrow derived cells and the action of IL-12. The maintenance of bacterial numbers and formation of granulomas require sustained production of TNF [221], without which bacterial numbers cannot be suppressed. The plateau level of bacteria can last from one to several weeks, depending on the mouse strain and the strain of *S. tytphimurium* used [222]. The final stage of infection is characterized by the generation of an acquired immune response able of eliminating *S. typhimurium* and long-lasting immunity against reinfection. The Figure 4 illustrates the several phases of the immune response against *Salmonella spp.* in the mouse typhoid model.

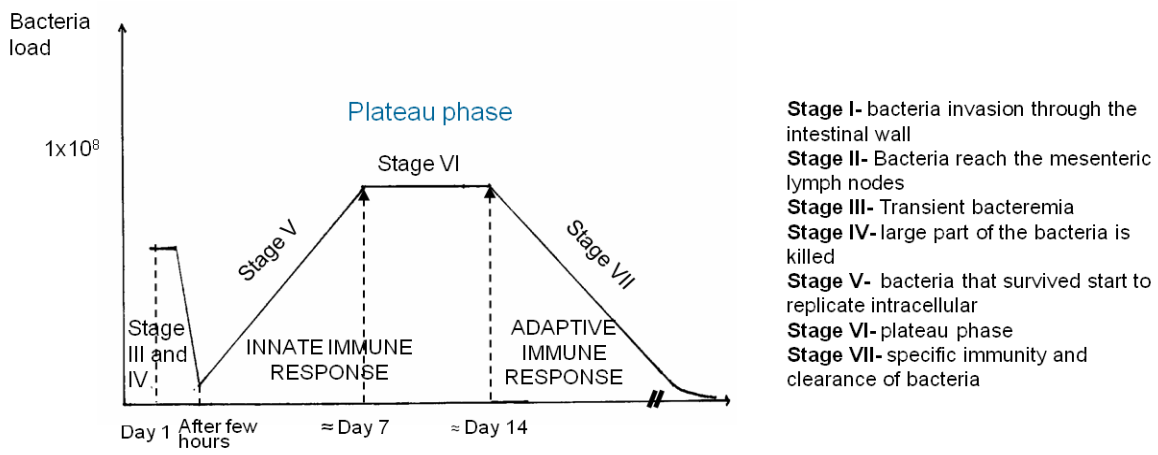

Figure 4: Representation of the mouse typhoid model. The scheme starts with stage III, which represents the transient bacteremia. This scheme just represents an approximation of the reality, the scale and all the time points are estimated.

Mouse resistance or susceptibility to *Salmonella* infection is controlled by the Natural resistance associated macrophage protein (*Nramp 1*). Nramp 1 is a transmembrane protein with divalent cation transporter functions localized to the endosomal/lysosomal compartment of the macrophage [223] and dendritic cells [224, 225]. Susceptible mouse strains usually have a mutation in the Nramp 1 gene (Nramp-1^s) and are not able to synthesize a functional Nramp 1 protein. These mice after infection with virulent *Salmonella* show a high susceptibility due to the uncontrolled replication of the bacteria and usually die within 6 days after infection while resistant mice, which have a functional Nramp 1 protein, can control the infection. The infection with virulent *Salmonella* of Nramp-1^s mice, such as C57BL/6 mice, provides only the possibility to investigate the *Salmonella* virulent factors and the host immune response in the early time points. In order to investigate the adaptive immune response against *S. typhimurium*, the mice have to express a functional Nramp 1 protein (Nramp-1^r) or in case of susceptible mice the infection has to be done with attenuated *Salmonella*. The attenuated *Salmonella* strain AroA$^-$ shows a mutation in genes which encode the pathway of aromatic biosynthesis [226]. Thus, it makes the pathogen dependent on supply of aromatic substances from the host. Therefore the *Salmonella* has a limited ability to replicate. Nramp-1^s infected mice with this strain of *Salmonella* enable the mice to survive and clear the infection. Thus it allows monitoring the adaptive immune response in these mice.

1.3.2 The immune response to *Salmonella*

The *S. typhimurium* infection involves several mechanisms in the innate and adaptive immune response that differ in their importance during distinct infection stages. The initial

stages of infection are characterized by an innate immune response triggered by host recognition of several microbial structures including PAMPs such as flagellin, lipopolysaccaride (LPS), and lipoproteins [227, 228]. Interaction of these microbial structures with specific host receptors ultimately leads to stimulation of innate immune functions of epithelia and phagocytes. Macrophages phagocytize *S. typhimurium*, and this process is facilitated by receptor-mediated uptake after opsonization of *Salmonella* with antibodies and complement [229]. Bacterial uptake is not a process done only by phagocytic cells. *Salmonella typhimurium* has the ability to invade host cells via the expression of a type-three secretion system (TTSS) encoded within the *Salmonella* pathogenicity island I (SPI-1) that injects virulence factors such as SipA and SopE into target cells and drives cytoskeleton rearrangements and phagocytosis, this promotes intracellular survival of the pathogen, or causes apoptosis of macrophages [230].

During the initial stages of infection TNF-α, IFN-γ, IL-12 and IL-18 are important for the host survival [231, 232]. Monocytes are recruited and they are the major producers of TNF-α and iNOS [233], the later being crucial for producing reactive nitrogen species important in *Salmonella* killing [234, 235]. Macrophages and dendritic cells produce IL-12 and IL-18 these cytokines are important to induce the expression of IFN-γ produced by NK cells, macrophages and B cells. The IFN-γ production will also induce the expression of IL-12 through a feedback loop [236, 237]. The recruitment and activation of phagocytes by chemokines and cytokines respectively characterizes the initial stages of *Salmonella* infection. As a consequence, large numbers of bacteria are eliminated and *Salmonella* infection is controlled until a certain degree. Then, only the response of antigen specific cells from the adaptive immunity can allow an effective eradication of bacteria and provide protection against a secondary infection with *S. typhimurium.*

The $CD4^+$ T cells have been shown to be crucial for the resolution of *S. typhimurium* infection [99, 219, 222, 238, 239]. *Salmonella typhimurium* induces a strong T-helper 1 (T_H1) response, characterized by the production of large amounts of IFN-γ [240-242] and TNF-α [238, 243-246]. These cytokines activate bactericidal mechanisms in macrophages and clearly improve the capacity of these cells to kill *S. typhimurium*. Additional functions of $CD4^+$ T cells is the regulation of antigen-specific B cell activation and maturation, either by direct cell-cell contact or by cytokine production. T cell help is essential for B cell differentiation such as isotype switch and affinity maturation. $CD4^+$ T cells also help in the generation of *Salmonella*-specific $CD8^+$ T cells and organization of granuloma formation to control bacterial spreading [219]. The mechanisms by which $CD8^+$ T cells control *S. typhimurium* infection are less well understood. $CD8^+$ T cells are able to lyse infected cells and release bacteria from their protective environment. The bacteria will be then accessible to activated phagocytes or

bactericidal molecules such as granulysin [247]. Similar to $CD4^+$ T cells, $CD8^+$ T cells are also IFN-γ producers and are able of phagocyte activation [222].

Salmonella infection induces a profound antibody response against multiple antigens, including the outer membrane proteins (OMP), flagellin and LPS [227, 228]. Antibodies against flagellin and LPS are largely T-independent, while the response against OMP is T cell dependent [228]. B cell-mediated immune reaction starts with an early antibody response, mostly of IgM, IgG3 and IgA isotypes, with the later one found principally in the intestinal lumen. The initial antibody production is usually provided by short-lived plasma cells. The systemic production of antibodies can drive the activation of complement and also opsonisation, thereby enhancing bacteria endocytosis. B cells later provide long-lasting humoral protection following the accumulation of antigen-specific long-lived plasma cells in the bone marrow. This anti-*S. typhimurium* humoral response is protective since transfer of serum from infected mice can protect recipient naïve mice from an otherwise lethal *S. typhimurium* challenge [248, 249].

2 Aims of this thesis

B cells are known to mediate suppression in autoimmune diseases through inhibition of inflammatory T cell responses of T_H1 and T_H17 [170, 199]. This suppression of autoimmune responses can be mimicked in vitro using supernatants from B cells cultivated with LPS from gram negative bacteria. LPS activates B cells via TLR4 and MyD88 to secrete IL-10, which inhibits the DCs to secrete immune stimulatory cytokines, such as IL-6, IL-12 and TNF-α [198]. IL-10 from B cells also inhibits the capacity of DCs to activate T cells for their proliferation and for their production of inflammatory cytokines [54]. These data show that B cell-mediated suppression depends on the provision of microbial products that can activate signalling via TLR4 and MyD88. Thus, microbial products drive the suppressive function of B cells. The present thesis investigated whether MyD88-signalling in B cells affects the immune response to an infection by *Salmonella typhimurium*.

The specific aims were to:

I. Assess the production of IL-10 by B cells upon *Salmonella typhimurium* stimulation in vitro
II. Which receptors are involved in B cell activation by *Salmonella typhimurium*
III. Effects of S. typhimurium on cytokine production by other APCs, such as dendritic cells and macrophages
IV. Investigate the role of IL-10 production by B cells in T cell response upon *Salmonella* infection in vivo.
V. Determine the role of cell-autonomous MyD88-triggering in B cells for B cell activation, germinal center formation, plasma cell differentiation, antibody production, and generation of long-lived plasma cells during *Salmonella* infection
VI. Characterize the contribution of MyD88-triggering in B cells to innate immune responses during *Salmonella* infection
VII. Analyse the influence of MyD88-triggering in B cells on $CD4^+$ T cell and $CD8^+$ T cell responses.
VIII. Analyse the role of MyD88 expressed in other cells than B cells in innate immune response and in adaptive immune response against S. typhimurium infection.
IX. Investigate the role of MyD88-triggering in B cells upon infection with virulent *Salmonella*
X. Investigate the protective role of MyD88 in B cells in mice vaccinated with attenuated *Salmonella* and then infected with virulent *Salmonella*.

3 Material

Addresses and contact information for suppliers are in section 9.

3.1 Equipment and material

Microscope slides, Super Frost plus 25 × 75 × 1.0 mm	Menzel Gläser
Cover slips, 24×60 mm	Menzel Gläser
Tissue-Tek® Cryomold intermediate	Sakura Finetek Europe B.V.
Centrifuge Heraeus, Biofuge fresco WCP	Heraeus
Centrifuge Heraeus, Multifuge 3 L-R	Heraeus
Microtome, Microm-HM 500 OM	Carl Zeiss
Microscope Zeiss 7082	Carl Zeiss
Microscope Leica DM IRE2	Leica
Irradiator Gammacell 40 Exactor	MDS Nordion
Real-Time PCR system Mx3000P	Stratagene
FACScalibur	Becton Dickinson
MACS- Magnetic cell sorting	Miltenyi Biotech GmbH
ELISA- Reader Spectra Max 250	Molecular Devices GmbH
Neubauer improved counting chamber	Carl Roth
96-well micro titre plates (u-, flat-bottomed)	Greiner bio-one
96-well micro titre plates (v-bottomed)	Corning , Costar
96-well micro titre plates (high binding EIA/RIA plates)	Corning , Costar

96-well PCR plate	Biozym Scientific GmbH
Safe seal tips, 10 µl, 20 µl, 200 µl and 1000 µl	Biozym Scientific GmbH
Safe lock tubes, 0.5 µl	Eppendorf
Thermo-Tube, 0.2 µl (DNase; RNase free)	Rapidozym
15ml and 50ml conical polypropylene tubes	Greiner bio-one
Pre-separation filters	Miltenyi Biotec
Liquid sample bags, Whirl-Pak®	Carl Roth

3.1.1 Reagents

Bovines Serumalbumin (BSA)	PAA Laboratories
Diethanolamin	Merck KgaA
Ethanol	Carl Roth
EDTA	Carl Roth
Tween 20	Carl Roth
Acetone	Merck KgaA
Difco™ MacConkey Agar	BD Pharmingen
Baytril 2.5%	Bayer
Tissue-Tek® O.C.T. Compound	Sakura
Dako Pen	Dako
Fluoromount G	Southern Biotech
Percoll	GE Healthcare
Tryptan blue (0,4%)	Sigma-Aldrich Chemie GmbH
Propidiumiodid (PI)	Sigma-Aldrich
Extravidin-Alkaline phosphatase	Sigma-Aldrich

para-nitrophenylphosphate (PNPP)	Sigma-Aldrich
BD Cytofix/Cytoperm Plus Kit	BD Bioscience
Tri-Reagent	Sigma- Aldrich
Chloroform	Merck
Isopropanol	Roth
Glycogen	Roche
Nuclease free water	Qiagen
DNA Free Kit	Applied Biosystems
Collagenase (from *Clostridium histolyticum*)	Sigma-Aldrich
Deoxyribonuclease I (from bovine pancreas)	Sigma-Aldrich

3.2 Buffers and solutions

Solutions were made up in H_2O prepared with Millipore water purifier, unless stated otherwise. The solutions were sterilized by autoclaving for 25 min at 121°C, or filter-sterilized through a 0.2 µm membrane.

3.2.1 Buffers / Solutions and media

Description	Composition
PBS-Buffer	130 mM NaCl, 10 mM sodium - phosphate buffer (100 mM Na_2HPO_4, 20 mM NaH_2PO_4), pH 7.4
MACS-Buffer	PBS-Buffer with 0.5% (w/v) BSA, pH 8.0
FACS-Buffer (PBA)	PBS-Buffer with 0.5% (w/v) Bovine Serum albumin (BSA) and 0.1% (w/v) NaN_3
PBS/3% BSA	PBS-buffer with 3% (w/v) BSA
PBS/1% BSA	PBS-buffer with 1% (w/v) BSA
PBS/0.05% Tween	PBS-buffer with 0.05% Tween20 (Roth)
PBS/0.1% Tween	PBS-buffer with 0.1% Tween20 (Roth)

Description	Composition
Carbonate Buffer	0.05 M Na_2CO_3, pH 9
ELISA-Buffer	500 ml buffer contain 24.5mg $MgCl_2.H_2O$ and 48ml diethanolamine, pH 9.8
Erythrocytes Lysis Buffer	0.83% NH_4Cl (w/v), pH 7.3
Serum-free medium	RPMI 1640 + GlutaMax-I (Invitrogen, Gibco)
Cell-culture medium	RPMI 1640 + GlutaMax-I (Invitrogen, Gibco), 50 µM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% Fetal calf serum (FCS), 1mM sodium pyruvate D-MEM, 50 µM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% Fetal calf serum (FCS), 1mM sodium pyruvate
Luria Bertani (LB) Medium	1% (w/v) Bacto-Tryptone, 0.5% (w/v) yeast extract, 85.5 mM NaCl, pH 7.5

3.3 Reagents for cell stimulation

Heat-killed *Salmonella* (HKS) from Max PIanck Institute provided by Ph.D. Sabine Seibert. TLR agonist Lipopolysaccharide (LPS) from *E. coli* O55:B5 purchased from Sigma-Aldrich were used to stimulate cells *in vitro*.

3.4 Antibodies

3.4.1 Antibodies for flow cytometry

The monoclonal antibodies (mAb) used for flow cytometry are in the table below. The mAb were conjugated with the fluorescent dyes FITC, PE, PerCP and Cy5 or APC. Biotinylated antibodies were detected in a second step using streptavidin (SA) coupled to FITC, PE, APC or PerCP. The mAb marked with an asterisk (*) were used as isotype controls.

Specificity	Clone	Source
B220 (CD45R)	RA3.6B2	DRFZ/ BD Pharmingen
CD1d	CD1.1, Ly-38; 1B1	BD Pharmingen
CD3 (CD3ε chain)	145-2c11	BD Pharmingen
CD4	GK1.5/4	DRFZ
CD8α	53-6.7	DRFZ/ BD Pharmingen
CD11b	Mac-1 α chain, M1/70	DRFZ/ BD Pharmingen
CD11c	N418/ HL3	DRFZ/ BD Pharmingen
CD19	1D3	BD Pharmingen
CD21/CD23	CR2/CR1, CD21a/CD21b	BD Pharmingen
	7G6	
CD23 ($Fc_{\varepsilon}RII$)	B3B4	BD Pharmingen
CD80 (B7.1)	16-10A1	BD Pharmingen
CD86 (B7.2)	GL1	BD Pharmingen
CD138 (Syndecan-1)	281-2	BD Pharmingen
CD154	MR1	Miltenyi Biotec
Fas	CD95 (Fas)	BD Pharmingen
Gr-1	RB6-8C5	BD Pharmingen
GL7	GL7-Ly 77	BD Pharmingen
IgM	Human Goat anti-mouse	BD Pharmingen
MHCII	M5/114	DRFZ
TCR β chain	H57-597	BD Pharmingen
IFNγ	XMG1.2	BD Pharmingen
IL-2	JES6-5H4	BD Pharmingen
IL-6	MP5-20F3	BD Pharmingen
IL-10	JES5-16E3	BD Pharmingen
TNFα	MP6-XT22	BD Pharmingen
Rat IgG1, κ*	R3-34	BD Pharmingen
Rat IgG_2b, κ*	A95-1	BD Pharmingen
Streptavidin APC	Streptavidin	BD Pharmingen
Streptavidin FITC	Streptavidin	BD Pharmingen
Streptavidin PE	Streptavidin	BD Pharmingen
Streptavidin PerCP	Streptavidin	BD Pharmingen

3.4.2 Antibodies for Elisa

Specificity	Clone	Source
IL-10	JES5-2A5 (capture)	BD Bioscience
IL-10-Biotin	SXC-1 (detection)	BD Bioscience
IFNγ	R4-6A2 (capture)	BD Bioscience
IFNγ-Biotin	XMG1.2 (detection)	BD Bioscience
IgM- AP	Goat anti-mouse	BD Bioscience
Ig(H+L)-UNLB	Goat anti-mouse	BD Bioscience
IgG1-AP	Goat anti-mouse	Southern Biotech
IgG2b- AP	Goat anti-mouse	Southern Biotech
IgG2c-Ap	Goat anti-mouse	Southern Biotech
IgG3-AP	Goat anti-mouse	Southern Biotech
IgG-AP	Goat anti-mouse	Southern Biotech

3.5 Recombinant Cytokines

Recombinant cytokines as ELISA standards were from R&D Systems: rmIL-6, rmIL-10 and rmIFNγ.

3.5.1 Magnetic Beads

Microbeads for magnetic-activated cell sorting (MACS) were purchase from Miltenyi Biotec: anti-CD43, anti-CD11c, anti-CD90 microbeads.

3.6 Polymerase Chain Reaction (PCR)

3.6.1 Reagents

Description	Source
Oligo (dT) primer	Promega

Description	Source
dNTP mix	Promega
Reverse transcription 10x buffer	Promega
$MgCl_2$	Promega, Sigma
Recombinant RNasin ribonuclease Inhibitor	Promega
AMV reverse transcriptase	Promega
PCR 10x buffer	GenExpress
dNTP mix	Sigma
Taq polymerase	DRFZ
Gel marker VI	Boehringer Ingelheim
LightCycler® FastStart DNA Master^{PLUS} SYBR Green I	Roche

3.6.2 Primers

The table below shows the sequences of the synthetic oligonucleotides used for Real Time PCR and conventional PCR. All primers were obtained from MWG and diluted to 100 µM stock solutions. The working solutions were prepared from stock solutions according to the requirements.

Primer-name	5'-3' sequence
β-Actin sense	TGG AAT CCT GTG GCA TCC ATG AAA C
β-Actin antisense	TAA AAC GCA GCT CAG TAA CAG TCC
IFN-γ sense	GCT CTG AGA CAA TGA ACG CT
IFN-γ antisense	AAA GAGA ATA ATC TGG CTC TGC
TNF-α sense	TCT CAT CAG TTC TAT GGC CC
TNF-α antisense	GGG AGT AGA CAA GGT ACA AC
GM-CSF-sense	GGC CTT GGA AGC ATG TAG AG
GM-CSF-antisense	CCG TAG ACC CTG CTC GAA TA

3.7 Mice

C57Bl/6, C57B/6 JHT $^{-/-}$ [250], MyD88$^{-/-}$, IL-10$^{-/-}$ [251] TLR2/4$^{-/-}$ [252], TLR9$^{-/-}$ were kept under special pathogen-free conditions in the mouse facility of the DRFZ located at the “Bundesamt für Risikobewertung (BfR)”, Berlin. All mice were used at 6-10 weeks of age. Animals were treated according to the requirements of the German legislation.

4 Methods

4.1 Bacterial culture

Stocks from attenuated *S. typhimurium* (SL7207) and virulent *S. typhimurium* (SL1344) were provided by Max Plank Institute, Department of Immunology. Bacteria were inoculated in 150ml of LB broth and incubated at 37°C (with shaking) for 3 - 4h then OD^{600} was measured and the growth was stopped at OD=0.7. Bacteria were washed twice in sterile cold PBS. Aliquots of bacteria were in PBS/15% of glycerol frozen at - 80°C. The OD^{600} was re-measured in the end and the bacteria were also platted in LB agar and MacConkey agar plates to confirm the final concentration of live bacteria.

4.2 Generation of bone marrow chimeric mice

The use of knockout mice is an important tool in biological research, which allows us to analyse precisely the role of specific molecules. In knock-out mice one or more genes are turned off through a gene knockout, which are genes genetically engineered to become inactivated and then are introduced in cultures of embryonic stem cells where the homologous recombination is carried out. Those embryonic stem cells will then be isolated and introduced into a blastocyt, which will be transplanted into a female mouse. In the end a mutant mouse is created with a deficiency for the respective gene in all cells. However, if we want to target a gene in a specific cell type another approach has to be followed.

A way to analyse a restricted deficiency to a particular cell type is the use of bone marrow chimera system. Here this system was used to restrict a genetic deficiency to B lymphocytes as already previously described [253]. This is achieved by elimination of the haematopoietic system of recipient B cell deficient (JHT) mice by lethal irradiation with a 7.2Gy from a γ-source. The next day the recipient mice are reconstituted with a mixture of haematopoietic stem cells from B cell-deficient (JHT) mice (80%) and from MyD88-deficient mice (20%). In the resulting chimera, all the B cells are $MyD88^{-/-}$ while all the other haematopoietic cell types are wild-type phenotype. Similarly, control mice were obtained by reconstituting irradiated B

cell-deficient (JHT) mice with a mixture of bone marrow cells from C57BL/6 (80%) and from MyD88-deficient mice (20%). In these mice, all the haematopoietic cell types, including B cells are mostly of wild type phenotype. The haematopoietic stem cell preparations were prepared aseptically and depleted of T cells by magnetic separation prior to transfer in order to eliminate the possibility of graft-versus-host disease. In total, a mixture of 6×10^6 cells (4.8×10^6 cells of 80% donor mice and 1.2×10^6 cells of 20% donor mice) were transferred in a volume of 200 µl of sterile PBS into the recipient mice. After six weeks when the hematopoietic system of the recipient mice is fully reconstituted the chimeric mice are ready to be used for infection. To prevent any risk of infection by opportunistic bacteria during the period of immune dysfunction mice are kept on antibiotic Baytril supplied in 1:250 dilution in the drinking water from one week before irradiation until four weeks after irradiation when the immune system is completely reconstituted.

A unique feature of this system is the total absence of leakiness i.e. no wild type B cells can be found in the chimera mice, which would be an unavoidable limitation with conditional mutagenesis based on the Cre/LoxP technology.

4.3 Mice infection

Mice were infected with the virulent *S. typhimurium* strain (SL1344) and also with the attenuated *strain* Aro A^- SL7207. $AroA^-$- *Salmonella* which shows a mutation in genes which encode the pathway of aromatic biosynthesis [226]. Such mutation makes the pathogen unable to supply aromatic substances. Those compounds are not freely available in the host thus, the deficiency limits the replication ability of *Salmonella*.

For infection, aliquots were thawed and appropriately diluted in sterile PBS. Mice were infected by i.v. injection of 1×10^6 *S. typhimurium* (SL 7207) and/or by i.v. injection of wild-type *Salmonella* (1×10^2). Bacteria were injected in a total volume of 200 µl of sterile PBS via the intravenous route through the lateral tail vein of the mice. Bacterial titres were determined by plating serial dilutions on LB agar plates before infection.

4.4 Serum preparation from mouse blood

Blood was taken from the tail vein, incubated overnight at 4°C and then centrifuged for 20 min at 13000 rpm. The serum was collected and stored at -20°C.

4.5 Organ preparation and confirmation of bacterial titres

For removal of spleen, liver and bone marrow, mice were killed by cervical dislocation. Bacterial burden was determined by platting serial dilutions of homogenized spleen and liver on MacConkey agar plates, and colonies were counted after overnight incubation at 37°C.
For the isolation of lymphocytes in the spleen, single cell suspensions were prepared by straining the organs through a 24G needle. Erythrocytes were lysed in spleen and bone marrow preparations by incubation in hypo-osmotic erythrocyte lysis buffer for 3 minutes (min) at room temperature (RT).

For liver preparation, mice were perfused by injection of 3ml PBS into the hepatic vein. Lymphocytes and hepatocytes were first separated by several centrifugations followed after by a centrifugation in 70% and 40% percoll in serum-free medium at 1900rpm for 25 min at 21°C. Afterwards, the interface was collected and the erythrocytes were lysed as explained above.

For Bone Marrow preparation, femur and tibia were removed and flushed out using RPMI serum-free medium. Single cell suspensions were prepared using a 25G needle. And the erythrocytes were lysed as described above.

4.6 Flow cytometry and FACS analysis

Flow cytometry is a very important technique used for enumerating and analysing distinct characteristics of single cells in a diversified population. To study the properties of different cell subsets, monoclonal antibodies (mAb) are used to identify specific protein markers of cells. These are detected with the specific monoclonal antibodies labelled with fluorescent dyes, or by specific antibodies followed by labelled anti-immunoglobulin antibodies.

During the analysis, cells undergo through a hydrodynamic focusing and each single cell is analyzed separately. Cells pass through a laser light with a specific wavelength and at this time several detectors are aimed in the direction of the light beam. One of the detectors is positioned in line with the light beam called forward scatter (FCS) gives information about the cell size and another is sideward scatter (SSC) positioned perpendicular to the light beam

and gives the information about granularity (internal complexity) of the cell. In addition to these are also the fluorescent detectors.

The fluorescent dyes can be a protein (PerCP, APC, PE) or a small organic molecule (FITC, Cy5). The chemical principle is when the cells passes through a laser beam it scatters the laser light, and an electron of the dye is excited to a higher energy level by absorption of a photon. When the electron relaxes and returns to the ground state a photon can be emitted as fluorescence. The amount of the emitted photons is related to the amount of antibodies bound per cell. All the fluorescent dyes absorb the same light wavelength but emit light at different wavelengths that are detected by fluorescent detectors. In FACSCalibur there are four detectors for the different fluorochromes and wavelengths (Table 1).

Laser	**Detectors and fluorochromes**	**Excitation-Maximum (λ in nm)**	**Emission-maximum (λ in nm)**
Argon (488 nm)	FL1, Fluoresceinisothiocyanat, (FITC)	495	519
	FL2, R-Phycoerythrin, (PE)	480; 565	578
	FL3, Propidiumiodid, (PI);	536	617
	Peridinin Chorophyll protein (PerCP)	490	675
Diode (635 nm)	FL4, Allophycocyanin, (APC)	650	660
	Cyanine 5, (Cy5)	695	670

Table 1- The different lasers and fluorochromes with emission-maximum wavelengths detected by four fluorescent detectors.

Flow cytometry does not only permit to analyze different cells but also allows sorting them from a heterogeneous mixture according to the fluorescent characteristics of each cell. This specialized flow cytometry technique is called fluorescence-activated cell sorter (FACS). The stream of liquid containing the cells is forced by an electrical charge to break into individual droplets and each drop should contain just one cell. Drops containing the charge can be deflected from the main stream of droplets by electrostatic forces into different containers. In this way, specific subpopulations of cells can be distinguished.

The procedure followed for Flow cytometric analyses is described below. For all the stainings, cells from single cell suspensions were resuspended in PBS/0.2%BSA and transferred to 96-well V-bottomed plates.

4.6.1 Cell surface staining

In order to prevent unspecific binding of mAb, cells from single cell suspension were incubated with 30µl/ml anti-FcγReceptor blocking in PBS/0.2%BSA for 20 min at 4°C. Afterwards samples were stained with the appropriate mAb conjugated to fluorescent dyes or biotin to cell surface antigens for 15min at 4°C in the dark (Fluorescent dyes are light sensitive). All the antibodies were used at a concentration determined previously usually at 1-2µg/ml. Cells were washed to remove unbound antibodies. When biotinylated mAbs were used, another step of incubation with FITC streptavidin, APC streptavidin or PerCP streptavidin was performed under the same conditions as described above. In order to exclude dead cells, propidium iodide (PI) was added to the samples immediately before data acquisition, except for the four color-stained cell samples, in FACSCalibur.

4.6.2 Intracellular staining for cytokines and CD40L

One very important method to measure cytokine production on a single cell level is by intracellular cytokine staining. This method requires an in vitro stimulation and a blocking substance that inhibits protein export from the cell. The cytokine thus accumulates within the endoplasmatic reticulum (ER)/Golgi since this pathway is blocked by substances like monensin or brefeldin A. In this work we used Heat Killed *Salmonella* (HKS) to induce cytokine production by T cells in the presence of antigen specific cells (APC) and stainings were done using the Cytofix/Cytoperm kit (BD biosciences). Splenocytes were diluted in complete RPMI medium and 3×10^6 cells were cultured in a volume of 1ml at 37°C, 5% CO_2 with or without the addition of HKS (1×10^8/well). After 1h when APCs had time to process the antigen, GolgiStop was added and incubated for four hours and half. Then surface staining was done as described before, and cells were fixed and permeabilized using the kit solution fixation/permeabilization for 20 min at 4°C. Cells were washed twice in perm/wash buffer and incubated in 100µl Perm/wash buffer containing the anti-cytokine mAb (2µg/ml) for 30min at 4°C. Cells were washed with Perm/Wash buffer and resuspended in 200µl PBS/BSA for acquisition at FACSCalibur.

4.7 Cell purification

4.7.1 General procedures

Spleen and bone marrow were removed from naive mice and single cell suspensions were done as described in 4.5 under sterile conditions. Afterwards, cells were purified by magnetic cell sorting (MACS). Cell specific antigens are labelled with magnetic beads and separated over a MACS column placed on a MACS separator. Labelled cells are retained in the column (positive fraction) and unlabelled cells pass through the column (unlabelled fraction). The positive fraction is then recovered when the column is detached from the magnet. An autoMACS device was used for the automatic separation of the cells.

4.7.2 Purification of B cells

There are two methods to purify B cells by MACS, one is by depletion of $CD43^+$ cells; this way allows the obtention of naive B cells, since $CD43^+$ is not expressed on conventional peripheral resting B cells. The second method is the purification by positive selection of $CD19^+$ B cells and allows the isolation of mature naive and memory B cells since $CD19^+$ is expressed in both populations.
Spleens of naive C57BL/6, $MyD88^{-/-}$, $TLR9^{-/-}$, $TLR2/4^{-/-}$ and $IL\text{-}10^{-/-}$ mice were removed to make single cell suspensions. Cells were incubated with 33µl of anti-CD19 or 30µl of anti-CD43 Microbeads per 10^8 cells in 297µl or 300µl PBS/BSA respectively for 15min at 4°C, then cells were washed and filtered. Cells were selected by autoMacs using the PosselD2 program for $CD19^+$ sort or the program depleteS for $CD43^-$ sort. Purity was checked by FACS staining for surface $CD19^+$ or $B220^+$ and was routinely above 95%.

4.7.3 Purification of Dendritic cells

Dendritic cells were selected by positive selection of $CD11c^+$ cells. Spleens from naïve C57BL/6 mice were removed and digested with 1mg/ml Collagenase and 0.5mg/ml DNase in serum-free medium for 30min at 37°C. After single cell suspension, cells were incubated in 25µl of anti-CD11c Microbeads in 225µl PBS/BSA per 10^8 cells for 15min at 4°C, and then cells were washed and filtered. Cells were selected by autoMacs using the PosselD2 program. Purity was checked by staining for surface $CD11c^+$ and MHC class II and was routinely above 95%.

4.8 Bone marrow derived macrophages

For the generation of BM derived macrophages, BM cells were plated in a cell culture petri dish and incubated overnight. Therefore, differentiated cells adhere more rapidly and precursors can be removed and plated in 10 ml of RPMI medium containing 10% FCS/ 1% Pen-Strep, 2-ME, L-glutamine supplemented with 10% of growth factor M-CSF (produced in supernatant of X-63 cell line) in a bacteria petri dish. At day 3 and day 6 of culture, cells were washed in order to remove non-adherent cells and new culture medium was added. On day 7, cells were harvested, counted and cultured under stimulating conditions in 96-well plated at $1.25x10^5$ cells per well in 50µl of RPMI.

4.9 In vitro assays

Cells were cultivated in growth medium at 37°C and 5% CO_2 atmosphere saturated with water vapour. Cell counts were calculated using a Neubauer improved counting chamber. Dead cells were stained with 10% trypan blue in PBS solution.

4.9.1 Culture of bone marrow cells

For the analysis of non antigen-specific IgM and IgG antibodies, $8×10^5$ bone marrow cells from naïve and infected chimera mice were cultivated in 200µl cell culture medium in 96-well flat-bottom microtitre plates at 37°C and 5% CO_2 atmosphere. Cultures were maintained for 8 days without changing the medium. At that time point, supernatants were collected for antibody detection by ELISA.

4.9.2 Stimulation of B cells and dendritic cells

For the analysis of IL-10, IL-6 and IFN-γ production, $5×10^5$ of purified B cells and dendritic cells were cultivated in 200µl of RPMI in 96-well flat-bottom microtitre plates at 37°C and 5% CO_2 atmosphere. Cells were stimulated with 10µg/ml and 5µg/ml of LPS and/or a Multiplicity of infection (MOI) of 100, 50, 25, 12.5 and 6.25 of HKS. After 72 h the supernatants were collected and cytokines were analysed by cell-based ELISA.

4.9.3 Assay to test the influence of supernatans from HKS activated B cell in the stimulation of activated macrophages

For the analysis of IFN-γ and IL-6 production by HKS-activated macrophages with activated B cell supernatants, macrophages were cultivated in 50 µl RPMI in 96-well flat-bottom microtitre plates at 37°C and 5% CO_2 atmosphere. Then, 100 µl of B cell supernatants (obtained from stimulated B cells at MOI 1:50 of HKS as described on 4.9.2) or medium, were added to the macrophages. After 2 hours, cells were stimulated with a Multiplicity of infection (MOI) of 25, 12.5 and 6.25 of HKS or medium at 37°C and 5% CO_2 atmosphere, 24 hours later, supernatants were collected and cytokines were analysed by ELISA. These samples were diluted 1:5 for the ELISA.

4.10 ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) is a method to detect the presence of an antigen or an antibody in a sample. Usually at least one antibody with specificity for a particular antigen is used to perform the ELISA. The capture antibody or an antigen is coated to the surface of high binding 96-EIA/RIA microtitre plate. Free binding sites are blocked by incubation with blocking buffer. The sample is added and the antigen-specific antibodies are bounded to the plate. A separate labelled antibody that recognizes a different epitope to the immobilized first antibody is then used to detect the bound antigen. The detection antibody can be covalently linked to an enzyme or can itself be detected by a secondary antibody which is linked to biotin. Biotin is then bounded by streptavidin coupled to an enzyme that catalyses a colorimetric reaction of its substrate. In this work the detection antibodies are linked to the enzyme alkaline phosphatase that hydrolyzes the para-nitrophenyl phosphate (pNPP) into yellow coloured para-nitrophenol and phosphate. Para-nitrophenol absorbs light between 405nm and 410nm and can be quantified in a photometer.

4.10.1 Cell-based IL-10 ELISA

If cytokines are of difficult detection due to a very low secretion rate by the cells or if they are quickly consumed a very sensitive method that permits to detect cytokines in these conditions is the Cell-based ELISA. This procedure is done in sterile conditions until the step of the cytokine detection. High binding 96 well EIA/RIA plates were coated with 4µg/ml anti-IL-10 mAb in carbonate buffer overnight at 4°C and blocked afterwards with PBS/3% BSA

buffer for 1h at 37°C. Plates were washed first in PBS/0.1%Tween and in the end in PBS to remove any antibody not specifically bounded. After 72h of B cells and dendritic cells stimulation as described in 4.9.2 cells were transferred into the high binding plates and incubated overnight at 37°C and 5% CO_2 atmosphere. During incubation the IL-10 present in the culture medium and also IL-10 produced by the cells is captured immediately and consumption is prevented. Recombinant IL-10 mAb was used as standard ranging from 100ng/ml to 0.195ng/ml. For detection 2µg/ml of biotinylated anti-IL-10 mAb in PBS/1%BSA was added and incubated for 1h at RT. Subsequently ExtrAvidin-Alkaline phosphatase diluted 1:10000 in PBS/1%BSA were added to the plate and incubated for 30 min at RT. All incubations steps were followed by repeated washing with PBS/0.1%Tween. 1mg/ml of substrate pNPP was dissolved in ELISA buffer and added to the plate. Then the measurement was done at 405nm.

4.10.2 IFNγ and IL-6 ELISA

For IFNγ and IL-6 cytokine detection we used the cell culture supernatants after the 72h stimulation of B cells and dendritic cells as described before. The high binding 96 well EIA/RIA plates were coated separately with 2µg/ml anti-IFNγ mAb and/or 4µg/ml anti-IL-6 mAb in carbonate buffer overnight at 4°C and blocked afterwards with PBS/3% BSA buffer for 1h at 37°C. Afterwards samples and recombinant IFNγ mAb and/or recombinant IL-6 mAb used as standard ranging from 100ng/ml to 0.195ng/ml were incubated for 2h at 37°C. For detection 1µg/ml of biotinylated anti-IFNγ mAb and/or 2µg/ml of biotinylated anti-IL-6 in PBS/1%BSA were used and incubated for 1h at RT. Subsequently ExtrAvidin-Alkaline phosphatase diluted 1:10000 in PBS/1%BSA were added to the plate and incubated for 30 min at RT. All incubations steps were followed by repeated washing with PBS/0.1%Tween. The development of the plates was done as described in 4.10.1.

4.10.3 Serum antibody ELISA

This method is used to detect *Salmonella*-specific and non-specific antibodies in mouse serum. High binding 96 well EIA/RIA plates were coated with 50µl of heat killed *Salmonella* (HKS) (1×10^8/ml) in PBS overnight at 4°C. After plates were washed in PBS and blocked with PBS/1%BSA at 4°C overnight or for 3h at 37°C. Then a standard sample and serum pre-diluted in PBS was added and two fold dilution series were performed and incubated for 2h at RT. As detection antibody an alkaline phosphatase conjugated IgM, IgG, IgG1, IgG2b, IgG2c and IgG3 diluted 1:1000 in PBS/0.05% Tween were used and incubated for 1h at RT. All incubations steps were followed by repeated washing with PBS/0.1%Tween followed by a

last wash in PBS. Further steps were as described before. The relative titre was calculated by comparing measured samples to a standard.

To determine antibody titres (including *Salmonella* specific and non-specific) in serum, the plates were coated with 1.5µg/ml anti-Ig(H+L) diluted in carbonate buffer overnight at 4°C. The procedure was done as described before.

4.10.4 Supernatant antibody ELISA

Supernatants from bone marrow cells obtained as described in 4.9.1 were incubated for 2h at RT in high binding 96 well EIA/RIA plates previously coated with 1.5µg/ml anti-Ig(H+L) diluted in carbonate buffer overnight at 4°C. As detection antibody an alkaline phosphatase conjugated IgM, IgG, IgG1, IgG2b, and IgG2c diluted 1:1000 in PBS/0.05% Tween were used and incubated for 1h at RT. Further steps were performed as described in 4.10.3.

4.11 Histology

Organs were embedded in tissue-tek (Sakura), frozen in dry ice and kept at -80°C. Using a cryostat, sections from the organs were cut between 5 microns and 7 microns. Sections were left air dry and then fixed in cold acetone for 8min left them dry again for 40min and finally stored at -20°C.

4.11.1 Staining for germinal centre B cells

Sections were defrosted and re-hydrate for 20min in PBS. After, were stained with IgM FITC or B220 alexa 488 diluted 1:200 in PBS and PNA rhodamine diluted 1:500 in PBS for 90min at RT in a staining box to maintain a humidified atmosphere and dark environment. Subsequently, sections were washed three times 10min each in PBS with magnetic stirrer. Finally sections were mounted with fluoromout G and a cover slip. During the staining, sections were protected from light. Stained sections were analysed microscopically and digital pictures were taken.

4.12 Molecular biological methods

4.12.1 Isolation and purification of RNA from splenocytes

RNA was isolated by the Tri-reagent RNA preparation method (Sigma-Aldrich). Briefly, between 6×10^6 and 1×10^7 splenocytes were homogenized in 1ml Tri-Reagent by repeated pipetting and stored at -70°C. After defrosting samples, 200µl of chloroform was added and mixed for 15 sec and left for 15min at RT. The mixture was centrifuged at 4°C at 13000rpm for 15min. The aqueous phase was transferred to a new tube and 500µl isopropanol was added and left for 10min at 4°C. In order to increase the yield, 1 µl of glycogen was added as a carrier. Then, RNA was precipitated by centrifugation for 15 min at 13000rpm at 4°C. The RNA pellet was washed in 75% ethanol, air-dried and dissolved in RNAse free water.

4.12.2 Generation of cDNA

Initially purified RNA samples were treated with DNAse (Applied Biosystems) to eliminate genomic DNA contamination. For this, 8µl of RNA sample (up to 1µg of RNA), 1µl of 10×DNase I buffer and 1µl of rDNAse were mixed and incubated for 30min at 37°C. The reaction was stopped by the addition of 1µl DNase inactivation reagent followed by centrifugation at 13000rpm for 1min at 4°C the DNase was inactivated by incubating the mixture at 65°C for 10min. After incubation the mixture was centrifuged at 13000rpm for 2min. Later, 9µl of it were used for reverse transcription. For that 1µl oligo(t)N Primer was added, the mixture was incubated for 10min at 55°C and then placed at RT for 15min. After, a reaction mix containing 4µl $MgCl_2$ (25mM), 2µl 10× Rev. Trans. Buffer, 2µl dNTP (10mM), 1µl nuclease free water, 0.5µl Rnase inhibiter and 0.5µl AMV-RT (reverse transcriptase) were added. This mixture was incubated for 3h at 42°C and heated for 99°C for 5min to inactivate the enzymes and cooled for 5 minutes at 4°C. Finally 30µl of water were added to cDNA.

4.12.3 Real-time polymerase chain reaction (RT-PCR)

RT-PCR is based in the amplification and simultaneously quantification of DNA. The principle is that the amplified DNA is quantified as it accumulates in the reaction in *real time* after each amplification cycle. This quantification is done with fluorescent dyes that bind to all double-stranded DNA, as the DNA accumulates in each cycle the fluorescence intensity increases also. For semi-quantitative real-time PCR total RNA was extracted from organs as described

in section 4.12.1 and transcribed to cDNA as described in section 4.12.2. In order to measure the differential mRNA expression of cytokines, the cDNA concentration of all samples was equilibrated using the housekeeping gene β-Actin as a reference. All PCRs were run for 45 cycles with 15sec 95°C, 15sec annealing temperature and 15sec 72°C using ABI Prism optical 96-well plates. Reaction mixtures were set up in 10µl final volume using 0.6µl of each primer (10mM), 2µl template cDNA, 2µl SYBR-Green master mix and finally 4.8µl of water. The PCR annealing temperature is summarized in Table 2. The crossing points (Cp) of all measured cytokines were then related to β-Actin using the following equation: $2^{Cp(b\text{-}Actin)- Cp(cytokine)}$

β-Actin	IFNγ	TNFα	GM-CSF
55°C	65°C	64°C	61°C

Table 2- PCR annealing temperatures used in light cycler

4.13 Statistical analysis

Statistical significance of results was determined with the statistic program included in the GraphPad Prism program (version 4.0; GraphPad, San Diego, CA). Student's unpaired t test was used to assess statistical significance where appropriate. Kaplan-Meier plots and log-rank tests were used so that the survival differences after virulent *S. typhimurium* infection could be assessed.

5 Results

5.1 Effects of heat-killed *Salmonella* in B cells, DCs and macrophages *in vitro*

5.1.1 HKS induces cytokine production by B cells

Previous studies have shown that IL-10 producing B cells are required for EAE recovery [170]. The production of IL-10 by B cells in response to microbial products suppressed T cell activation by inhibiting the response of DCs to TLR agonists. This was confirmed in EAE studies showing that activation of B cells via TLR2/4 and MyD88 were required for EAE recovery. This suggests that the TLR agonists controlling the suppressive activity of B cells are most likely provided by components of Mycobacterium tuberculosis. That gives an idea that microbes can control the regulatory function of B cells.

In order to investigate the role of B cells during infections, using the *S. typhimurium* mouse model, it was decided to test first, whether the B cells are able to produce IL-10 and IL-6 *in vitro,* upon nonviable heat-killed *Salmonella* (HKS) stimulation. Therefore, $CD19^+$ B cells were isolated from C57BL/6 and stimulated *in vitro* with HKS or LPS as a control (Figure 5). Purity of B cells as checked by FACS analysis was routinely higher than 97%.
Stimulation of B cells with HKS induces the same ranges of IL-10 amount as with LPS (Fig.5A and 5C, respectively). IL-6 and IFN-γ were also tested in order to identify if production of pro-inflammatory cytokines by B cells will be affected during *Salmonella* stimulation. It was observed that HKS triggered naive B cells to produce 0.5-0.75 ng/ml of IL-6 (Figure 5-B) and IFN-γ production by LPS and HKS activated B cells was not observed (data not shown). Conclusively, HKS can induce IL-10 and IL-6 by B cells *in vitro*.

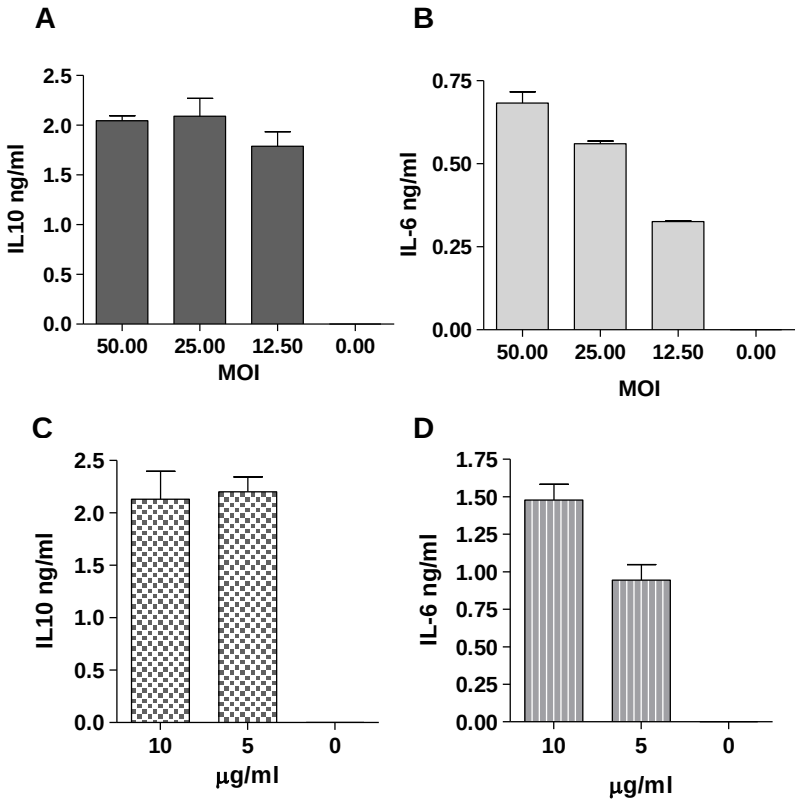

Figure 5: HKS and LPS induce IL-10 production in B cells. B cells were isolated from splenocytes of naïve C57/6 mice by positive selection of $CD19^+$ cells and stimulated in vitro. A) and B), B cells stimulated with HKS. C), and D), B cells stimulated with LPS. IL-10 and IL-6 production were measured by cell-based ELISA at day 4. Error bar, SEM.

5.1.2 *Salmonella typhimurium* activates B cells through TLR2/4 and MyD88

The microbial components of *S. typhimurium* provide various TLR agonists, which activate the immune cells and induce an appropriate immune response [254].

In order to analyse the relative contributions of TLRs to the bacterial induction of IL-10 in B cells, splenic B cells from C57BL/6 mice, TLR-$9^{-/-}$ deficient mice, TLR2/4 deficient mice and MyD88 deficient mice were stimulated *in vitro* with nonviable *S. typhimurium* (Figure 6). HKS triggers IL-10 production by B cells via TLR-2/4 and MyD88 showing that the principal TLR agonists in B cells of *S. typhimurium* are LPS, lipoproteins and other PAMPs, excluding the bacterial DNA, which is a TLR-9 agonist. This observation is not surprising, since Weiss et al. showed that TLR4, TLR2 and MyD88 are involved in host defence against *Salmonella typhimurium* [51].

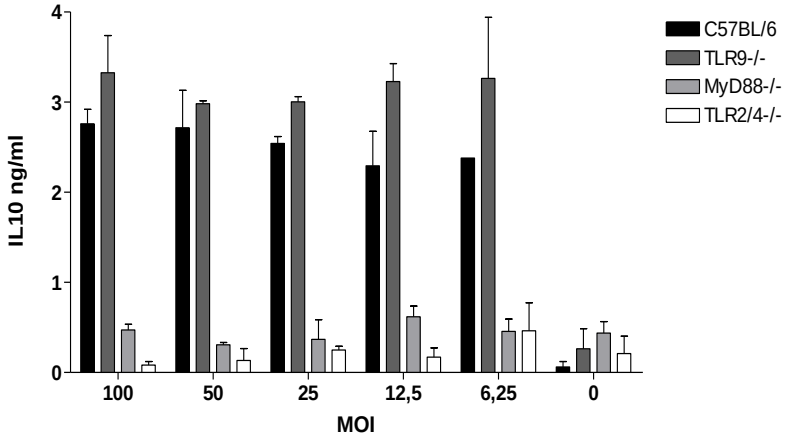

Figure 6: HKS induces IL-10 production by naïve B cells via TLR2/4 and MyD88. Splenic B cells from C57BL/6 mice (black bars), TLR-9$^{-/-}$ mice (dark gray bars), TLR-2/4$^{-/-}$ mice (light gray bars) and MyD88$^{-/-}$ mice (white bars) were sitmulated with HKS for 3 days, and IL-10 was determinate at day 4 by cell-based Elisa. Error bar, SEM.

5.1.3 HKS induces cytokine production by dendritic cells

In previous studies it was reported that B cells and DCs provide cytokine environments with different impact on T cell activation following TLR stimulation. Supernatants taken from LPS-activated B cells repress T cell activation by DC. In contrast, supernatants from TLR activated DC lead to a stimulation of T cell response [54]. Therefore, DCs and B cells appear to provide different cytokine milieu upon TLR-triggering.

To further investigate the role of DCs upon HKS stimulation, splenic DCs were isolated with a purity superior to 97% from C57BL/6 mice and stimulated with HKS or LPS as control (Figure 7). HKS and LPS trigger IL-6 and IFN-γ by DCs but not IL-10. DCs produce 8 - 8.6 ng/ml of IFN-γ upon HKS stimulation, which is higher compared to the LPS stimulation. IL-6 production by DCs reaches values of 1.5 – 2.5 ng/ml upon HKS stimulation. These observations show that DCs after HKS stimulation are able to produce high amounts of inflammatory cytokines.

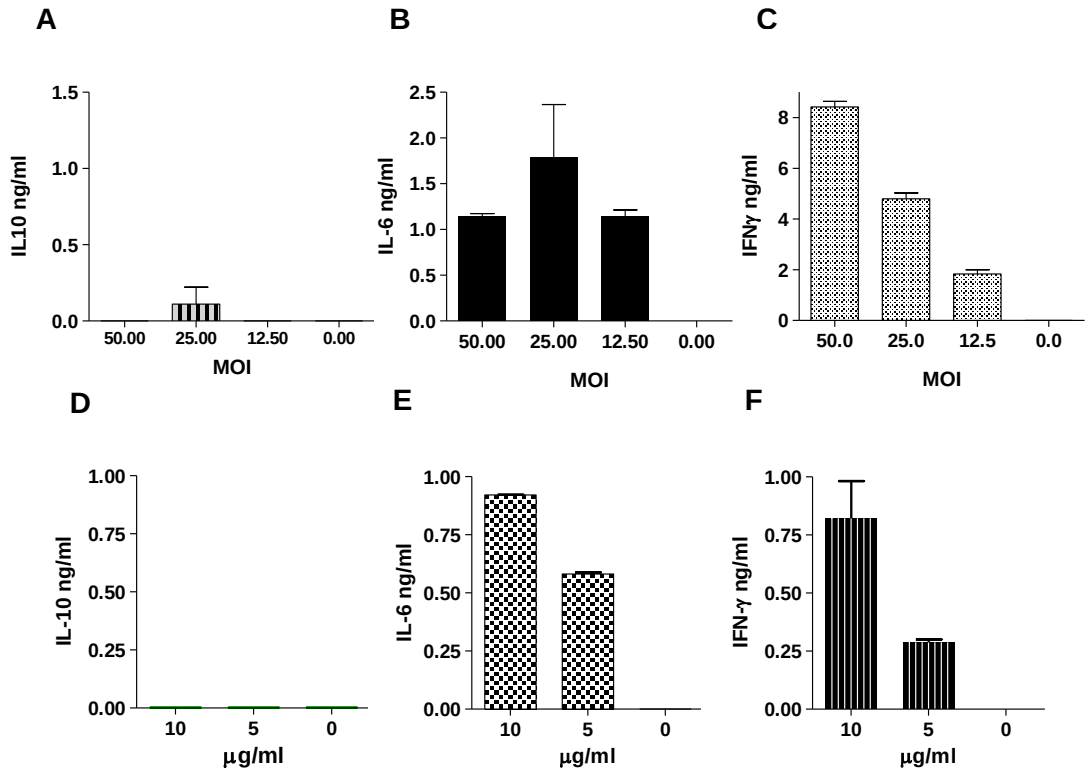

Figure 7: HKS and LPS induce IL-6 and IFN-γ production in DCs. Splenic Dendritic cells were isolated from naïve C57BL/6 mice by positive selection of $CD11c^+$ cells and stimulated in vitro. A), B) and C), DCs stimulated with HKS. D), E) and F), DCs stimulated with LPS. IL-10, IL-6 and IFN-γ were measured by ELISA at day 4. Error bar, SEM.

5.1.4 HKS-stimulated B cells inhibits activation of HKS-stimulated bone marrow derived-macrophages

Macrophages are known to play a crucial role in immune defence against bacterial infections. TLR stimulation increases the phagocytic activity of macrophages and promotes phagosome maturation allowing efficient capture and destruction of microbes [255]. Furthermore, TLR-activated macrophages secrete inflammatory cytokines driving formation of granuloma [256], which limits dissemination of the microbe, and also produce effector substances directly associated with microbe killing [257]. In the next step, the effect of cytokines produced by B cells in response to heat-killed *Salmonella* on macrophages activated with HKS was evaluated in an *in vitro* assay. B cells from spleens of C57BL/6 mice, MyD88-deficient mice and IL-10-deficient mice were purified by CD43 depletion and stimulated during 3 days with heat-killed *Salmonella.* Subsequently, B cell supernatants were obtained, and added to macrophages generated *in vitro* from bone marrow cells. After 2 hours, HKS was added to the culture and 72 hours later supernatants were harvested and assessed for cytokines by ELISA. It was observed that macrophages are able to produce IL-6 and IFN-γ upon HKS stimulation (Figure 8-A and B). Supernatants from wild-type B cells (WT) suppressed the

secretion of IL-6 by bone marrow derived-macrophages, and this was also observed with supernatants from MyD88-deficient B cells (MyD88$^{-/-}$) although to a lesser extent (Figure 8-A and B). WT-B cell supernatants suppressed almost 2 fold more the amount of IL-6 secreted by HKS-activated macrophages compared to MyD88-deficient B cell supernatants (Figure 8-A). In the case of IFN-γ, MyD88-deficient supernatants were found to further increase its production, whereas WT-supernatants had no effect (Figure 8-B). According to these results it seems that the suppression is not solely MyD88-dependent. In order to investigate if suppression of macrophages is due to IL-10 production by B cells, B cells from IL-10$^{-/-}$ mice were also tested. The amount of IL-6 production by bone marrow derived-macrophages with IL-10$^{-/-}$ B cell supernatants was similar to the MyD88-deficient B cell supernatants (Figure 8-A). IFN-γ production by macrophages was higher with IL-10$^{-/-}$ B cell supernatants than the MyD88$^{-/-}$ B cells supernatants (Figure 8-B).

This suggests that the increased IFN-γ production by macrophages with IL-10$^{-/-}$- B cell supernatants can be due to the presence of IFN-γ in IL-10$^{-/-}$ B cell supernatants. Since, IL-10$^{-/-}$ B cell supernatants had higher amounts of IFN-γ compared to the other strains (Figure 8-C and D). The high levels of IL-6 and IFN-γ in IL-10$^{-/-}$ B cell supernatants are also not surprising, considering that the signal transduction pathway by MyD88 it is not blocked compare to the MyD88 deficient B cell supernatants. Myeloid differentiation factor 88 (MyD88) is critical for TLR-mediated activation of the transcription factor NF-κB and hence the induction of pro-inflammatory cytokines [258].

Conclusively, HKS-activated B cells are able to suppress HKS-activated bone marrow derived-macrophages. This suppression can be due to IL-10 most likely via MyD88-dependent, but also a strong component of suppression is MyD88-independent. Possibly other anti-inflammatory cytokines are also produced by B cells. As reported before, LPS-activated B cells are able to produce TGF-β, which is an anti-inflammatory cytokine [259].

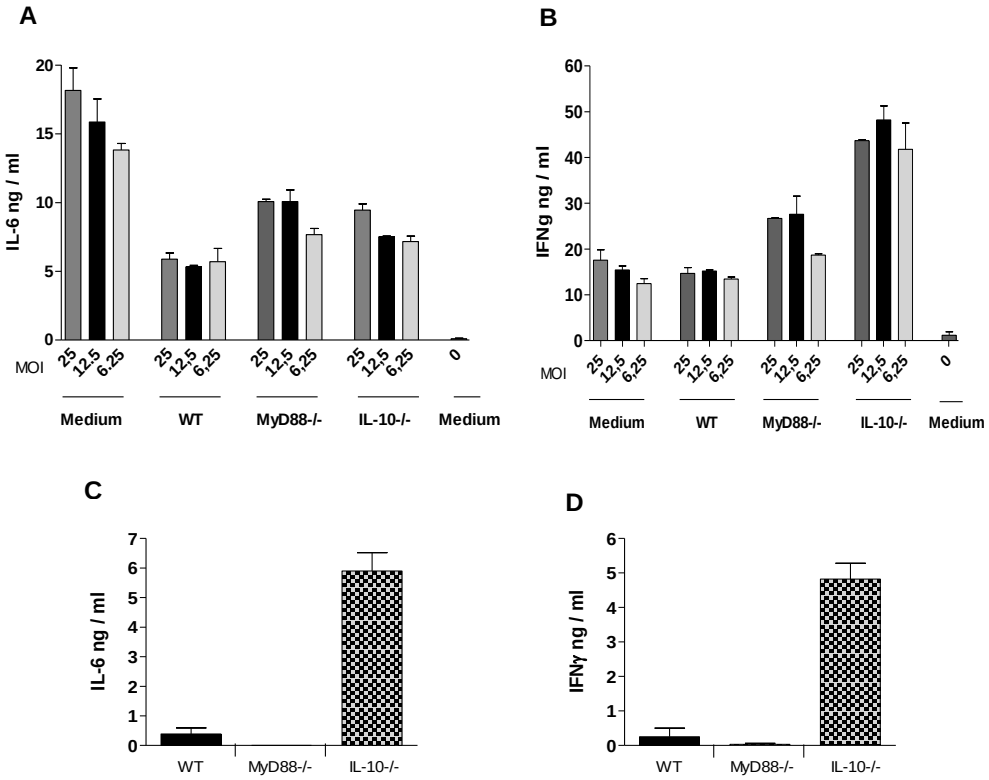

Figure 8: **Effect of HKS-Activated B cell supernatants on bone marrow-derived macrophages activated with HKS**. B cells from the indicated mouse strains were activated with HKS at a MOI of 50:1. Supernatants were collected at 72hours and added to bone marrow-derived macrophages, then HKS at MOI of 25:1, 12.5:1, 6.25:1 and 0:1 was added to the cultures. A) IL-6 production by BM macrophages. B) IFN-γ production by BM macrophages. C) and D), IL-6 and IFN-γ measurement of B cell supernatants from the indicated mouse strains

5.2 Role of IL-10 in B cells during *S. typhimurium* infection

B cells activated through TLRs can inhibit through the secretion of IL-10 the production of inflammatory cytokines by dendritic cells and macrophages [54]. It is also reported that blockade of IL-10 with antibody *in vivo* augments TNF-α production and increases resistance of mice to *Salmonella* infection [260]. In order to address the question whether IL-10 production by B cells plays a direct role in *Salmonella* infection, mice with IL-10 deficiency restricted to B cells were generated using an established bone marrow chimera system [253]. Briefly, B cell-deficient JHT mice were irradiated and reconstituted with a mixture of 80% bone marrow cells from JHT mice (these bone marrow cells cannot produce B cells because of a genetic deletion in the immunoglobulin heavy chain locus) and 20% bone marrow cells carrying the genetic deletion of interest. In the resulting reconstituted animals, JHT bone marrow progenitors provide the majority of haematopoietic cells (except B cells),

which are therefore of wildtype phenotype because defects in immunoglobulin genes only affect the B cells development. In contrast, all the B cells are derived from the bone marrow cells lacking the gene of interest (IL-10 or MyD88). A unique feature of the chimera system is the total absence of leakiness i.e. no wild type B cells can be found in the chimeric mice. In order to investigate the functions of IL-10 in B cells, chimera mice lacking IL-10 in B cells (B-IL-$10^{-/-}$) and chimera mice with wildtype B cells (B-WT) were used .and infected with *S. typhimurium*.

5.2.1 IL-10 deficient B cells induce a stronger immune response against *Salmonella typhimurium*

The previous observations showed that B cells stimulated by HKS produce large amounts of IL-10 *in vitro*, in a TLR2/TLR4-MyD88 dependent manner, and these B cells are able to suppress HKS-activated macrophages *in vitro*. In order to corroborate these observations, IL-10-deficient B cell mice were infected with attenuated *S. typhimurium* and sacrificed at day 21 to better see the direct influence in the adaptive immune response.

Macrophages and dendritic cells accumulate in spleen on day 21 after infection (Figure 9:-A and B). IL-10-deficient B cell mice had more macrophages and dendritic cells in the spleen than control mice. This difference was more evident for macrophages than dendritic cells.

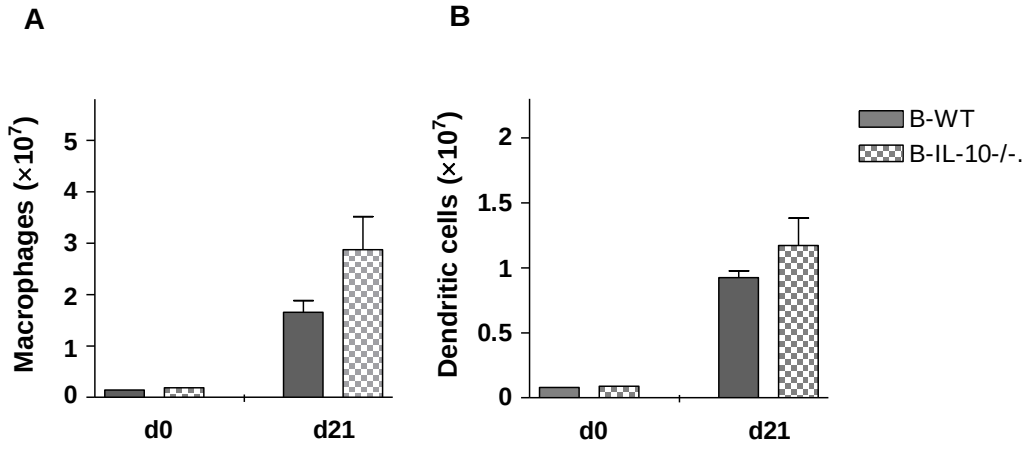

Figure 9: Absolute cell numbers of macrophages and dendritic cells in the spleen of naïve and infected mice. B-WT (gray bars) mice and B-IL-$10^{-/-}$ (squared bars) mice were infected with 10^6 attenuated *S. typhimurium* i.v.. Mice were sacrificed at day 21 after infection. A), numbers of macrophage. B) numbers of dendritic cells. These results represent just one experiment. Mean±SEM; naïve mice: n=1 ; infected mice n=5.

These observations suggest that there is a stronger $CD4^+$ T cell response in B-IL-10 mice. In order to test this, *Salmonella*-specific $CD4^+$ T cells, IFN-γ and TNF-α producing $CD4^+$ T cells,

were determined in naive and infected mice by flow cytometry after a short *ex vivo* re-stimulation of splenocytes with HKS. *Salmonella*-specific $CD4^+$ T cells were identified by co-expression of CD4 and CD154, which is a marker for antigen-reactive helper CD4 T cells [261, 262]. According to this technology *Salmonella*-reactive $CD4^+$ T cells could be detected on day 21 after infection, showing a clearly stronger accumulation in B-IL-$10^{-/-}$ mice compared to control mice (Figure 10-A). As expected, IL-10 in B cells reduces the inflammatory response against *Salmonella* infection (Figure 10-B and C). However, it was not possible to detect any differences in the bacterial numbers (data not shown). B-IL-$10^{-/-}$ mice had 2-fold more IFN-γ and TNF-α producing $CD4^+$ T cells than controls (Figure 10-B). These observations show for the first time that IL-10 in B cells inhibits an adaptive $CD4^+$ T cell response in an infection model using *Salmonella typhimurium*.

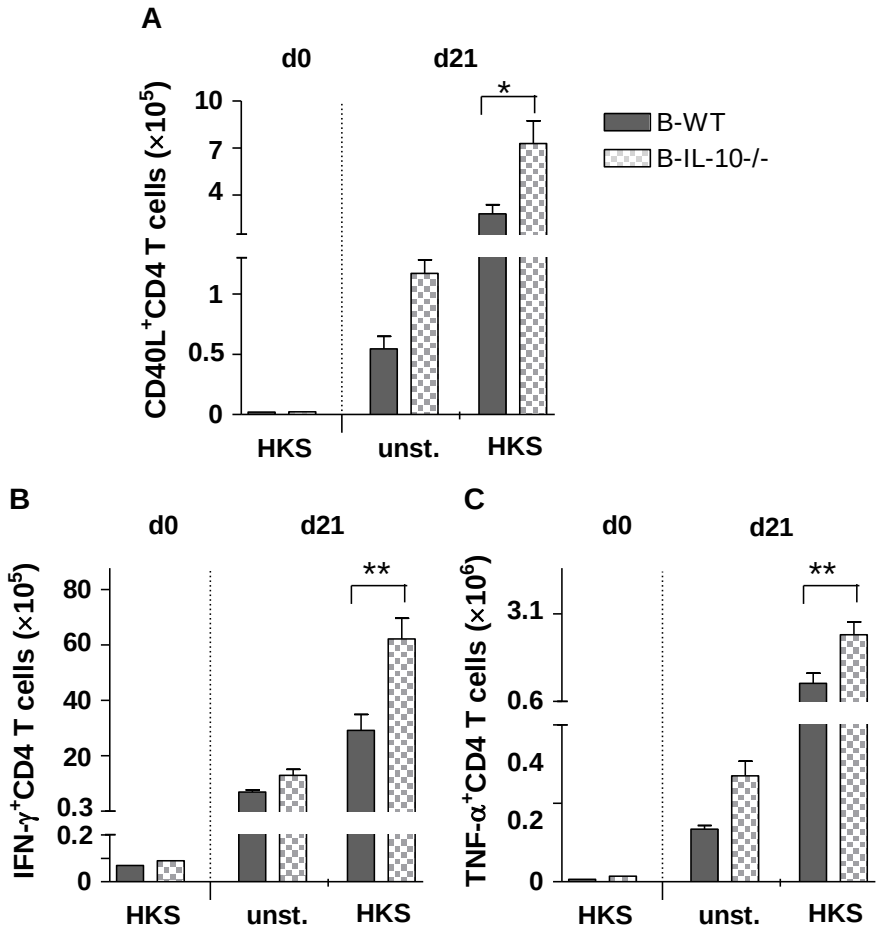

Figure 10: Numbers of *Salmonella* -specific $CD4^+$ T cells (A) and IFN-γ (B) and TNF-α (C) production by $CD4^+$ T cells in the spleen of naïve and infected mice. B-WT (gray bars) mice and B-IL-$10^{-/-}$ (squared bars) mice were infected with 10^6 attenuated *S. typhimurium* i.v.. Mice were sacrificed at day 21 after infection. These results represent just one experiment. Mean±SEM; naïve mice: n=1 ; infected mice n=5. (* for $p<0.05$ and ** for $p<0.01$).

5.3 Role of MyD88-signalling in B cells during *Salmonella typhimurium* infection

It has been demonstrated that B cells activated by microbial products through MyD88 induce IL-10 production, and could inhibit T cell-mediated immune responses [54]. In this work it has been observed that B cells produce IL-10 through TLR/MyD88 activation using heat-killed *Salmonella* and that IL-10 production by B cells inhibits $CD4^+$ T cell response during *Salmonella* infection *in vivo*. Therefore, to assess the effect of MyD88 signalling in B cells during *Salmonella* infection, mice with MyD88 deficiency restricted to B cells were generated using the established bone marrow chimera system [253] explained previously. In order to investigate the functions of MyD88 in B cells, control C57BL/6, MyD88-deficient mice, chimera mice lacking MyD88 in B cells (B-$MyD88^{-/-}$) and chimera mice with wildtype B cells (B-WT) were used and infected with *S. typhimurium*.

5.3.1 Course of *S. typhimurium* infection in MyD88-B cell deficient mice

B-$MyD88^{-/-}$ and B-WT chimera mice were infected via the intravenous route with 1×10^6 *S. typhimurium* SL7207. This route of infection mimics the systemic phase of disease.
In order to have a complete understanding of the different stages of the immune response to *S. typhimurium*, previously clarified on section 1.3.1, the infection course was monitored at several time points (Figure 11) of disease.

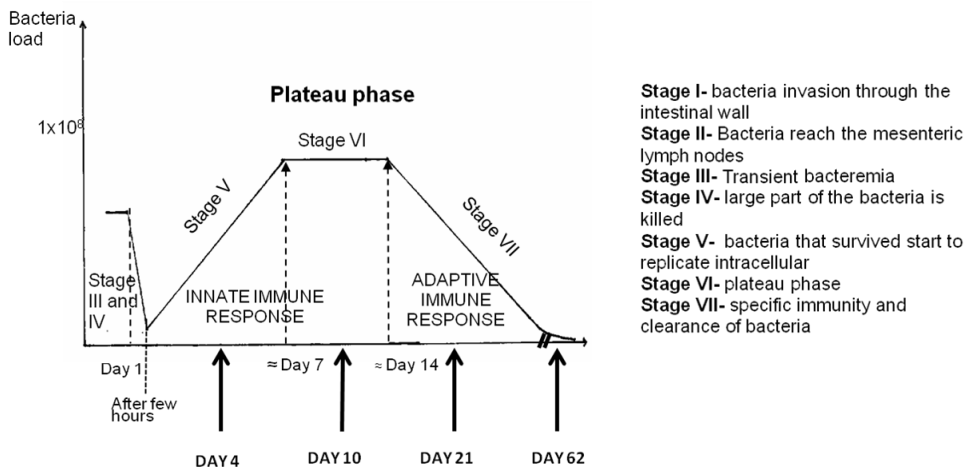

Figure 11: Representation of the mouse typhoid model. The scheme starts with stage III, which represents the transient bacteremia. Mice were sacrificed at day 4, during the innate immune response, at day 10 during the plateau phase, at day 21 where the adaptive immune response has been established and at day 62, when a big part of bacteria has been cleared.

Both groups of mice, B-WT and B-MyD88$^{-/-}$, were sacrificed at different days and the bacterial load was determined (Figure 12). At day 62, both groups of mice effectively cleared attenuated *Salmonella* from the liver, whereas bacteria clearance from the spleen was only completed by day 90, again equally between the two groups (data not shown). Conclusively, MyD88 in B cells is not required for the control of the bacterial load during primary infection with attenuated *Salmonella* in the affected organs.

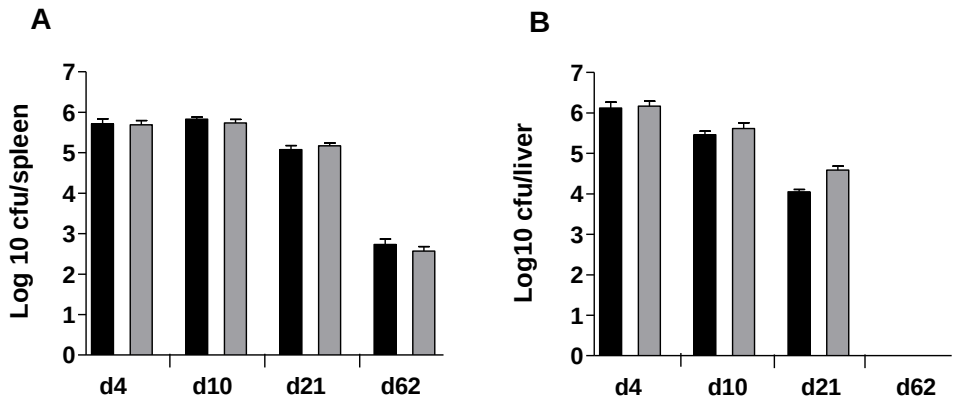

Figure 12: Baterial titres in spleen and Liver after infection of B-WT (black bars) and B-MyD88$^{-/-}$ (gray bars) with *S. typhimurium*. Mice were infected with 1×10^6 SL 7207 intravenous and on the described days (d4, d10, d21 and d62) after infection the mice were sacrificed. Spleen (A) and liver (B) were homogenized and plated on MacConkey agar plates and the colonies forming unit (CFU) were counted. Mean±SEM; spleen n > 14; liver n > 13.

5.3.2 B cells deficient for MyD88 produce a delayed and attenuated humoral response to *S. typhimurium*

In B cells, MyD88 is considered as a cell autonomous amplifier of humoral immunity. B cell activation through TLRs leads to a polyclonal activation and production of low affinity IgM antibodies [263], and it is also reported that MyD88 is required for the generation of long-term humoral immunity during live virus infection [214]. To assess the importance of MyD88 function in B cells during *S. typhimurium* infection, the dynamic of the B cell response in spleen and bone marrow was followed.

5.3.2.1 Numbers of conventional B-2 cells, follicular B cells and marginal zone B cells in B-MyD88$^{-/-}$ and in B-WT during *Salmonella* infection

The spleen is a principal site for the induction of antibody responses to blood-borne pathogens. B cell-mediated immune reaction starts with an early antibody response, which is provided by short-lived plasma cells mainly generated from marginal zone B cells, and follicular B cells, which usually provide long-lasting humoral protection following the accumulation of antigen specific long lived plasma cells in bone marrow.

In order to obtain a complete survey of the B cell compartment in B-WT and B-MyD88$^{-/-}$ mice, the absolute numbers of B-2 cells, follicular B cells and marginal zone B cells were determined (Figure 13) by flow cytometry.

Salmonella stimulated a rapid accumulation of B cells in spleens (Figure 13-A). At the peak of the response, B cell numbers had approximately doubled in both types of mice, although B-MyD88$^{-/-}$ had fewer B cells than B-WT mice. After day 4, the numbers of splenic B cells progressively declined. This decrease was specific to B cells, because the numbers of total splenocytes continued to increase until day 21 post-infection (data not shown). Follicular B cells increased in the spleen at day 4 (Figure 13-B), followed by a decrease until day 62. B-MyD88$^{-/-}$ have less follicular B cells than the B-WT in all the time points. Marginal zone B cells were rapidly activated and accumulated at the early stage of the immune response, day 4, but after the number decreased until day 21. At day 62 MZ B cells increased again for both groups of mice. In contrast to follicular B cells, MZ B cells are 2 fold more in B-MyD88$^{-/-}$ mice on day 4 and on day 10 compare to the B-WT mice. Altogether it turns out that B-MyD88$^{-/-}$ mice have fewer splenic B cells. Wether this difference has implications for humoral immune response will be shown in the next paragraphs.

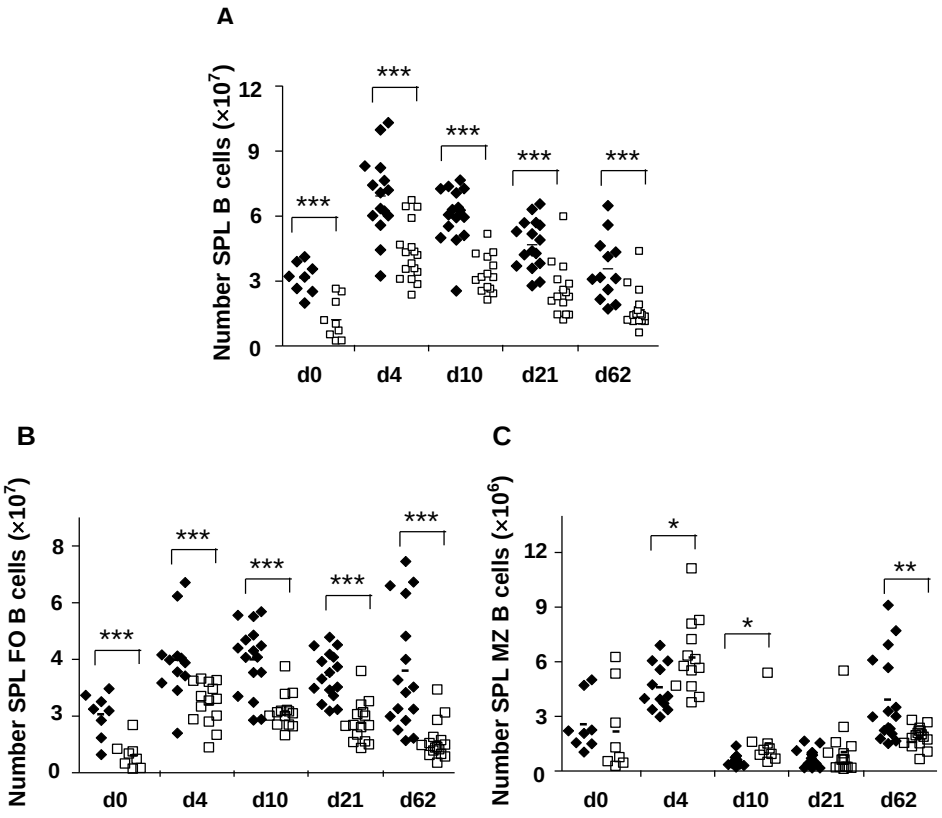

Figure 13: Numbers of total B cells and B cell subsets in spleen of naïve and infected mice. B-WT mice (♦) and B-MyD88$^{-/-}$ (□) were infected i.v. with 10^6 live attenuated *S. typhimurium*. A), total numbers of B220$^+$ B cells in spleen. B), total numbers of B220$^+$ CD23^{hi} CD21$^+$ follicular B cells. C), total numbers of B220$^+$ CD23$^{lo/-}$ CD21^{hi} marginal zone B cells in spleen. Data represent the compilation of three independent experiments; mean±SEM; n> 8, (* for p<0.05, ** for p<0.01 and *** for p<0.0001).

5.3.2.2 Less germinal centre B cells in MyD88-B cell deficient mice

Long-lived plasma cells and memory B cells develop by differentiation of B cells that have proliferated and undergone affinity maturation in germinal centres (GC).

In order to investigate whether MyD88 in B cells has a role in the formation of germinal centres, B-MyD88$^{-/-}$ and B-WT GC B cells were determined by FACS and by histology.

GCs could be detected already at day 10 after infection in B-MyD88$^{-/-}$ and B-WT mice (Figure 14-A and B). This response was decreased in B-MyD88$^{-/-}$, which contained only 0.89±0.16% (mean±SEM) of GC B cells compared to 1.33±0.16% (mean±SEM) of GC B cells in B-WT mice at day 10. Thus, MyD88-signaling in B cells contributes to the GC reaction during *Salmonella* infection.

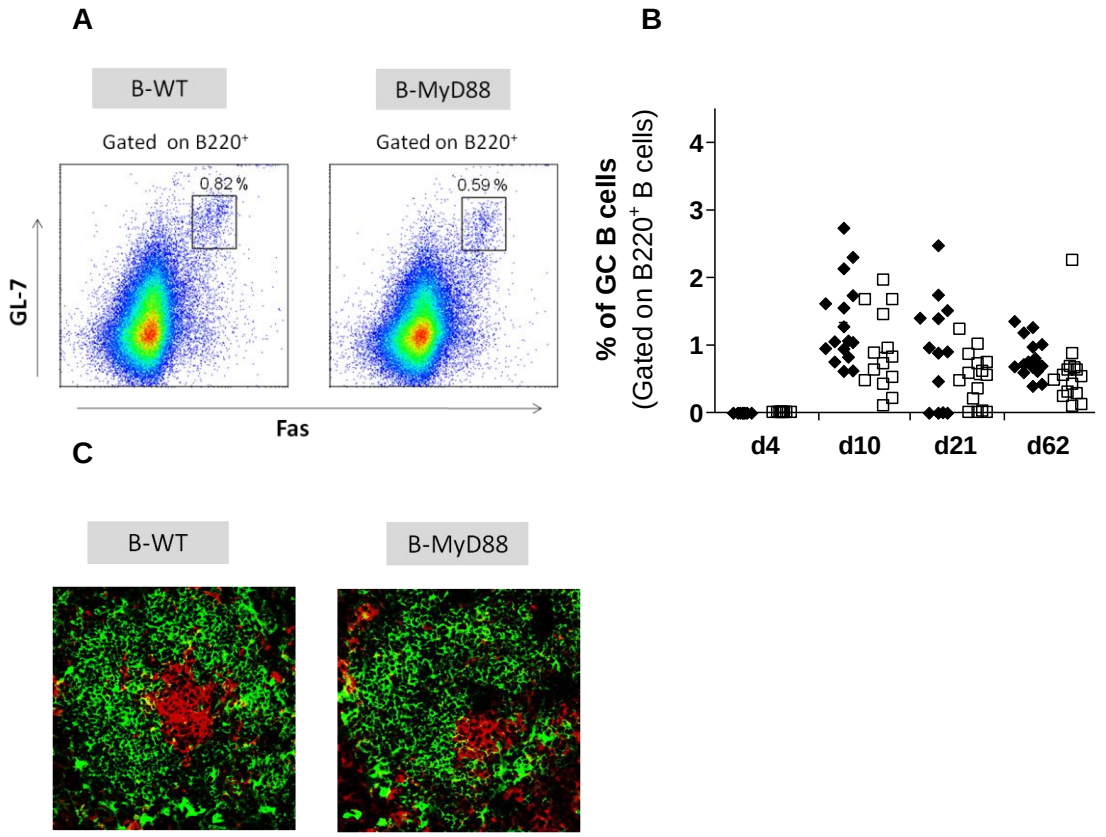

Figure 14: Reduced germinal centre B cells in B-MyD88$^{-/-}$ mice. A), Representative staining for splenic germinal centre B cells of infected mice on day 10. B), Frequency of B220^{+} GL7^{+} Fas^{+} germinal centre B cells of B-WT (♦) and B-MyD88$^{-/-}$ (□) infected mice with 10^{6} attenuated *S. typhimurium* in spleen. C), Representative staining of germinal centres from splenic sections prepared 10 days after immunization by immunofluorescence staining with PNA (red) and IgM Alexa 488 (green). Graph B), represents the compilation of three independent experiments; mean±SEM; n> 8.

5.3.2.3 MyD88 in B cells amplifies humoral immune response

After an immune challenge, some activated B cells differentiate into short-lived plasma cells mostly secreting IgM antibodies that supply an early layer of humoral protection. The mechanisms involved in this response are not yet fully discovered. In B-WT mice, we observed a striking accumulation of MHC-II^{int} CD138^{+} plasma cells at day 4 after infection. The number of these plasma cells then progressively decreased over time (Figure 15-B). The kinetic of this response was altered in B-MyD88$^{-/-}$ mice, in which the number of plasma cells was significantly reduced at day 4. It then continued to increase until day 10 when it reached control levels. Thus, MyD88 functions in B cells act as an accelerator and an amplifier of the early plasma cell response. This may be particularly important for protection from infections primarily controlled by antibodies. Regarding to the previous results, GC B cells were decreased in MyD88-deficient B cell mice, which suggests that MyD88 potentiates B cell activation at an early stage, possibly at the initiation of the B cell priming i.e. before the cell fate decision to become either a short-lived plasma blast or a GC B cell has been made.

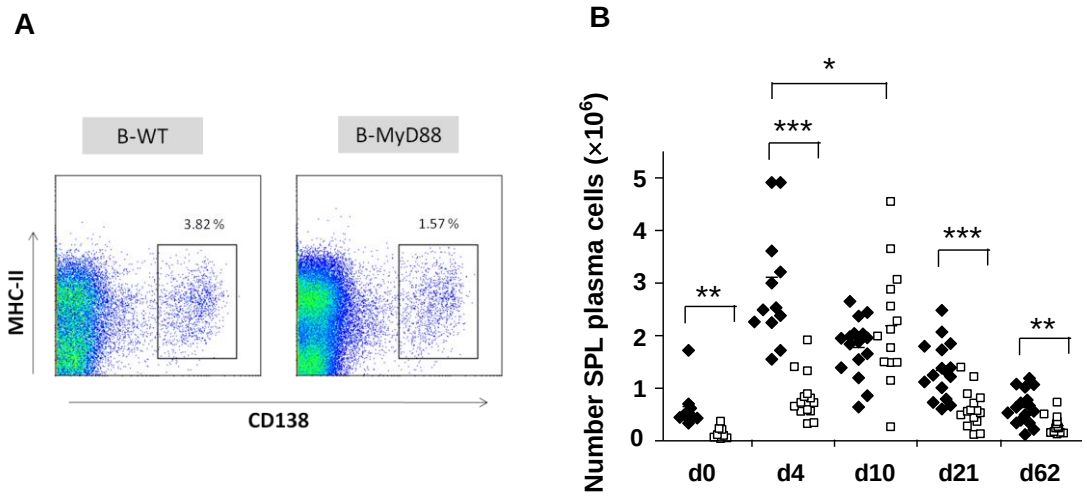

Figure 15: Reduction of plasma cells in B-MyD88$^{-/-}$ mice in early response to *Salmonella*. A), Representative staining for frequency of plasma cells in spleen at day 4. B), Total numbers of MHC-II^{int} CD138^{+} plasma cells of naïve and infected B-WT (♦) and B-MyD88$^{-/-}$ (□) mice with 10^6 attenuated *S. typhimurium* in spleen. mean±SEM; n> 8, (* for p<0.05, ** for p<0.01 and *** for p<0.0001).

Antibodies are important for protection from *Salmonella* particularly during the early stages of infection, while they have little effects at later phases when the bacteria are already intracellular [264]. The kinetics of the specific antibody response in B-WT and B-MyD88 were followed. The natural amounts of IgM before immunization as well as levels of *Salmonella* reactive IgM antibodies were significantly reduced in B-MyD88$^{-/-}$ mice (Figure 16-A and B). These mice also showed a delayed *Salmonella*-specific IgM response, which took 3 weeks to reach control levels (Figure 16-A). This may be due to the impairment of their early immune response in spleen (Figure 15-B). The *Salmonella*-specific IgG response became detectable around two weeks after infection, and it was also delayed in B-MyD88$^{-/-}$ mice (Figure 16-C). Beyond these initial differences, the 2 types of mice had similar titres of *Salmonella*-specific IgM and IgG antibodies from day 30 to day 62. The analysis of the different IgG isotypes revealed an enhanced *Salmonella*-specific IgG1 response in B-MyD88$^{-/-}$ mice (Figure 16-F), while the IgG2b and IgG2c titres were similar to controls. In contrast, B-MyD88$^{-/-}$ mice produced less *Salmonella*-specific IgG3 (Figure 16-G).

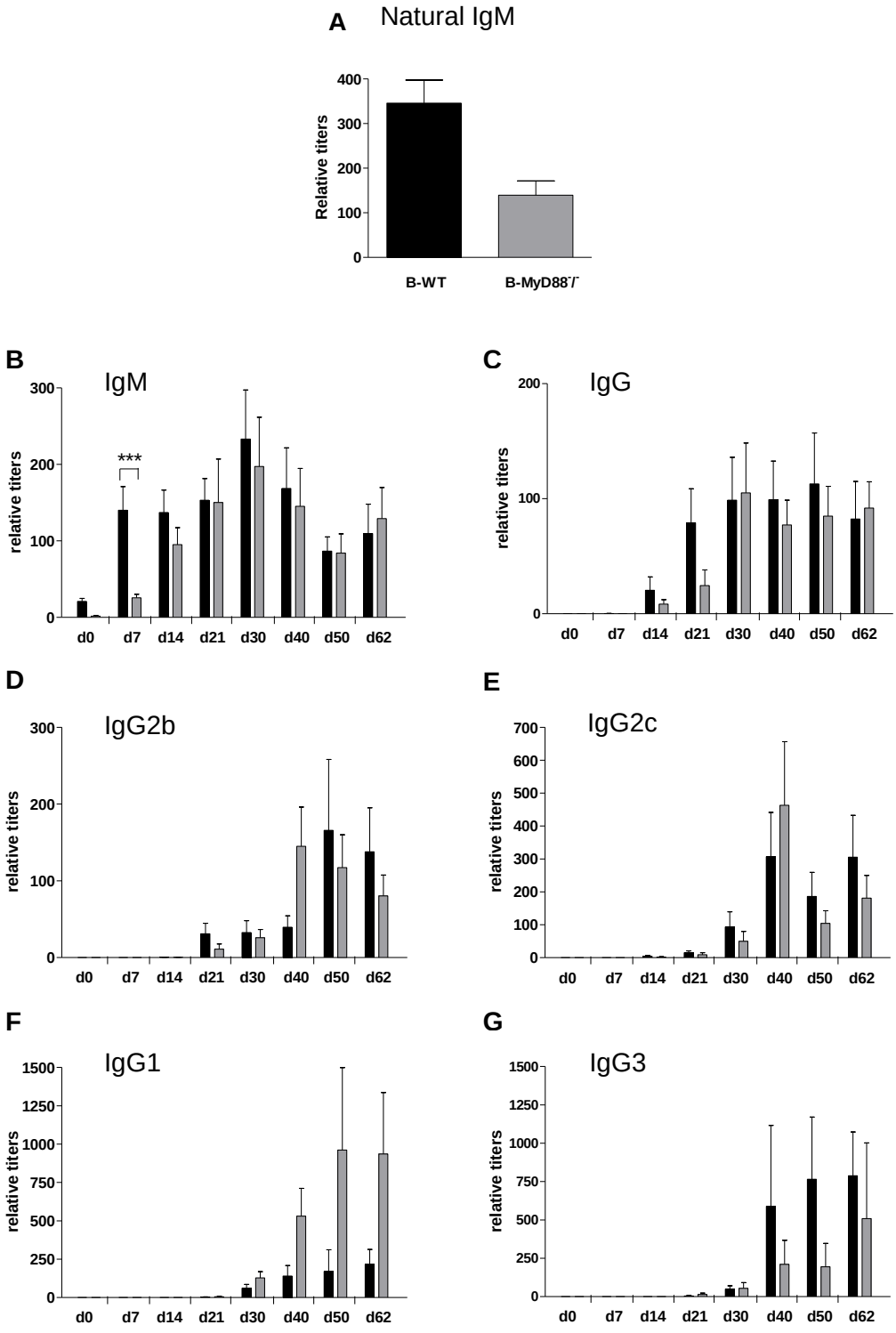

Figure 16: Relative titers of serum from naïve and infected B-WT (Black Bars) mice and B-MyD88$^{-/-}$ (gray bars) mice. Mice infected with 10^6 attenuated *Salmonella* SL 7207and serum levels were determined at day 7, 14, 21, 30, 40, 50 and 62. A), Natural IgM antibodies. B), C), D), E) and G) *Salmonella* -specific IgM, IgG, IgG2b, IgG2c, IgG1 and IgG3 respectively. Data represent the compilation of 3 independent experiments. Mean±SEM, (*** for $p<0.0001$).

Conclusively, MyD88 signalling in B cells was not strictly required for any of the isotypes investigated. MyD88 acts as a cell autonomous amplifier for all the facets of the B cell

response. Nevertheless, none of the parameters analysed were completely blocked in the absence of MyD88 in B cells, indicating that other signalling pathways are sufficient to produce some B cell response, and remarkably, specific antibody titres eventually reached almost normal levels in B-MyD88$^{-/-}$ mice.

5.3.2.4 Reduction of bone marrow plasma cells in MyD88-deficient B cell mice

Long-lived plasma cells that reside in bone marrow are important for long term protection [265]. To examine whether there is an accumulation of plasma cells in bone marrow of mice lacking MyD88 in B cells, MHC-II^{int} CD138^{+} plasma cells in bone marrow were monitored by flow cytometry at various time points.

Plasma cells accumulated in bone marrow at later time points (Figure 17-B). At day 62, B-MyD88$^{-/-}$ mice had about 3-fold less plasma cells than B-WT mice (Figure 17-B).

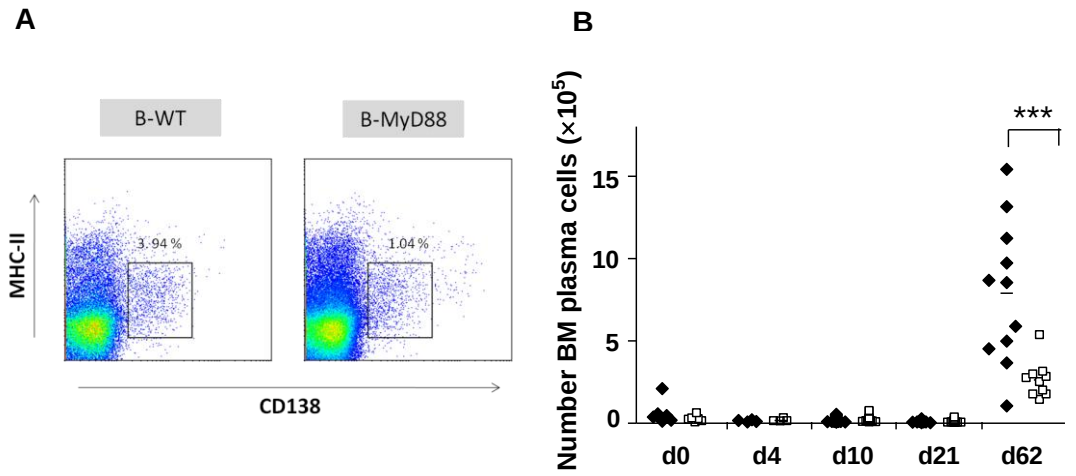

Figure 17: Reduction of plasma cells in B-MyD88$^{-/-}$ bone marrow mice. A), Representative staining of frequency of plasma cells in bone marrow at day 62. B), total numbers of MHC-II^{int} CD138^{+} plasma cells of B-WT (♦) and B-MyD88$^{-/-}$ (□) infected mice with 10^6 attenuated *S. typhimurium* in spleen. mean±SEM; n> 8, (*** for p<0.0001).

Consistent with this, the production of antibodies by bone marrow cells cultivated *in vitro* from B-MyD88$^{-/-}$ mice was reduced compared to cells from B-WT mice (Figure 18). All the antibody isotypes, except IgG1, were reduced in MyD88-deficient B cell mice. The isotype IgG1 was the only antibody increased in the earlier time points of infection in the B-MyD88$^{-/-}$ mice but this level was reached by the control mice at day 62. Finally, MyD88 signalling in B cells may be important for the long-lived humoral response.

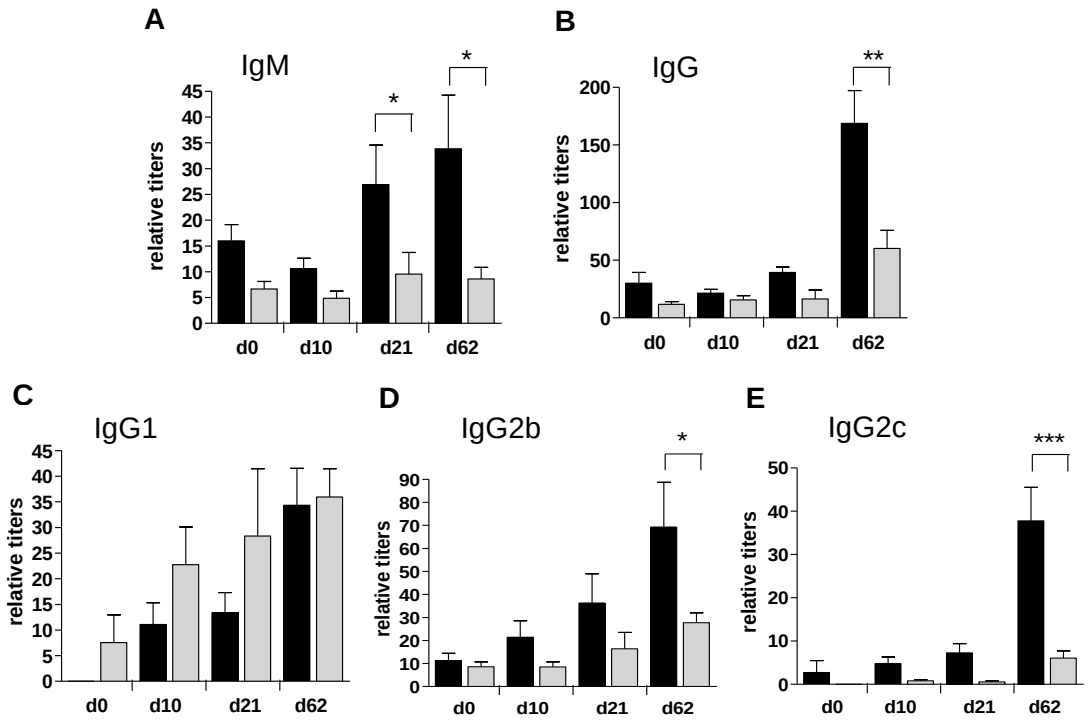

Figure 18: Relative titers of antibodies in bone marrow supernatants from naïve and infected B-WT (black bars) mice and B-MyD88$^{-/-}$ (gray bars) mice. The mice were infected with 10^6 attenuated *Salmonella* SL7207and sacrificed at day 10, day 21 and day 62. Antibody levels were measured in bone marrow supernatants from cultured bone marrow cells. A), B), C), D), and E) Non antigen-specific IgM, IgG, IgG1, IgG2b and IgG2c respectively. Data represent the compilation of 3 independent experiments. Mean±SEM, (* for $p<0.05$, ** for $p<0.01$ and *** for $p<0.0001$).

5.3.3 Dual roles of MyD88 in innate immunity

In previous studies it was shown that MyD88-activated B cells produce cytokines such as IL-10 that can inhibit DC *in vitro* [54]. This suggested that MyD88-signalling in B cells could suppress the innate immune response to *Salmonella.*

Neutrophils are the first cells recruited at sites of infection. In spleen, Gr-1^{high} MHC-II^{-} neutrophils increased until day 21 for both chimera mice after infection (Figure 19-A). Notably, the early phase of this response was significantly amplified in B-MyD88$^{-/-}$ mice, which had more neutrophils than B-WT mice at day 4 (Figure 19-A). This tendency was still evident at day 10 but no longer statistically significant. Similar results were obtained using CD11b and Gr-1 to identify neutrophils (data not shown). This elevated response was not due to differences in bacterial burdens, because they were similar in both types of mice (Figure 12). Therefore, MyD88-signalling in B cells inhibits the accumulation of neutrophil in infected spleens. In contrast, the recruitment of neutrophils was severely impaired at day 4 in MyD88-deficient mice. However it reached control levels at day 21 after infection (Figure 19-

B). This reduced response in MyD88-deficient mice was visible in higher bacterial burden in the spleen (data not shown). Together, these results suggest a dual role for MyD88 in the neutrophil response: MyD88-signalling in some cells promotes the accumulation of neutrophil at infected sites, but MyD88-signalling in B cells specifically antagonizes this activity.

The accumulation of macrophages at infected sites is required to stop the expansion of *Salmonella* [266, 267]. Interestingly, both groups of chimera mice show an enlarged splenic macrophage compartment already at day 4 after infection (Figure 19-C). Moreover, B-MyD88$^{-/-}$ mice contained significantly more splenic macrophages than B-WT mice on day 10 and on day 21, indicating that MyD88-signalling in B cells inhibits the accumulation of macrophages in infected spleens. In contrast, the macrophage response was strongly impaired at day 4 in MyD88-deficient mice, although it reached control levels at day 21 (Figure 19-D). Consequently, MyD88-signalling in some cells controls the accumulation of macrophages at infected sites, but MyD88-signalling in B cells limits this innate immune response.

NK cells play an important role in immune defence against *Salmonella* [268, 269]. Among other functions, NK cells are considered to be as the major source of.macrophage-activating cytokine IFN-γ. Given the accumulation of macrophages in spleen it was therefore important to investigate the IFN-γ production by NK cells. *Salmonella* stimulated a rapid IFN-γ production by NK cells at day 4 (Figure 19-E). Remarkably, B-MyD88$^{-/-}$ cell deficient mice had significantly more IFN-γ-producing NK cells than control mice on day 4 (Figure 19-E), while the total numbers of NK cells were comparable (data not shown). This result, suggests that MyD88-signalling in B cells strongly inhibits the production of IFN-γ by NK cells. In MyD88-deficient mice, the NK cell response was almost completely impaired at day 4 and day 21. This suggests that MyD88-signalling in some cells abrogates NK cell activation, while MyD88-signalling in B cells reduces NK cell activation possibly by producing inhibitory signals via B cells.

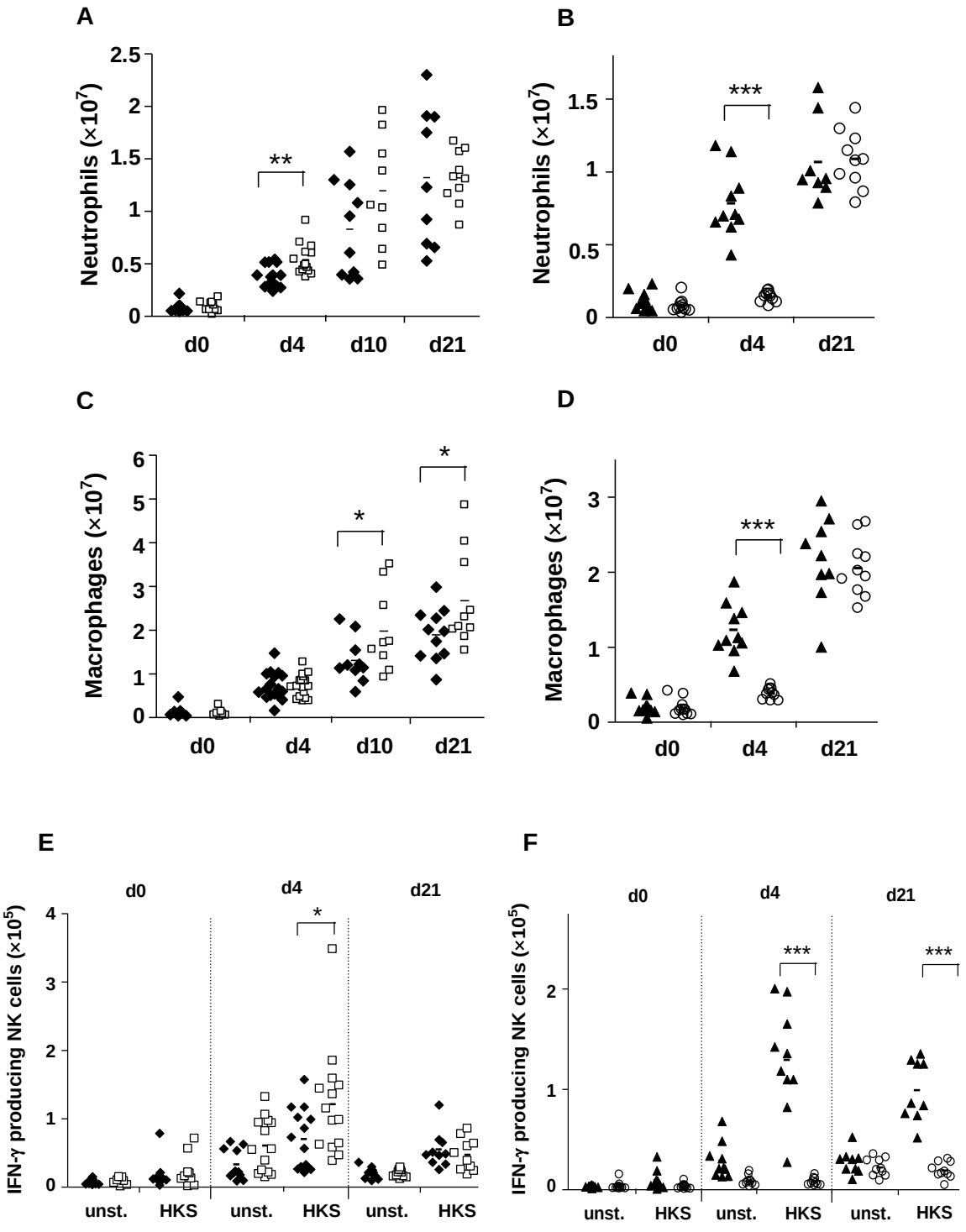

Figure 19: Mice in which B cells lack MyD88 have an increased innate immune response contrary to the MyD88-deficient B cell mice. B-MyD88$^{-/-}$ (□) and B-WT (♦) chimera mice, and C57BL/6 (○) and MyD88$^{-/-}$ (▲) mice were infected with 10^6 live attenuated *S. typhimurium* i.v.. A) and B), Total numbers of neutrophils in spleen. C) and D), Total numbers of splenic CD11b^{bright} macrophages. E) and F), Total numbers of IFN-γ$^+$ producing NK cells, splenic cells were stained for DX5 and TCR-β. IFN-γ production was determined by intracellular cytokine analysis after short-term culture with and without HKS. Mean±SEM, n > 9, (* for p<0.05, ** for p<0.01 and *** for p<0.0001).

5.3.3.1 The role of MyD88 in B cells in dendritic cell numbers

Dendritic cells are critical for both innate and adaptive immunity. They are able to secrete high amounts of IL-12 and TNF-α, which stimulate IFN-γ production by NK cells [270, 271], and IL-6 [231] and IFN-γ [272].

Salmonella infection stimulated a progressive increase in the number of splenic DCs in both B-WT and B-MyD88$^{-/-}$ mice (Figure 20-A). Notably, B-MyD88$^{-/-}$ mice had slightly more splenic DC than B-WT mice on day 10. This difference became statistically significant on day 21 after infection. On the contrary, in MyD88-deficient mice the number of DCs was significantly lower on day 4, reaching control levels at day 21 (Figure 20-B).

Lack of MyD88 in B cells results in less B cells, but slightly more DCs and macrophages (Figure 20-C). DCs and macrophages are important APCs during bacterial infections and this increase might represent a stronger induction of T cell responses through antigen presentation and cytokine production [51, 273-278].

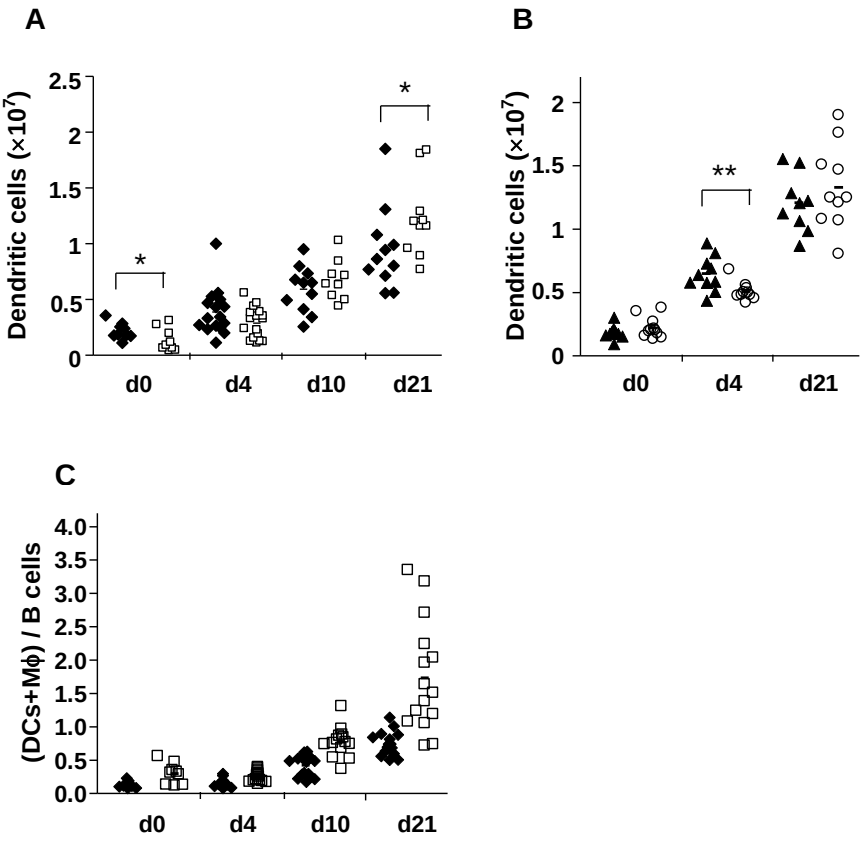

Figure 20: B-WT (♦) and B-MyD88$^{-/-}$ (□) chimera mice, and C57BL/6 (▲) and MD88$^{-/-}$ (○) mice were infected with 10^6 live attenuated *Salmonella* i.v..A) and B), total numbers of CD11c$^+$ dendritic cells determined by flow cytometry. C), ratio of DCs and macrophages to B cells. The ratio was calculated with the absolute cell numbers. Mean±SEM, n>9, (* for $p<0.05$ and ** for $p<0.01$).

5.3.3.2 Effects of MyD88-signalling in B cells on GM-CSF mRNA level in splenocytes from naïve and infected mice.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a protein secreted by several cells, as for example, T cells and macrophages. It acts on bone marrow cells to stimulate the production of macrophages, granulocytes and dendritic cells.

To investigate the underlying events that lead to the increased numbers of neutrophils, macrophages and dendritic cells in MyD88-deficient B cell mice, the expression of GM-CSF level was measured. For this purpose, splenocytes from naïve and infected mice at day 4 were obtained and their RNA was reverse transcribed to cDNA. With this cDNA as template, a real-time PCR was performed for the amplification GM-CSF. Cytokine mRNA levels were always calculated relative to the expression level of the β-actin of the respective mouse. GM-CSF was upregulated in all mice after infection (Figure 21). B-MyD88$^{-/-}$ mice had higher levels of GM-CSF compare to the B-WT mice. In contrast, the level of GM-CSF in MyD88-deficient mice was much less than in wild type mice (Figure 21). Conclusively, MyD88 signalling in B cells inhibits GM-CSF production.

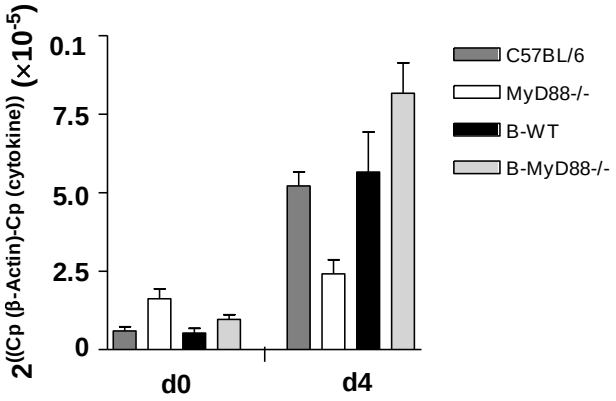

Figure 21: GM-CSF production by splenocytes from naïve and infected mice. C57BL/6 (dark gray bars) and MD88$^{-/-}$ (white bars) mice, and B-WT (black bars) and B-MyD88$^{-/-}$ (light gray bars) chimera mice, were infected with 10^6 live attenuated *Salmonella* i.v and sacrificed at day 4. Quantification was performed by Real-time PCR. All samples were referred to β-actin. The error bars represent the maximal differences between 5 mice per group. Only one experiment was done.

5.3.4 Dual roles for MyD88 in adaptive T cell response to *Salmonella*

5.3.4.1 Role of MyD88 in splenic CD4$^+$ T cell compartment

CD4$^+$ T cells are of particular importance for the sterile resolution of *Salmonella* infection. It has been shown that MyD88-signalling in B cells inhibits T_H1 response during EAE, suggesting that MyD88 coordinates inflammatory T cell responses via a combination of

inhibitory and stimulatory signals [54]. In order to test whether B cells operate a similar mechanism during *Salmonella* infection, the chimera mice were infected as described before and T cell response was determined by flow cytometry.

The numbers of splenic $CD4^+$ T cells increase progressively after infection until day 21 for all groups of mice (Figure 22). B-$MyD88^{-/-}$ mice accumulated significantly more $CD4^+$ T cells on day 21 compared to the B-WT mice (Figure 22-A). Thus, MyD88-signalling in B cells inhibits the expansion of $CD4^+$ T cell compartment in infected spleens. It seems that B cells are the principal mediators of this effect, since the MyD88-deficient mice had significantly fewer $CD4^+$ T cells than the control mice at day 21 (Figure 22-B).

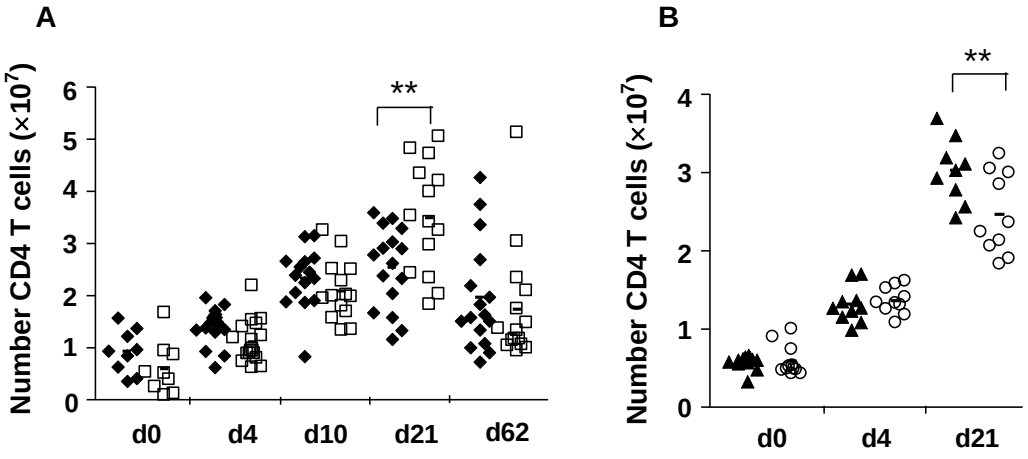

Figure 22: Increase of $CD4^+$ T cells during infection of *Salmonella typhimurium*. B-WT (♦) and B-$MyD88^{-/-}$ (□) chimera mice, and C57BL/6 (▲) and $MD88^{-/-}$ (○) mice were infected with 10^6 live attenuated *Salmonella* i.v..A) and B), total numbers of $CD4^+$ T cells determined by flow cytometry. Mean±SEM, n> 9, (** for p<0.01).

5.3.4.2 MyD88-signalling in B cells influences IL-2 secretion by $CD4^+$ T cells at later time point of infection

IL-2 is secreted by activated T cells and is a potent inducer of T cell proliferation, and T_H1 and T_H2 effector T cell differentiation [102]. In addition to its effects on $CD4^+$ and $CD8^+$ T cells, IL-2 also stimulates NK cells to proliferate and induces cytolytic activity when present at high levels. Furthermore IL-2 is known to be required for the development of memory T cells [279].

To investigate the underlying events that lead to the observed increased number of $CD4^+$ T cells as well NK cells in MyD88-B cell deficient mice, the IL-2 production by $CD4^+$ T cells was quantified in infected mice. For this purpose, splenocytes were re-stimulated with *Salmonella*

for 6 hours *in vitro*, and then analysed co-expression of CD4 and IL-2 by flow cytometry. In B-WT and B-MyD88$^{-/-}$ mice, it was detected high numbers of IL-2 producing CD4 T cells at day 10 and at day 21 after infection, and MyD88-signalling in B cells had no influence on this response (Figure 23-A). The number of IL-2-producing CD4^{+} T cells then decreased significantly at day 62 for both groups. Interestingly, B-MyD88$^{-/-}$ mice had significantly more IL-2-producing CD4^{+} T cells than B-WT mice at day 62. This observation suggests that B-MyD88$^{-/-}$ mice still had a stronger induction of inflammatory response at day 62 compared to the B-WT mice, since both groups of mice still had bacteria in the spleen at this time point. In contrast, it was detected less IL-2 secreting CD4^{+} T cells in MyD88-deficient mice than in wild-type mice, although this response progressively emerged, demonstrating that other signalling pathways can partially compensate for the absence of MyD88 (Figure 23-B).

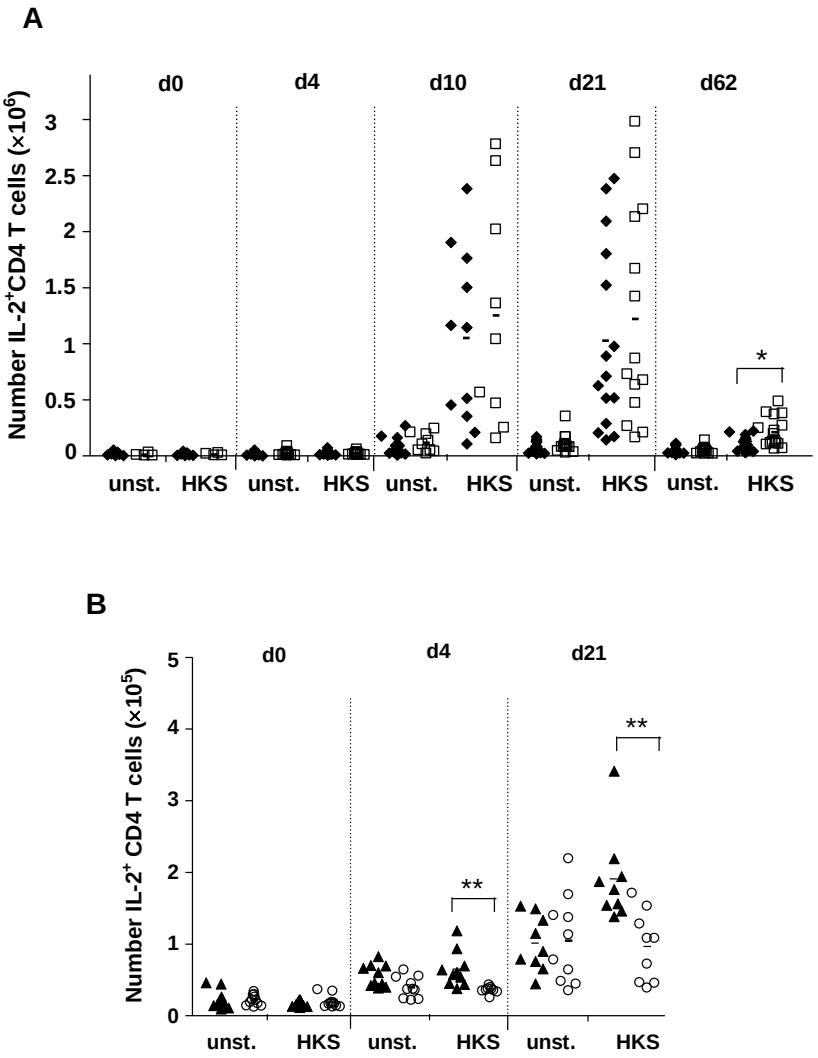

Figure 23: Increased of IL-2 production of CD4$^+$ T cells after *Salmonella* infection. B-WT (♦) and B-MyD88$^{-/-}$ (□) chimera mice, and C57BL/6 (▲) and MD88$^{-/-}$ (○) mice were infected with 10^6 live attenuated *Salmonella* i.v..A) and B), total numbers of IL-2 producing CD4$^+$ T cells determined by flow cytometry. Mean±SEM, n> 9, (* for p<0.05 and ** for p<0.01).

5.3.4.3 Activation of *S. typhimurium*-specific CD4$^+$ T cells

It has been described in other studies that after attenuated *Salmonella* infection of susceptible mice there are large numbers of *Salmonella* -specific CD4$^+$ T cells and CD8$^+$ T cells [280, 281]. To examine if MyD88-signalling in B cells affects CD4$^+$ antigen specific T cells in infected mice, the splenocytes were restimulated with *Salmonella* for 6 hours *in vitro*, and then analysed for co-expression of CD4 and CD154. According to this technology, *Salmonella* -reactive CD4$^+$ T helper cells could be detected at day 10 after infection, reaching a peak at day 21 in B-MyD88$^{-/-}$ and B-WT mice, with no influence from MyD88-signalling in B cells (Figure 24-B). B-WT and B-MyD88$^{-/-}$ mice still had detectable numbers of antigen-reactive CD4$^+$ T helper cells on day 62. Contrary, the MyD88-deficient mice had only very

few CD154-expressing $CD4^+$ T cells in all the time points (Figure 24-C), this confirms that MyD88 plays an essential role for $CD4^+$ T cell activation only in some specific cells.

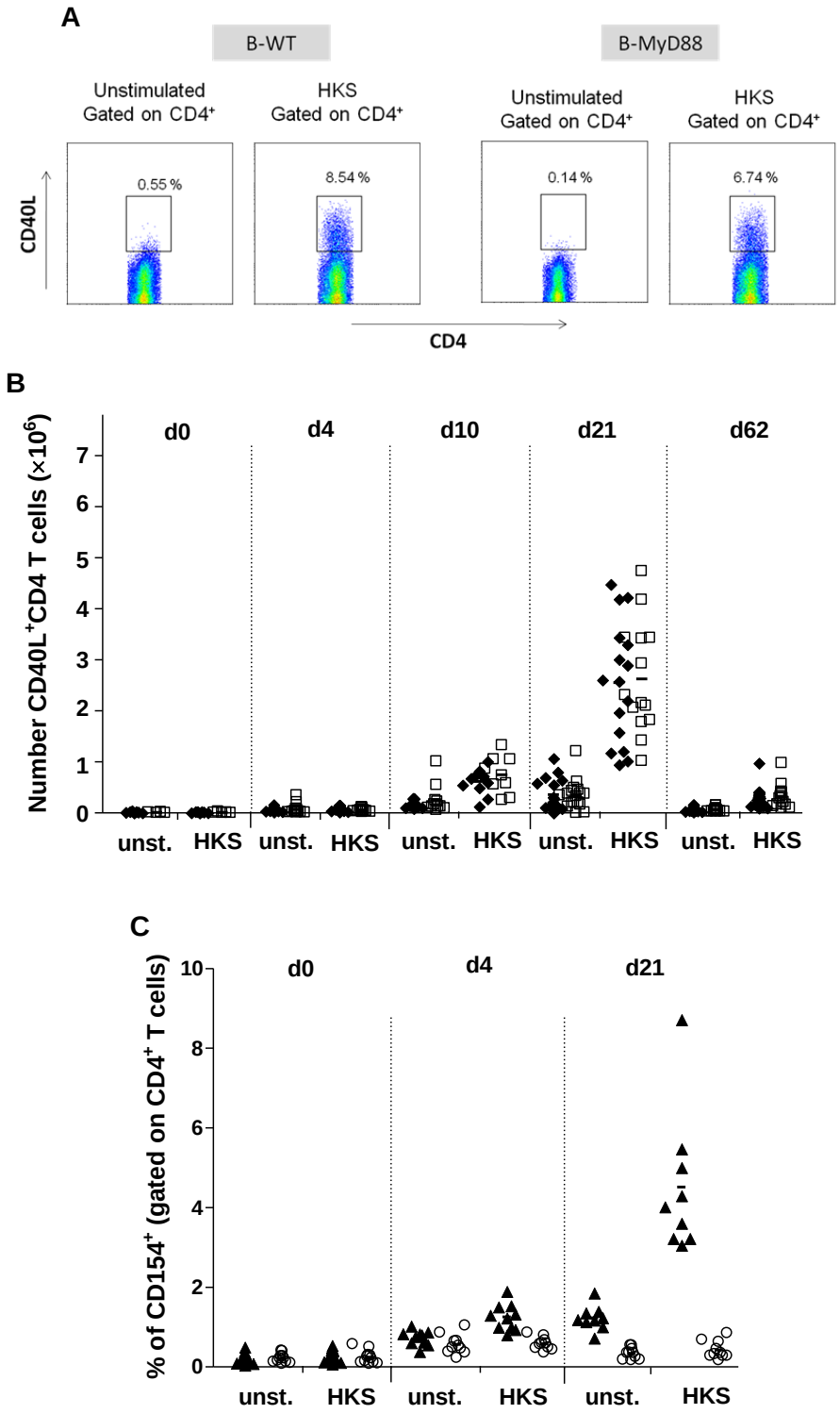

Figure 24: Activation of *Salmonella* -specific $CD4^+$ T cells in mice. B-WT (♦) and B-$MyD88^{-/-}$ (□) chimera mice, and C57BL/6 (▲) and $MD88^{-/-}$ (○) mice were infected with 10^6 live attenuated *Salmonella* i.v.. A), Representative staining for CD40L (CD154) of unstimulated and stimulated cells with HKS. B) and C), Frequency numbers of $CD40L^+$ among $CD4^+$ T cells. Data shown represent the compilation of at least two experiments. Mean±SEM, n>9

5.3.4.4 MyD88-signalling in B cells supresses IFN-γ and TNF-α production by $CD4^+$ T cells

S. typhimurium induces a strong T_H1 response, in which cytokines, such as IFN-γ and TNF-α play an important role in controlling *Salmonella* infection [222]. In order to assess the consequences of MyD88-activation in B cells for $CD4^+$ T cell response, IFN-γ and TNF-α production were determined in naive and infected mice by flow cytometry after a short *ex vivo* re-stimulation of splenocytes with heat-killed *Salmonella*. MyD88 signalling in B cells suppressed the accumulation of IFN-γ and TNF-α producing $CD4^+$ T cells on day 21 (Figure 25-B and E, respectively). These data confirm previous studies performed in the EAE model, where mice lacking MyD88 selectively in B cells make stronger T_H1 and T_H17 responses than control mice [54]. IL-17-producing $CD4^+$ T cells were also assessed, but were undetectable (data not shown), supporting the fact that IL-17 has mild roles during *Salmonella* infection [114]. As expected, MyD88-deficient mice had fewer IFN-γ and TNF-α producing $CD4^+$ T cells than control mice at day 21 (Figure 25-C and F). Surprisingly, in MyD88-deficient mice it was possible to detect much more IFN-γ producing $CD4^+$ T cells than CD154-producing $CD4^+$ T cells, suggesting that this cytokine response was stimulated independenty of the TCR. It is also possible that these two assays have different sensitivities for antigen-reactive CD4+ T cells. Conclusively, MyD88-signalling in B cells downregulates the inflammatory response of $CD4^+$ T cells during *Salmonella* infection.

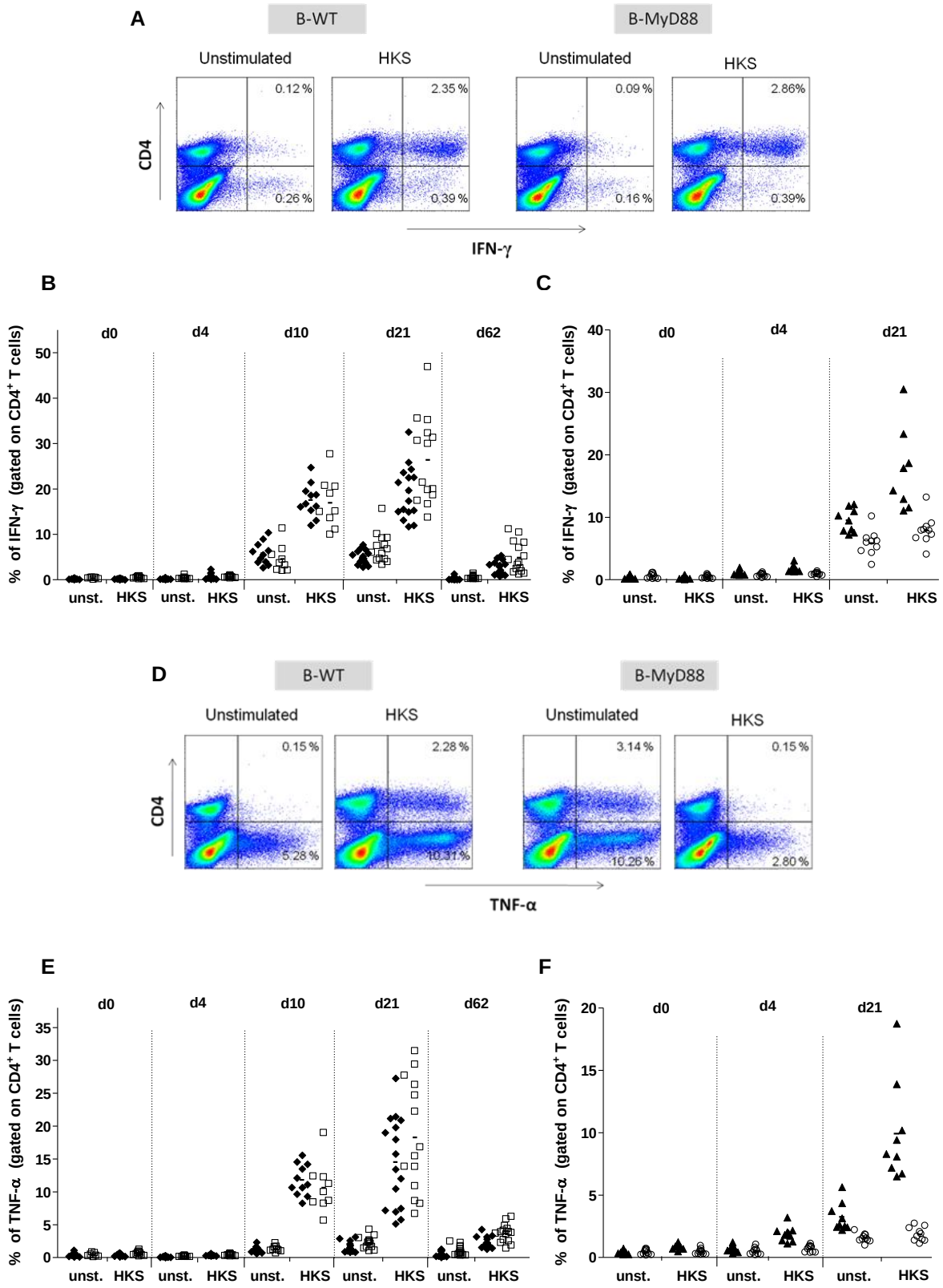

Figure 25: Inflammatory response of CD4$^+$ T cells during *S. typhimurium* infection. B-WT (♦) and B-MyD88$^{-/-}$ (□) chimera mice, and C57BL/6 (▲) and MD88$^{-/-}$ (○) mice were infected with 10^6 live attenuated *Salmonella* i.v.. A) and D) Representative staining for IFN-γ and TNF-α of unstimulated and stimulated splenocytes with HKS, respectively. B) and C) Frequency numbers of IFN-γ$^+$ among CD4$^+$ T cells. E) and F), Frequency numbers of TNF-α$^+$ among CD4$^+$ T cells Data shown represent the compilation of at least two experiments. Mean±SEM, n>9.

5.3.4.5 CD8$^+$ T cell response during *S. typhimurium* infection

Salmonella stimulates a specific CD8 T cell response, which provides some protection during primary infection, and particularly during secondary challenge [121, 238]. To examine the role of MyD88-signalling in B cells on CD8 T cell responses, the number of CD8$^+$ T cells and IFN-γ production by these cells was determined by flow cytometry. B-MyD88$^{-/-}$ mice accumulated more CD8$^+$ T cells than B-WT mice in infected spleens at day 21 (Figure 26-A). MyD88-signalling in B cells also inhibited the production of IFN-γ by *Salmonella*-stimulated CD8 T cells at day 21 after infection (Figure 26-D). In contrast, the CD8$^+$ T cell response was impaired in MyD88-deficient mice (Figure 26-E). Collectively, these data support the concept that MyD88-signalling in B cells antagonizes the T cell activation stimulated by MyD88 signalling in other cells, such as DC and macrophages.

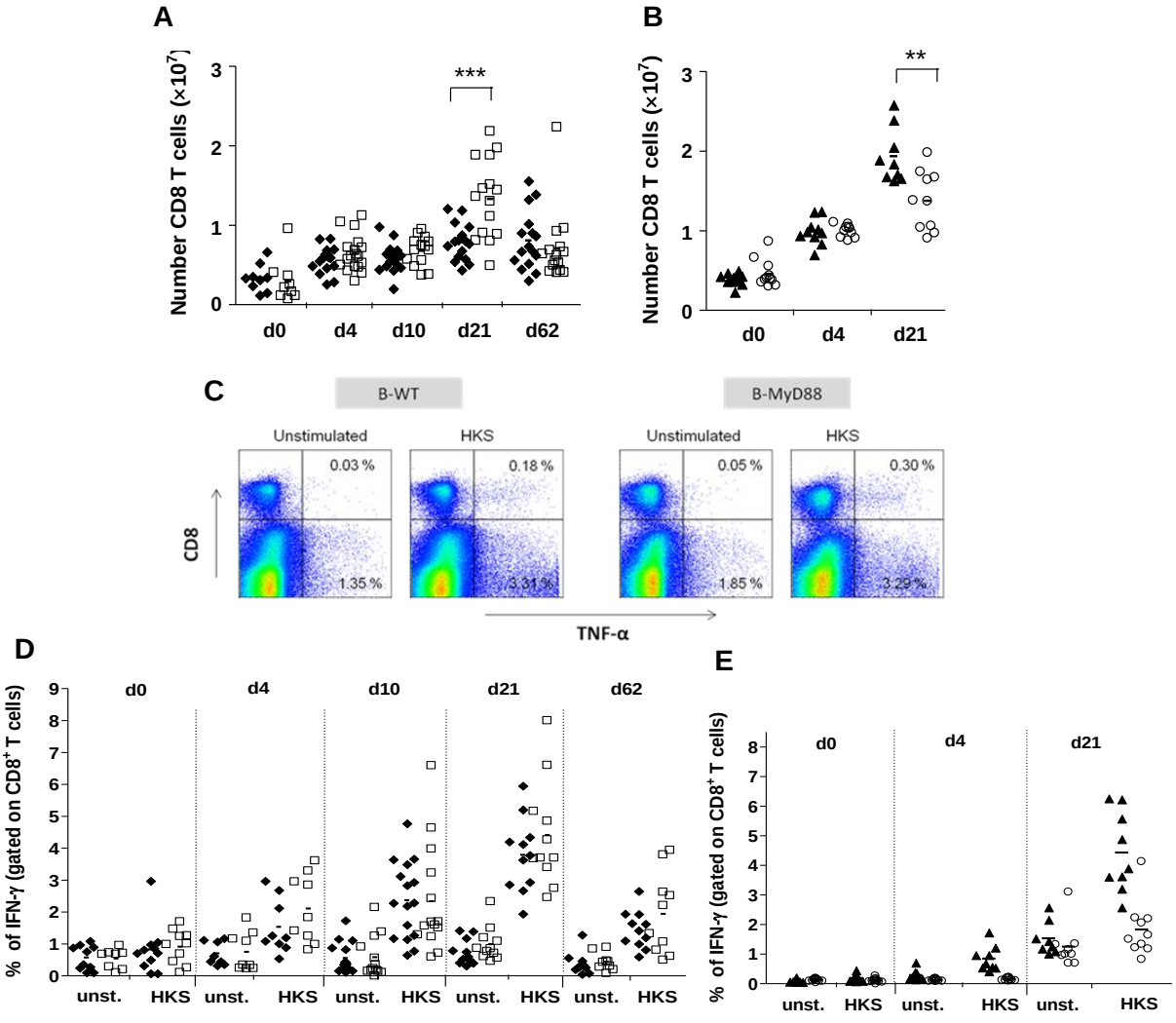

Figure 26: CD8$^+$ T cell response during *S. typhimurium* infection. B-WT (♦) and B-MyD88$^{-/-}$ (□) chimera mice, and C57BL/6 (▲) and MD88$^{-/-}$ (○) mice were infected with 10^6 live attenuated *Salmonella* i.v.. A) and B), Total numbers of CD8$^+$ T cells determined by flow cytometry. C), Representative staining for IFN-γ unstimulated and stimulated splenocytes with HKS. D) and E), Frequency numbers of IFN-γ$^+$ among CD8$^+$ T cells. Data shown represent the compilation of at least two experiments. Mean±SEM, n>9, (** for p<0.01 and *** for p<0.0001)

5.3.4.6 Summary of absolute cell numbers of *Salmonella* -specific $CD4^+$ T cells, and cytokines production by $CD4^+$ T cells and $CD8^+$ T cells.

In addition to the analysis of frequencies of $CD40L^+$ $CD4^+$ T cells, IFN-γ and TNF-α producing $CD4^+$ T cells, and IFN-γ producing $CD8^+$ T cells, the absolute cell numbers were also determined in order to obtain a complete analysis of B-WT, B-MyD88$^{-/-}$ mice, C57BL/6 and MyD88$^{-/-}$ mice. Table 3 and Table 4 summarize the absolute cell numbers in the spleen for the B-WT and B-MyD88$^{-/-}$ chimera mice and C57BL/6 and MyD88$^{-/-}$ mice respectively.

Salmonella -specific $CD4^+$ T cells increased progressively during infection for all mice (Table 3 and Table 4). However the difference was not statistically significant for the MyD88 deficient B cell mice and B-WT mice (Table 3). On the contrary MyD88 deficient mice had very few *Salmonella*-specific $CD4^+$ T cells compare to the WT mice (Table 4). By comparison to B-WT mice the average number of IFN-γ and TNF-α producing $CD4^+$ T cells was increased from 5.2×10^6 to 1.0×10^7 and 4.1×10^6 to 7.5×10^6 in spleens of B-MyD88$^{-/-}$ mice on day 21, corresponding to a 92% and 83% of increase, respectively (Table 3). For IFN-γ producing $CD8^+$ T cells, MyD88-deficient B cell mice had an increase of 104% compared to B-WT mice at day 21 (Table 3).

		$CD4^+$ $CD40L^+$	$CD4^+$ IFN-γ^+	$CD4^+$ TNF-α^+	$CD8^+$ IFN-γ^+
Day 4	B-WT	8.5×10^4 ± 1.2×10^4	7.4×10^4 ± 1.3×10^4	5.2×10^4 ± 3.9×10^3	8.6×10^4 ± 1.8×10^4
	B-MyD88$^{-/-}$	6.1×10^4 ± 8.4×10^3	6.3×10^4 ± 6.7×10^3	3.8×10^4 ± 3.1×10^3	1.5×10^5 ± 3.4×10^4
Day 10	B-WT	6.1×10^5 ± 7.5×10^4	3.4×10^6 ± 3.4×10^5	2.7×10^6 ± 2.6×10^6	1.4×10^5 ± 2.9×10^4
	B-MyD88$^{-/-}$	7.5×10^5 ± 1.2×10^5	4.4×10^6 ± 4.8×10^5	2.4×10^6 ± 2.7×10^5	1.7×10^5 ± 2.8×10^4
Day 21	B-WT	2.5×10^6 ± 2.9×10^5	5.2×10^6 ± 7.7×10^5	4.1×10^6 ± 7.4×10^5	3.1×10^5 ± 4.5×10^4
	B-MyD88$^{-/-}$	2.6×10^6 ± 2.9×10^5	1.0×10^7 ± 1.5×10^6	7.5×10^6 ± 1.3×10^6	6.2×10^5 ± 1.1×10^5
	P value summary	ns	**	*	**

Table 3: Numbers of total *Salmonella* -specific $CD4^+$ T cells, and IFN-γ and TNF-α producing $CD4^+$ T cells, and IFN-γ producing $CD8^+$ T cells in spleen for B-WT and B-MyD88$^{-/-}$ infected mice at day 4, day 10 and day 21 with attenuated *S. typhimurium*, were determined by FACS. Mean±SEM; n>14, (ns for p>0.05; * for p<0.05 and ** for p<0.01).

MyD88 deficient mice showed reductions between 65% and 85% compared to C57BL/6 mice in IFN-γ and TNF-α producing $CD4^+$ T cells at day 21 and IFN-γ production $CD8^+$ T cells show a reduction of 70% (Table 4).

These results confirm again that MyD88 plays an important role specifically in some cells for the activation of adaptive $CD4^+$ T cell immunity.

		$CD4^+ CD40L^+$	$CD4^+ IFN\text{-}\gamma^+$	$CD4^+ TNF\text{-}\alpha^+$	$CD8^+ IFN\text{-}\gamma^+$
Day 4	C57BL/6	$1.6\times10^5 \pm 1.8\times10^4$	$2.3\times10^5 \pm 2.8\times10^4$	$2.4\times10^5 \pm 3.4\times10^4$	$8.7\times10^4 \pm 1.9\times10^4$
	$MyD88^{-}/^{-}$	$7.9\times10^4 \pm 8.9\times10^3$	$1.2\times10^5 \pm 1.2\times10^4$	$9.1\times10^4 \pm 1.2\times10^4$	$1.5\times10^5 \pm 1.6\times10^4$
	P value summary	***	**	***	***
Day 21	C57BL/6	$1.3\times10^6 \pm 1.9\times10^5$	$5.4\times10^6 \pm 7.4\times10^5$	$3.0\times10^6 \pm 4.5\times10^5$	$8.3\times10^5 \pm 7.0\times10^4$
	$MyD88^{-}/^{-}$	$9.7\times10^4 \pm 1.6\times10^5$	$1.8\times10^6 \pm 1.9\times10^5$	$4.4\times10^5 \pm 5.4\times10^4$	$2.5\times10^5 \pm 3.5\times10^4$
	P value summary	***	***	***	***

Table 4: Numbers of total *Salmonella* -specific $CD4^+$ T cells, and IFN-γ and TNF-α producing $CD4^+$ T cells, and IFN-γ producing $CD8^+$ T cells in spleen for C57BL/6 and $MyD88^{-/-}$ infected mice at day 4 and day 21 with attenuated *S. typhimurium*, were determined by FACS. Mean±SEM; n>8, (** for $p<0.01$; *** for $p<0.0001$).

5.3.4.7 Effects of MyD88-signalling in B cells on cytokine mRNA levels in splenocytes from naïve and infected mice.

To corroborate the previous data and to have an overview of all the cells in spleen, RNA of splenocytes from naïve and infected mice at day 4 and day 21 was extracted and transcribed to cDNA as described on section 5.3.3.2. IFN-γ and TNF-α were measured by real-time PCR. IFN-γ was upregulated in all mice after infection (Figure 27-A). In contrast, TNF-α upregulation was evident only for the B-WT and B-$MyD88^{-/-}$ mice (Figure 27-B). MyD88-deficient B cell mice had increased levels of IFN-γ and TNF-α than B-WT mice on day 21, although this difference was not statistically significant. Further repetition of this exeperiment is necessary to statistically validate these results.

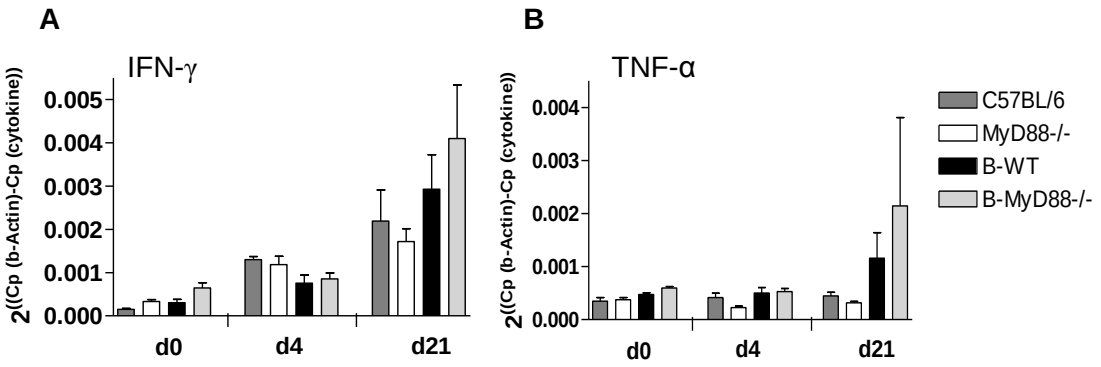

Figure 27: Relative levels of IFN-γ and TNF-α mRNA in spleens from naïve and infected mice. C57BL/6 (dark gray bars) and MD88$^{-/-}$ (white bars) mice, and B-WT (black bars) and B-MyD88$^{-/-}$ (light gray bars) chimera mice, were infected with 10^6 live attenuated *Salmonella* i.v and sacrificed at day 4 and day 21. Quantification was performed by Real-time PCR. All samples were referred to β-actin. The error bars represent the maximal differences between 5 mice per group. Only one experiment was done.

5.3.5 MyD88-signalling in B cells impairs mice survival after a virulent *Salmonella* infection

Cellular immunity (mediated by neutrophils, macrophages, NK cells, DCs, CD4$^+$ T_H1 T cells and CD8$^+$ T cells), and humoral factors (antibodies) synergistically protect the host from *Salmonella* infection. The observations obtained so far showed that apart from the T cell response, innate immunity is also significantly increased in mice lacking MyD88 selectively in B cells. To better document this effect, infection with virulent salmonella was used, which usually leads to mortality of B-WT mice within 10 days. During this short period, it is unlikely that an antigen-specific T cell response is fully developed and could account for this short term host resistance. Rather, the initial response to this acute infection is likely mediated by innate immunity. Therefore, B-MyD88$^{-/-}$ and B-WT mice were infected via the intravenous route with 100 *Salmonella* of the virulent strain SL1344. B-WT started to die on day 6, which is 4 days earlier than B-MyD88$^{-/-}$ mice (Figure 28-A). The median survival time was 8 days for B-WT mice and 12 days for B-MyD88$^{-/-}$ mice. Earlier mortality correlated with higher bacterial loads. In particular, on day 6 B-WT mice had on average over 100-fold more bacteria in their spleens than B-MyD88-/- mice, and 10-fold more bacteria in their livers (Figure 28-B). The lower bacterial load in B-MYD88-/- mice suggests that their innate immune defence was better able to control the initial expansion of the bacteria than the innate immunity in B-WT mice. These data demonstrate that MyD88-signalling in B cells significantly impairs host survival during infection with virulent Salmonella. They further support the notion that MyD88 has a dual role in host protection: MyD88 signalling in some cells extends survival of the infected mice, but MyD88 signalling in B cells has the opposite effect, so that mice lacking My D88 in B cells survive better against S. typhimurium infection.

Together with the observations on the T cell reponse to attenuated strain, they further suggested that B-MyD88-/- mice vaccinated with attenuated Salmonella would be better protected upon rechallenge than the WT mice. To address the question whether MyD88-signalling in B cells induces protection after vaccination, B-WT and B-MyD88$^{-/-}$ mice were initially vaccinated intravenously with 10^6 attenuated *Salmonella* and 90 days later, when mice had already cleared the infection (data not shown), were challenged with 100 virulent *Salmonella* via the intravenous route. At day 60 following challenge, 50% of B-WT mice had died from the virulent infection. Extraordinarily, all the mice with MyD88-deficient B cells were still alive and healthy. In conclusion, this results show that MyD88-signalling in B cells impairs host resistance to *Salmonella* infection.

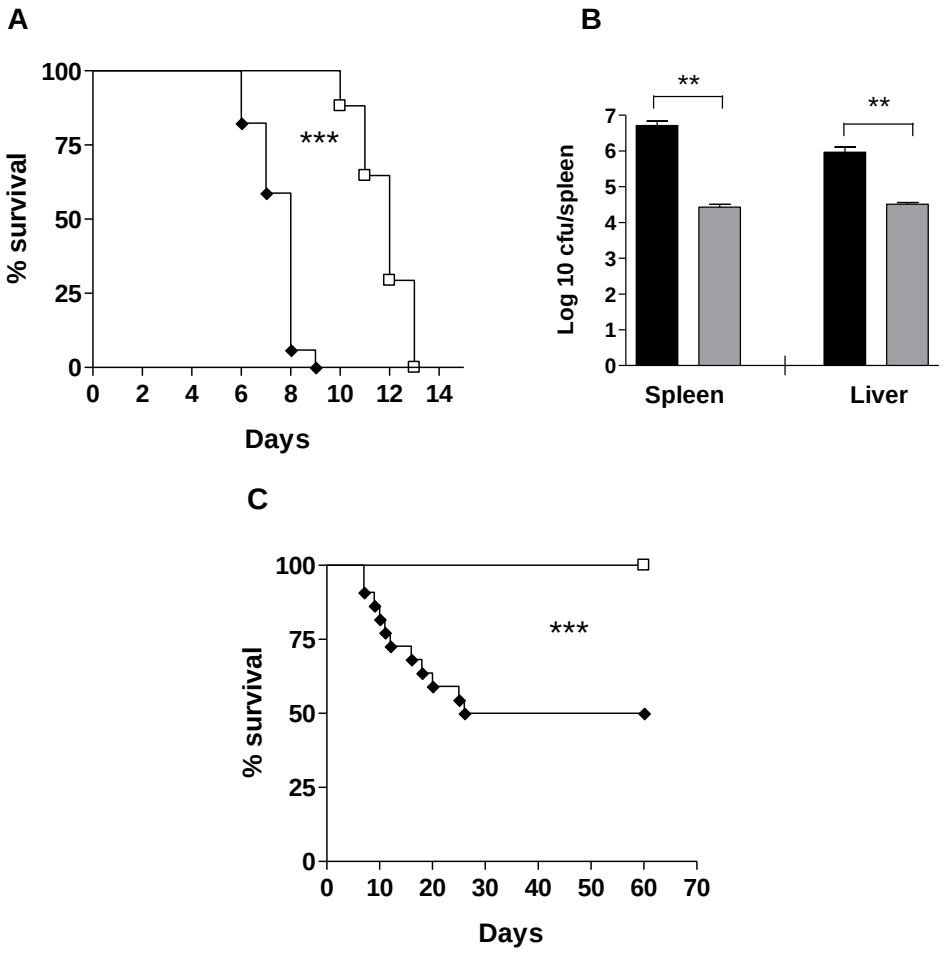

Figure 28: Susceptibility of B-MyD88$^{-/-}$ (□) and B-WT (♦) chimera mice to i.v. challenge with virulent *S. typhimurium* SL1344. A) Naïve and vaccinated (C) were infected i.v. with 100 organisms of *S. typhimurium* SL 1344. B) Spleen was homogenized and plated on MacConkey agar plates and the colonies forming unit (CFU) were counted on day 6. C), Both groups were infected 90 days after infection and autopsy confirmed that both groups of mice had overwhelming *Salmonella* infection in liver and spleen (data not shown). Survival was monitored daily until time of death. Survival curves were analyzed with the log rank test, n>17. B) Experiment done once, mean±SEM n> 3 (** for p<0.01 and *** for p<0.0001).

6 Discussion

The work here presented addressed the role of MyD88-signalling in B cells upon *Salmonella* infection. MyD88 is the major adaptor protein involved in the signalling pathway of the Toll-like receptors upon pathogen recognition. This activation in some innate immune cells can potentiate inflammatory response, but on the other hand can drive suppression by the provision of IL-10 by B cells [54]. The major aim of this thesis was the analysis of this inhibitory circuit and their consequences in the immune response during *Salmonella* infection.

In the first part of this study *in vitro* data with HKS activated B cells, DC and macrophages revealed production of high amounts of IL-10 by B cells through TLR2/4-MyD88 dependent manner. On the contrary, DC and macrophages produced big amounts of pro-inflammatory cytokines upon *Salmonella* activation. Importantly mice with IL10-deficient B cells showed an increased inflammatory response by T cells upon *Salmonella* infection.

In the second part it was demonstrated that MyD88-signalling in B cells inhibits the innate immunity and adaptive T cell immunity in response to *Salmonella typhimurium* infection. In particular, MyD88-signalling in B cells impairs survival of the mice exposed to a primary as well as to secondary infection by virulent *Salmonella*. This MyD88-mediated suppression was specifically elicited by B cells, as total MyD88-deficient mice show impaired protective immunity against Salmonella.
Thus, MyD88 exerts opposite effects in distinct cells types creating a controlled balance in the host response against *Salmonella* infection.

6.1 Role of B cells during infections

B cells are known to have an important role in immune defence against microbial infections. Infections with *Chlamydia trachomatis, Francisella tularensis, Leishmania major, Plasmodium chabaudi, Pneumocystis carinii, Mycobacterium tuberculosis* and *Salmonella typhimurium* have been reported to enhance susceptibility in B cell deficient mice [282-289].
During *Salmonella* infection, B cells are known to induce a strong antibody response [227]. In humans, vaccination with purified polyssacharide Vi antigen from serovar Typhi stimulates antibody production and provides protection [290, 291]. In mice the transfer of serum from infected mice can transfer protection from an otherwise lethal *Salmonella* challenge [248, 249]. Furthermore, B cells are required for protection against oral infection with virulent *Salmonella*, and vaccination with attenuated *Salmonella* is less protective in B cell deficient mice [292, 293]. Urgrinovic and colleagues have described that B cell deficient mice infected with attenuated *Salmonella typhimurium* showed an impaired T_H1 T cell response [294].
The role of B cells has been studied during *Salmonella* infection using B cell deficient mice. However, beside the fact that mice with targeted deletions that ‘’knockout’’ specific genes are useful tools in immunology, they also can lead to inaccurate conclusions regarding the role of a particular component of immunity, if not interpreted carefully. In order to have a better approach for studying the role of molecules of interest in B cells during infection, chimeric mice were generated in which a genetic deficiency of interest, e.g MyD88 or IL-10, was restricted solely to B cells. We observed that B-MyD88$^{-/-}$ mice were more resistant to primary infection with virulent *Salmonella* than mice with wild-type B cells. This observation contrasts other studies where a ‘’knockout mouse’’ was used. For instance, B cell deficient mice have been reported to succumb to virulent *Salmonella* infection within the same time range as wild type mice [292]. MyD88-deficient mice died much earlier after oral infection with virulent *Salmonella* than the wild-type mice [295].

6.1.1 Effects of MyD88 in B cells

B cell development is dependent upon and closely regulated by the expression of the antigen receptor genes [296]. We have observed that naive MyD88-deficient B cell mice have fewer B cells than control mice. This observation is maintained after infection with *Salmonella typhimurium*. Is MyD88 important in the homeostasis and development of the B cell

compartment? Some studies have shown that TLR-4 and TLR-9 are expressed on the surface of B cells during early stages of development [297] suggesting that these receptors have a role in the development of B cells. In vitro studies with pre-B cell line showed that LPS can provide stimulus for B cell differentiation [298]. Moreover, Hayashi and colleagues have shown that TLR-4 signalling favours B lymphocyte maturation, whereas TLR-2 retards this process [299]. However other studies have shown that MyD88 and TLR-9 deficient mice have normal B cell populations [27, 300, 301]. This is in agreement with our observation, as MyD88 knockout mice have normal B cell numbers (data not shown). This suggests that maybe in knockout mice the cell numbers are under homeostatic control. The exact reasons for our observations in the MyD88-deficient B cell mice are still unclear, but further analysis of homeostatic survival and proliferation of B cells should shed light on the possible role of MyD88 in B cell populations. There are possible endogenous ligands for the regulation of these processes since TLR can recognize self-antigens, such as heat-shock proteins [302, 303].

In addition to the role served by BCR signals in shaping the development and function of B cells. Innate immune receptors, such as TLRs have also important roles. TLRs recognize specific microbial products that also help to sculp humoral immune response. B cells express most TLR and can respond to a variety of TLR ligands. Their response to these stimuli can be to proliferate, to differentiate into antibody secreting plasma cells, to become more efficient antigen presenting cells (APC) or to secrete cytokines [304, 305].

The role of TLRs for antibody responses is still object of controversy. Pasare and Medzhitov reported that T-dependent antigen-specific antibody responses require activation of TLRs in B cells [215], but Gavin and colleagues have later reported that B cells can produce antibodies independently of TLR activation [306]. Furthermore, Ruprecht and Lanzavechia demonstrated that naive human B cells require TLR signals for productive T cell-dependent activation [169]. Several studies have evaluated humoral immune responses in mice with MyD88 deficiency restricted to B cells. In particular, it was found that mice with MyD88-deficient B cells mount a weaker antibody response to antigen mixed with LPS than mice carrying wild type B cells [307]. MyD88-deficient B cells also form less germinal centres and produce less specific antibodies than wild-type B cells in chimera mice harbouring both types of B cells following infection with murine gamma herpes virus 68 [213].

Our results show that MyD88-deficient B cell mice infected with *Salmonella typhimurium* have less germinal centres but they are able to mount a normal specific antibody response of all antibody isotypes with exception of IgG1 and IgG3. MyD88-deficient B cell mice had a

slightly increase of IgG1 production and a delayed IgG3 production compared to the control mice. These observations are in agreement with other studies, which demonstrated that B cells from MyD88-deficient mice resulted in a biased-T_H2 response with increased IgG1 production and decreased levels of IgG3 [27, 308-310]. However, Barr and colleagues have shown that MyD88-deficient B cell mice infected with *Salmonella* show loss of IgG2c and IgM, which we do not [311]. Nevertheless they demonstrated that MyD88 B cell-deficient mice have less germinal centres and were detected on day 14 after infection [311]. Interesting are also the findings of Cunningham, which show that in WT mice infected with attenuated *Salmonella*. Plasma cells appear in spleen by day 3 and germinal centres one month later when the infection has been resolved [228].

The natural IgM pool is largely derived by the B1 population of B cells [312, 313]. Barr et al. also demonstrated that natural antibodies, such as IgM depends on B cell intrinsic MyD88-transduced signals [311]. We also observed a decrease of natural IgM in the MyD88 deficient mice, and this observation is not surprising considering that B1 population in peritoneal cavity was reduced in MyD88 deficient B cell mice (data not shown). Despite the fact that we observed a decrease in natural IgM, the mice were able to restore the levels of antigen specific antibodies at later time points of infection. Our work indicates that in mice infected i.v. with *Salmonella typhimurium*, the antibodies mainly derive from follicular and marginal zone B cells. We had observed that MyD88-deficient B cell mice had fewer follicular B cells but more MZ B cells after infection compared to the control mice. Both groups showed a decrease in the MZ B cell population after a late time point of infection. A decrease in marginal zone B cells has been associated to antigen transport after exposure to opsonised antigen [314, 315]. Furthermore, LPS induces a reduction of MZ B cells, associated with their migration into follicles [314, 316, 317]. Oliver et al. also have demonstrated that stimulation with LPS of MZ B cells results in their rapid proliferation, IgM and IgG3 antibody secretion and up-regulation of the co-stimulatory molecule CD86 [166, 167]. This observation suggests that since MZ B cells from MyD88-deficient B cell mice cannot be efficiently stimulated, this leads to a decrease in the levels of IgM and IgG3 in the beginning of the immune response against to *Salmonella*, but this levels are restored at a later time point after infection. MZ B cells differentiate into antibody-forming cells during the first week after infection whereas, follicular B cells respond slower. Both populations are known to undergo GC reactions that give rise to memory B cells and long-lived plasma cells [166]. We observed that B-MyD88$^{-/-}$ have fewer long-lived plasma cells in the bone marrow and as well as, reduced levels of all antibody isotypes except IgG1. This observation is in agreement with Guay and colleagues, who showed that MyD88-deficient mice infected with polyoma virus can form germinal

centres and mount early specific antibody responses, but they fail to generate bone marrow plasma cells and to maintain long-term humoral immunity [214].

Overall these data suggest that MyD88-signalling in B cells acts as a cell autonomous amplifier for all the facets of B cell response. MyD88-dependent signalling in B cells is not totally required for primary B cell immune response, but it seems necessary for effectively generating long-term antibody responses to *Salmonella* infection.

6.1.2 Link between MyD88-activated B cells and innate immunity

The innate immune system provides the first layer of protection and MyD88 is a crucial component of this response. MyD88 stimulates resident tissue macrophages to produce neutrophil chemoattractants [56, 57]. The recruitment of neutrophils to infected tissues can be or is impaired in MyD88-deficient mice [57, 318-320]. We observed that in the absence of MyD88, the accumulation of neutrophils and macrophages within infected spleens was severely delayed. However they reached control levels by day 21 after infection, indicating that other signalling pathways can substitute for MyD88. In contrast, the lack of IFN-γ-producing NK cells was not recovered even at later time points in MyD88-deficient mice. Given that the early provision of IFN-γ by NK cells is essential for macrophage activation and for protection of *Salmonella* [99], it is likely that this defect contributes to the impaired survival of MyD88-deficient mice after infection by virulent *Salmonella* [51]. The activation of NK cells has been described in mice infected with *Listeria monocytogenes* [321]. Myeloid cells and NK cells are recruited by $CD11c^+$ DC into clusters localized around the central arterioles in the T cell zones of the white pulp areas in spleen, where the bacteria is trapped. In these clusters, IL-12 and IL-18 production by DCs stimulates NK cells to produce IFN-γ, which is necessary also for the activation and maturation of colocalized monocytes into TNF- and iNOS-producing DCs [321]. MyD88 plays also an important role in this response, since NK cells from MyD88-deficient mice do not form clusters and do not produce IFN-γ upon *Listeria* infection [321]. It is possible that a similar process occurs during *Salmonella* infection. MyD88 would drive the NK cell response by stimulating their recruitment around central arterioles, and by inducing signals via IL-12 and IL-18 that are necessary for IFN-γ expression.

Remarkably, multiple aspects of the innate immune response were increased in the absence of MyD88-signalling selectively in B cells: the numbers of neutrophils and IFN-γ-producing NK cells were higher at day 4, and the numbers of macrophages were higher at day 10 and at day 21. The innate immune response was therefore stronger during the whole course of

infection in B-MyD88$^{-/-}$ mice, although different players were involved at different time points. This increased innate response most likely explains why B-MyD88$^{-/-}$ were more resistant to a primary infection by virulent *Salmonella* than mice with wild-type B cells. These observations show that absence of MyD88-signalling restricted to B cells augmented the immune response against *Salmonella*, whereas the absence of MyD88-signalling in other cells has opposite effects. How MyD88-signalling B cells influences the innate immune cells? Neutrophils are known to be crucial in survival of infected mice with *Salmonella* [322]. The recruitment of these cells at the site of infection is orchestrated by several chemokines, such as CXCL1, CXCL2, CCL2 and CCL3 [56, 57, 323, 324]. Recently it has been described that IFN-γ coordinates CCL3-mediated neutrophil recruitment *in vivo* [323]. This suggests that IFN-γ production by NK cells can affect the recruitment of neutrophils. Furthermore, IFN-γ is secreted by NK cells in response to the IL-12 produced by dendritic cells and macrophages [269, 325]. Macrophages provide an important line of defense against *Salmonella* [221]. Supernatants from *Salmonella* TLR-activated B cells can suppress production by macrophages of proinflammatory cytokines that are usually produced by classically activated macrophages. This suppression can be due to presence of IL-10 in the supernatants since B cells stimulated with *Salmonella typhimurium* produces large amounts of IL-10 *in vitro*, in a TLR2/TLR4-MyD88 dependent manner. Furthermore, it has been reported that IL-10 can polarize monocytes into alternatively activated macrophages, which play a role in resolution of inflammation, accompanied by reduced pro-inflammatory cytokine secretion [326]. Therefore it is possible that B cells influence the polarization of activated macrophages. For example, it has been reported that IL-10 inhibits TNF-α and IL-12 production by macrophages and their stimulatory effect on IFN-γ production by NK cells [270, 327]. In general, these observations suggest that MyD88-activated B cells can regulate the response of macrophages and dendritic cells, which are able to control activation of NK cells and neutrophils.

6.1.3 Effect of MyD88-activated B cells on T cell immunity

In antigen-presenting cells (APCs), TLRs can induce signals that, through expression of cytokines and co-receptors, are capable of activating T cell immunity [3, 328]. Mice lacking MyD88 show impaired T_H1 responses to antigens emulsified in complete Freund's adjuvant, whereas T_H2 responses were normal [328]. This effect was also observed for *Toxoplasma gondii* and *Leishmania major* [329, 330]. More specifically, MyD88-deficient dendritic cells migrate normally into lymph nodes and up-regulate co-stimulatory receptors as efficiently as wild type DCs [331], but they do not secrete cytokines that are essential for T cell immunity [332].

According to our results, the absence of MyD88 leads to delayed accumulation of dendritic cells within infected spleens. Furthermore, *Salmonella*-specific inflammatory T_H1 response was strongly impaired by means of IFN-γ and TNF-α production by $CD4^+$ T cells in MyD88 deficient mice compared to wild-type mice. This observation is in contrast to previous findings, which demonstrated that MyD88-deficient mice can mount T_H1 responses against some microbes, such as *Listeria monocytogenes* [333], *Mycobacterium tuberculosis* [334], or influenza virus [335], implying that MyD88-independent receptors are able to recognize these pathogens and initiate T cell priming. Possibly, MyD88-deficient mice remain less resistant to these microbes, because of defective innate immune responses.

Interesting, it was the observation done in the B-MyD88$^{-/-}$ mice, these mice had stronger *Salmonella* -specific inflammatory T_H1 response compared to B-WT mice. This corroborates the previous findings, which show that absence of MyD88 in B cells results in heightened T_H1 and T_H17 responses during EAE, leading to a chronic form of disease [54]. During *Salmonella* infection, deficiency of MyD88 signalling in B cells does not increase the number of antigen-reactive $CD4^+$ T cells, as determined by staining intracellular CD154 or IL-2, but rather enhance the polarization of the reactive $CD4^+$ T cells towards the T_H1 pathway characterized by the secretion of IFN-γ and TNF-α. These results are in agreement with the observations made by Lampropoulou et al. and Fillatreau et al., demonstrating that the B cell-mediated regulation of EAE does not influence the production of IL-2 by antigen-reactive T cells, but specifically suppress the inflammatory T_H1 and T_H17 responses [54, 170]. This B cell-mediated regulation of T_H1 immunity could be mimicked *in vitro*, and IL-10 was identified as a key suppressive mediator [54]. During *in vitro* culture, B cells stimulated through MyD88 using TLR ligands, such as LPS, secrete large amounts of IL-10 and supernatants from these B cells inhibit in an IL-10-dependent manner the differentiation of naive $CD4^+$ T cells into T_H1 cells [54]. This inhibition operates indirectly via suppression of the production of IL-12, IL-6, IL-23 and TNF-α by DCs [54]. Therefore, TLR activated B cells can control T_H1 immunity by inhibiting via IL-10 the production of inflammatory mediators such as IL-12 by DC [170, 336]. There is evidence for this regulatory circuit *in vivo*, since mice with B cell-restricted deficiency in IL-10 make stronger T_H1 responses than mice with wild-type B cells, and DCs isolated from B cell-deficient mice produce higher amounts of IL-12 and induce stronger T_H1 responses than DCs from wild-type mice upon adoptive transfer [170, 336]. Maybe IL-10 is involved in the suppressive function of B cells during *Salmonella* infection because heat-killed *Salmonella* induces IL-10 secretion by B cells via a MyD88-dependent pathway *in vitro* and mice with IL-10-deficiency restricted to B cells have also a stronger T_H1 response with higher amounts of IFN-γ and TNF-α during *Salmonella* infection. Other studies, dissecting the role of IL-10 and other cytokines during bacterial infections showed

that IL-10 suppresses IFN-γ and TNF-α production by T cells [270, 327]. Sashinami et al. reported that neutralization of endogenous IL-10 reduced bacterial growth and this can be due to the increase of IFN-γ and TNF-α production [337]. B cells can interfere with the activation of T_H1 cells by suppressing and/or modulating the function of DCs indirectly by their production of IL-10. If this mechanism is important, where and when can B cells instruct DC function *in vivo*? There is evidence that B cells interact directly with DC at a very early stage of the immune response. Following activation, B cells rapidly up-regulate the chemokine receptor CCR7, which confers them responsiveness for the T cell zone specific chemokines CCL19 and CCL21 [338]. The newly equipped B cells then migrate into the T cell area, where they accumulate at the periphery of the periarteriolar lymphoid sheath and near the terminal arteriolar branches. It has been previously shown that primed B cells start to proliferate there, within clusters of DC and T cells, within few days after immunization [339, 340]. In this microenvironment, B cells can directly interact with DC via both antigen-independent and antigen-dependent mechanisms [341, 342]. Even though this interaction has been regarded principally as a place where DC can provide B cells with signals facilitating antibody responses [343, 344], it is possible that B cells equally modulate the functions of DC, possibly through cytokines such as IL-10. The suppression of DC function through the production of IL-12, could explain why B-MyD88$^{-/-}$ mice show enhanced NK cell response already at day 4 after infection with *Salmonella*.

Beside DCs, macrophages are also an important APC in the combat of bacterial infections by phagocytosing and destroying bacteria and presenting bacteria-derived antigens to T cells. We have previously shown that B cells can modulate macrophage function in the production of cytokines, such as IL-6 and IFN-γ. Maybe B cells influence the T cell response trough macrophages via IL-10. For instance, it has been shown that B1 cells are able to control the effector functions of macrophages via IL-10 secretion [345] and Fiorentino and colleagues demonstrated that IL-10 inhibits the ability of LPS-activated macrophages to produce inflammatory cytokines, such as IL-1, TNF-α and IL-6 leading to the inhibition of T cell activation [346]. If this is true, where and when macrophages interact with B cells? In the spleen, the marginal zone has two main populations of macrophages: the marginal-zone metallophilic macrophages and marginal-zone macrophages. The latter population, is characterized by expression of DC-SIGN and the type I scavenger receptor MARCO (macrophages receptor with collagenous structure). MZ macrophages are located together with the marginal zone B cells. It has been suggested that MZ B cells can be activated by marginal zone macrophages and that MZ macrophages are able to transfer processed antigens to MZ B cells [347]. Moreover, Koppel and colleagues have shown that SINGR1-expressing macrophages interact with MZ B cells and that are involved in the early

production of antibodies [348]. Maybe, in this microenvironment B cells can simultaneously secrete IL-10 and modulate macrophages function.

However we have to consider that maybe it is not only IL-10 secretion by MyD88-activated B cells regulating innate and adaptive immune responses. For instance, B cells can suppress inflammation in bowel disease by innate immune cells via IL-10-independent mechanism [349]. Moreover, Tian and colleagues have shown that LPS-activated B cells can inhibit T cell immunity via TGF-β secretion in NOD mice [259]. Scott et al. have described that LPS-activated B cells can directly suppress T cells independently of IL-10, via antigen presentation involving MHC-II and CD86 [350]. Overall, these studies suggest that MyD88-signalling in B cells can induce several molecular mechanisms to suppress immunity. During *Salmonella* infection, we observed that the absence of MyD88 in B cells induces an increase in immune response involving different cell populations: neutrophils and NK cells, macrophages and DC, and finally CD4 and CD8 T cells. These effects had a dramatic consequence on the protection of vaccinated mice.
We have observed that all vaccinated B-MyD88$^{-/-}$ mice survived an infection with virulent *Salmonella* while half of the B-WT succumbed to the infection. Several studies have described the role of B cells in the formation and maintenance of T cell memory and in protection against infection [284, 351-353]. For example, it has been demonstrated that antigen presentation and antibody production are necessary to maintain memory CD4^{+} T cells [284, 354]. Other studies show the contrary, that for instance, B cell-deficient mice infected with *Listeria monocytogenes* have defective CD4^{+} T cell responses, although B cells do not express listerial antigens [355]. Moreover, Whitmire and colleagues have shown that B cells are required for CD4^{+} T cell memory generation in infection with lymphocytic choriomeningitis virus (LCMV) independently of antibody production [353]. These studies imply that maybe B-cell functions other than antibody production are important for memory CD4^{+} T cell responses.

With regard to the role of B cells in CD8^{+} T cell memory, several studies have demonstrated that CD8 memory can be maintained in the absence of B cells [356-358]. Moreover, CD8 memory can be maintained in the absence of specific antigens [359-361] and in the absence of cross-reactive antigens and MHC class I [362]. However, other studies have shown the opposite. For example, Shen and colleagues have demonstrated that B cells influence the pool of CD8 T cell memory, since in absence of B cells there is an increased death of activated CD8^{+} T cells in contraction phase, leading to a decrease of *Listeria monocytogenes*-specific CD8^{+} T cell memory [352].

In addition studies have revealed a role for MyD88 in the generation and maintenance of T cell memory. Rahman et al. have demonstrated that MyD88-deficent mice can provide $CD8^+$ T cell response in the early stages of LCMV infection, but afterwards showed a reduced accumulation of $CD8^+$ T cells [363]. Moreover, Quigley and colleagues have shown that activation of TLR2-MyD88 signalling pathway is critical for CD8 T cell clonal expansion and memory formation [364]. Pasare and Medzhitov demonstrate that TLRs contribute to cell memory because MyD88-deficient mice show weak secondary responses [331].

Collectively, our studies demonstrate that MyD88-signalling in B cells decreases the inflammatory T cell response against *S. typhimmurium* and this is reflected in the protection of vaccinated mice against subsequent challenge.

6.1.4 Summary

Here we identified that MyD88-signalling in B cells functions as a regulator of inflammation during infection.
MyD88-deficient B cell mice develop a stronger innate and T_H1 immune response to *Salmonella* than mice with wild-type B cells. In contrast, mice lacking MyD88 in all cells show an impaired immune response. Remarkably, B-$MyD88^{-/-}$ mice were more resistant to a primary virulent *Salmonella* infection than control mice. Moreover, these mice were more resistant to secondary infection with the virulent *Salmonella* after vaccination with attenuated *Salmonella* that the control mice.

The mechanism involved in immunosuppression of the immune response via MyD88-signalling in B cells during *Salmonella* infection is still not completely clear. One possibility could be a set of distinct processes acting at different time points or a central mechanism acting at the initiation of the immune response. All our observations indicate that it is a central property of B cells. TLR/MyD88 activated B cells produce IL-10 upon infection resulting in the inhibition of the immune response. If so, why upon infection B cells would produce an anti-inflammtory cytokine via MyD88 inhibiting the immune response, while in other cells, such as macrophages and dendritc cells, MyD88 functions as an activator of the immune response? Can this mechanism result in an effective immune response and have benefits to the organism? These questions are addressed in the next chapter.

7 Conclusion

B cells are known to have a suppressive function through the provision of IL-10, which plays an important role in resolution of EAE. Beside the fact that suppressive functions of activated B cells are beneficial to the host in autoimmune diseases, they also can be favourable in the combat against pathogens by improving the dynamic of the response and the robustness of pathogen sensing.

In the case of EAE model, TLR-agonists were identified as inducers of IL-10 production in naive B cells. *In vivo* experiments showed that mice in which only B cells lack MyD88 or TLR2/TLR4 develop a chronic form of EAE [54]. These data suggest that microbial products are required for the recovery from EAE, by stimulating MyD88, TLR2 and TLR4 on B cells. However, MyD88 signalling in different cell types, such as DCs stimulates pathogenic T cell responses and induces EAE [54]. This indicates that TLR agonists have a different impact on different cells of the immune system, which drive the induction and the resolution of this autoimmune pathology.
The present study demonstrated that MyD88-mediated B cell suppression operates also during bacterial infection with *Salmonella*. In vitro experiments showed that heat-killed *Salmonella* induce IL-10 production by B cells through MyD88 and TLR2/TLR4. Mice in which only B cells lack MyD88 or IL-10 mount an intense T cell response of T_H1 type, suggesting that TLR-activated B cells have a suppressive effect also in a infection model. On the other hand the same pathogens induce DCs and macrophages to produce pro-inflammatory cytokines, which presumably stimulates T cell response helping in the clearance of the pathogen.
Collectively, these two models demonstrate the identification of an immunosuppressive role of MyD88-activated B cells, as a common mechanism of immunoregulation.

In fact this form of regulation fits to a known network motif often found in transcription networks, and termed as a type 1-incoherent feedforward loop (I1-FFL) in system biology [365]. The I1-FFL is made of 3 elements X, Y, and Z, in which X is the input signal and stimulates directly Z, but also Y, which regulates the stimulatory function by inhibiting Z. This network motif is defined as “incoherent” because it includes opposite types of connections, stimulatory from X to Z and from X to Y, and inhibitory from Y to Z. In our case, microbial products (X), stimulate DC or macrophages (Z), and induce B cells (Y) to secrete IL-10 that

inhibits DC and/or macrophages. During infections, macrophages also play an important role in the orchestration of immunity. Therefore, the regulatory functions of MyD88-activated B cells mediated by IL-10 can operate through an inhibition of the inflammatory effects of DC or macrophages exposed to TLR agonists [54, 170, 211, 345, 366, 367]. Consequently, we consider Z as a DC or a macrophage, since it is not clarified yet which cellular components are involved in this regulatory mechanism.

This network motif can provide the immune system with advantageous properties. Since in the I1-FFL, the microbial input (X) will directly activate Z and concomitantly trigger Y. The activation of Z will induce an increased immune response by production of pro-inflammatory cytokines, promotion of T_H1 responses and also an immunological memory. In a I1-FFL this activation can exceed and be transiently superior to the final state. This can occur, since the input signal X rapid stimulates a powerful induction of Z and Y which after a certain time progressively reduces the intensity of response until a defined steady state. Thus, toxic side effects inherent to inflammation and the risk of immunopathology can be avoided [368]. For instance, it was previously described that the disruption of the inhibitory loop can lead to a severe immunopathology: mice lacking MyD88 or IL-10 restricted in B cells develop a chronic EAE, while mice with wild-type B cells rapidly recover after a short-time of paralysis [54, 170]. Another feature of an effective immune response is the induction of a fast response. This means that Z can respond rapidly to the input signal X. The intensity of the final steady state, and the time until it is reached is controlled by the Y inhibitory loop. The result is an accelerated induction and intense response that is transiently superior to the steady state, until the inhibitory arm regulates the response. In contrast, a simple regulation from X to Z has slower kinetics of induction due to the limitations imposed by the steady state [369].
Furthermore, I1-FFL provides also the immune system with increased robustness in the microbial sensing. Microbes acquiring mutations that reduce the affinity of their agonists for their receptor would become less immunogenic and could easily escape the host defence mechanism. The I1-FFL may limit this risk. In fact, the reduced positive signal produced by a mutated agonist is compensated by a proportional reduction of the inhibitory signals, so that the equilibrium remains, and an effective immune response can still develop. This confers robustness to the process of microbial recognition by innate receptors because it allows to protect the system from fluctuations imposed on it by the environment.
Altogether, the I1-FFL described here, shows that IL-10 production by B cells regulates the response presumably of DC and/or macrophages to TLR stimulation with *Salmonella* typhimurium. However it would be interesting to test this regulatory function of MyD88-activated B cells in infections by other pathogens, including different bacteria and viruses,

which induce distinct immune responses and cause immunopathologies other than the ones induced by the specific microorganism studied in this work.

Furthermore, this network motif helps to clarify the fact that microbes can also have a protective effect in the development of autoimmune diseases in the so-called ''hygiene hypothesis'' [366]. It has been described that infection of NOD mice with attenuated *Salmonella typhimurium* can reduce the incidence of type 1 diabetes (T1D) [370].

Conclusively, together with previously reported findings, the current work points out that a main function of MyD88-activated B cells is to regulate the magnitude of innate and adaptive immune responses, thereby protecting the host from excessive immunopathology.

8 References

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11 Publications

Lampropoulou V.*, K. Hoehlig *, T. Roch*, P. Neves, E. Calderón Gomez, C. H. Sweenie, Y. Hao, A. A. Freitas, U.Steinhoff, S. M. Anderton, and S. Fillatreau, (* equally contributing authors), *Toll-like receptor-activated B cells suppress T cell-mediated autoimmunity*. J Immunol, 2008. **180**(7): p.4763-73.

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