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ROLE OF MYD88 PROTEIN IN THE MAINTENANCE OF IMMUNOLOGICAL SELF-TOLERANCE: INDUCTION OF AUTOIMMUNITY BY SALMONELLA INFECTION CONSEQUENT TO MYD88 DEFICIENCY

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This dissertation is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Under the direction of Professor Basel K. al-Ramadi

May 2014

DECLARATION OF ORIGINAL WORK

I, Jincy Merin Issac, the undersigned, a graduate student at the United Arab Emirates University (UAEU) and the author of the dissertation entitled "Role of MyD88 protein in the maintenance of immunological self-tolerance: Induction of autoimmunity by *Salmonella* infection as a consequent to MyD88 deficiency", hereby solemnly declare that this dissertation is an original research work done and prepared by me under the guidance of Prof. Basel K. al-Ramadi, in the College of Medicine and Health Sciences at UAEU. This work has not been previously formed as the basis for the award of any academic degree, diploma or similar title at this or any other university. The materials borrowed from other sources and included in my dissertation have been properly cited and acknowledged.

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ABSTRACT

Activation of the innate immune system is a prerequisite for the induction of adaptive immune responses to both infectious and non-infectious agents. Toll-like receptors (TLRs) are a family of proteins important for recognizing pathogen associated molecular patterns. MyD88 is an adaptor molecule whose function is critical in TLR signaling. MyD88-deficient ($MyD88^{-/-}$) mice exhibit heightened susceptibility to infections, even by attenuated strains of *Salmonella enterica* serovar Typhimurium. Paradoxically, despite their hypersusceptibility, infected $MyD88^{-/-}$ mice produce elevated serum levels of anti-*Salmonella* antibodies regardless of the route of infection. This hypergammaglobulinemia was observed with both Th1-driven (IgG2c and IgG3) and Th2-driven (IgG1) antibody isotypes. The dysregulated antibody responses also led to the production of autoantibodies as demonstrated by reactivity to dsDNA and thyroglobulin, positive nuclear staining with HEp2 cells, and immune complex deposition in the kidneys of $MyD88^{-/-}$ mice. Utilizing intracellular cytokine staining, real-time PCR and 6-color flowcytometric analysis, we demonstrated that these autoimmune responses correlate with the activation of distinct populations of IFN-γ^+/IL-4^+ and IFNγ^+/IL-10^+ T helper lymphocytes. Further analysis revealed that these most likely represent a specialized family of cells known as follicular helper T cells (T_{FH} cells) which function to promote B cell activation and antibody production. The aberrant expansion of these T_{FH}-like cells could underlie the activation of autoreactive B cells leading to humoral autoimmunity. Our findings highlight a critical role for TLR-MyD88 pathway in controlling reactivity to self-antigens and provide a potential mechanism for the inter-relationship between microbial infections and autoimmune disease development in humans.

Keywords:

Innate immunity; Toll like receptors; hypergammaglobulinemia; *Salmonella* infection; SLE; autoimmune disease.

الملخص

تحفيز جهاز المناعة الفطري شرط أساسي لتنشيط جهاز المناعة التكيفي تجاه العوامل المعدية وغير المعدية. المستقبلات شبيهة التول (TLRs) هي مستقبلات مهمة لاستشعار الأنماط الجزيئية المرافقة للكائنات الممرضة و التي تعتمد على الجزيء الناقل Myd88 لنقل الإشارة داخل الخلية. عند إجراء التجارب على الفئران المفتقرة لMyD88 (MyD88-/-) وجد أن لديهم قابلية عالية للموت لدى إصابتهم بالعدوى حتى بأنواع مضعفة من السالمونيلا Typhimurium *Salmonella enterica serovar*. بعد إصابة هذه الفئران بعدوى السالمونيلا، لوحظ أنهم أنتجوا كميات كبيرة من الأجسام المضادة ضد السالمونيلا من كلا نوعي الخلايا المساعدة Th-1 وTh-2 بغض النظر عن طريقة العدوى و هذه الظاهرة تعرف بفرط جاما جلوبين الدم (hypergammaglobulinemia). فرط جاما جلوبين الدم ترافق مع نشاط بعض هذه الأجسام المضادة إلى مولد الضد الذاتي حيث تفاعلت مع الحمض النووي و الثايروجلوبين، كما وجدت ترسبات للمتراكبات المناعية في الكلية. باستخدام عدة تقنيات، تمكنا من تحديد علاقة بين الاستجابة الناعية الذاتية و وجود نشاط لمجموعة مميزة من الخلايا التائية المساعدة $IFN\gamma^+/IL\text{-}4^+$ و$IFN\text{-}\gamma^+/IL\text{-}10^+$ ، كذلك تبين لنا أن هذه الخلايا على الأغلب هي من عائلة متخصصة تسمى بالخلايا الجريبية التائية المساعدة (T_{FH} cells) و التي لها دور في تعزيز نشاط الخلايا البائية و دفعها لإنتاج الأجسام المضادة. و قد يفسر وجود شذوذ في توسع للخلايا شبيهة الخلايا الجريبية التائية المساعدة نشاط الخلايا البائية النشطة ذاتيا المسببة لأعراض أمراض المناعة الذاتية. في الختام، دراستنا توضح أهمية مسار المستقبلات الشبيهة بالتول و بروتين MyD88 في التفاعل مع مولدات الضد الذاتية و تقترح آلية تربط الإصابة بالعدوى و نشئة أمراض المناعة الذاتية عند الإنسان.

كلمات دلالية:

المناعة الفطرية، المستقبلات الشبيهة بالتول، فرط جاما جلوبين الدم، عدوى السالمونيلا، الذئبة الحمراء الجهازية (SLE) ، أمراض المناعة الذاتية.

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Being miles apart, my sincere love goes to my husband Rewin for being my pillar of strength and for patiently waiting for me to complete my PhD.

DEDICATION

Dedicated to my parents and husband

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1. INTRODUCTION

The two main elements of the host immune defense system include the innate and adaptive immunity whose components target infectious agents like microbes and non-infectious agents such as adjuvants. Effective host defense against microbial infections is dependent on prompt recognition of pathogens, rapid induction of immediate anti-microbial defense mechanisms and ultimately the activation of strong, antigen-specific, lymphocyte responses. The innate immune system is an evolutionary conserved system that recognizes and responds to a wide range of microbial pathogens. It plays a crucial role in the initial control of infections and is mediated by phagocytic cells and antigen presenting cells (APCs). These cells recognize evolutionary conserved components of the pathogen essential for their survival and pathogenicity known as pathogen associated molecular patterns (PAMPs) through the host germline encoded pattern recognition receptors (PRRs). Several families of PRRs have been identified which include TLRs (Toll like receptors), RIG (retinoic acid-inducible gene 1 like receptors) and NLRs (nucleotide-binding oligomerization domain like receptors) all of which play a central role in the host defense (Kumar et al., 2011).

1.1. Toll like receptors

TLRs, a family of one of the most extensively studied PRRs, is a class of conserved type 1 transmembrane structures that identify PAMPs through the process of pattern recognition (Takeda et al., 2003). This in turn leads to the initiation of a series of signaling cascades, resulting in the transcriptional expression of genes that code for inflammatory cytokines, chemokines and co-stimulatory molecules (Akira et al., 2006); (Kopp and Medzhitov, 2003);

(Medzhitov, 2007) depending on the type of cells activated. Some examples include secretion of TNF-α by murine macrophages on activation of TLR4 and TLR2 (Jones et al., 2001) On recognition of viral associated patterns, TLR activation on NK cells leads to production of GM-CSF (granulocyte-monocyte colony stimulating factor) and IFN-γ (Adib-Conquy et al., 2013).

The first member of the TLR family was a protein identified in Drosophila melanogaster (fruit fly). Drosophila Toll was found to play a crucial role in host response to fungal infections as well as dorso-ventral patterning during embryogenesis in the fruit fly (Lemaitre et al., 1996). Several mammalian homologues of the Drosophila Toll protein have been identified and shown to induce the expression of genes involved in the inflammatory response. One such example is TLR1-5 that has been found to be more closely linked to the Toll homologs of Drosophila (Rock et al., 1998); (Medzhitov et al., 1997).

About 13 mammalian TLRs have been cloned in mammals of which TLR1-9 are conserved between mouse and human. Each of them recognizes distinct molecular patterns derived from viruses, pathogenic bacteria, pathogenic fungus and parasitic-protozoa through their PRRs (Akira et al., 2006); (Janeway, 1989). They are expressed in many immune cells such as B cells, dendritic cells (DCs), macrophages (antigen presenting cells) and certain types of T-cells as well as non-immune cells like fibroblast and epithelial cells (Iwasaki and Kelsall, 1999); (Iwasaki and Medzhitov, 2004); (Akira et al., 2006). Most TLRs are expressed on the cell surface, which include TLR 2, 4, 6, 8 and 10 while some like TLR3, 7 and 9 are expressed intracellularly (Akira et al., 2006); (Kawai and Akira, 2010) (Table 1). Members of the TLRs subfamily within the cell are localized on the membrane of intracellular vesicles, such as endosomes and lysosomes where they

identify the nucleic acid components of pathogenic organisms (Medzhitov, 2007). TLRs are found as individual units or as pair as well as with a range of other signaling molecules.

TLR	Ligand	Microbial source
TLR1	triacyl lipopeptides	bacteria
TLR2	peptidoglycans, lipoproteins, atypical LPS, zymosan, phospholipomannan, GPI anchors, heat-shock proteins (e.g. HSP 70)	bacterial, fungi, protozoa, host cells
TLR3	poly I:C, dsRNA	virus
TLR4	LPS, mannan, glucuronoxylomannan, glycoinositolphospholipids, RSV fusion protein, heat-shock proteins (e.g. HSP 60)	bacterial, fungi, protozoa, virus host cells
TLR5	flagellin	bacteria
TLR6	diacyl lipopeptides	bacteria
TLR7	synthethetic imiazoquinoline-like molecules, ssRNA	virus
TLR8	ssRNA	virus
TLR9	CpG DNA	bacteria, protozoa, virus
TLR10	function and ligand unknown	-
TLR11 (mouse only)	components of uropathogenic bacteria, profillin like molecules	bacteria, protozoa
TLR12-13 (mouse only)	function and ligand currently unknown	-

Table 1: Toll like receptors and pathogen recognition (Adapted from Chong D.L.W. Sriskandan S. Herwald H, Egesten A (eds): Sepsis – Pro-Inflammatory and Anti-Inflammatory Responses. Contrib Microbiol. Basel, Karger, 2011, vol 17, pp 86–107)

These evolutionarily conserved proteins are characterized by an extracellular ligand binding N terminus domain made up of leucine rich repeats (LRRs) and an intracellular C terminus domain homologous to IL-1 receptor (Bowie and O'Neill, 2000); (Medzhitov, 2007). The intracellular Toll IL-1 receptor (TIR) region consists of around 200 amino acids and is involved in signal transduction (Bauer et al., 2008); (Bowie and O'Neill, 2000). Protein–protein interaction is mediated by the TIR domain, one of the earliest motifs to be involved in signaling

(Kimbrell and Beutler, 2001). Binding of the TLR to its ligand reveals the presence of an infection which leads to the activation of the immune system and elimination of the pathogen.

Upon binding to their respective ligands, TLRs generate signals through the recruitment of adaptor molecules such as MyD88 (myeloid differentiation primary response 88), TRIF (TIR-domain-containing adapter-inducing interferon-β), TIRAP (TIR domain containing adaptor protein) and TRAM (TRIF related adaptor molecule) via the TIR cytoplasmic domain. This stimulates the production of reactive oxygen and nitrogen intermediates (ROI and RNI). TLR signaling induces the production of pro-inflammatory cytokines, type 1 interferons as well as enhances the expression of co-stimulatory molecules. This leads to the activation of APCs, subsequently initiating the adaptive immune response (Werling and Jungi, 2003).

The MyD88 (myeloid differentiation factor 88) is the central adaptor molecule used by all TLRs except TLR3. The pathway initiates the expression of NF-kB or interferon-regulatory transcription factor (IRF-1) that are involved in the regulation of inflammation and activation of pathogen specific adaptive immunity (Kumar et al., 2009); (Akira, 2003). MyD88 is also involved in the signaling pathway of IL-1 and IL-18 receptors. This adaptor molecule recruits IL-1 receptor-associated kinase-4 (IRAK-4) to TLRs through the death domain interaction of both molecules. By phosphorylation, IRAK-1 is activated and induces the interaction of TNF-receptor associated factor (TRAF6). TRAF6 then activates TAK1 complex through ubiquitination. TAK1 in turn leads to the subsequent activation of IκB kinase *(*IKK) complex. This catalyzes IκB and its degradation results in the release of NF-κB. The transcription factor once liberated

translocates into the nucleus and induces gene expression of the target inflammatory cytokines to the infection (Akira, 2003). TAK1 also activates the mitogen activated protein (MAP) kinase pathway that eventually leads to the activation of activator-protein (AP-1) and NF-κB. Meanwhile, TLR4 possesses a MyD88 dependent and a TRIF dependent pathway, whereas TLR3 signal only through TRIF. Signaling through TRIF adaptor molecule leads to the recruitment of TBK1 together with IKKi that result in the phosphorylation of IRF3. Once phosphorylated, IRF3 dimerizes and translocates to the nucleus and binds to the DNA. TRIF can also bind to TRAF6 and receptor-interacting protein (RIP-1) that mediates the activation of NF-κB. For the induction of type1 interferons (IFNs), activation of IRF3 and NF-κB are required (Fig. 1). On stimulation through TLR ligands, DCs and macrophages are the major producers of inflammatory cytokines.

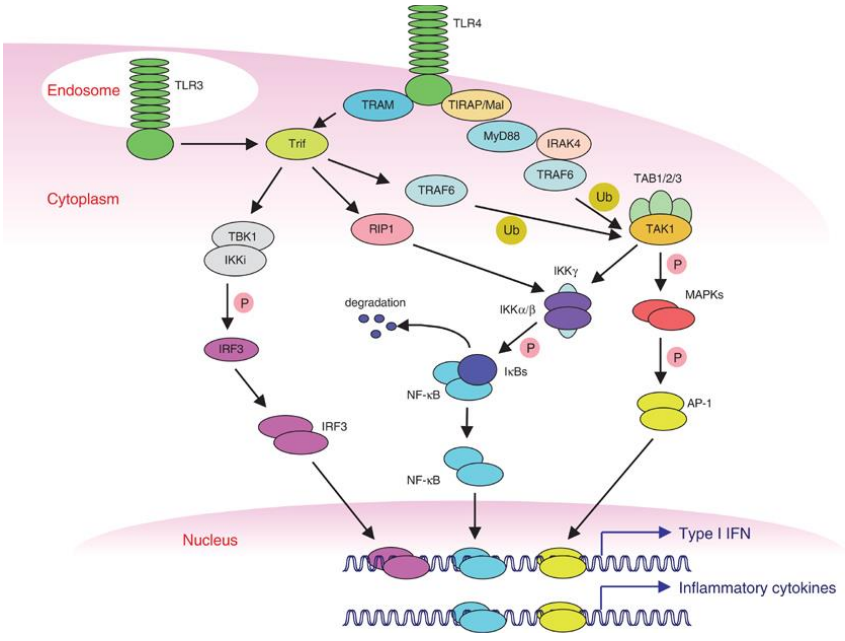

Fig. a. Toll like receptor signaling. T Kawai and S Akira. Cell Death and Differentiation (2006) **13,** 816–825.

1.1.1. Control of adaptive immune response by TLRs

Activation of the innate immune cells by TLR ligands initiates adaptive immune responses (Fig.2). On recognition of PAMPs through TLRs on dendritic cells and macrophages, costimulatory molecules and major histocompatibility complex (MHCII) molecules are upregulated. Signaling through the receptors induces the production of cytokines like IL-12, chemokines and nitric oxide. The increased expression of CD80/86 and CD40 on DCs along with the presence of IL-12 leads to the activation of $CD4^+$ T cells and its differentiation to T helper-1 (Th1) cells (Werling and Jungi, 2003). TLR4 ligand like LPS can direct dendritic cell maturation through enhanced expression of CD86 which is important for the development of Th2 responses (Dabbagh et al., 2002). TLR2 activation through peptidoglycan and zymosan ligands induce increased IL-10 production from DCs (Qi et al., 2003) that is involved in the differentiation of Th2 cells (Iwasaki and Kelsall, 1999). The differentiation of $CD4^+$ T cells to effector T cells is controlled by transcription factors. A very recent study has shown that IRF4 transcription factor in DCs drives Th2 differentiation (Williams et al., 2013). GATA-3 is the master regulator for Th2 responses promoting IL-4 production (Zheng and Flavell, 1997), while T-bet drives Th1 lineage and IFN-γ production (Szabo et al., 2000). Loss of T-bet impairs Th1 response and by default commits to Th2 cell lineage (Matsui et al., 2005). TLR2 signaling has been shown to be involved in expansion of Treg cells (Netea et al., 2004) and TLR2 and TLR4 in promoting Th17 responses (Reynolds et al., 2010); (Higgins et al., 2006). Synergistic effect of TLR2 and TLR7/8 agonists on DCs drives T cell differentiation to Th2/Th17 cells (Matsui et al., 2005). This is due to the lack of production of IL-12p70 on activated DCs (Wenink et al., 2009). TLR7 activation on DCs reduced Foxp-3

levels and the suppressive function of regulatory T cells through IL-6 production (Hackl et al., 2011). Th1/Th2 differentiation is also determined by the amount of antigen involved in the TLR activation of DCs (Sun and Pearce, 2007). At high doses of Ova-peptide, splenic DCs induced Th1 development, while low doses induced Th2 responses (Boonstra et al., 2003). In another study, high doses of LPS through TLR4 failed to induce Th2 cell responses but rather induced IL-12 production resulting in Th1 response (Eisenbarth et al., 2002). Thus, the activation of T cell subset largely depends on the TLR signals on APCs, the cytokine milieu and the dose of antigen involved.

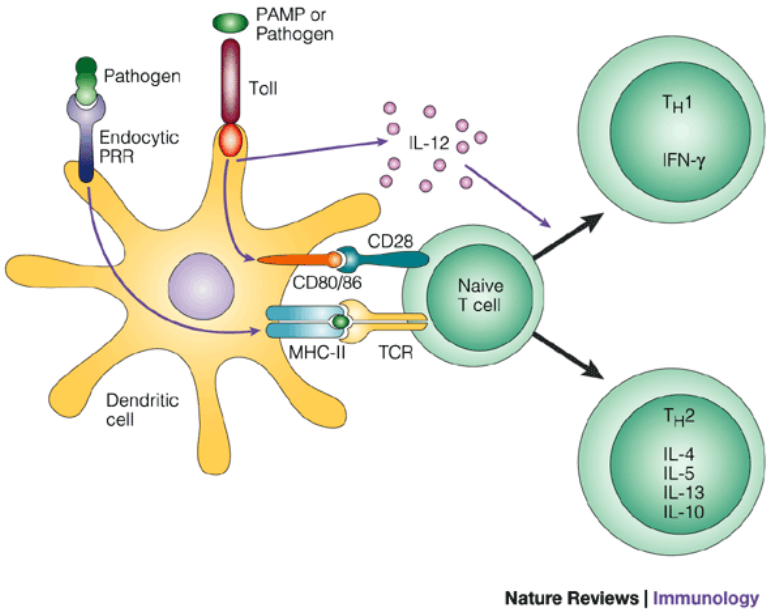

Fig. b. Toll-like receptors and innate immunity. Ruslan Medzhitov. *Nature Reviews Immunology* 1, 135-145 (November 2001)

Some TLRs are expressed on T cells, though their level of expression is variable. They are found to behave as costimulatory molecules and have a direct effect on T cell responses (Rahman et al., 2009); (Komai-Koma et al., 2004). One such example is the engagement of TLR1/2 through Pam_3CysSK_4 ligand on activated $CD8^+$ T cells that leads to enhanced $CD8^+$ T cell proliferation and

survival (Cottalorda et al., 2006). Stimulation of TLR5 on Tregs with flagellin enhances the expression of Foxp-3 and Treg suppressive activity (Crellin et al., 2005). The type of T cell differentiation generated largely depends on the category of molecular pattern/ligand that is recognized by its respective TLR (Tipping, 2006).

Since APCs are involved in initiating the adaptive immune response, it is of relevance to investigate the role of TLRs in altering the adaptive immunity. A defect in one or more components of the pathway exhibited, increased susceptibility to a variety of pathogens examined in mice and humans which underlines the importance of TLR-MyD88 interaction in recognition of microbial pathogens (Campos et al., 2004); (Scanga et al., 2004).

1.2. Autoimmunity

The concept of autoimmunity as a cause for the development of disease in humans has been accepted into the field of medical science since the 1950's. It was first predicted in the beginning of the twentieth century by the Nobel Laureate Paul Ehrlich in his proposed idea of 'horror autotoxicus' wherein an immune response against one's own body tissues does not occur in a 'normal' being. An autoimmune disease develops when the immune system starts attacking self-tissues as a result of failure of self-tolerance, resulting in an aberrant immune response. There is no individual theory that can fully explain the phenomenon of autoimmunity or the development of autoimmune diseases. Several factors are implicated in autoimmunity which include environmental triggers, immunological and hormonal factors and genetic variants. Most autoimmune diseases can only be treated but not cured because their target of self-antigen cannot be eliminated. Self-tolerance can be broadly classified into central tolerance and peripheral

tolerance. In central tolerance the immature self-reactive lymphocytes are deleted by apoptosis in the primary lymphoid organs, like bone marrow and thymus. Apoptosis is driven by the high affinity of T cells to MHCII in combination with self peptides and high affinity of B cell receptor (BCR) to naïve antigen for B cells. Although the process of central tolerance is efficient, it cannot fully eliminate all self-reactive lymphocytes partly because not all self antigens are expressed in the central site of lymphocyte development. Autoimmune regulator gene (AIRE) mainly expressed on medullary thymic epithelial cells (Kyewski and Klein, 2006) induces the expression of tissue-specific antigens, necessary for the negative selection of autoreactive T cells. In peripheral tolerance, the self antigens that are encountered by the mature self-reactive B and T lymphocytes in peripheral organs are eliminated through anergy (intrinsic biochemical changes that reduce the activation of self-reactive receptors on cells), deletion or suppression by Treg cells (Rioux and Abbas, 2005); (Gregersen and Behrens, 2006); (Xing and Hogquist, 2012). These cellular strategies act as checkpoints in the pathway for the differentiation of lymphocytes, production of antibodies and effector T cells. When self-reactive lymphocytes escape these mechanisms of tolerance and get activated, autoimmune disease develops.

In the lymphoid organs, the major B cell subsets are the transitional (newly emigrant) B cells, marginal zone and follicular B cells. The main mechanisms in regulating B cell tolerance during its development include deletion, receptor editing and anergy. Rag genes that catalyze VDJ recombination are persistently expressed, enabling BCR receptor editing (Jankovic et al., 2004) in the immature B cells. Rag expression is first induced during gene assembly of immunoglobulin heavy chain (Allman et al., 1999); (Alt et al., 1984) and second, when pre-B cells

stop their division during genetic recombination of Ig light chain (Li et al., 1993). As B cells mature, their level of Rag expression is lowered. The self-reactive BCR do not undergo prolonged receptor editing. Some reports have also highlighted the expression of Rag in the germinal centers of spleen and Peyer's patches (Han et al., 1996) as well as in B cells activated *in vitro* and *in vivo* (Hikida et al., 1996). Through the process of receptor editing which is at a frequency of 30-35%, those cells with non self-reactive Ig L and H chain are positively selected. Mature BCR is expressed on immature B cells in the form of IgM. Those immature B cells with high avidity interactions with multivalent membrane bound self-antigens are arrested and undergo elimination through apoptosis within the bone marrow (Hartley et al., 1993a). During B cell development in the bone marrow, the majority of antibodies from early immature B cells were autoreactive with anti-nuclear affinity (Wardemann et al., 2003). Several studies have shown that about 80% of the immature B cells are deleted before they reach the mature B cell compartment in the spleen, suggesting a large population of autoreactive B cells in the initial repertoire (Pieper et al., 2013); (Sidman and Unanue, 1975); (Norvell et al., 1995); (Pieper et al., 2013). In the process of central B cell tolerance, the immature B cell with prolonged and excessive levels of BCR cross linking to self antigens, promptly internalizes the BCR thereby arresting its maturation (Hartley et al., 1993b); (Nemazee and Hogquist, 2003). In the course of their halt, the acquisition of adhesion molecules, such as CD62L homing receptors, which are necessary to migrate to lymph nodes, is blocked (Hartley et al., 1993a). Nemazee and colleagues have shown that gene rearrangement can edit the specificity of autoreactive BCR, thus rescuing the cells from clonal deletion (Nemazee and Hogquist, 2003). Other mechanisms of B cells escaping the primary lymphoid

organ include intermediate BCR signals and the ignorant state of self-reactive B lymphocytes to antigens. The majority of the immature B cells undergo receptor editing. However, this process also selects B cells that express two or more Ig L and H chains that largely react to self-antigens. As a result, B cells that co express autoreactive and non autoreactive BCRs get in to the mature peripheral B cell populations (Li et al., 2002); (Liu et al., 2005). B cell activating factor (BAFF) is crucial for peripheral B cell survival and their maturation. The level of expression of BAFF-R on immature non self-reactive B cells is associated with their tonic BCR signaling. Unlike non autoreactive B cells, immature autoreactive B cells express low levels of BAFF-R that correlate with their insufficient BCR tonic signaling (Rowland et al., 2010). Enhanced BAFF expression that causes increased B cell expansion is known to be linked to autoimmunity. Elevated level of the cytokine is observed in the serum of autoimmune patients (Migita et al., 2007); (Pers et al., 2005); (Mackay et al., 2003); (Mackay and Browning, 2002).

During thymic development, T cells are selected based on their interaction with the MHC protein-bound with the self peptide fragments of antigen. Those developing T cells that express TCR with intermediate affinity to self-peptide MHCII complex are positively selected and mature to $CD4^+$ T/$CD8^+$ T cells. Double positive thymocytes that express TCR that do not bind to the self-peptide MHC complex are unable to survive and die by apoptosis. Polyclonal thymocytes that bind to self antigens are deleted in the cortex but those tissue specific or circulating antigens are deleted in the thymic medullary region. The positively selected thymocytes in the cortex move to the medulla trigger signals that induce expression of homing receptors and TCRs. On moving to the medulla, the thymocytes are still tested for their self reactivity where the medullary epithelial

cells and dendritic cells express costimulatory molecules like CD80, CD86 and CD28L for T cells (Derbinski et al., 2005). In this stage, those cells that bind strongly to self-peptide MHC complexes under the control of autoimmune regulatory AIRE gene, undergo clonal deletion (Goodnow et al., 2005). It has been shown that AIRE-deficient mice possessed disordered medulla (Gillard et al., 2007); (Dooley et al., 2008) with altered maturation of thymocytes and reduced accumulation of Treg cells (Lei et al., 2011). Another process of tolerance mechanism is through clonal diversion where self-reactive clone from T cell repertoire induce Treg differentiation thus attaining immunoregulatory function (Xing and Hogquist, 2012).

T cell differentiation and their effector functions require two signals, the first of which is the antigen and the secondary signal is provided through costimulatory molecules on APCs. Costimulatory signals largely determine the activation or deletion of T lymphocytes. Not all self-antigens are expressed in the primary lymphoid organs; some can be expressed from sequestered tissues or through developmental antigens. Thus, tolerance mechanisms of deletion and anergy of self-reactive clones take place in the periphery. In the process of peripheral tolerance as mentioned earlier, a state of unresponsiveness or anergy can be generated in self-reactive lymphocytes. This is mainly through the repression of IL-2 expression and TCR signaling, as a result of interaction with APCs that do not possess adequate levels of costimulatory molecules and through activation of CTLA-4 on Tregs. Mice deficient in CTLA-4 develop spontaneous autoimmunity (Waterhouse et al., 1995); (Tivol et al., 1995). An increased degree of autoreactive T cells accumulate in lymphoid tissues in the absence of CTLA-4 (Walker and Abbas, 2002). Furthermore, several nutrient sensing pathways have been

identified in mediating T cell anergy. Some of these include activation of ATP-deprivation sensor, adenosine signals and others. Tolerogenic DCs that arise from incomplete maturation also regulate tolerance. These peripheral tolerogenic DCs fail to provide effective signals for T cell activation and proliferation (Xing and Hogquist, 2012). Peripheral deletion of autoreactive lymphocytes is mediated through Fas (death-domain-containing receptor) and Bim (BCL-2-interacting mediator of cell death) apoptotic pathways. As T helper cells are required for the activation of B cells, T cell tolerance is the most crucial process to maintain peripheral B cell tolerance (Goodnow et al., 2005); (Xing and Hogquist, 2012), as the B cells remain functionally unresponsive or undergo death by apoptosis due to specific T helper cells that are either deleted or anaergic. Newly formed immature B cells on migration to the spleen can encounter self antigens that are absent in the bone marrow. Similarly these cells are rapidly deleted due to high avidity interaction with the peripheral self-antigens (Goodnow et al., 2005). Also, B cells that have low affinity for self antigens can be rendered unresponsive through inhibitory receptor engagement and lack of signaling through NF-ĸB. In a varied repertoire of B cell population, mature B cells that have escaped clonal deletion in the bone marrow have a greater requirement for BAFF for their survival and being weak competitors, they are unable to express high levels of BAFF-R and are thus deleted (Lesley et al., 2004). Within the germinal centers, somatic hypermutation and affinity maturation increases B cell specificity. These processes however, can result in the generation of self-reactive receptors from formerly productive ones. The survival of these cells is based on their affinity to self-antigens. They can be eliminated because of competition for DC antigen presentation or T cell help (Basten and Silveira, 2010).

Genetic factors that lead to disease susceptibility are mainly caused by complex traits that involve multiple genotypes and environmental factors such as pathogen exposure, smoking etc. However, there are several single gene disorders that have led to experimental models for the study of autoimmune diseases. The genetic variants that lead to autoimmunity can be identified through various genetic resources and tools. Genomic and proteomic approaches have made it possible to understand the molecular basis of the diseases. Microarray analyses are useful in determining the expression of hundreds of genes at the same time, providing crucial information in the pathogenesis of the disease, its progression and treatment (Fathman et al., 2005). Use of protein microarrays and assays based on flow cytometry has been greatly adapted in proteomic studies in autoimmunity. Numerous studies in complex human traits have reported the mapping of susceptible loci. Some of these include identification of CTLA-4 locus in Grave's disease and type1 diabetes, PTPN22 locus in SLE, RA (Rheumatoid arthritis) and type1 diabetes and Nod2 susceptible gene in Crohn's disease (Rioux and Abbas, 2005). Also, most autoimmune diseases have been strongly associated with the MHC region containing the HLA genes. Various animal models have been valuable in investigating the general mechanism involved in these diseases. One challenge in identifying genetic disorders is the product combination of genetic repertoire and environmental factors. The versatility of the techniques in the study of autoimmunity is limited and a multidisciplinary approach is required.

1.2.1. TLR signaling in B cell development

TLRs are expressed on early hematopoietic progenitor cells that help in distinguishing between the self and non-self in the course of their development.

TLR signaling commits the differentiation of myeloid progenitors to monocytes or macrophages. MyD88-dependent signaling commits lymphoid biased progenitors to potential DCs through cytokine production that influence differentiation patterns (Nagai et al., 2006). An earlier study has implicated that signaling through TLR7/9/ MyD88 is not essential for the positive and negative selection of the B cell repertoire. They are however necessary for germinal center reaction and antigen induced activation of B cells (Silver et al., 2006). On the other hand, study by Isnardi et al. has identified the involvement of TLR signaling pathway during B cell development where expression of TLR7, TLR10, IRAK and MyD88 was detected in B cell fractions. They found that IRAK and MyD88 deficient B cells had altered receptor editing leading to B cell precursor expressing ANA. Moreover IRAK/MyD88/UNC93B1 deficient patients possess increased frequency of naïve mature autoreactive B cells in the periphery (Isnardi et al., 2008). These data suggest the role of TLR signaling in the removal of autoreactive B cell lymphocytes and in maintenance of central and peripheral B cell tolerance.

1.2.2. Autoimmune mouse models

Autoimmune diseases can be classified into organ specific, that target individual organs/tissues and systemic autoimmune diseases, characterized by autoantibodies against wide variety of autoantigens. The development of several inbred strains of mouse models, as a result of genetic and immunological manipulation, have contributed significantly in understanding the pathogenesis involved in autoimmune diseases. In spontaneous mice models, the autoimmune disorder develops in a given mouse strain or as a result of crosses between different strains, e.g non obese diabetic (NOD) mouse. In an induced model from physical, chemical or biological factors, the animals generally develop

autoimmunity, as a result of exposure to high concentrations of the self antigens activating an immune response. Some examples of this model are experimental autoimmune encephalomyelitis (EAE) for study of multiple sclerosis and collagen induced arthritis to study RA (Morel, 2004).

1.2.2.1. Systemic lupus erythematosus (SLE)

SLE is a complex multifactorial chronic autoimmune disease involving a combination factor of genetic and environmental variants. The disease is characterized by a systemic inflammation as a result of loss of B and T cell tolerance. The exact mechanism in the occurrence of the disorder is challenging to elucidate because of the combination of several susceptible gene loci. SLE presents itself depending on the humoral and cellular responses established. The distinct features of the disorder involve swollen joints, glomerulonephritis, proteinuria, skin rash and respiratory disorders. Females are more susceptible to the disease in both humans and mouse models of SLE. In order to understand the genetic and pathological features of the disease, induced and spontaneous mouse models of SLE have been studied. The spontaneous mouse strain models include the New Zealand Black and New Zealand White (NZB/W) mice, MRL/lpr mice, and BXSB/Yaa mice (Perry et al., 2010). Immunological manifestations in these mice include B cell and T cell hyper activation, production of ANAs (anti-nuclear autoantibodies), immune complex deposition and glomerulonephritis. Among the three strains BXSB/Yaa mice exhibit a more severe glomerulonephritis. The disease development in NZB/W mice is dependent on several susceptible genes on both MHC and non-MHC loci (Kotzin and Palmer, 1987); (Kono et al., 1994); (Rahman et al., 2002). The other two strains of the SLE model possess single gene mutation. The MRL/lpr mice are developed from numerous crosses of inbred

strains of mice. Its phenotype was characteristic to an lpr mutation of the fas gene located on chromosome 19. As a result, these mice show defective lymphocyte apoptosis leading to massive lymphadenopathy and high amounts of circulating immunoglobulins, including autoantibodies. The enlarged lymph nodes include accumulation of double negative $CD4^{-}CD8^{-}$ $B220^{+}$ T cells. FasL gene mutation located on chromosome 1 also called generalized lymphoproliferative disease (gld) has shown to cause autoimmunity similar to that of an lpr mutation (Takahashi et al., 1994). Overexpression of cytokines like IL-12 and NO has been associated with pathogenesis in lpr syndrome (Huang et al., 1996). Also IL-4 and IFN γ though play opposing roles in antibody production; their isotype switching is indispensable in the development of autoimmunity in this spontaneous model. Knockout mice on either of these cytokines produce significantly reduced titers of their respective antibody isotypes along with decreased lymphadenopathy,end organ disease and early mortality (Peng et al., 1997). IFN-γ dependent autoimmune pathology has also been based on the hyper responsiveness of lpr/lpr cells to IL-18 (Neumann et al., 2001). In BXSB mice, the disease is accelerated in males due to mutation in the Yaa (Y linked autoimmune accelerator locus) - containing Y chromosome. The genetic cause for Yaa involves the translocation of TLR7 from the X chromosome onto the Yaa-containing Y chromosome. Along with abnormal B and T cell activation, these mice display monocytosis and lymphoid hyperplasia. Furthermore, it is the only model among the three that develop antibodies to nuclear components of Smith (Sm) and ribonucleoprotein. As a result of the genomic duplication, TLR7 ligand induced hyper responsiveness has been reported. However, since multiple other genes are also being duplicated, the exact role of this allele in accelerating the disease remains uncertain (Rottman

and Willis, 2010). Through TLR7 transgenic mice, TLR7 overexpression leads to spontaneous autoimmunity characterized by glomerulonephritis, autoantibodies to RNA components and dendritic cell expansion. Moreover, a reduction in the TLR7 gene dosage in the Yaa strain of mice abolishes their phenotype (Deane et al., 2007). BXSB male mice deficient in IL-21R lacks the abnormal characteristics of the Yaa mutant strain and seem highly resistant to the disease. This suggests the IL-12 signaling pathway in playing an essential role in the pathogenesis of the autoimmune disease in this SLE model (Bubier et al., 2009). Quantitative analyses have revealed the susceptibility loci of BXSB on chromosomes 1, 3, and 13 (Bxs1-6) (Haywood et al., 2000). Some induced models of lupus include pristane induced model and induced graft versus host disease. Intraperitoneal injections of pristane into Balb/C mice generate autoantibodies and immune complex deposition similar to that found in MRL/lpr mice (Satoh et al., 1995). Disease is accelerated when pristane is administered to lupus-prone NZB/W F1 mice leading to enhanced IL-12 secretion and autoantibodies to nuclear components (Yoshida et al., 2002). The development of disease in this model is cytokine dependent as IL-6 or IFN-γ deficient mice abrogate the SLE phenotype but not IL-4 deficient mice (Richards et al., 1998); (Richards et al., 2001). Chronic graft versus host disease (cGVHD) is induced by the transfer of mature T cells into immunocompromised or semiallogenic recipient causing a cytokine storm ensuing a chronic GVHD (Reddy P, 2009). $CD4^+$ T knockout mice do not develop autoantibodies or renal disease on allogeneic splenocyte transfer highlighting the role of host endogenous T cells in cGVHD (Chen et al., 1998). Several potential targets have been identified in therapy for SLE mouse models. Some of these include the use of monoclonal antibodies against BAFF/BAFFR and cytokines

that are known to accelerate disease such as IL-6, IFN-γ and TNF-α.

1.2.2.2. Multiple Sclerosis

Multiple Sclerosis is a complex inflammatory neurodegenerative disease of the central nervous system. Some of the major theories that explain the cause of MS include immunological as it is an immune mediated process, environmental variations, infectious agents as trigger factor and genetic predisposition. The most widely used and well known model of multiple sclerosis in humans is the experimental autoimmune encephalomyelitis (EAE) in mice or rats. The disease is induced in the animals by administration of myelin derived peptides in Freund's complete adjuvant, such as myelin oligodendrocyte derived protein (MOG) in C57BL/6 mice (Farine, 1997); (Miller and Karpus, 2007). It can also be induced in naïve recipients by adoptive transfer of encephalitogenic T cell blasts. In the process, injection of the myelin antigens causes a breakdown of peripheral tolerance leading to myelin specific T cell activation (Perl et al., 2004). The disease is T cell mediated involving mononuclear cell infiltration of B cells, T cells and macrophages in the brain and spinal cord through the blood-brain barrier (Perl et al., 2004), by the help of expression of integrins (Yednock et al., 1992). These infiltrating cells produce inflammatory cytokines and reactive oxygen species that lead to the destruction of neuronal myelin sheath. The passive form of induction of EAE that involves adoptive transfer of T cells from immunized animals, helps in directly understanding the effector phase of the disease in the CNS (Katsnelson, 2012). Theiler's Murine Encephalitis Virus-Induced Demyelinating Disease (TMEV-IDD) is another related murine model for MS. The principal mononuclear cells infected as a result of the virus are the macrophages or the oligodendrocytes (Clatch et al., 1990). Owing to the

complexity of the disease, EAE has its own limitations, however, these mouse models of demyelinating disease have played a significant role in better characterizing the T cell mediated demyelination and CNS tissue injury.

1.2.2.3. Type1 diabetes (T1D)

Type1 diabetes mellitus also called juvenile diabetes (because of its early onset of age) is another chronic organ specific autoimmune disorder that occurs in genetically susceptible individuals through environmental factors. The disease is due to the lack of insulin as a result of damage to the pancreatic β cells. The inflammatory infiltrate in insulitis compromises mainly mononuclear cells, $CD4^+$ and $CD8^+$ T cells. The cause of destruction of the β cells has been strongly linked to the highly polymorphic HLA class II molecules. Some of the autoantigens recognized by the islet cell antibodies are the glutamic acid decarboxylase (GAD65), protein tyrosine phosphatase–like molecule (IA-2) and thirdly insulin protein. This has made possible it to identify individuals who are at a high risk of developing T1D, before the clinical symptoms come in play. However, the contribution of these antibodies in the pathogenesis is not clearly understood (Notkins and Lernmark, 2001). The best known models of human type1 diabetes in rodents are the non-obese diabetic (NOD) mouse and the BioBreeding (BB) rat. The susceptibility to T1D in the NOD mice has been linked to the genetic loci of the insulin-dependent diabetes (Idd) region, compromising multiple genes, highlighting a large number of potential targets in the development of the disease (Driver et al., 2010). In the spleen and lymph nodes of the NOD mice, the reactive T cell clones to the islets has been known to be from both $CD4^+$ T and $CD8^+$ T cells (Van Belle et al., 2009). In the NOD mice IL-2 defective production leads to intra-islet Treg cell dysfunction, causing a breakdown of peripheral tolerance

establishing a loss of balance between Treg cells and Teffector cells (Tang et al., 2008). Another susceptibility of the NOD mice to the disease, is the variation in the CTLA-4 gene that correlated with low mRNA levels of the CTLA-4 splicing, contributing to the tissue damage (Ueda et al., 2003). The effector functions of β cell autoreactive $CD8^+$ T cells are MHCI dependent and can be negatively selected by interaction with MHCII variants (Serreze et al., 2004). Cyclophosphamide, when administered at high doses in NOD mice, leads to an aggravation of T1D and this has been associated with reduced $CD4^+CD25^+Foxp3^+$ Treg population (Brode et al., 2006). A pathogen induced model of diabetes is the lymphocytic choriomeningitis virus (LCMV) infection of the RIP-GP transgenic mice under the control of the rat insulin promoter that result in the LCMV glycoprotein expression in the pancreatic cells. As a result, the virus specific T cells recognize the infected cells as well as the glycoprotein from the β cells leading to an immune attack on these cells, causing an instant progress of the disease (Van Belle et al., 2009). The NOD is the best used model to understand the genetic causes of the human disease as well as to reverse the disease after hyperglycemia appears. The disease can be prevented by cytokine alteration or by activation of Treg suppressor cells so as to hinder effector cell functions (Farine, 1997).

1.2.2.4. Rheumatoid arthritis (RA)

RA is a heterogenous disease involving induced and spontaneous synovial inflammation. No single gene is responsible for the expression of the disease due to the polygenic genetic susceptibility, but genetics contributes to the overall disease. Several inflammatory cytokines, like TNF-α, IL-1 and IL-6, are expressed at high levels in the joints of individuals with RA (Lindqvist et al., 2002).

Genetically, RA is linked with MHCII molecule HLA-DR4. The MrL/l mice developed first by Murphy and Roths is a useful model for SLE, as mentioned earlier. These mice exhibited IgM and IgG related rheumatoid factors (RF) and polyarthritis, along with histological evidence of joint inflammation which are among the features of human RA. The synovial immune response is a result of interaction between T cells, B cells and macrophages and the joint disease was closely linked with increase amounts of IgM RF and joint pathology (Hang et al., 1982). Other transgenic mouse models that develop RA spontaneously are (a) K/BxN mice in which immune response is elicited to enzyme G6P1 in a T cell dependent manner and (b) human-TNF transgenic mice that express excess TNF-α that is involved in the pathogenesis of arthritis (Bevaart et al., 2010). Some of the induced animal models that have proved to be efficient in the study of RA in humans are rat adjuvant arthritis, rat type II collagen arthritis and mouse type II collagen arthritis. Adjuvant arthritis is developed by injecting mycobacterium in a Freund's adjuvant like mineral oil in the tail of the male Lewis rats (Farine, 1997). Though this model develops robust polyarticular inflammation, it also shows bone resorption and periosteal bone formation. The bone resorption can be inhibited by inhibition of IL-1 in this model. In rat type II collagen arthritis, an emulsion of bovine type II collagen in adjuvant is intradermally injected. This type of arthritis is characterized by cartilage damage, immune complex deposits, bone resorption and proliferation which are mediated by both B cells and T cells. Treatments with soluble TNFR and IL-1ra have been useful as combination therapies (Bendele, 2001); (Bendele et al., 2000). This treatment is also useful for the other RA mouse model of mouse type II collagen arthritis. In this model, the susceptibility has been linked to the expression of MHCII haplotypes, particularly the H-2^{q}

haplotype (Bevaart et al., 2010). Another joint specific antigen like cartilage proteoglycan is a candidate for inducing arthritis in mice in a Th1 cell mediated disease and it resembles RA both genetically and pathologically (Otto et al., 2000). Several treatments for RA have been approved some of which are the use of cytokine inhibitors (against TNF-α, IL-6, IL-1) as well as T cell targeted therapies.

1.2.3. Influence of infections in the development of autoimmunity

As we know, several environmental and genetic factors contribute to the development of immune response to self antigens in the host body. Among the environmental factors, infectious agents play a role as triggers of autoimmunity. Cells of innate immune system, like the antigen presenting cells and B cells possess receptors on the surface which can recognize or bind to the molecular patterns of the pathogen. For an adaptive immune response to occur, an antigen specific and a non-antigen specific signal is required. The antigen specific signal is through the concept of molecular mimicry in which the antigenic patterns of the invading pathogen resembles/mimics the antigenic determinants/self peptides of the host tissue (Rose and Mackay, 2000). Such an infection causes the activation of the local APCs, which in turn leads to the presentation of the self peptides. This presentation results in the activation and expansion of T cells that cross react to the self antigen, directly lyse the tissue cells and produce cytokines that activate cells like macrophages and further mediate tissue damage and activate pathogen specific B cells. This effect can also lead to the phenomenon of 'epitope spreading' in the case of chronic infection of target organs or in chronic autoimmune diseases. In this phenomenon an immune response that is initiated by the existing pathogen or direct lysis by the pathogen leads to tissue damage. These

antigens released from the injured tissue are engulfed by the APCs eliciting an expanded immune response to the protein, leading to an intramolecular or intermolecular epitope spreading of different self antigens. Acute phase proteins, such as complement proteins, opsonize the micro-organism and have the ability to bind to cellular debris of apoptotic cells, thus activating an antibody mediated effector response (Gregersen and Behrens, 2006). In bystander activation, pathogen specific T cells once activated, cause inflammation that injures the host tissue and activates autoreactive T cells in a non-host specific manner (Delogu et al., 2011). Another way that infections can lead to autoimmune response is through changes in protein (post translational modification, misfolding or mutations due to oxidative stress/free radical production) and exposure of cryptic antigens to T cells that have escaped central and peripheral tolerance which were invisible to the immune system and have now become immunogenic (Sfriso et al., 2010). Proteins produced from microorganisms of bacteria, mycoplasma and virus infected cells, called superantigens can directly bind to the TCR, regardless of antigen specificities, resulting in the activation of a large number of T cells of different antigenic specificities and functioning as a strong immune stimulating molecule (Samarkos and Vaiopoulos, 2005). Overproduction of various inflammatory cytokines including type1 interferon enhances the adaptive immune responses. Viruses are known to initiate signaling through endogenous PRRs, eliciting type1 IFN cytokines that have been associated with various autoimmune diseases.

One of the well-known associations of autoimmunity in humans is infections with viruses and bacteria. The concept of molecular mimicry was first noted in the 1980s where, by using monoclonal antibodies it was determined that the measles

virus phosphoprotein and a human simplex virus (HSV) protein cross reacted with different antigenic determinants of the intermediate filament protein in humans (Fujinami et al., 1983). Human cytomegalovirus (HCMV) is a common opportunistic pathogen with a long latency that targets several immune cells. This virus induces immunological abnormalities like RF, ANAs and cryoglobulins and lymphocytosis that has been linked to autoimmune diseases like RA, Sjodren's syndrome, Crohn's disease and others. Increase disease activity in SLE patients has also been associated with increase viral activity (Sfriso et al., 2010). In one finding, about 15% of patients with T1D showed the presence of HCMVin the lymphocytes which induces islet cell-specific antibodies that react with the human islet cell protein (Pak et al., 1990). Through the mechanism of molecular mimicry the epitope derived from the HCMV once processed and presented by the DCs is recognized by GAD65 cross-reactive T cells (Hiemstra et al., 2001). A possible association of Coxsackie B virus (CVB) with T1D has been suggested due to T cell cross reactivity of GAD amino acid (enzyme expressed in the brain and β cells in the pancreas) and P2-C protein (involved in viral replication) of the virus due to their sequence similarity (stretch of 6 amino acid sequences) (Samarkos and Vaiopoulos, 2005); (Tian et al., 1994). In rheumatic fever, infection with Group A Streptococcus can lead to inflammation of the heart where myosin in the heart has been known to be the dominant autoantigen. In this disease peripheral T cell clones against the *Streptococcal* M protein were established to be cross reactive and hence, proliferate in response to human myosin and laminin (Ellis et al., 2005). Monoclonal antibodies that recognize the M protein and *Streptococcus* carbohydrate epitope GlcNAc (Adderson et al., 1998) also cross-react with myosin as well as DNA and keratin which was confirmed by the affinity of the

non-myosin specific purified Abs from rheumatic fever patients to the myosin proteins (Cunningham et al., 1988). Further supporting the concept of molecular mimicry, Lunardi and group in their study identified a sequence of peptide that shared homology with the VP1 protein of HCMV and human cytokeratin as well as transcription factor GATA1 (Lunardi et al., 2008). GATA1 plays a crucial role in megakaryopoiesis and erythropoiesis. The antiviral peptide antibodies were also specific to collagen type II, single-stranded DNA, and cardiolipin (Sfriso et al., 2010). Significant homology has been revealed between the myelin basic protein and nucleoprotein of the hemagglutinin of influenza virus, hepatitis B virus polymerase and others (Oldstone, 1998). In a murine model of herpes simplex keratitis (HSK), local infection of HSV leads to inflammatory T mediated disease in the cornea. Similarly in a murine myocarditis model, induction of Chlamydial peptides that shared a similar sequence with the myosin peptide, caused inflammatory heart disease in BALB/c mice (Wucherpfennig, 2001); (Bachmaier et al., 1999). Another study revealed the neutralizing effects of monoclonal antibodies against myocardial CVB_3. These antibodies can recognize discontinuous epitopes on the viral capsid proteins as well as human/mouse cardiac myosin and cardiac fibroblast, inducing chronic myocardial inflammation (Gauntt et al., 1995).

Superantigens are able to bind to a range of class II MHC molecules and TCR in a Vβ specific manner. Epstein Barr virus causes a lymphoproliferating disease that leads to vast T cell activation of human $VI3^+$ T cells due to possible induction of K18.3 that possess superantigenic properties (Sutkowski et al., 1996). Staphylococcal toxic shock syndrome toxin-1 and staphylococcal enterotoxin A are the most commonly secreted T cell superantigens of the bacteria which

additionally produce protein A, which is a B cell superantigen (*Staphylococcus aureus* nasal carriage in rheumatoid arthritis). In one study, chronic exposure to small amounts of superantigen-producing *Staphylococcus aureus* caused an inflammatory systemic disease similar to SLE, characterized by infiltration of mononuclear cells predominantly $CD4^+$ T cells bearing TCR Vβ8 accompanied by ANAs and glomerular deposits (Chowdhary et al., 2012). The enterotoxin A can also trigger encephalitogenic MBP-specific non-$V\beta8^+$ T cells to cause experimental allergic EAE in PL/J mice (Soos et al., 1995). In chronic diseases like Crohn's disease, the role of superantigens have been emphasized due to the increased percentages of $V\beta8^+$ T cells in the mesenteric lymph nodes that drain the inflammatory lesions (Posnett et al., 1990). In one finding Crohn's disease has been associated with I2 bacterial gene sequence that could be linked with disease pathogenesis in patients (Sutton et al., 2000). This was confirmed in mice where I2 was evidenced as a superantigenic stimulus that is a target of $CD4^+$ Treg type cells (Dalwadi et al., 2001). Some superantigens are also virally encoded such as the protein coded by the open reading frame (ORF) gene of MMTV (Kay, 1995).

In other modes of development of autoimmunity from infections, Coxsackie virus B3 can infect and lyse the cardiac myocytes directly, causing myocardial destruction in SCID mice that can lead to autoimmunity through epitope spreading. TMEV virus, a natural mouse pathogen, has been known to induce $CD4^+$ T cell mediated demyelinating autoimmune disease due to the release of sequestered self-antigens that activate T cells which initially react to an immunodominant peptide from PLP and later to peptides from myelin basic protein and myelin oligodendrocyte (Miller et al., 1997).

1.2.4. Influence of innate immunity in development of autoimmune diseases

An over activated response of the immune system to endogenous and exogenous ligands contribute to the development of autoimmunity. A large number of published data have established the involvement of the innate immune system, particularly the TLRs, in the pathophysiology of autoimmune diseases. Pattern recognition receptors like TLRs present on innate immune cells recognize PAMPs and signal through the common adaptor MyD88 molecule, except for TLR3 that signals in a MyD88 independent manner, leading to the downstream transcription of interferon inducible genes (Interferon α/β). TLR9 recognizes hypomethylated CpG motifs and TLR7 identifies viral ssRNA. Other intracellular non TLR associated cytoplasmic receptors, like MDA-5 and RIG-1, recognize viral components of dsDNA/dsRNA and activate the innate immune system. Damage associated molecular patterns (DAMPs) released from dying cells contain high mobility group box1, heat shock proteins, uric acid and other proteins which function as alarmins and signal through TLRs inducing an inflammatory response. Pathogen mediated cell damage or damage as a result of action of cytotoxic lymphocytes, can enhance the antigen processing of the self peptides and increase expression of costimulatory molecules that effectively activate the T cell responses. Peripheral tolerance and activation of autoreactive T cells strongly depend on the innate immune cell activation.

Some studies have highlighted the role of signaling through TLR3, TLR7 and TLR9 in the production of autoantibodies and development of autoimmunity in animal models of SLE. TLR3 expression was revealed in the glomerular mesangial cells of the MRL/lpr mice and on injection with viral dsRNA, the ligand was taken up by the mesangial cells and the infiltrating immune cells. This

leads to an increase in the serum cytokine levels of IL-6, IL-12p70, and IFN-α, eventually aggravating renal damage and proteinuria in the autoimmune mice (Patole et al., 2005). TLR7 and TLR9 have been closely associated with disease pathogenesis in SLE, one reason for this is their contribution to IFN-α production. In a model of AM14 B cell transgenic cell line, immune complex containing DNA/RNA gets internalized and the complex that triggers TLR7/9 results in B cell activation and early autoantibody production. Dual TLR and BCR signaling have been implicated in the activation of auto-reactive B cells in AM14 B cells activation through MyD88 dependent TLR9 signaling (Rawlings et al., 2012). A class of immature dendritic cells, called plasmacytoid DCs expressing both TLR7 and TLR9 take up SLE-associated immune complex, leading to high levels of IFN-α production. The FcγR-mediated nucleic acid delivery to the TLR7/TLR9 of the DCs plays a vital role in the pathogenesis of SLE (Marshak-Rothstein, 2006). The influence of TLR9 in autoimmune MRL/lpr mice has been conflicting in various published data. TLR9 deficient lpr mice display a different HEp-2 immunofluroscent staining pattern consisting of a nuclear speckled pattern (antibodies reactive to snRNPs), However, a homogenous one is observed in the TLR9-intact lpr mice (Marshak-Rothstein and Rifkin, 2007). $TLR9^{-/-}$ MRL/lpr mice have increased autoantibody titers with massive lymphadenopathy, severe renal disease, proteinuria and mortality in comparison to the TLR9-sufficient mice suggesting a protective role in TLR9 signaling through a potential Treg cell function (Wu and Peng, 2006). A Yaa mutation results in expression of double the levels of TLR7 that drives the B6.Sle1.Yaa mice to produce high titers of IgG autoantibodies to RNA-associated autoantigens and increased numbers of activated T cells, mononuclear neutrophils and monocytes (Marshak-Rothstein

and Rifkin, 2007). In several lupus prone mouse models, deficiency of MyD88 abrogates the disease. One of the examples is an SLE mouse model that involves a single mutation of the protein tyrosine Lyn gene (a negative regulator of BCR signaling). In this model, disrupting the MyD88 signaling completely abolished the IgG autoantibodies to nuclear antigens along with prevention of splenomegaly and glomerulonephritis. A reduction in these mice serum antibody titers of IgM and IgG2a similar to those of the wild-type mice, was reported with decreased expression of maturation markers in dendritic cells and $CD4^+$ T cell populations when compared to the $Lyn^{-/-}$ mice (Silver et al., 2007). Teichmann and colleagues, established the role of MyD88 in B cells but not in DCs in the progression of lupus using the MRL.Fas^{lpr} model. Moreover, the frequency of follicular T helper cells as well as IFN-γ producing $CD4^+$ T cells were substantially reduced in MyD88 deficient B cell and DCs in the lupus mice (Teichmann et al., 2013).

Other biologically active autoantigens include heat shock proteins (hsp), glucosaminoglycans, high mobility group box 1 (HMGB1) and others. In one study generation of hsp, gp96 cell surface expressing transgenic mice develop hyper functional and over activated DCs in a MyD88 dependent manner leading to spontaneous development of SLE like autoimmune disease (Liu et al., 2003). Antibodies to Hsp60 and Hsp90 are commonly present in patients with SLE. High mobility group box1 is a DNA binding protein that facilitates transcription and its exposure to neutrophils/macrophages leads to increased production of inflammatory cytokines through increased nuclear translocation of NF-ĸB. HMGB induced activation of the myeloid cells has been demonstrated to involve the interaction with TLR2 and TLR4 that generates inflammatory responses (Park et al., 2004).

In other autoimmune models such as autoimmune myocarditis, depletion of C3 leads to reduced production of IL-1 and TNF-α and prevents the development of the disease. The same model revealed a subset of $CD44^{high}$ $CD62L^{low}$ expressing complement receptors (CR1 and CR2), that are involved in the activation of T cells through the upregulation of costimulatory molecules, highlighting the role of complement pathway in the induction of myocarditis (Zouali et al., 2005). In EAE mice models, complete resistance to the disease was observed in $MyD88^{-/-}$ mice along with absence of proinflammatory cytokines and hence, no brain inflammation. Though TLR2 is dispensable for induction of EAE, absence of TLR4 aggravated the disease (Marta, 2009). Moreover, TLR4 and TLR9 have a regulatory role in this Th17 mediated autoimmune disease where $TLR4^{-/-}$ and $TLR9^{-/-}$ mice possess increased expression of IL-6 and IL-23, whereas absence of MyD88 exhibited reduced levels of these cytokines along with IL-17 (Marta et al., 2008). Several *in vitro* and emerging *in vivo* research have highlighted the role of TLRs in disease progression and appropriate manipulation of these components in the treatment of chronic inflammatory conditions.

1.3. TLRs and their role in microbial infections

TLRs can bind to multiple components of bacterial cell wall such as recognition of LPS of Gram-negative bacteria by TLR4, peptidoglycan from Gram-positive bacteria (detected by TLR2), lipoarabinomannan (LAM) from mycobacteria (detected by TLR2), diacyl or triacyl lipopeptides from bacteria, mycobacteria and mycoplasma (detected by TLR2/1 or TLR2/6), flagellin from flagellated bacteria (detected by TLR5) and genomic DNA rich in unmethylated CpG DNA (detected by TLR9) from bacteria (Kumar et al., 2009). Ligand binding leads to activation of antigen presenting macrophages and DCs that can recognize

these ligands through the TLRs. This also directs their maturation to professional APCs which initiates the adaptive immune response.

TLR2 plays a potent role in detecting Gram-positive bacterial components particularly peptidoglycans and lipoproteins that are potent immunostimulants. Infections with *Staphylococcus aureus* or *Streptococcus pneumoniae* caused increased morbidity in $TLR2^{-/-}$ mice (Takeuchi et al., 1999); (Echchannaoui et al., 2002). TLR2 reportedly has also shown to detect LPS in non enterobacteria such as *Porphyromonas gingivalis* and *Helicobacter pyroli* but the LPS they detect is structurally different from the typical LPS of Gram-negative bacteria (Hirschfeld et al., 2001); (Smith et al., 2003). TLR2 identifies a large range of microbial components as they form heterophilic dimers with other structurally related TLRs like TLR1/ TLR6. Impaired TLR2 function in murine macrophages using gene knockouts or antibody blocking, on exposure to the bacterial cell wall components lead to diminished production of TNF-α and IL-6, in comparison to the normal cells (Takeuchi et al., 1999); (Takeuchi et al., 2000); (Torres et al., 2004). Thus several studies have demonstrated that TLR2-MyD88 dependent signaling contribute significantly to host resistance.

LPS endotoxin, a major constituent of the outer wall of Gram-negative bacteria interacts with CD14 (a soluble protein) and MD-2 (myeloid differentiating factor 2) to bind to TLR4 and activates signaling through MyD88, inducing a strong inflammatory response. TLR4 mutations have found to be associated with hyporesponsiveness to LPS (Hoshino et al., 1999). TLR4 mutant mice (C3H/HeJ mice) were highly susceptible to *Mycobacterium tuberculosis* infection due to impaired macrophage recruitment and proinflammatory cytokine response, leading to chronic infection of the bacteria (Abel et al., 2002). Impaired

phagosome maturation has been observed in the absence of signaling through MyD88 or TLR2/4 (Blander and Medzhitov, 2004). Infection with *Candida albicans* in TLR4 null mice exhibited reduced macrophage inflammatory protein-2 (Mip-2) and chemokine secretion (Netea et al., 2002). Moreover, a vast number of gene expression in the lung as a result of *Klebsiella pneumonia* infection, is controlled by TLR signaling that provides host survival (Schurr et al., 2005). On the other hand, in response to LPS, MyD88 independent pathway has been found to be activated in MyD88 deficient cells. These cells activate IRF3 which induce production of IFN inducible genes. However, MyD88 dependent pathway is crucial for the production of inflammatory cytokines to LPS through TLR4 signaling (Kawai et al., 2001).

Other TLRs, like TLR9 in co-operation with TLR2 have been found to provide effective resistance to *M. tuberculosis* infection, with inflammatory cytokine responses (IL-12p40, TNF, and IFN-α) and IFN-γ production (by $CD4^+$ T cells) to be largely TLR9 dependent (Bafica et al., 2005). NF-ĸB activation and chemokine gene expression to *Helicobacter pyroli* infection requires expression of TLR2 and TLR5 (Smith et al., 2003). In some infections like *Borrelia burgdorferi*, MyD88 signaling is critical for control of pathogen burden (Liu et al., 2004). Control of intracellular bacteria like *Brucella*, is also largely dependent on MyD88 for efficient pro-inflammatory cytokine production like TNF-α and IL-12p40 from DCs. This adaptor molecule is also crucial for DC maturation and activation that is essential for IFN-γ production by antigen specific T cells (Macedo et al., 2008). In *Chlamydial muridarum* infection, reduction in IFN-γ levels in natural killer cells has been associated with reduced inflammatory cytokines, as a result of lack of MyD88 (Nagarajan et al., 2011). Several *in vitro*

studies and *in vivo* studies have demonstrated the function of TLRs in recognition of *Salmonella* PAMPs and their response through the induction of pro-inflammatory cytokine gene expression. Previously, we have confirmed the role of signaling through TLRs in the survival of MyD88 deficient mice to both oral and intraperitoneal infection with an attenuated strain of *Salmonella* Typhimurium (Issac et al., 2013).

1.4. *Salmonella* infection

Salmonella of the family enterobacteriaceae are Gram-negative, flagellated, rod shaped, facultative intracellular bacteria first discovered in 1885 in pigs. About 2500 strains of the bacteria have been identified, most of which are pathogenic. The bacilli vary depending on their capsular antigen, flagellar antigens or envelope antigens. *Salmonella* species cause typhoid fever and gastroenteritis in humans and front a worldwide threat to human health. *S. choleraesuis* is associated with bacteremia, *S. typhi* and *S. paratyphi* which produce enteric fever and *S.* Typhimurium and *S. entiriditis* which produce gastroenteritis. Typhoid fever is the best studied enteric fever caused by *S. typhi. S. typhi* infects approximately 20 million people per year and is responsible for about 200,000 deaths per year (Broz et al., 2011). Infection is characterized by fever, abdominal pain and diarrhea within 6-72 hours that continues up to a week. *Salmonella* enteric species are generally contracted through contaminated poultry food or fruits and vegetables. With the increasing number of *Salmonella* strains, an emerging concern is their resistance to multiple antibiotics used to control or prevent it. So far multi-drug resistant *Salmonella* strains have been found to be resistant to fluroquinolones and third generation cephalosporins (World health organization 10-14). Infection of *Salmonella enterica* serotype Typhimurium in

mice results in a disease that resembles human enteric fever disease and is a widely used model for the study of systemic *Salmonella* infection in humans.

The first stage of the infection which lasts for a few hours, involves rapid clearance of the bacteria from the blood by phagocytic cells of the spleen and liver, where large number of organisms are killed due to the bactericidal activity of macrophages. In the second phase of infection, bacteria that have survived undergo intracellular multiplication in the target organs and an exponential growth phase occurs which is characterized by the production of inflammatory cytokines like TNFα and IFN-γ that is vital for the induction of macrophage bactericidal activity to suppress infection (Mittrucker and Kaufmann, 2000); (Sebastiani et al., 2002). The host is then able to curb the bacterial growth and reach a plateau phase with high bacteria levels. Once the acquired immunity involving both B cells and T cells develop, particularly the activation and expansion of Th1 cells the bacterial infection is efficiently cleared. The main route of entry is through specialized epithelial cells of the Peyer's patches called M cells. *Salmonella* then further propagates to the mesenteric lymph nodes and eventually through the blood stream to the spleen and liver causing disease. In the initial stages of bacterial infection after ingestion of contaminated food, bacteria adhere and cause subsequent invasion in the intestine. Bacterial adherence is mediated through type1 and type IV fimbriae, autotransporter adhesins as well as type 1 repetitive adhesins (Linke et al., 2011). *Salmonella* gain access into the macrophages through phagocytosis and receptor mediated uptake. Alongside, the injection of the secretory proteins by the bacteria enhances uptake and drives internalization (Finlay and Brumell, 2000). Virulence proteins called type3 secretion system (T3SS) encoded by *Salmonella* pathogenicity island (SPI) promote bacterial

uptake into the host cells and its survival. T3SS coded by SPI-1 in the intestinal lumen is involved in bacterial invasion into the epithelial cells and host cells, while T3SS encoded by SPI-2 is involved in the systemic phase of intracellular bacterial replication and survival as well as dissemination to the organs (Salcedo et al., 2001). Proteins coded by SPI-2 inhibit the fusion of *Salmonella* containing vacuoles in the macrophages with the lysosomes and endosomes (Uchiya et al., 1999); (Tobar et al., 2006). This is facilitated by the ability of the SPI-2 TSS to thwart the NADPH oxidase assembly at the phagosomal membrane thus evading oxidative damage (Gallois et al., 2001). The effector proteins also counteract the localization of iNOS to the *Salmonella* containing vacuole thus, avoiding death by the combined microbicidal effect of reactive oxygen and nitrogen species (Chakravortty et al., 2002). The induction of these proteins plays a vital role in determining the virulence of *Salmonella* and its systemic spread into the host tissues.

Several mouse strains vary in their susceptibility to *Salmonella* infection depending upon their status of their Nramp1 (natural resistance-associated macrophage protein 1) gene expression. This macrophage specific transmembrane protein acts as a channel for divalent cations into the phagosome and controls intracellular microbial replication during the early phase of infection. A wild-type allele of the gene is present in the mouse strains of C3H and 129, while C57BL/6 and BALB/c carry a mutant allele of the gene making them 1000 fold more susceptible to *Salmonella* infection (al-Ramadi et al., 2001); (al-Ramadi et al., 2002); (al-Ramadi et al., 2004); (Gerold et al., 2007). The Nramp1 gene provides natural resistance to several intracellular infections and any mutation in the gene

reduces the efficacy to kill the pathogen and thus has been associated with increased susceptibility (Skamene et al., 1998).

1.5. Role of TLRs in *Salmonella* infections and its association with autoimmunity

The main ligands of *Salmonella* that recognize and activate TLRs are the lipopolysaccharide, lipoproteins, CpG DNA and flagellin which are recognized by TLR4, TLR2, TLR9 and TLR5 respectively (Hoshino et al., 1999); (Gewirtz et al., 2001); (Feuillet et al., 2006); (O'Brien et al., 1980); (Vazquez-Torres et al., 2004). Csg component of the fimbriae that forms a structural characteristic of *Salmonella* Typhimurium is recognized by TLR2, eliciting an IL-8 host immune response (Tukel et al., 2005). LPS was postulated to being the major virulence factor since direct injection of LPS into the blood stream caused shock. Endotoxin containing LPS and BLP of Gram-negative bacteria can induce a strong inflammatory response. TLR4 responds to LPS-binding protein in association with CD14 and MD-2 proteins (Vazquez-Torres et al., 2004). A dominant missense mutation in TLR4 signaling in C3H/HeJ strain leads to impaired production of TNF-α, a cytokine that is crucial for protective function in response to *Salmonella* infection, when compared to wild-type mice (Li and Cherayil, 2003). $TLR4^{-/-}$ mice were more susceptible to infection than $TLR2^{-/-}$ mice which were similar to the WT mice, suggesting that TLR4 seems to a dominant role over TLR2, mainly due to its earlier expression in the immune response to *Salmonella*. However, $MyD88^{-/-}$ mice harbored the most bacterial burden due to defective macrophage killing and TNF-α production (Weiss et al., 2004). Moreover, TLR4 signaling through MyD88 and not TRIF, is essential for protection by establishing a plateau phase of bacterial growth in the typhoid model, as negligible levels of NO and TNF-α

production were observed in MyD88$^{-/-}$ mice (Talbot et al., 2009). Activation of TLR9 enhances the antigen presentation by DCs and killing of *Salmonella* organisms in a ROS dependent manner (Lahiri et al., 2010). Furthermore, interaction of TLR5 of the lamina propria DCs with the *Salmonella* organisms, mediate migration of the bacteria across intestinal mucosa to the mesenteric lymph nodes (Uematsu et al., 2006). On the other hand, TLR11 blocks the entry of highly invasive *Salmonella* into the murine Peyer's patch and the systemic spread, highlighting the potential of TLR11$^{-/-}$ mice as *S.* Typhimurium murine model (Shi et al., 2012). Interestingly, a study has also shown that *S.* Typhimurium failed to form *Salmonella* containing vacuole and multiply in TLR2/4/9$^{-/-}$ and MyD88$^{-/-}$ bone marrow derived macrophages, suggesting a pre-requisite of TLR signaling in the induction of virulent gene expression for bacterial survival (Arpaia et al., 2011b). Furthermore, inflammation in *Salmonella* colitis as a result of induction of T3SS by SPI-2, is solely dependent on MyD88 signaling while SPI-1 induced T3SS causes inflammation in a MyD88 independent manner (Hapfelmeier et al., 2005).

MyD88 as an adaptor molecule, plays a crucial role in the acute phase of infection as MyD88$^{-/-}$ mice are hyper-susceptible to even an infection with an attenuated strain of *S.* Typhimurium, due to increased bacterial burden in target organs, delayed recruitment of phagocytes and production of inflammatory cytokines (Issac et al., 2013). Several studies have confirmed the generation of antibody responses to *S.* Typhimurium, even in TLR mutant mice strains. Generally, *Salmonella* infection in wild-type mice is characterized by a Th1 mediated IFN-γ response that drives B cells to the production of IgG3 and IgG2c humoral response. However, enhanced *Salmonella* specific IgG1 responses were

found to take place independently of signaling through MyD88 and TLR4 (Seibert et al., 2010). This was confirmed in another study where oral infection with recombinant attenuated *S.* Typhimurium (RASV) elicited dominant Th2 responses in MyD88$^{-/-}$ mice, suggesting that signaling through the adaptor molecule may be dispensable for induction of LPS specific antibody responses (Ko et al., 2009). Moreover, in our previous study we verified dysregulated *Salmonella* specific antibody responses of all isotypes (both Th1 and Th2 responses) being produced in the MyD88 deficient mice in response to intraperitoneal infection of *Salmonella,* establishing a state of hypergammaglobulinemia. The memory T cells produced as a result of the bacterial dose, however failed to protect the MyD88 knockout mice on a challenge to the virulent strain of the bacteria, further emphasizing the vitality of efficient signaling through the MyD88 for protection against *Salmonella* infection (Issac et al., 2013).

Several studies, as briefly described earlier, have confirmed TLRs to be actively involved in the pathogenesis of autoimmune diseases. Irrespective of the specificity of B cell receptor, TLR expression in B cells activates autoreactive B cells in autoantibody production (Soulas et al., 2005). A dramatic IgM hypergammaglobulinemia response, as a result of B cell hyper activation was observed in MyD88$^{-/-}$ mice, in response to *Borrelia burgdorferi* infection. This contributed to autoreactivity of IgM antibodies to autoantigens at levels similar to those observed in a lupus prone MRL/lpr mouse strain (Woods et al., 2008). Similarly, oral administration of RASV in MyD88$^{-/-}$ mice induces hyper-IgG autoimmune response in a T cell dependent manner, involving increased dsDNA autoantibody levels along with deposition of immune complex in the kidneys. Oral infection also leads to systemic inflammation characterized by increased

levels of inflammatory cytokines from enhanced inflammatory myeloid cell populations along with expansion of the B cell and T cell compartment compartment (Ko et al., 2011).

2. AIMS OF THE THESIS

The overall aims of the thesis were to understand the potential mechanism involved in the observed phenomenon of hypergammaglobulinemia and to test the loss of immunological self-tolerance in MyD88-deficient mice following infection with *Salmonella* Typhimurium.

The specific aims were:

1. To define the underlying mechanisms involved in the induction of hypergammaglobulinemia in mice lacking MyD88 following infection with attenuated *S.* Typhimurium.
2. To characterize the extent and specificities of autoantibody production in *Salmonella*-infected MyD88$^{-/-}$ mice and define the parameters required for their synthesis.
3. To uncover the cellular and molecular mechanisms responsible for the dysregulated antibody production.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Summary of materials used and suppliers:

Materials	**Company**
PBS (-$CaCl_2$ – $MgCl_2$)	GIBCO
10X PBS (-$CaCl_2$ – $MgCl_2$)	GIBCO
BSA	Sigma
SS Agar	Mast Group
Trypticase soy agar	Mast Group
Trypticase soy broth	Oxoid
RPMI 1640 medium (-L glutamine)	HyClone
Ethanol	Carlo Erba
H_2SO_4	Readel-de Haen
Tryptan blue solution (0.4%)	Sigma
DPX	Panreac
Ampicillin	Sigma
Streptomycin	Sigma
NaCl	Panreac
NaH_2PO_4. H_2O	Panreac
Na_2HPO_4	Panreac
Na_2CO_3	AnalaR
NaN_3	AnalaR
$NaHCO_3$	GibcoBRL

NH_4CL	Sigma
$KHCO_3$	Sigma
EDTA	Sigma
H_3BO_3	Sigma
96 well Maxisorp nunc immuno plate	Thermo Scientific
24 well tissue culture plates	Becton Dickenson
96 well Microtest U bottom culture plates	Becton Dickenson
Microscope slides	Fischer Scientific
7-AAD Viability Staining Solution	e-Bioscience
Streptavidin HRP	R&D
TMB	Becton Dickenson
Xylene	Panreac
Heamotoxylin	Panreac

3.1.2. Standard solutions

3.1.2.1. 10x Phosphate buffered saline (PBS) for antibody determination by ELISA

87.66 g of NaCl, 2.56g of NaH_2PO_4, 11.94g of Na_2HPO_4 dissolved in 1L d.H_2O, pH 7.2.

3.1.2.2. Phosphate buffered saline (PBS) with 0.05% Tween-20

1x PBS (made from home-made 10xPBS) with 0.05% tween-20, pH 7.2- 7.4

3.1.2.3. Trypticase soy broth (TSB)

3.1.2.4. RBC lysis buffer

8.3NH_4Cl, 1g $KHCO_3$ 1.3ml of 5%EDTA dissolved in 1L distilled water and filtered through 0.2µm filter

3.1.2.5. Blocking buffer for pathogen specific antibody detection by ELISA (1% BSA, 5% sucrose, 0.05% NaN_3 in PBS)

5g of BSA, 25g of sucrose, 0.25g NaN_3 in 500ml 1xPBS (made from home-made 10xPBS), pH 7.2-7.4

3.1.2.6. Reagent diluent for antibody determination ELISA (1%BSA)

1g Bovine serum Albumin dissolved in 100ml 1xPBS (made from home-made 10xPBS), pH 7.2-7.4

3.1.2.7. 0.05M Carbonate Buffer for autoantibodies detection by ELISA

0.16g Na_2CO_3 and 0.3g $NaHCO_3$ dissolved in 100ml PBS, pH 9.6

3.1.2.8. Borate buffer saline (BBS)

200mM of boric acid, 75mM NaCl in 1L d.H_20, pH 8.4

3.1.2.9. BBT

0.5%BSA, 0.4%tween in BBS

3.1.2.10. BBS: BBT

0.5%BSA, 0.1% NaN_3 and 0.4%tween in BBS

3.1.2.11. 2N H_2SO_4

6ml of conc.H_2SO_4 was dissolved in 100ml d.H_20

3.1.2.12. Staining Buffer for FACS (PBS/1% FCS/0.1% NaN_3)

1ml Fetal calf serum and 0.1g NaN_3 was dissolved in 100ml PBS

3.1.2.13. 4% paraformaldehyde for fixation

100ml 37% formaldehyde in 400ml d.H_2O and 500ml 0.2M Phosphate buffer

3.1.2.14. AutoMACS buffer for cell purification

0.5% BSA, 2mM EDTA in 1xPBS, pH 7.2

3.1.3. List of antibodies used in ELISA and cell culture:

Antibody	Catalogue #	Company	Concentration / Dilution used
Rat anti-mouse IgG3 (Biotin)	1191-08	Southern Biotech	1:5000
Goat anti-mouse IgG1 (Biotin)	1070-08	Southern Biotech	1:20,000
Goat anti-mouse IgG2c (Biotin)	1079-08	Southern Biotech	1:20,000
Goat anti-mouse IgM (Biotin)	115-066-075	Jackson Immunoresearch	1:10,000
Purified mouse IgG1	0102-01	Southern Biotech	50ng/ml
Purified mouse IgG2c	0122-01	Southern Biotech	50ng/ml
Goat anti-mouse IgM+IgG+IgA (H+L)	1010-01	Southern Biotech	0.1µg/ml
Goat anti-mouse IgG, F(ab´)$_2$ fragment-specific	115-006-006	Jackson Immunoresearch	1µg/ml
Biotin labeled-goat anti-mouse IgG (Fcγ-specific)	115-066-008	Jackson Immunoresearch	1:10,000
FITC-labeled goat anti-mouse IgG (Fcγ-specific)	31547	Pierce	1:100
Purified anti-mouse CD3 (clone 2C11)	8310	CJ Lab	1-5µg/ml
Hamster anti-mouse CD28 (clone 37.N.51)	12.96	CJ Lab	10-20µg/ml
Mouse IgG	115-000-003	Jackson Immunoresearch	1µg/ml
Mouse IFN-γ set	555138	Becton Dickenson	
Mouse IL-4 set	555232	Becton Dickenson	
Mouse IL-10 set	555252	Becton Dickenson	

3.1.4. Antibodies used for FACS staining

Antibody	Conjugate	Company	Catalogue	Dilution used
Rat anti-mouse CD3	FITC	BDPharmingen	553063	1:200
Rat anti-mouse CD4	PE-Cy7	Biolegend	100528	1:200
Rat anti-mouse CD4	APC-Cy7	BDPharmingen	552051	1:100
Rat anti-mouse CD4	APC	e-Bioscience	17004182	1:200
Rat anti-mouse CD8a	APC-Cy7	BDPharmingen	557654	1:100
Rat anti-mouse CD11b	APC-eFluor780	e-Bioscience	47011282	1:500
Rat anti-mouse CD19	PE-Cy7	BDPharmingen	552854	1:200
Rat anti-mouse Sca-1	PE	BDPharmingen	553336	1:100
Rat anti-mouse Ly6G/C	FITC	e-Bioscience	11593182	1:200
Hamster anti-mouse CD80	APC	BDPharmingen	560016	1:200
Rat anti-mouse CD86	FITC	e-Bioscience	11086282	1:100
anti-mouse CD25	APC	BDPharmingen	557192	1:100
Armenian Hamster IgG1 Isotype Control	Alexa Fluor 488	e-Bioscience	12488883	1:200
Rat IgG2a Isotype Control	PE-Cy7	e-Bioscience	25432182	1:200
Rat IgG2a Isotype Control	PE	e-Bioscience	12432182	1:200
Rat IgG2a Isotype Control	APC-eFluor780	e-Bioscience	47432182	1:200
Rat IgG2b Isotype Control	FITC	e-Bioscience	11403182	1:200
Rat IgG2b Isotype Control	APC	e-Bioscience	17403182	1:200
Rat IgG2b Isotype Control	APC-eFluor780	e-Bioscience	47403182	1:200

Rat IgG2a Isotype Control	FITC	e-Bioscience	11432182	1:200
Rat IgG1 Isotype Control	APC	Biolegend	400412	1:200
Rat anti-mouse IFN-γ (for intracellular staining)	PE-Cy7	Biolegend	505826	1:100
Rat anti-mouse IL-4 (for intracellular staining)	APC	Biolegend	504106	1:100
Rat anti-mouse IL-10 (for intracellular staining)	PE	Biolegend	505008	1:100
Rat IgG1 Isotype Control	PE-Cy7	Biolegend	400416	1:200
Rat IgG2b Isotype Control	PE	Biolegend	400608	1:200
Anti-mouse CD16/32	-	e-Bioscience	14016185	1:100

3.1.5. Mice

C57BL/6 mice, obtained from Harlan Olac (Bicester U.K.) and MyD88-deficient ($MyD88^{-/-}$) mice provided by Dr. S. Akira (Osaka University, Japan) (Adachi et al., 1998a; Kawai et al., 1999) through Dr. Richard Flavell (Yale University School of Medicine, USA) were bred in our animal facility and maintained in filter-topped isolator cages on Bactrim-supplemented water (for $MyD88^{-/-}$ mice only). $CD154^{-/-}$ mice ($CD40L^{-/-}$) mice on C57BL/6 background were provided by Dr Richard Flavell's lab (al-Ramadi et al., 2006); (Xu et al., 1998). Mice were taken off antibiotics for at least 7-10 days before use in any experiment. All animals were routinely used at 8-12 weeks of age. All studies involving animals were conducted in accordance with and after approval of the animal research ethics committee of the College of Medicine and Health Sciences, United Arab Emirates University.

3.2. Methods

3.2.1. Bacterial strains used and preparation

For the current studies, we used an attenuated, double auxotrophic, strain of *Salmonella enterica* serovar Typhimurium (hereafter referred to as *S.* Typhimurium), with deletions in the aroA and aroD genes which are critical for the synthesis of essential prokaryotic aromatic compounds. The *aroA*$^{-}$/*aroD*$^{-}$ mutant strain, designated BRD509, expressing the empty nirB plasmid vector was used throughout (al-Ramadi et al., 2001); (al-Ramadi et al., 2004). It acquired resistance to ampicillin, which is encoded by the nirB plasmid, and can therefore be easily selected. IFN-γ and IL-2 cytokine expressing strains of BRD509, designated GIDIFN and GIDIL2, have been previously described (Al-Ojali et al., 2012a); (al-Ramadi et al., 2002); (al-Ramadi et al., 2001). The expression of IFN-γ and IL-2 is under the control of the nirB promoter (Xu et al., 1998). In some studies, we also utilized a strain of *Acinetobacter baumanii* (designated NM97), which is a clinical isolate from Tawam hospital, and was kindly provided by Dr. Tibor Pal (Department of Microbiology & Immunology, CMHS). Aliquots of BRD509 (or its derivative strains) were grown on *Salmonella* Shigella (SS) agar [with ampicillin (100μg/ml conc.)]. *A.baumanii* (NM97) strain was grown on trypticase soy agar (TSA). For the BRD509 (or its derivative strains) bacterial dosage preparation, about 4-5 CFUs were cultured in 10 ml trypticase soy broth (TSB) containing ampicillin (1μg/ml) overnight and then diluted 1:10 in fresh TSB [with ampicillin (1μg/ml)] and grown for 2 hours (shaking) at 37°C. At the end of this incubation, the OD_{600} nm optical density of the bacterial suspension was measured at 600nm. 1ml of the culture was read on a spectrophotometer

against TSB as blank. The bacterial concentration was estimated based on the formula of OD_{600} nm of 1=1.2x10^9/ml bacterial CFUs. Log-phase bacterial suspensions were prepared in pyrogen-free saline and injected intraperitonealy (i.p) in 0.5ml volume at the indicated dose. For the NM97 bacterial dosage preparation, about 4-5 CFUs were cultured in 5ml TSB overnight. The next day, 5 ml bacterial culture was poured into a conical flask containing 45ml fresh TSB and kept for shaking at 37°C for 4 hours. The bacterial culture was then transferred to a 50 ml tube, centrifuged at 3500 rpm for 15 minutes and re-suspended in 1xPBS. The culture at 1:100 dilution was read on a spectrophotometer against 1xPBS as blank. Log-phase bacterial suspensions were prepared in pyrogen-free saline and injected intraperitonealy in 0.5 ml volume at the indicated dose. For all infections, the bacterial doses were verified by CFU plate counts.

3.2.2. Enumeration of bacterial load and IgA extraction from fecal pellets

Procedures for determination of CFU have been described previously (al-Ramadi et al., 2001). Groups of mice (5-10 mice per group) were sacrificed at different time points after i.p infection. Feces were freshly collected, weighed and suspended in 1xPBS. The suspension was vortexed and an aliquot at an appropriate dilution was streaked on SS agar plate containing both ampicillin (100μg/ml) and streptomycin (200μg/ml) and the CFU counts were determined after overnight incubation at 37°C. To determine the bacterial loads in spleen and liver, target organs were aseptically removed at the indicated time points and homogenized in 1 ml of cold sterile saline in an Ultra-terrax T25 tissue homogenizer (Janke & Kunkle, Staufenim Breisgau, Germany). An aliquot of

appropriate dilution was streaked on SS agar (with ampicillin) plates for BRD509 and the CFU counts were determined after overnight incubation at 37°C. Spleen weights were also determined at the indicated time points post inoculation.

For secretory IgA extraction, fecal pellets were suspended at a concentration of 250 mg/ml of cold extraction buffer consisting of 1×PBS solution, 30 mM EDTA, 1% BSA, and 1/100 dilution of protease inhibitor cocktail stock (Calbiochem, Germany). The suspension was then vortexed and supernatants were collected after centrifugation at 10,000 rpm for 7 minutes at 4^{o}C and stored at −20^{o}C until assayed for IgA levels.

3.2.3. Peritoneal exudate cell collection

Groups of mice (5–10 mice per group) were sacrificed at different time points after infection. To harvest peritoneal lavage, 10 ml of cold $Ca2^{+}$-, $Mg2^{+}$-free saline was injected into the exposed peritoneal cavity and the fluid was withdrawn through the anterior abdominal wall with a 20-gauge needle. The fluid was then spun down at 2500 × *g* for 5 minutes and the cell pellet was resuspended in distilled water.

3.2.4. Spleen cell preparation

Single cell suspensions were prepared by gently teasing the spleen between frosted ends of microscope slides. Cells were then spun down at 1200 rpm for 5 minutes at 4°C. The spleen cell suspension was depleted of red blood cells by incubation in RBC Lysis buffer (10-12 ml/spleen sample) for 5 minutes, after which they were spun down at 1200 rpm for 5 minutes and suspended in 1xPBS. The erythrocyte-depleted spleen cells were counted on a hemocytometer and cell viability was determined by trypan blue dye exclusion.

3.2.5. Spleen cell purification

Purification of $CD4^+$ and $CD11b^+$ spleen cell subpopulations was done using magnetic bead separation on an autoMACS cell sorter (Miltenyi Biotec, Germany), following manufacturer's procedure. For the purification of T lymphocytes, spleen cells (10 x 10^6 cells per run) were centrifuged at 300 x *g* for 10 minutes and cell pellets were re-suspended in 90µl of autoMACS buffer and 10µl of anti-CD4 mAb-coated magnetic beads (GK 1.5 clone, Miltenyl Biotec). In case a higher cell number was needed for purification, the volume of buffer and magnetic beads were adjusted accordingly, while maintaining the cell: magnetic beads ratio. After incubation with mAb-coated magnetic beads for 15 minutes at 4-8°C, spleen cells were washed with 1ml of buffer and centrifuged again at 300 x *g* for 10 minutes. Cells were then re-suspended in 500µl buffer and CD4-positive cell fraction was collected using the Possel program on the autoMACS cell sorter. The extent of cell purity was verified independently by staining with non-cross-reactive CD4-specific mAb (RM 4-5) and analysis on a FACSCanto (BD). Routinely, purified cells were confirmed to be 90-95% $CD4^+$ T cells.

The $CD4^+$ T cell-negative fraction of the spleen cell suspension was also collected, and cell number was determined, for subsequent purification of $CD11b^+$ myeloid cells. For this purification, 10 x 10^6 spleen cells were re-suspended in 90µl of autoMACS buffer and incubated with 10µl of anti-CD11b mAb-coated magnetic beads (Miltenyl Biotec) for 15 minutes at 4-8°C. After incubation, the cells were washed with 1ml of the buffer and centrifuged again at 300 x *g* for 10 minutes. Cells were then re-suspended in 500µl buffer and magnetic separation was carried out with the autoMACS separator. CD11b-positive cell fraction was collected using the Possel program on the magnetic cell sorter. Routinely, the cells

collected were composed of 75-85% $CD11b^+$ cells as confirmed by staining with fluorochrome conjugated anti-CD11b antibodies.

3.2.6. Flow cytometry

Spleen cell suspensions were prepared from animals infected intraperitonealy 21 days previously with a dose of about 3000 BRD509/mouse. Processing of spleen cells for FACS analysis was conducted, as described previously (al-Ramadi et al., 2001). Whole spleen cells and PECs obtained from the peritoneal wash, as described above were re-suspended in staining buffer (SB) (PBS/1% FCS/0.1% NaN_3**)** at a concentration of 0.5 x 10^6 cells/well in U-bottom 96-well plate (BD) and incubated with 50µl/well of anti-mouse CD16/CD32-specific mAb (clone 2.4G2) for 30 minutes at 4°C to block FcγRIII/II sites in order to avoid nonspecific binding. The plate was then centrifuged at 750 rpm for 3 minutes at 4°C. After decanting the buffer, cells were stained with appropriate dilutions, of directly-conjugated monoclonal antibodies (mAbs; all purchased from eBioscience or BD or BioLegend, as previously mentioned and detailed in Table 3.1.4) in a total volume of 100µl/well and analyzed by 6-color FACS. In all staining groups, 7-AAD dye (e-Bioscience) was included in order to exclude non-viable cells from the analysis. All antibodies were pre-titrated in preliminary experiments and used at saturating concentrations. Cells were incubated with a mixture of appropriately-diluted mAbs at 4°C for 30 minutes followed by two wash cycles with SB. The first wash was with 100 µl/well of SB where the cells were mixed well with the pipette and centrifuged. In the second wash, 200 µl/well of SB was added and mixed well followed by centrifugation. Cells were finally re-suspended in 200µl/well of SB and fixed by adding 100µl of 4%

paraformaldehyde per well. Data were collected on 30,000 cells using BD FACSCantoII and analyzed using BD FACSDiva software.

For intracellular cytokine staining, 100µl of anti-mouse CD3 (clone 2C11) at 5µg/well and 100µl of anti-mouse CD28 (clone 37.N.51) at 20µg/well in 1xPBS were added into 24-well tissue culture plates and incubated at 37°C with 5% CO_2 for 2 hours after which the solution was aspirated from each well. Enriched $CD4^+$ T cells (1 x 10^6 cells/ml/well) were suspended in RPMI supplemented with 5%FCS, l-glutamine, sodium pyruvate, essential amino acids, non-essential amino acids, pen/strep, gentamicin, and 2-ME (all reagents from Gibco BRL) for stimulation at 37°C with 5%CO_2 for 24 hours. Purified $CD4^+$ T cells incubated in the absence of anti-CD3/CD28 mAb were also included and used as non-stimulated control. Four hours before harvesting the cells, 1 µl of BD GolgiPlug (Brefeldin A) was added per well. Intracellular staining for cytokines was carried out following staining for cell surface antigens as described earlier, after which cells were permeabilized using permeabilization/fixation buffer (BD Cytofix/Cytoperm Plus #555028) for 20 minutes at 4°C. Cells were then incubated with 100µl/well of antibody conjugates of IFN-γ, IL-4 or IL-10 for 30 minutes at 4°C and washed with 1x Perm/Wash solution. Isotype controls for the respective cell surface and intracellular antibodies were used to determine nonspecific binding. Data were collected on 30,000 cells and analyzed using BD FACSDiva software.

3.2.7. Preparation of heat killed bacteria

Bacterial strains were grown to log-phase, as described above. After taking an aliqout for verification plate counting, bacterial suspension was washed in

pyrogen-free saline, centrifuged at 4000 rpm for 15 minutes at 4°C and finally re-suspended in 5 ml PBS. The samples were then placed in a water bath at 65^0C for 1 hour, following which aliquots (100µl) were removed and streaked on SS agar or TSA plates for BRD509 or NM97 bacterial strains, respectively, to confirm that all bacteria were heat killed. The remainder of the heat-treated suspension was aliquoted in 0.5 ml volume and stored at -20°C until use.

3.2.8. Measurement of pathogen specific antibodies in serum

Serum samples were obtained at the indicated time points post i.p infection with ~200 CFUs/mouse. Blood was collected on the day of sacrifice from the vena cava, left at room temperature to clot for 30-60 minutes, spun down at 2500 rpm for 5 minutes, following which sera (~200-300µl) were collected. Serum samples were stored at -20°C till further analysis. The presence of *Salmonella*-specific as well as Acinetobacter-specific antibodies of IgG1, IgG2b, IgG2c, IgG3, and IgM isotypes were determined by an established ELISA protocol (Issac et al., 2013). Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated overnight with 100 µl/well of heat-killed bacteria (1×10^6 cells/well). Wells were then washed 3 times with PBS-T and blocked for 2 hours at room temperature using 300µl/well of the blocking buffer (1%BSA, 5%Sucrose, 0.05%NaN_3 in PBS). To generate a standard curve, wells were coated with 0.1µg/ml of goat anti-mouse Ig(H+L) (Southern Biotech), and blocked with 300µl/well of 2% BSA in 1xPBS (7.2 pH). After one wash cycle, serum samples from individual animals were serially titrated in the plate (range 1/100 to 1/3200 dilution, in duplicates). For the standard wells, mouse isotype-specific antibody (Southern Biotech) (starting at 50ng/ml and titrated in 1:2 serial dilutions) was added in a final volume of 100µl/well. The standards were prepared in separate tubes and added in 100µl

aliquots to appropriate wells. For the serum samples, dilutions were carried out directly in the plate. The test plates were then incubated at room temperature for 2 hours. After extensive washing, secondary antibodies (biotin-conjugated, isotype-specific antibodies; dilutions indicated in table 3.1.3) were added (100µl/well) and incubated for another 2 hours at room temperature. After another series of washes, streptavidin–HRP (100µl/well; R&D #DY998) was added at a final dilution of 1/200 and incubated for 40 minutes. Wells were washed again and developed by adding 100µl/well of the substrate 3,3′,5,5′-tetramethylbenzidine (TMB; Becton Dickenson #555214) and incubated for 10-30 minutes. The reaction was stopped by adding 50µl/well of 2N H_2SO_4. Plates were read at 450 nm using a TECAN microplate reader (Maennedorf, Switzerland).

3.2.9. Total IgG isotypes

Nunc MaxiSorp 96-well plates were coated with 100µl of 1µg/ml goat anti-mouse IgG $F(ab')_2$ (Jackson Immunoresearch) in 0.5M sodium bicarbonate (pH=9.6) and incubated at 4°C overnight. After washing, plates were blocked for 2 hours with 200µl/well of 3% BSA in 1xPBS (pH 7.2). Plates were washed and serially diluted samples along with standard dilutions of mouse isotype specific antibody (Southern Biotech) in 1% BSA were added at a final volume of 100µl/well. The standards were prepared in individual eppendorf tubes and 100µl aliquots were transferred to the wells. For the test samples, serial dilutions were carried out directly in the plate, followed by incubation for 2 hours at room temperature. After a series of washes, 100µl of biotin-labeled goat anti-mouse IgG (isotype specific) (Southern Biotech) antibody was added and incubated for an additional 2 hours followed by streptavidin-HRP, as mentioned earlier. TMB

substrate solution was added for color development and stopped with 2N H_2SO_4. The plates were then read at 450nm using TECAN reader.

3.2.10. Cytokine determination

PECs obtained from the peritoneal wash, were also resuspended in supplemented RPMI medium and kept at 4 ◦C until placed in tissue culture plates. Cells were cultured, without further stimulation, at a concentration of 1.5 × 106/ml (PECs) in 24-well plates and incubated for 24–48 hours at 37 ◦C with 5% CO2. Culture supernatants were then collected, spun free of any cells, and kept at −20 ◦C until assayed. Production of NO was measured by the accumulation of $NO2^-$ in culture supernatants by the Griess reaction, following a procedure previously published (al-Ramadi et al. 1992). Production of IL-6 and IL-12 was quantitated in culture medium following in vitro culture of peritoneal, or spleen cell population after 2, 24 or 48 hours of infection.

Spleen cells were stimulated by plate-bound mAbs specific to murine CD3 and CD28 molecules. For Ab immobilization, anti-mouse CD3ε (clone: 2C11; 50µl/well; 1µg/well) and anti-mouse CD28 (clone 37.N.51; 50µl/well; 10µg/well) mAbs were diluted in 1xPBS and added into 96-well U bottom plates (BD) and incubated at 37^0C with 5% CO_2 for 2 hours after which, the solution was aspirated from each well. Purified $CD4^+$ splenic T cells (1 x 10^5 cells/200µl), resuspended in complete medium (RPMI + 5%FCS + supplements), were then added and incubated at 37^oC in a humidified incubator with 5% CO_2 for 24 hours. As controls, purified $CD4^+$ T cells were incubated without anti-CD3/CD28 mAb stimulation. Cells were spun down at 3,000 rpm for 7 minutes and supernatants were collected. Cell-free culture supernatants were analyzed for IFN-γ, IL-4 and

IL-10 cytokines using specific ELISA kits from BD Biosciences (as mentioned in table 3.1.3.) according to manufacturer's instructions. For the IFN-γ ELISA, test supernatants were run at final dilutions of 1:10-1:20. For IL-4 detection, all samples were run undiluted. For IL-10 cytokine detection, test supernatants were run either undiluted (for C57BL/6 group) or 1:2 dilution (for the infected $MyD88^{-/-}$ groups). Test supernatants from all non-infected control groups were run undiluted for all ELISA determinations.

3.2.11. Measurement of autoantibodies in serum

The production of autoantibodies specific to dsDNA, thyroglobulin and mouse IgG (rheumatoid factor) was assessed by ELISA following a published method with modification (Chen et al., 1998). For the detection of anti-dsDNA antibodies, Maxisorp Nunc ELISA plates (Thermo Scientific) were coated with dsDNA at a final concentration of 1µg/ml (from calf thymus; Sigma #D4522) diluted in borate-buffered saline (BBS) and incubated overnight at 4°C. Following 3 washes with BBS, wells were blocked with 200µl/well of BBS supplemented with Tween and BSA (BBT:BBS, 0.4%Tween 80, 0.5%BSA, and 0.1%NaN_3) for 1 hour at room temperature. Serum samples diluted to 1/1000 in BBT were added in duplicate, serially titrated on the plate (to a final volume of 100µl/well) and incubated for 2 hours at room temperature. After washing with BBS, the plates were incubated for 2 hours at room temperature with biotinylated goat anti mouse IgG (Fcγ-specific; Jackson Immunoresearch), diluted 1/10,000 in BBT (100µl/well). The plates were finally washed and incubated with streptavidin-HRP and developed using TMB, as mentioned above. Reaction was stopped with 2N

H_2SO_4 and read at 450 nm using a TECAN microplate reader (Maennedorf, Switzerland).

For the detection of RF and anti-thyroglobulin antibodies, Maxisorp plates were coated overnight at 4°C with 100µl of 1µg/ml of mouse IgG (Jackson Immunoresearch Laboratories) for RF assay or 100µl of 1µg/ml of thyroglobulin (from bovine thyroid; Sigma #T1001) diluted in 0.05M carbonate buffer (pH=9.6). Following 3 washes with PBS containing 0.05% Tween (PBS-T; pH =7.2), the plates were blocked with 200µl/well of 1% BSA in PBS-T at room temperature for 2 hours. Serum samples, diluted to 1/1000 in PBS-T containing 0.5M NaCl, were added in duplicate, serially titrated on the plate (to a final volume of 100µl/well) and incubated for 2 hours at room temperature. The plates were then washed and developed using the same procedure described above.

3.2.12. Detection of ANA by immunoflurosence using HEp-2 cells.

Serum ANA antibodies were detected by indirect immunofluorescence using HEp-2 ANA kit (INOVA Diagnostics, San Diego, CA, USA #708100), according to the manufacturer's protocol.

Diluted serum samples (1/80 dilution) and controls were added (25µl per reaction) and incubated for 30 minutes in a moist chamber at room temperature. After a gentle rinse with PBS, the slides were washed by immersing in a tray filled with PBS for 5 minutes. PBS was changed and the wash repeated. One drop of goat anti-mouse IgG-FITC (Pierce) was added into each well and incubated for 30 minutes. Slides were washed similarly in PBS and dried, following which the mounting media was added and coverslip placed. The fluorescence was visualized at 400x magnification using an Olympus BX51fluorescence microscope (Olympus Corporation, Japan)

3.2.13. Histopathological kindney analysis.

Kidneys were fixed in 4% paraformaldehyde and embedded in paraffin. To assay for immune complex deposition, paraffin-embedded 5μm tissue sections were dehydrated. The process of dehydration was done at room temperature by immersing the sections in xylene I solution for 5 minutes, xylene II for 5 minutes, absolute ethanol I for 3 minutes, absolute ethanol II for 3 minutes, 90% ethanol for 3 minutes, 80% ethanol for 3 minutes and finally, 70% ethanol for 3 minutes. Endogenous peroxidase activity was blocked using 1.5% H_2O_2 in PBS. After 3 wash cycles with PBS-T (5 minutes each), the slides were further blocked with 1%BSA in PBS for 45 minutes. The sections were then stained with biotin-goat anti-mouse IgG (Fcγ-specific; 1/2000 dilution) for 2 hours, followed by streptavidin-HRP (BD Pharmingen #550946) for 1 hour. Sections were washed again with PBS-T (3 times, 5 minutes each). The peroxidase activity was determined using DAB chromogen (BD #51-7549KC) for 5-10 minutes followed by washing with distilled water. Hematoxylin staining was done for 1 minute and sections were rehydrated. Rehydration was in the following order: 70% ethanol for 1 minute, 80% ethanol for 1 minute, 90% ethanol for 1 minute, absolute ethanol I for 1 minute, absolute ethanol II for 1 minute, xylene I for 2 minutes and xylene II for 2 minutes. The sections were mounted with DPX and viewed under 200x and 400x light microscope.

3.2.14. RNA extraction using Trizol

RNA was extracted from whole spleen cells, purified $CD4^+$ T cells, or $CD11b^+$ myeloid cells by the Trizol method. Cells (2-5 x 10^6 per sample) were pelleted and resuspended in 1ml Trizol (Invitrogen #15596-018), following which

200µl of chlorophorm was added and mixed well. Once the cells were spun down at 14000 rpm for 10 minutes, 3 phases were obtained from which the top clear RNA layer (~500µl) was transferred to a new tube. Equal volume of 2-propanol (~500µl) was then added and mixed well to precipitate the RNA. Tubes were spun again, supernatant was discarded and 500µl of 70% ethanol added. On flicking the tubes, RNA was observed as a white pellet. Tubes were spun again, supernatant was removed and RNA was finally suspended in 20µl nuclease free water and stored at -80°C.

In studies involving whole spleen cells, the isolated RNA was further treated with RNase-free DNase (Promega #M6101) to eliminate genomic DNA contamination. In this process 2µg of RNA was treated with 2µl DNAase and 2 µl DNAase buffer. Volume was made up to 20µl with nuclease-free water and incubated at 37°C for 30 minutes in a theromomixer (Eppendorf). Stop solution (2µl) was then added and incubated at 65°C for 10 minutes to inactivate the DNase. The quality and quantity of the RNA was determined using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA).

3.2.15. Reverse Transcription Reaction

RNA was reverse transcribed using TaqMan reverse transcription reagent (Applied Biosystems #N8080234). Each master mix reaction contained 10x RT buffer, 25mM $MgCl_2$, deoxy NTPs mixture (2.5mM), random hexamers (50µM), RNAase inhibitor (20U/µl) and MultiScribe RT enzyme. The master mix was aliquoted into separate PCR tubes. RNA was added (1µg/10µl per reaction) and total volume was made up to 50µl with nuclease-free water. The one step RT-PCR reaction was run on GeneAmp PCR System 2700 from Applied Biosystems, under the following conditions: hexamer incubation for 10 minutes at 25°C, reverse

transcription at 48°C for 30 minutes and reverse transcriptase inactivation at 95°C for 5 minutes. The samples were held at 4°C for a maximum of 1 hour until the samples could be removed and stored at -20°C

3.2.16. Real time PCR reactions

The real time PCR was performed using TaqMan gene expression assay as specified below and amplified using the 7500 Real Time PCR System (Applied Biosystems). Each 20µl PCR reaction contained 10µl of 2xTaqMan Universal Master Mix (Applied Biosystems #4440047), 1µl of 20x TaqMan assay Mix (Applied Biosystems #4331182), 2µl cDNA and 7µl nuclease free water. A negative PCR reaction was also carried out using only the reaction mixture without cDNA in order to ensure that there was no DNA contamination .The thermal cycling conditions were as follows: 95°C for 10 minutes (Inactivation of Reverse Transcriptase and activation of TaqMan polymerase), 95°C for 15 seconds (denaturation of dsDNA) and 60°C for 1 minute (annealing/extension-fluorescent data collected during this step) for a total of 40 cycles with the threshold set as 0.2. Data was analyzed using the Ct values for each sample that were in duplicates. Results were normalized to HPRT (Hypoxanthine-guanine phosphoribosyl transferase) and the mRNA fold change was determined using the following equation:

Fold change= $2\ [\triangle^{Ct\ (infected)}]\ /\ 2\ [\triangle^{Ct\ (control)}]$, where $\triangle$Ct(infected) = threshold cycle (Ct) for target gene after infection - Ct for HPRT after infection and$\triangle$ Ct(control) = Ct for target gene saline treated - Ct for HPRT saline treated. Control used was uninfected wild-type (C57BL/6).

The assay ID of the primers used are as follows:

HPRT (Mm01545399_m1), IL-10 (Mm00484683_m1), BAFF (Mm00446347_m1), APRIL (Mm03809849_s1), GATA-3 (Mm00484683_m1), IFN-γ (Mm01168134_m1), T-bet (Mm00450960_m1), RoRγ (Mm01261022_m1), IL-17A (Mm00439618_m1), IL-17F (Mm00521423_m1), IL-4 (Mm00445259_m1), Foxp3 (Mm00475162_m1), TGF-β (Mm01178820_m1), IL-21 (Mm 00517640_m1), CXCR5 (Mm00432086_m1), bcl-6 (Mm 00477633_m1)

3.2.17. Statistical analysis

Statistical significance was analyzed using Student's t-test or Mann-whitney test, using the statistical program of GraphPad Prism software (San Diego, CA). Differences between experimental groups were considered significant when p values were <0.05.

4. RESULTS

4.1. Susceptibility of MyD88$^{-/-}$ mice to infection

The BRD509 strain of serovar Typhimurium is attenuated as a result of deletions in the *aroA* and *aroD* genes. It is very efficient in affording protection against virulent *Salmonella* challenge, with an immunizing dose of 10^5 CFUs per mouse i.p., resulting in 100% protection against up to 10,000 LD50 dose of virulent *Salmonella*. In *Salmonella*-susceptible mouse strains of diverse genetic backgrounds, including C57BL/6 and BALB/c, the LD_{50} of the BRD509 strain is >2 x 10^6 CFUs per mouse when given i.p. This contrasts with the virulent, parental, bacterial strain, designated SL1344, which has a LD_{50} of <10 CFUs per animal. In order to assess the effect of MyD88 deficiency on susceptibility to *Salmonella* infection, MyD88$^{-/-}$ mice and their wild-type MyD88$^{+/+}$ counterparts were inoculated with different doses of BRD509 (range of 4 x 10^2 to 4 x 10^5 CFUs/mouse), following which animal survival was followed for up to 60 days. As shown in **Fig.1*A***, MyD88$^{+/+}$ mice exhibited 100% survival even at the highest inoculum dose used. In contrast, the survival rates of MyD88$^{-/-}$ mice were ~6%, 19%, 30% and 48% following inoculation with 4 x 10^5, 4 x 10^4, 4 x 10^3 and 4 x 10^2 organisms per animal, respectively. Moreover, the median survival for the different groups was 20, 31, 36 and 59 days, respectively (**Fig.1*A***). These findings demonstrate that MyD88$^{-/-}$ mice are markedly more susceptible even to a highly attenuated strain of *Salmonella* at a dose approximately 10,000-fold lower than the LD_{50} dose in MyD88$^{+/+}$ mice. The increased susceptibility of MyD88$^{-/-}$ mice to *Salmonella* correlated with an inability to control bacterial proliferation in target organs. In response to inoculation with a moderate dose of ~5 x 10^4 CFUs, MyD88$^{+/+}$ mice kept bacterial proliferation under control with less than 10-fold

the inoculum being detectable in spleen or liver at any time after infection (**Fig. 1*B-C***).

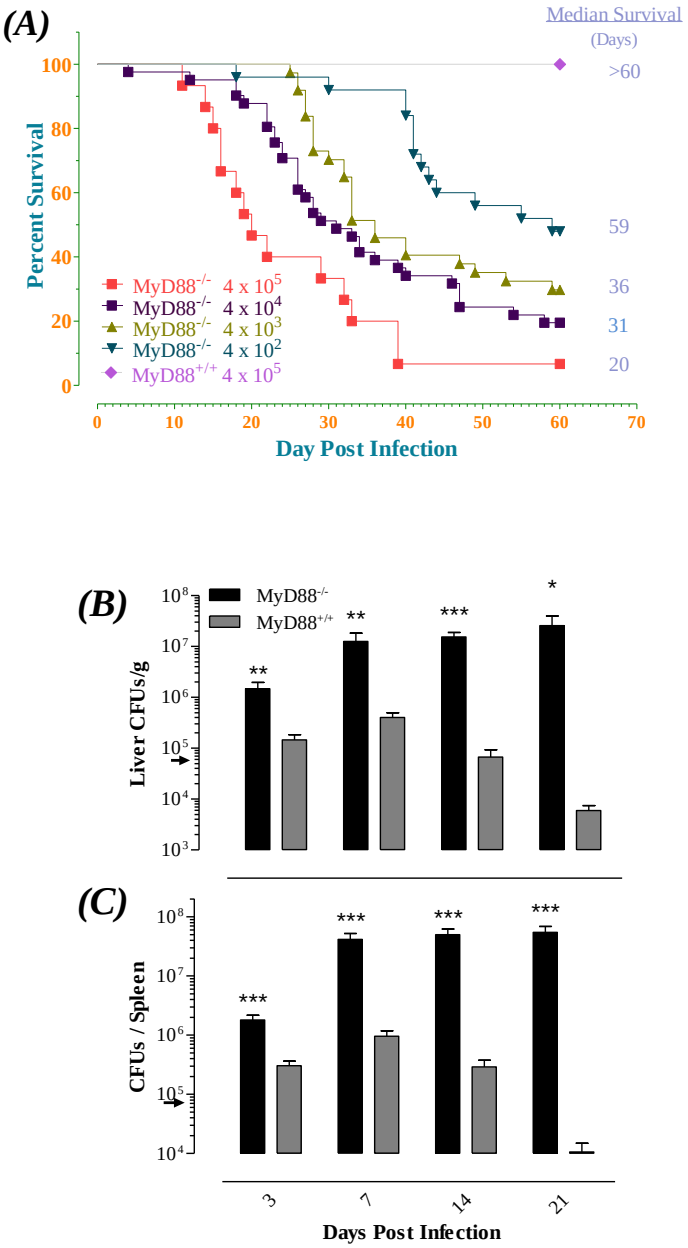

Fig. 1. MyD88-deficient mice are hypersusceptible to *Salmonella* infection. ***(A)*** Wild-type C57BL/6 (MyD88$^{+/+}$) and MyD88-deficient (MyD88$^{-/-}$) mice were inoculated i.p. with an attenuated strain of *S.* Typhimurium, designated BRD509, at the indicated doses. Animal survival was followed for up to 60 days. The calculated median survival (in days) for each group is shown. The results are pooled from 6 independent experiments. ***(B-C)*** Mice were inoculated i.p. with 5 ×10^4 CFUs of BRD509 strain. At the indicated days post inoculation, animals were sacrificed and the bacterial load in livers ***(B)*** and spleens ***(C)*** were enumerated. Each data point represents the mean ± SEM of 5 mice per group. Asterisks denote statistically significant differences between MyD88$^{-/-}$ and MyD88$^{+/+}$ mice (*p < 0.05; **p < 0.01; ***p < 0.001). The results are representative of 3 independent experiments. Data are based on Fig.1 of the paper Issac et al. 2013.

In sharp contrast, proliferation of attenuated *Salmonella* organisms increased over the 3 week-observation period, reaching approximately 1000-fold increase in spleen and liver by day 21 post-infection. The extent of the susceptibility of MyD88$^{-/-}$ mice was further examined by infecting the animals with a low dose of BRD509 (~200 CFUs/mouse) and following bacterial shedding in the feces over a 4 week-period. As shown in **Fig. 2*A***, a low level of detectable fecal shedding of *Salmonella* organisms (~0.01 CFUs/mg feces) was seen only in week 2 post-infection in MyD88$^{+/+}$ mice. By day 21 post-infection, as the infection had cleared, no further shedding was observed. In contrast, MyD88$^{-/-}$ mice exhibited a progressively worsening, persistent, infection accompanied by increasing levels of bacterial shedding over the 4-week observation period (**Fig. 2*A***). This also corresponded to increased bacterial burden in the spleen and liver of infected MyD88$^{-/-}$ mice where the bacteria recovered was about 100-200 fold greater than in infected MyD88$^{+/+}$ mice (**Fig. 2*B*-*C***). Thus, there is a failure to control bacterial replication in MyD88$^{-/-}$ mice even at a very low inoculum of an attenuated *Salmonella* strain.

4.2. Defective recruitment of inflammatory cells and cytokine response

Given the critical role of MyD88 adaptor in TLR signaling and the innate immune response, we reasoned that the failure to control bacterial proliferation may be due to an early defect in the response to *Salmonella* infection. A critical step in the innate immune response to an intraperitoneal route infection with *Salmonella* is the recruitment of inflammatory cells into the peritoneal cavity within the first 48 hours of infection. Analysis of peritoneal cell exudates (PECs) in infected mice revealed a delayed cellular influx in MyD88$^{-/-}$ mice (**Fig. 3*A***). At

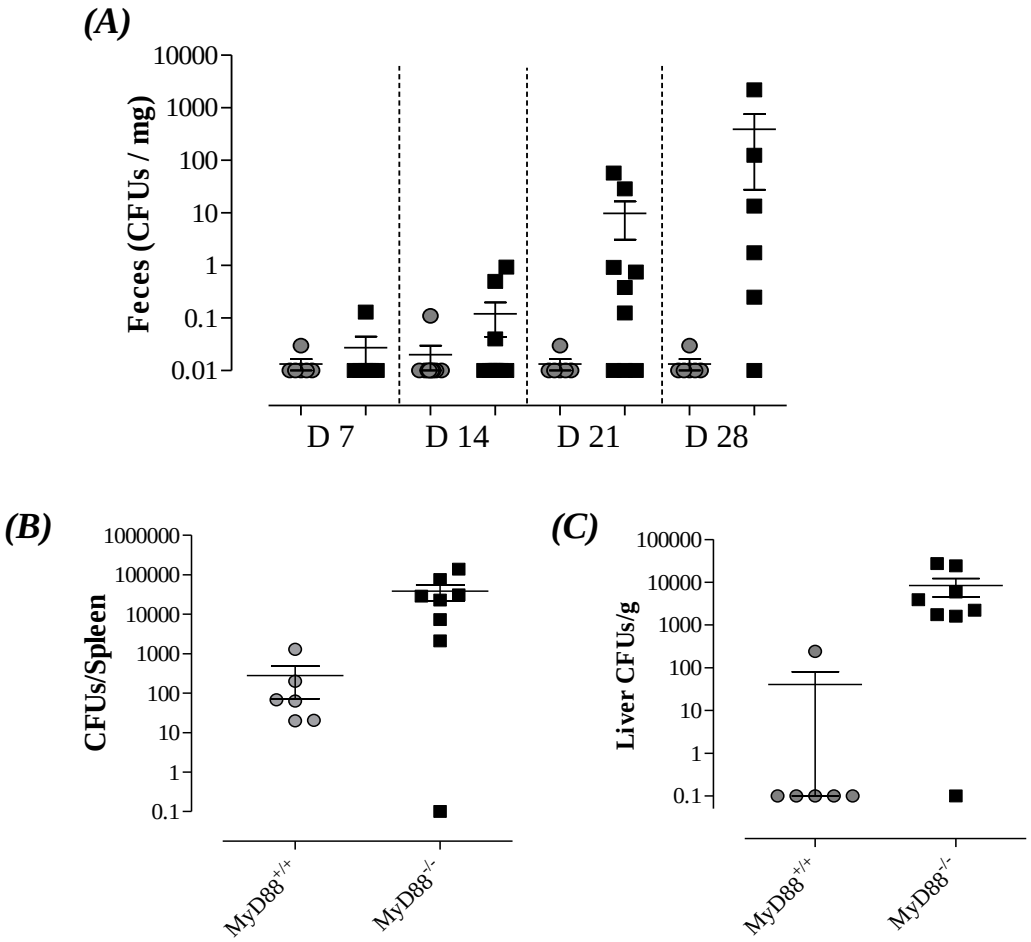

Fig. 2. Increased fecal shedding of *Salmonella* in infected MyD88$^{-/-}$ mice. Mice were inoculated i.p. with a low dose of BRD509 (~200 CFUs/mouse) and the extent of bacterial shedding in the feces (***A***) and bacterial load in spleen (***B***) and liver (***C***) were enumerated. Each data point represents the mean ± SEM of 6-10 mice per group. Asterisks denote statistically significant differences between MyD88$^{-/-}$ and MyD88$^{+/+}$ mice (*, $p<0.05$; **, $p<0.01$). The results are representative of 3 independent experiments.

2 hours post-infection, the number of PECs in MyD88$^{+/+}$ mice was 19% higher than that observed in MyD88$^{-/-}$ mice. By 24 hours, the mean PECs count in MyD88$^{+/+}$ mice was 5-fold higher than in MyD88$^{-/-}$ mice (8 x 10^6 vs 1.6 x 10^6 cells, respectively). Based on the kinetics of cell recruitment, the response peaked at 24 hours in wild-type mice, whereas it was minimal at 24 hours and was still rising at 48 hours in MyD88$^{-/-}$ mice.

In addition to this quantitative delay in cell recruitment, flowcytometric analysis of PECs collected 48 hours post-infection demonstrated a significant defect in recruitment of inflammatory cells, exemplified by CD11b^{+}Gr-1^{+} neutrophils, in MyD88$^{-/-}$ mice. In terms of absolute counts, these cells constituted up to 3 x 10^6 (~55%) of total PECs in infected wild-type mice but only 0.5 x 10^6 (~8%) of PECs in MyD88$^{-/-}$ mice (**Fig. 3*B***). These results reveal quantitative as well as qualitative alterations in inflammatory cell recruitment between infected wild-type and MyD88$^{-/-}$ mice.

Resistance to *Salmonella* infections is dependent on a robust Th1 response which leads to the activation of host macrophages and elimination of intracellular pathogens (Mastroeni et al., 1998). We therefore analyzed cytokine production in wild-type and MyD88$^{-/-}$ mice during the innate immune phase of the infection. Production of IL-6, IL-12, and nitric oxide (NO) was undetectable in *ex vivo*-cultured PECs from MyD88$^{-/-}$ mice within the first 48 hours of *Salmonella* infection (**Fig. 3*C*-*E***, respectively). This is in contrast to infected MyD88$^{+/+}$ mice, in which production of IL-6, IL-12 and NO was readily detectable at 48 hours post-infection. These results are in agreement with previous studies that documented a defect in production of IL-1β, TNF-α and IL-6 in MyD88$^{-/-}$ mice (Kawai et al., 1999); (Weiss et al., 2004).

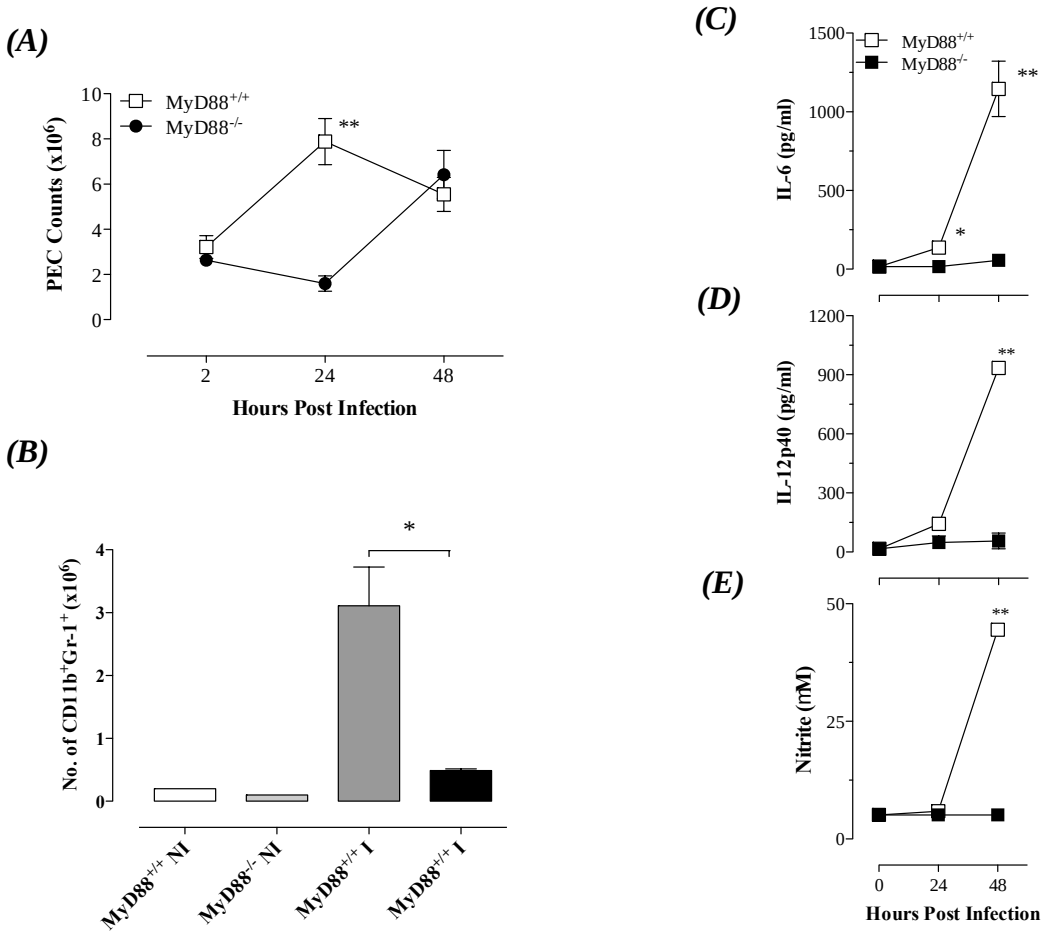

Fig. 3. Defective recruitment and activation of inflammatory cells in infected MyD88$^{-/-}$ mice. Following inoculation of BRD509 (~3 × 10^5/mouse), the number of total peritoneal exudate cells (PECs) were enumerated at 2, 24 and 48 hrs time points (***A***). After staining with mAbs to CD11b and Gr-1 and analysis by flow cytometry, the number of CD11b$^+$Gr-1$^+$ neutrophil population was determined at 48 hrs post-infection (***B***). Total PECs were collected from either saline-treated or *Salmonella*-infected mice at 24 and 48 hrs and cultured overnight without additional stimulation. Cell-free culture supernatants were tested for IL-6 (***C***), IL-12p40 (***D***) and nitric oxide (***E***). Each data point represents the mean ± SEM of 3–5 mice per group, compiled from 2 independent experiments. Date are based on Fig. 2A and 2D and Fig. 4 A-C from the paper Issac et al,2013.

4.3. MyD88$^{-/-}$ mice exhibit dysregulated antibody responses following *Salmonella* infection

Systemic infection with *Salmonella* organisms typically leads to the induction of Th1-driven antibody production, characteristically of the IgG3 and IgG2c isotypes in C57BL/6 mice (al-Ramadi et al., 2006); (Harrison et al., 1997). Given the increased susceptibilty of MyD88$^{-/-}$ mice to *Salmonella* infection, we reasoned that anti-*Salmonella* antibody responses would likely be also compromised in these animals. To test this hypothesis, mice were infected i.p. with a low dose of BRD509 (~200 CFUs/mouse) and sera collected 4-6 weeks later for the determination of isotype-specific, anti-*Salmonella* antibody levels. In C57BL/6 mice, BRD509 infection induced a predominantly Th1-driven antibody response, exemplified by IgG3 and IgG2c isotypes (**Fig. 4*B*-*C***). Infection of MyD88$^{-/-}$ mice induced the production of significantly higher levels of anti-*Salmonella* antibodies of IgG3, IgG2c as well as IgM isotypes (**Fig. 4*A*-*C***). Even more surprisingly, *Salmonella*-specific antibodies of the Th2-driven IgG1 isotype were detected in MyD88$^{-/-}$ mice, an isotype not commonly associated with *Salmonella* infections in wild-type, MyD88$^{+/+}$ mice (**Fig. 4*D***). The absolute levels of *Salmonella*-specific IgG1 and IgG2c were also determined using known standards (shown in **Fig. 5*A*-*B***) and the calculated concentrations in the various treatment groups were in agreement with the results shown in **Fig. 4**. Thus, despite their increased susceptibility to *Salmonella*, MyD88$^{-/-}$ mice exhibit a state of infection-induced hypergammaglobulinemia which was evident in all of the tested antibody isotypes.

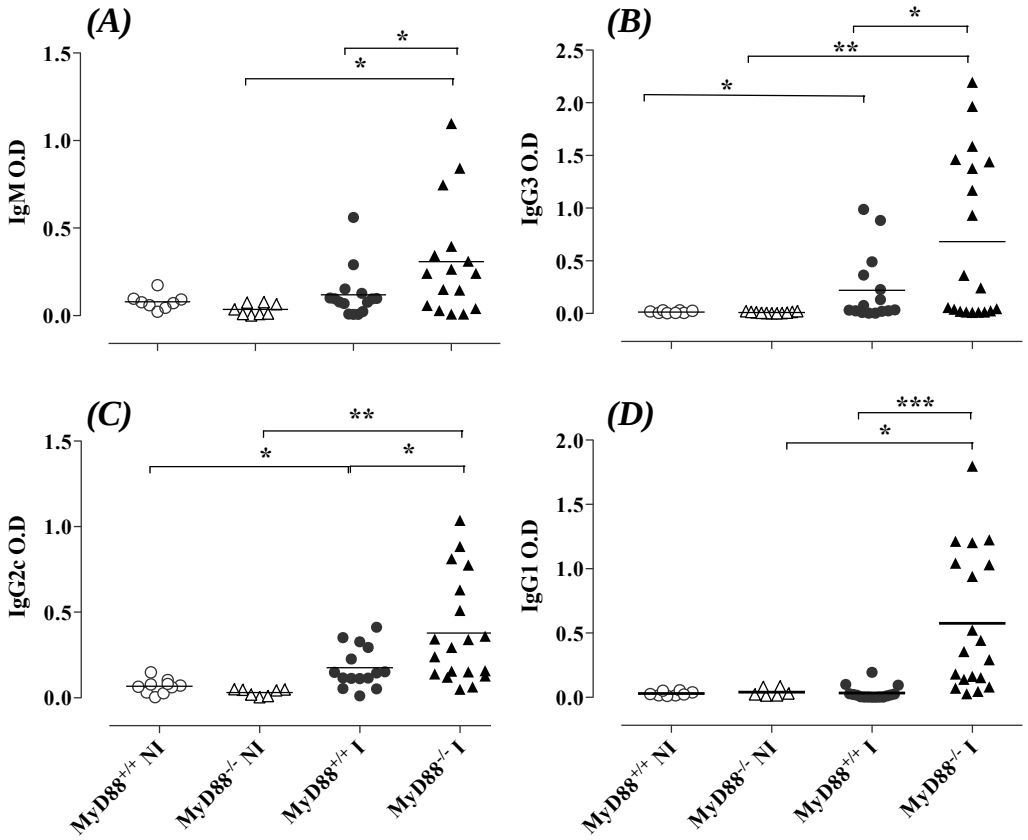

Fig. 4. Hypergammaglobulinemia in *Salmonella*-infected MyD88$^{-/-}$ mice. Mice were inoculated i.p. with BRD509 (~200 CFUs/mouse) and sera collected at 4-6 weeks and analyzed for the presence of *Salmonella*-specific IgM (***A***), IgG3 (***B***), IgG2c (***C***) and IgG1 (***D***) isotypes. Asterisks denote statistically significant differences between the indicated experimental groups (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Data are pooled from 3 independent experiments.

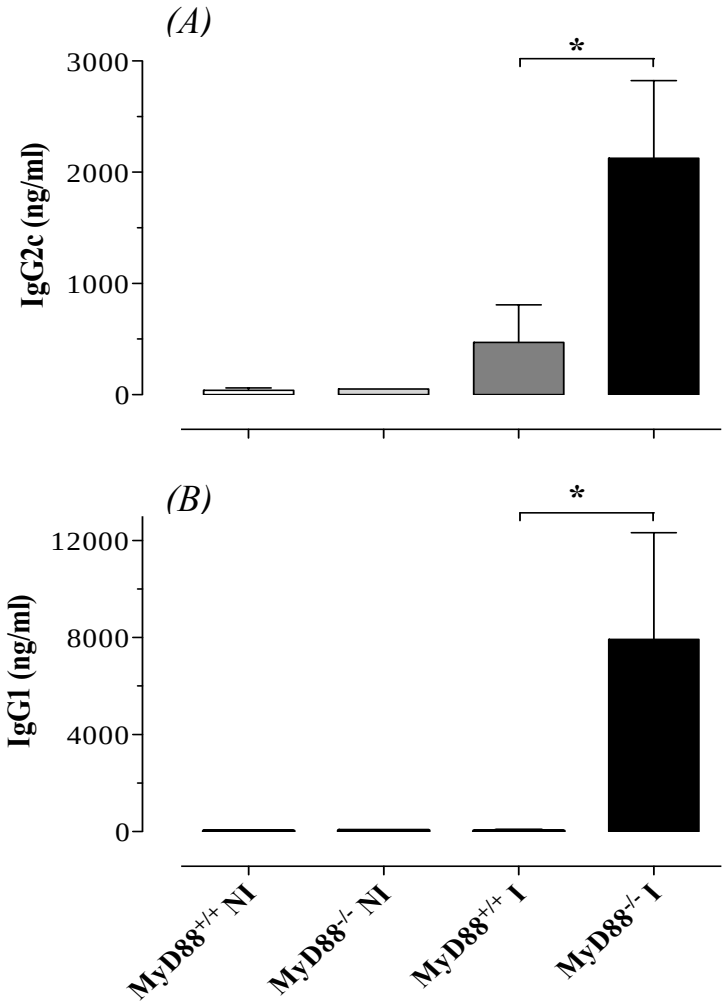

Fig. 5. Quantification of *Salmonella*-specific serum. IgG2c (***A***) and IgG1 (***B***) antibodies in non-infected (NI) and 3 week-infected (I) mice. Attenuated BRD509 strain was used for i.p. infection at a dose of (~200CFUs/m). Absolute antibody levels are expressed in ng/ml serum. Asterisks denote statistically significant differences between infected MyD88$^{-/-}$ and MyD88$^{+/+}$ mice (*, p<0.05). Data are pooled from 3 independent experiments.

The kinetics of the early development of isotype specific antibodies to *Salmonella* organisms were evaluated at weekly intervals following inoculation**.** Wild-type mice developed anti-*Salmonella* antibodies of IgG2c and IgG3 isotypes of increasing concentrations over the 3 week-observation period (**Fig. 6*A*-*B***). No significant *Salmonella*-specific antibodies of IgG1 isotype were observed in C57BL/6 mice (**Fig. 6*C***). In contrast, starting at 14 days post-infection, MyD88$^{-/-}$ mice secreted *Salmonella*-specific antibodies of all three (IgG2c, IgG3 and IgG1) isotypes at higher levels than seen in C57BL/6 mice (**Fig. 6*A*-*C***). Thus, the state of hypergammaglobulinemia in infected MyD88$^{-/-}$ mice became evident as early as 14-21 days post *Salmonella* inoculation and was still significant up to 2 months later (the longest period of observation).

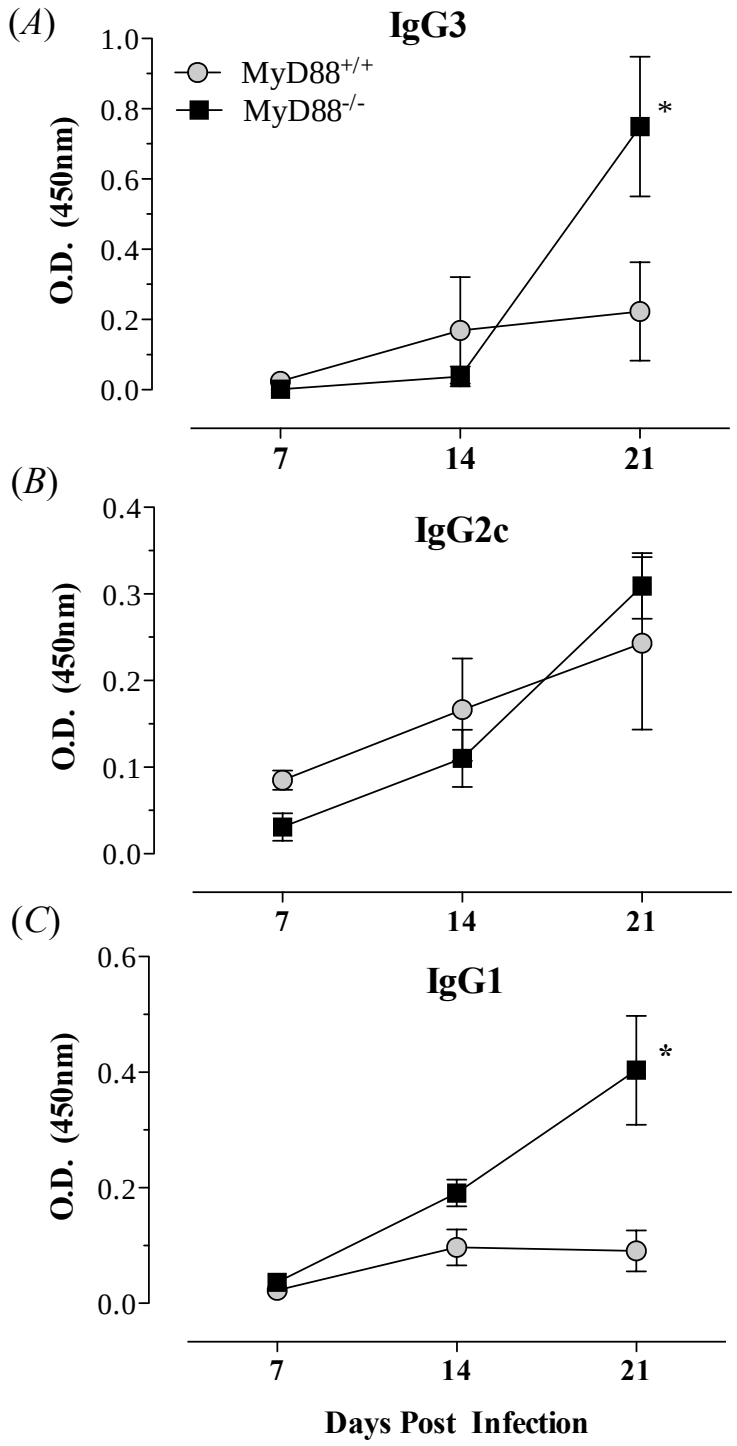

Fig. 6. Kinetics of development of anti-*Salmonella* antibodies. The development of antibodies to *Salmonella* infection was evaluated up to 3 weeks post i.p infection with a dose of 4000 CFUs/mouse. *Salmonella* specific IgG3 (***A***), IgG2c (***B***), and IgG1 (***C***) were determined. Data are compiled from 3 independent experiments. Asterisks denote statistically significant differences between $MyD88^{-/-}$ and $MyD88^{+/+}$ mice (*, $p<0.05$).

The effect of MyD88 deficiency on global antibody levels was evaluated by quantitating the concentrations of total serum IgG3, IgG2c, and IgG1 isotypes in both MyD88$^{+/+}$ and MyD88$^{-/-}$ mice before and after infection with *Salmonella*. In uninfected animals, relatively low levels of serum IgG3 and IgG2c were detected but they were equivalent in both MyD88$^{+/+}$ and MyD88$^{-/-}$ mouse strains (**Fig. 7*A*-*B***). Serum IgG3 concentrations were 45 ± 9 µg/ml (mean ± SEM) and 44 ± 27 µg/ml for MyD88$^{+/+}$ and MyD88$^{-/-}$ mice, respectively (**Fig. 7*A***). For IgG2c, serum concentrations were 554 ± 94 µg/ml and 307 ± 37 µg/ml for MyD88$^{+/+}$ and MyD88$^{-/-}$ mice, respectively (**Fig. 7*B***). In infected mice, serum IgG3 and IgG2c concentrations were increased significantly by an average of 3-5 fold over those in uninfected mice. In contrast, baseline serum IgG1 concentrations in uninfected MyD88$^{-/-}$ mice were significantly elevated compared to MyD88$^{+/+}$ mice (2.3+ 0.7 vs 0.63 + 0.1 mg/ml, respectively; **Fig. 7*C***). However, no significant changes in serum IgG1 concentrations were observed upon infection with *Salmonella* in both mouse strains. These findings indicate that the humoral immune response in MyD88$^{-/-}$ mice is constitutively skewed to Th2-induced IgG isotypes.

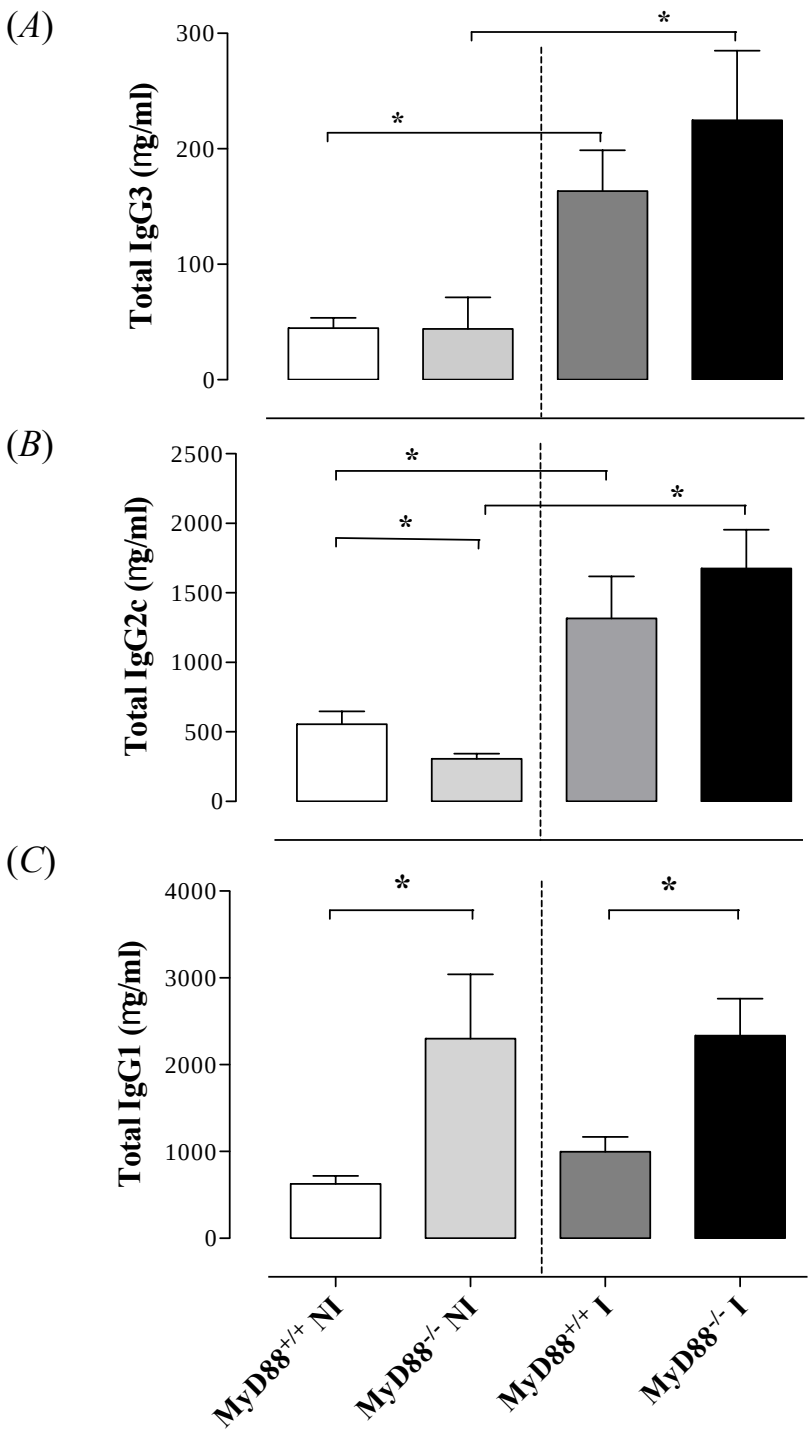

Fig. 7. *Salmonella*-induced dysregulation in total serum antibodies in MyD88$^{-/-}$ mice. Following infection with BRD509 (~200 CFUs/mouse), sera were collected 4-6 weeks later. Total serum IgG3 (***A***), IgG2c (***B***), and IgG1 (***C***) in both infected and non-infected mice were quantified by ELISA. Each data point represent mean ± SEM of 3–10 mice per group (*, $p<0.05$).

4.4. Oral *Salmonella* inoculation leads to persistent infection in MyD88$^{-/-}$ mice

Since MyD88$^{-/-}$ mice were hypersusceptible to systemic *Salmonella* infections, their response to an oral administration of BRD509 strain was investigated. Unlike systemic infections, mice inoculated orally with doses of up to 1 x 10^9 CFUs/animal exhibited 100% survival and had no signs of morbidity for up to 60 days post-infection (data not shown). Long-term persistence of oral infections was followed by quantitating the extent of bacterial shedding in the feces for 6 months. In MyD88$^{+/+}$ mice, fecal shedding of bacteria was evident up to 2 weeks post-infection with ~1 x 10^8 CFUs/mouse (**Fig. 8*A***). At later time points, no significant shedding was observed. In contrast, substantial shedding of *Salmonella* organisms was evident in infected MyD88$^{-/-}$ mice for the entire observation period (up to day 191 post-infection).

Bacterial loads were also determined in mesenteric lymph nodes (MLNs), livers and spleens of infected mice at different time points post oral infection (**Fig. 8*B*-*D***). Consistent with the findings from systemic infection, MyD88$^{-/-}$ mice harbor significantly increased loads (~10 to 100-fold) of BRD509 organisms in their tissues compared with wild-type mice over the first 3 weeks of infection. As expected, the highest concentration of bacteria was found in MLNs but even here differences between wild-type and MyD88-deficient mice were found throughout the observation period (**Fig. 8*B***), suggesting that MyD88 is also important for immune defense along mucosal tissues. Assessment of spleen weights in orally infected mice also reflected a pattern previously observed with systemic infections (**Fig. 8*E***). Thus, early on, spleen weights of infected MyD88$^{-/-}$ mice lagged behind those of wild-type counterparts, perhaps reflecting the defective recruitment of inflammatory cells. By day 21 post-infection, as bacterial proliferation was

brought under control, splenomegaly in wild-type mice began to normalize. However, the degree of splenomegaly continued to increase in infected MyD88$^{-/-}$ mice most likely as a reflection of their failure to control the infection (**Fig. 8*E***). Interestingly, while infected MyD88$^{-/-}$ mice exhibited continuous shedding of bacteria in the feces (**Fig. 8*A***), no *Salmonella* organisms were detectable in spleens and livers of these animals at day 191 after infection (data not shown). Therefore, in the absence of MyD88 protein, oral administration of an attenuated *Salmonella* strain leads to a long-term persistent infection that appears to be restricted to the mucosal tract.

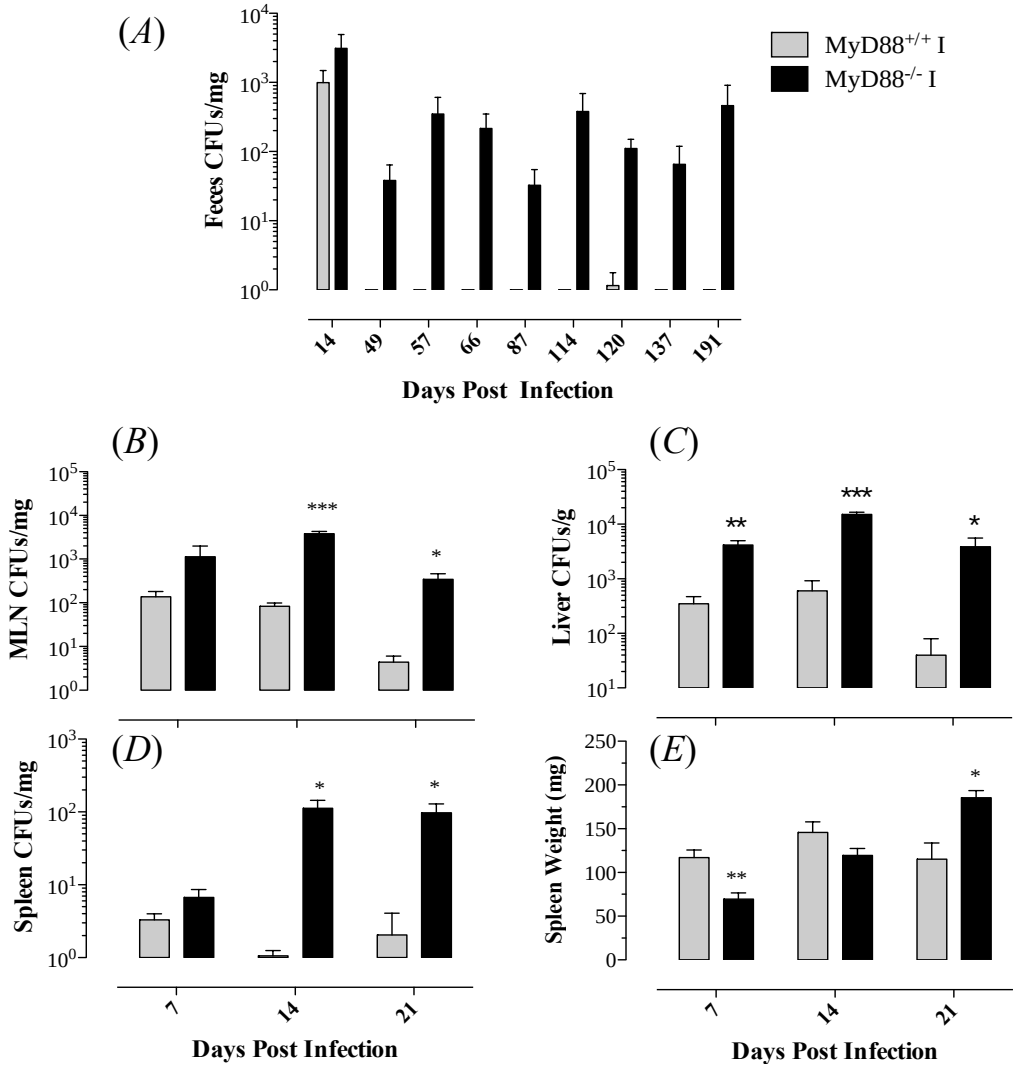

Fig. 8. Oral infection with attenuated *Salmonella* leads to persistent infection. Mice were inoculated orally with BRD509 (~1 x 10^8 CFUs/mouse). Shedding of *Salmonella* organisms in the feces was followed for up to 27 weeks (***A***). (***B-D***) Mice were inoculated orally with ~1 x 10^9 CFUs/mouse of BRD509 strain and bacterial load in mesenteric lymph nodes (MLNs; ***B***), liver (***C***) and spleen (***D***) was enumerated at the indicated time points. Degree of splenomegaly was also determined (***E***). Asterisks denote statistically significant differences between MyD88$^{-/-}$ and MyD88$^{+/+}$ mice (*, p<0.05; **, p<0.01; ***, p<0.001). The experiment was performed twice with similar results.

Next, the systemic and mucosal *Salmonella*-specific antibody levels were evaluated after oral infection in normal and MyD88$^{-/-}$ mice. Consistent with the findings of systemic infections, orally-infected MyD88$^{-/-}$ mice had elevated levels of *Salmonella*-specific IgG3, IgG2c and IgG1 antibodies (**Fig. 9*A*-*C***). In normal mice, the most significant rise in anti-*Salmonella* antibodies was in the IgG2c isotype (**Fig. 9*B***). No significant differences in serum IgA levels were observed between the mouse strains following infection (**Fig. 9*D***). However, a significantly stronger mucosal IgA response was observed in infected wild-type mice compared to MyD88$^{-/-}$ counterparts, despite the higher levels of bacterial persistence seen in the latter mouse strain (**Fig. 9*E***). These data demonstrate that the induced hypergammaglobulinemia in MyD88$^{-/-}$ mice is not restricted to systemic infections. In contrast, MyD88 protein appears to be essential for the induction of a robust mucosal IgA response. Taken together, these findings suggest that the systemic antibody response is fundamentally dysregulated in MyD88$^{-/-}$ mice.

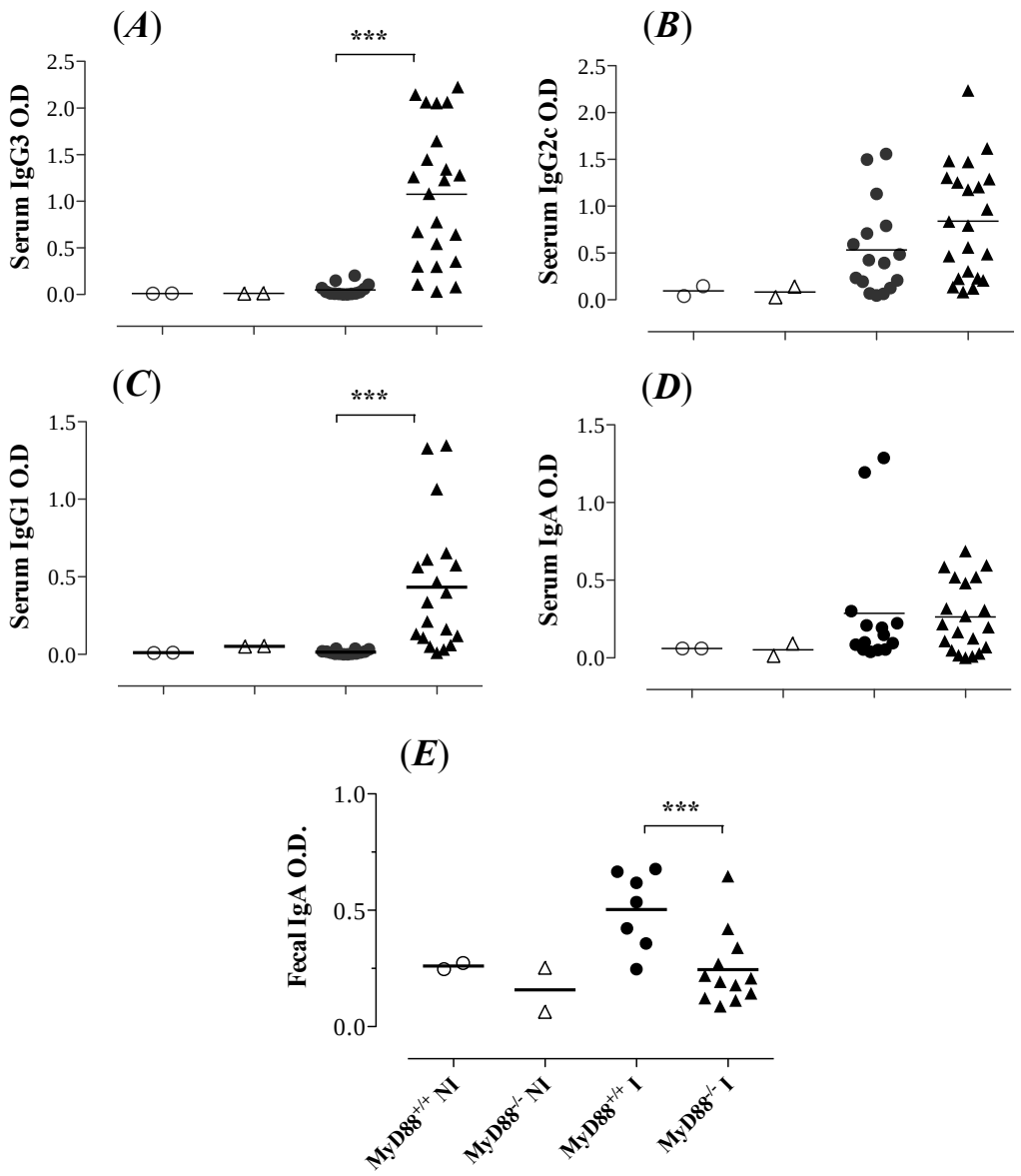

Fig. 9. Elevated serum but not mucosal antibody levels following oral infection with BRD509 in MyD88$^{-/-}$ mice. Animal sera were tested at 3-6 months after infection for the presence of *Salmonella*-specific IgG3 (***A***), IgG2c (***B***), IgG1 (***C***) and IgA (***D***) antibody isotypes. Mucosal IgA levels were also determined in fecal pellets at the same time (***E***). Data are compiled from 2 independent experiments. Asterisks denote statistically significant differences between MyD88$^{-/-}$ and MyD88$^{+/+}$ mice (***, $p<0.001$).

4.5. Breakdown of humoral self-tolerance in MyD88$^{-/-}$ mice following systemic *Salmonella* infection

Given the observed dysregulation in antibody synthesis in *Salmonella*-infected MyD88$^{-/-}$ mice, the potential generation of autoantibodies was examined in these mice. Polyclonal B cell activation in response to infections has been shown to cause hyper antibody responses that could lead to autoimmunity (Woods et al., 2008); (Ko et al., 2011). Sera were collected 4-6 weeks following i.p infection and tested for reactivity against a panel of self antigens including dsDNA, thyroglobulin and IgG. To rule out non-specific cross-reactivity, all autoantibody ELISAs were determined at a serum dilution of 1/1000 or greater. The data illustrate the presence of significant titers of autoantibodies to dsDNA, thyroglobulin and IgG (RF) in *Salmonella*-infected MyD88$^{-/-}$ mice (**Fig. 10*A*-*C***). Importantly, sera from uninfected MyD88$^{-/-}$ mice had no reactivity to any of the autoantigens. Similarly, no autoreactive antibodies were detectable in wild-type (MyD88$^{+/+}$) mice before or after infection. These findings provide evidence for the secretion of autoantibodies in MyD88$^{-/-}$ mice, but not for normal mice, following infection with *Salmonella*.

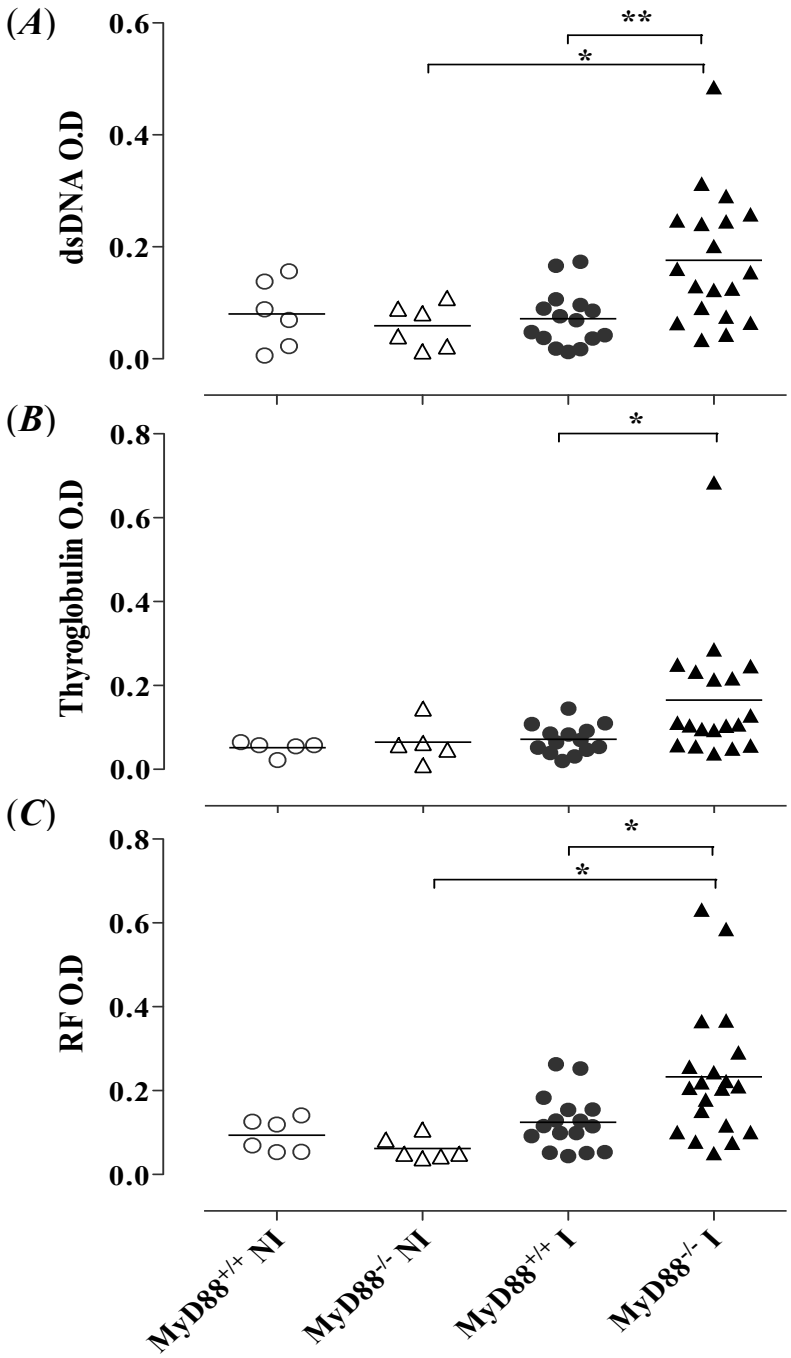

Fig. 10. Autoantibody reactivity of MyD88$^{-/-}$ sera after systemic *Salmonella* infection. Sera were collected 4-6 weeks following i.p. infection with BRD509 (~200 CFUs/mouse) and tested for reactivity with dsDNA (***A***), thyroglobulin (***B***) and rheumatoid factor (RF; ***C***) by specific ELISA. The cut-off for the detection of these autoantibodies was determined at 1/1000 dilution. Date compiled from 3 independent experiments. (*, $p<0.05$; **, $p<0.01$).

Autoantibodies targeting dsDNA, histones and other nuclear antigens can also be determined by immunofluorescence using HEp-2 human epithelial cells. The presence of anti-nuclear antibodies (ANA) has been strongly associated with various autoimmune mouse models (Silver et al., 2007); (Sadanaga et al., 2007); (Bolland et al., 2002). To determine the overall levels of ANA, we assayed the reactivity of serially diluted sera to fixed HEp-2 cells by immunofluorescence. Approximately 53% of sera (8 out of 15) collected from *Salmonella*-infected MyD88$^{-/-}$ mice showed strong reactivity with HEp-2 cells (**Fig. 11*G***). Importantly, these sera exhibited different staining patterns, such as homogenous and fine/coarse speckled (**Fig. 11*D*-*F***), reflecting the different nuclear antigens being recognized. In contrast, only about 8% of the sera from infected MyD88$^{+/+}$ mice (1 out of 12) were positive for HEp-2 staining (**Fig. 11*C, G***). Non-infected mice of both strains were all negative (**Fig. 11*A*-*B***). Positive ANA staining with HEp-2 cells generally correlated with high anti-dsDNA antibody levels.

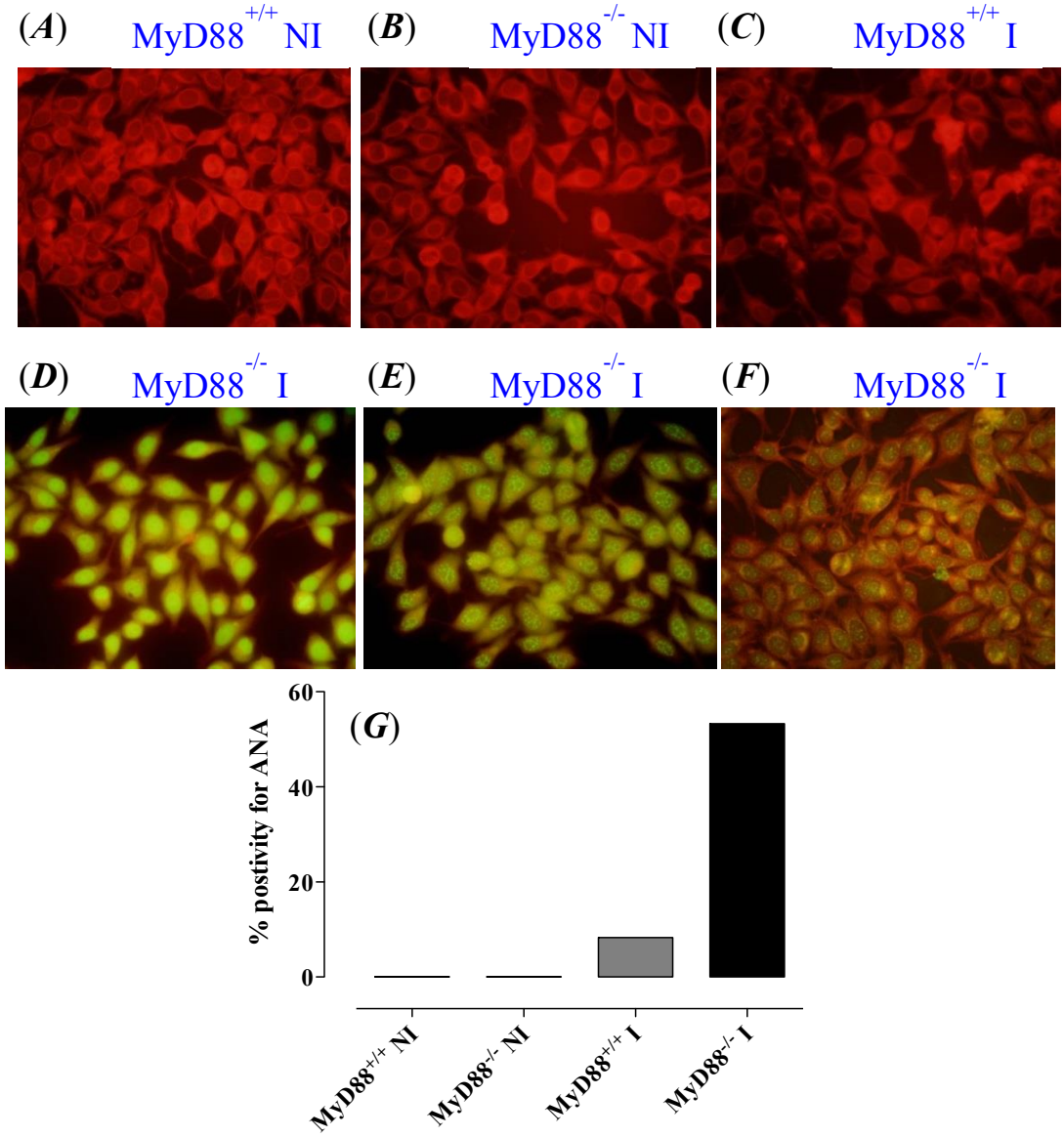

Fig. 11. Immunofluoresence detection of HEp-2 cell ANA reactivity of sera from *Salmonella*-infected MyD88$^{-/-}$. ANA staining obtained with sera from non-infected (***A-B***) and infected (***C-F***) MyD88$^{+/+}$ (***A, C***) and MyD88$^{-/-}$ (***B, D-F***) mice. Different staining patterns are detected, including homogenous (***D***), speckled (***E***), and cytoplasmic (***F***). Negative staining is depicted in panels ***A-C***. The percentage of different experimental groups (5-15 mice/gp) that were positive for ANA is summarized in panel ***G***. The fluorescence was visualised at 400x magnification using an Olympus fluorescent microscope. Data are compiled from 2 independent experiments.

Given the state of hypergammaglobulinemia and the increased levels of serum IgG autoantibodies in infected MyD88$^{-/-}$ mice, we next evaluated whether this was associated with more abundant deposits of immune complexes in kidney glomeruli. No evidence of staining was detected in the glomeruli of non-infected MyD88$^{-/-}$ or MyD88$^{+/+}$ mice (**Fig. 12*A, D***). In comparison, about 20% of infected MyD88$^{+/+}$ mice showed positive staining of glomeruli but those were generally of low level of intensity (**Fig. 12*E-F***). In sharp contrast, much brighter staining, indicating more deposits of immune complexes, was detected in ~69% of *Salmonella*-infected MyD88$^{-/-}$ mice (**Fig. 12*B-C, G***). Thus, the absence of MyD88 predisposes animals to hypergammaglobulinemia, production of autoantibodies, and deposition of immune complexes in kidney glomeruli following infection with *Salmonella*.

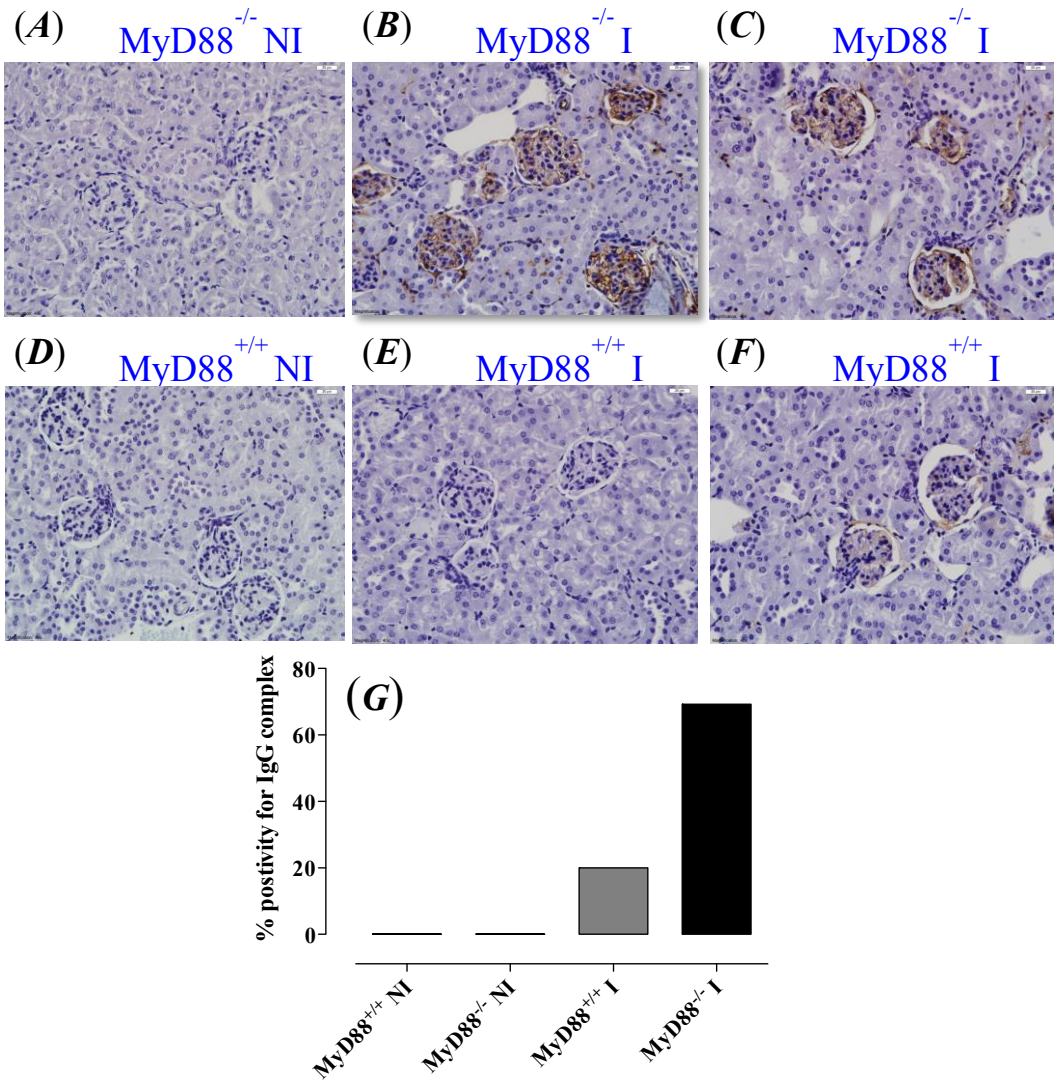

Fig. 12. Immunoglobulin deposition in kidney sections of *Salmonella* infected MyD88$^{-/-}$ mice. Kidney sections were prepared from non-infected (***A, D***) and infected (***B, C, E, F***) MyD88$^{-/-}$ (***A-C***) and MyD88$^{+/+}$ (***D-F***) mice and stained with anti-IgG antibody, as described in M&M. (***G***) The percentage of mice whose kidney sections scored positive for the presence of immune deposits by DAB staining. Images were taken at 400x magnification. Data are compiled from 2 independent experiments.

4.6. Requirements for infection-induced hypergammaglobulinemia and autoantibody production

The next series of experiments were designed to study the requirements for the observed hypergammaglobulinemia and autoantibody synthesis in MyD88$^{-/-}$ mice. We first evaluated whether infection with another Gram-negative bacterium, *Acinetobacter baumanii*, could lead to similarly dysregulated antibody production. Mice infected with a dose of ~1 x 10^5 organisms of *Acinetobacter baumanii* (strain NM970) still harbored bacteria in spleen and liver 2 months following inoculation, with higher bacterial loads than those being observed in MyD88$^{-/-}$ mice (**Fig. 13*A*-*B***). Infected wild-type mice were strongly positive for *Acinetobacter*-specific antibodies, predominantly of IgG3 isotype (**Fig. 13*C***). Interestingly, infected MyD88$^{-/-}$ mice also developed mainly IgG3 antibodies specific to Acinetobacter, but they were significantly lower than those observed in infected MyD88$^{+/+}$ mice (**Fig. 13*C***). No significant bacteria-specific IgG2c or IgG1 antibodies were detected (**Fig. 13*D*-*E***). Thus, infection with a non-intracellular Gram-negative pathogen failed to induce hypergammaglobulinemia in MyD88$^{-/-}$ mice, as observed with *Salmonella* bacteria.

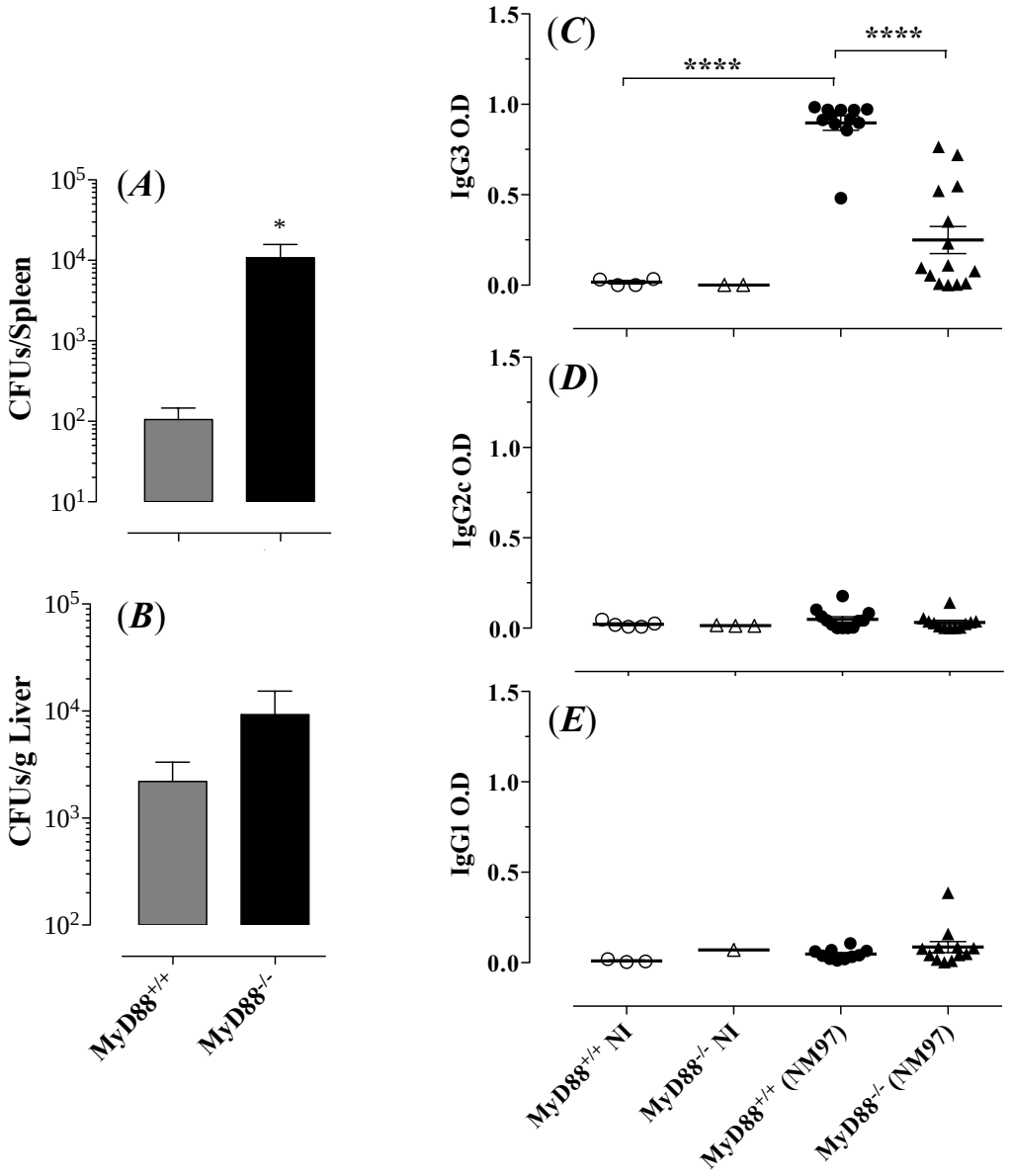

Fig. 13. No evidence for immunoglobulin dysregulation in MyD88$^{-/-}$ mice following infection with *Acinetobacter baumanii.* Two months post i.p. infection with a dose of ~1 x 10^5 CFUs/mouse of *A. baumanii*, bacterial counts in spleen (***A***) and liver (***B***) were enumerated. Serum levels of anti-acinetobacter IgG3 (C), IgG2c (D) and IgG1 (E) isotypes were determined at 6-8 weeks post -infection.(*, $p<0.05$, ****, $p<0.0001$). Data are compiled from 2 independent experiments.

The presence of antibodies specific to dsDNA and thyroglobulin was also tested in these mice following *Acinetobacter* infection. However, no autoantibodies were detectable in any of the experimental groups (**Fig. 14*A*-*B***). Furthermore, there was no evidence of antibody dysregulation or autoantibody production in MyD88$^{-/-}$ mice infected with the Gram-positive bacteria, Group B *Streptococcus* (*Streptococcus agalactiae*), or another Gram-negative pathogen *E. coli* (data not shown).

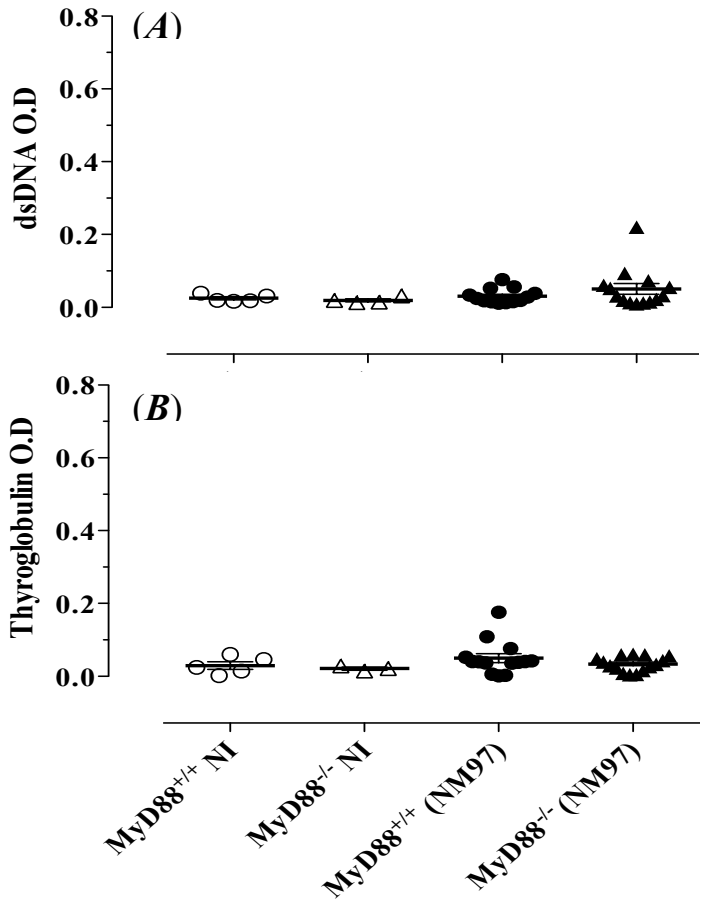

Fig. 14. Serum reactivity to dsDNA and thyroglobulin following *A. baumanii* infection. Serum reactivity to dsDNA (***A***) and thyroglobulin (***B***) were determined 6-8 weeks post-infection with *A. baumanii*. The cut off for the detection of these autoantibodies was determined at 1/1000 dilution. Data are compiled from 2 independent experiments.

The capacity of heat-killed (HK) *Salmonella* (strain BRD509) to induce a state of hypergammaglobulinemia was also tested. Following injection with 2 x 10^5 HK *Salmonella* organisms, low levels of IgG3 antibodies were detected in MyD88$^{+/+}$ mice which were similar to the levels produced after live infection (**Fig. 15*A***). In contrast to infection with live bacteria, however, no IgG2c antibodies were detected in response to HK bacteria in MyD88$^{+/+}$ mice (**Fig. 15*B***). Another difference in isotype profile, was the induction of IgG1 antibodies seen in MyD88$^{+/+}$ mice after injection of HK, but not live, *Salmonella* (**Fig. 15*C***). In sharp contrast to live *Salmonella* infection, MyD88$^{-/-}$ mice injected with HK BRD509 strain produced largely IgG1 but not any significant IgG3 or IgG2c isotype antibodies (**Fig. 15*A*-*C***). Thus, injection with HK bacteria favors the induction of predominantly *Salmonella*-specific IgG1 antibodies in both mouse strains with no evidence of hypergammaglobulinemia being observed in MyD88$^{-/-}$ mice.

In line with these observations, sera collected from HK *Salmonella*-injected mice also failed to show any reactivity to dsDNA. This was true for both wild-type and MyD88$^{-/-}$ mice (**Fig. 15*D***). Taken together, these results indicate that heat-killed *Salmonella* fail to induce dysregulated antibodies, suggesting the nature of the bacterial antigen is critical for the observed breakdown in self-tolerance that is observed in MyD88$^{-/-}$ mice.

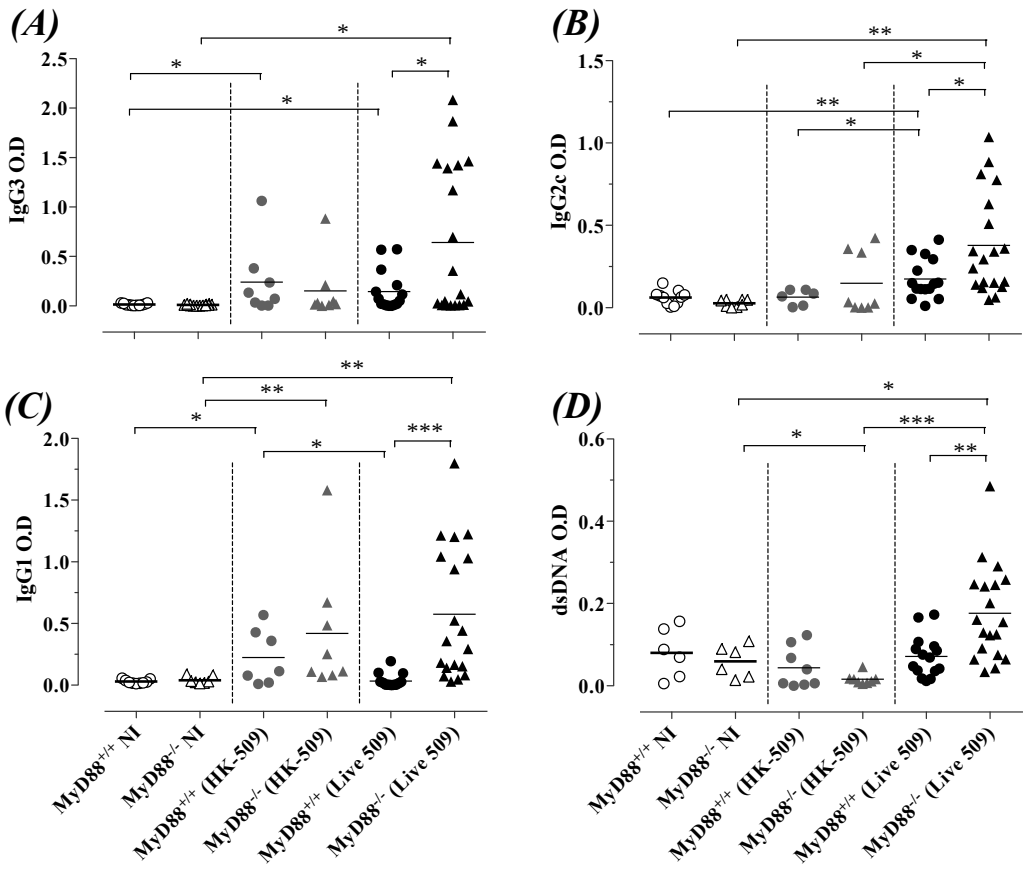

Fig. 15. Absence of immunoglobulin dysregulation and autoantibodies in MyD88$^{-/-}$ mice following injection of heat-killed BRD509. Serum levels of *Salmonella*-specific IgG3 (***A***), IgG2c (***B***) and IgG1 (***C***) antibodies were determined at 8 weeks post i.p inoculation of 2 x 10^5 heat-killed (HK) BRD509 in comparison to those observed in response to live BRD509 infection. (D) Serum reactivity to dsDNA in heat-killed vs live *Salmonella* infected mice. The cut-off for the detection of dsDNA was determined at 1/1000 dilution. Data are compiled from 2 independent experiments (*, p<0.05; **, p<0.01; ***, p<0.001).

4.7. Regulation of hypergammaglobulinemia by cytokine-expressing *Salmonella* strains

It is clear from the data thus far, that infection of MyD88$^{-/-}$ mice with an attenuated strain of *Salmonella* leads to dysregulated humoral immune responses in which pathogen-specific antibodies of all IgG isotypes are produced at high levels. Immunoglobulin class switching is largely regulated by T cell-derived cytokines such as IFN-γ and IL-4, which are produced by Th1 and Th2 cells, respectively. Our laboratory has previously demonstrated the potential of manipulating the anti-*Salmonella* immune response in wild-type mice by using recombinant *Salmonella* strains engineered to express specific murine cytokines (al-Ramadi et al., 2001); (Al-Ojali et al., 2012b). Two of these strains, GIDIL2 and GIDIFN, which express IL-2 and IFN-γ respectively, were therefore used to study their effect on humoral immune responses in MyD88$^{-/-}$ mice. These cytokines are important regulators of the Th1 response required for protection against intracellular bacterial infection in C57BL/6 mice. The bacterial load in the spleen and liver were determined at 4 weeks post-infection with a low dose of BRD509 (~200 CFUs/mouse) (as shown earlier) or GIDIFN. As described earlier, the bacterial loads in the spleen and liver of MyD88$^{-/-}$ mice after BRD509 infection were 100-200 fold greater than in the infected wild-type mice (**Fig. 16*A*-*B*).** Similarly, using the GIDIFN strain, the bacterial load recovered in the target organs of the MyD88$^{-/-}$ mice were 400-500 fold greater than in the wild-type mice (**Fig. 16*A*-*B*)**. Notably, the bacterial loads in GIDIFN-infected MyD88$^{-/-}$ mice were 1.5-2.6 fold lower than those infected with BRD509. In MyD88$^{+/+}$ mice, the differences in bacterial loads between BRD509- and GIDIFN-infected mice approached 4-9 fold (**Fig. 16*A*-*B***). This suggests that IFNγ-expressing

Salmonella strain is cleared more robustly by both mouse strains compared to BRD509. The observations in wild-type mice are consistent with previous data from our laboratory (Al-Ojali et al., 2012a).

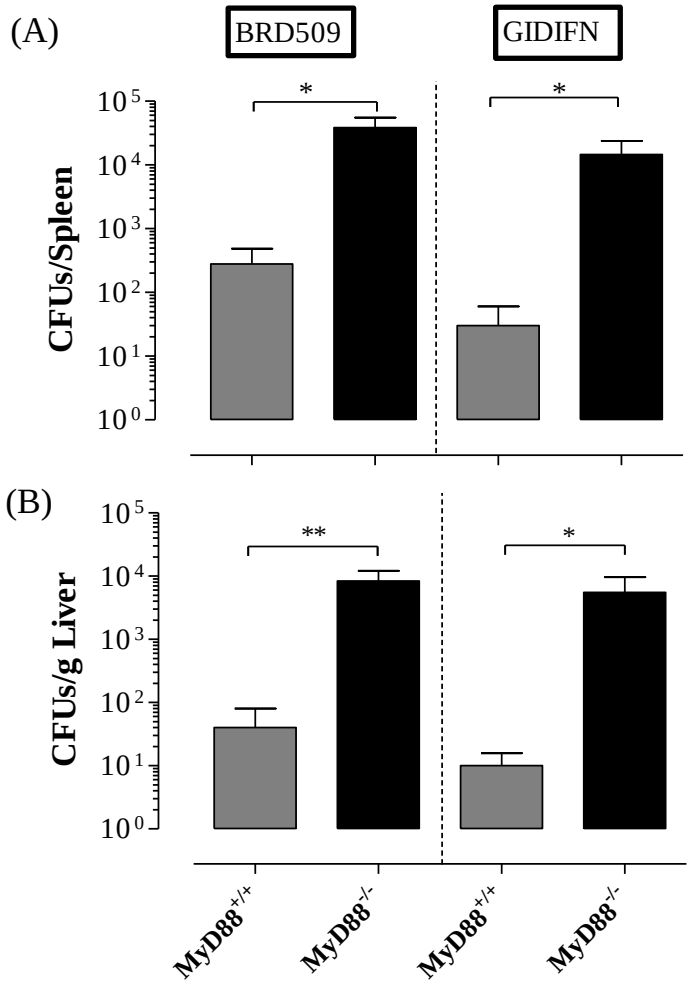

Fig. 16. Hypersusceptibility of MyD88$^{-/-}$ mice to infection with an IFN-γ-expressing *Salmonella* strain, GIDIFN. Mice were inoculated i.p. with GIDIFN or its parental strain BRD509 (~200 CFUs/mouse) Bacterial loads in the spleen (***A***) and liver (***B***) were enumerated at 4 weeks post-infection (*, $p<0.05$; **, $p<0.01$).

Salmonella-specific antibody responses were compared in normal or MyD88$^{-/-}$ mice 4-6 weeks following a low dose (~200 CFUs/mouse) infection with GIDIFN, GIDIL2 or BRD509 strain. In C57BL/6 mice, significant titers of IgG3 and IgG2c isotypes were observed after infection, with the former being predominant in BRD509-infected mice and the latter in mice infected with either GIDIL2 or GIDIFN strain (**Fig. 17*A*-*B***). Importantly, no IgG1 antibodies were detectable in wild-type mice following infection with any of the *Salmonella* strains (**Fig. 17*C***). In contrast, compared to wild-type animals, MyD88$^{-/-}$ mice consistently produced significantly higher levels of antibodies of all three isotypes to all *Salmonella* strains, with the sole exception of IgG2c isotype in GIDIL2-infected mice (**Fig. 17*A*-*C***). It is also interesting to note that infection with GIDIFN, but not GIDIL2, strain appeared to enhance Th1-driven isotypes (IgG2c and IgG3) and reduce Th2-driven IgG1 isotype in MyD88$^{-/-}$ mice. Overall, a state of hypergammaglobulinemia was still evident in MyD88$^{-/-}$ animals following infection with recombinant, cytokine-expressing, *Salmonella* strain.

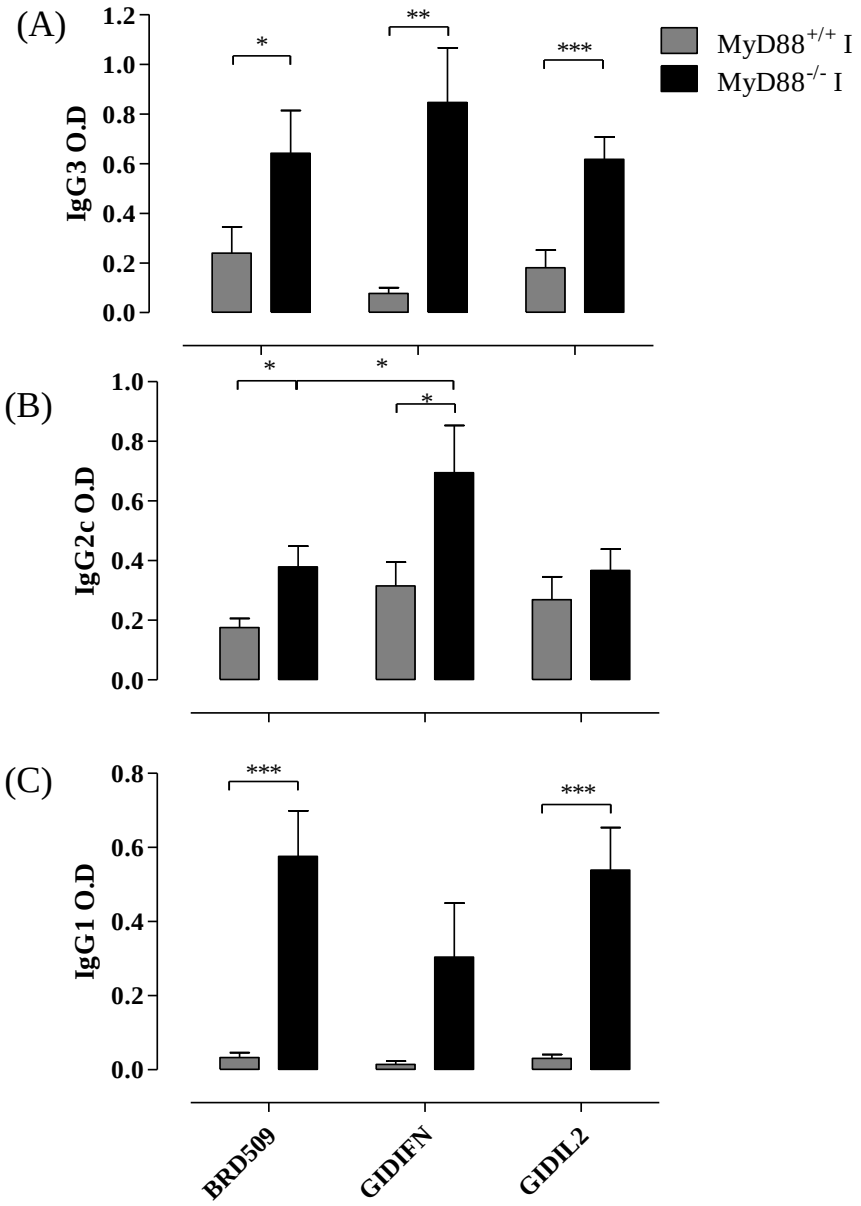

Fig. 17. Induction of hypergammaglobulinemia in MyD88$^{-/-}$ following infection with two different cytokine-expressing *Salmonella* strains. Mice were inoculated i.p. (~200 CFUs/mouse) with BRD509 or its recombinant derivatives, the IFNγ-expressing (GIDIFN) or IL-2-expressing (GIDIL2) strain. *Salmonella*-specific antibodies of the IgG3 (***A***), IgG2c (***B***) or IgG1 (***C***) isotypes were determined at 4-6 weeks post-infection. Data are compiled from 2 independent experiments for cytokine expressing strains and 3 independent experiments for the BRD509 strain (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

The presence of autoreactive antibodies specific to dsDNA and thyroglobulin was determined in sera of infected mice at 4-6 weeks post inoculation. As shown in **Fig. 18*A*-*B***, significant reactivity to the two self antigens was observed in MyD88$^{-/-}$ mice infected with the BRD509 strain. Sera of MyD88$^{-/-}$ animals infected with either GIDIFN or GIDIL2 failed to exhibit any significant reactivity to self antigens. Sera of infected wild-type mice, regardless of the *Salmonella* strain used, were also negative.

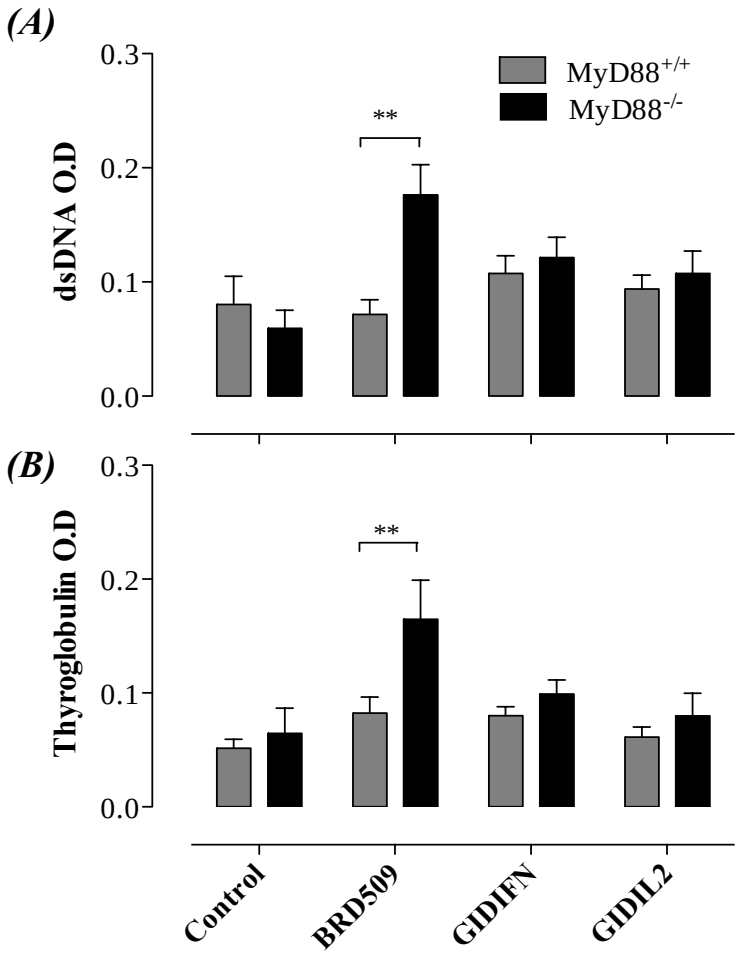

Fig. 18. No evidence for autoantibody induction in mice infected with cytokine-expressing *Salmonella* strains. Serum reactivity to dsDNA (***A***) and thyroglobulin (***B***) following infection with BRD509, GIDIFN or GIDIL2 *Salmonella* strains in WT and MyD88$^{-/-}$ mice. The cut off for the detection of autoantibodies was determined at 1/1000 dilution. Data are compiled from 2 independent experiments for cytokine expressing strains and 3 independent experiments for the BRD509 strain (**, p<0.01).

4.8. Flowcytometric analysis of the cellular alterations following infection of MyD88$^{+/+}$ and MyD88$^{-/-}$ mice

In an effort to better understand the underlying mechanism for the observed hypergammaglobulinemia in MyD88$^{-/-}$ mice, the cellular changes in the spleen following infection were characterized by flowcytometry. This analysis was carried out at 3 weeks post-infection since this is the time at which elevated serum antibodies began to be observed. Normal or MyD88$^{-/-}$ mice were infected with ~3000 CFUs/mouse of BRD509 strain and sacrificed at day 21 for analysis. As expected, inoculation of MyD88$^{-/-}$ mice with this rather low dose of attenuated *Salmonella* results in bacterial persistence in target organs as evidenced by the significantly larger loads in liver and MLNs (**Fig. 19*C*-*D***). This is accompanied by a protracted state of splenomegaly in MyD88$^{-/-}$ mice characterized by significantly larger (~3.8-fold) spleens compared to infected MyD88$^{+/+}$ mice (**Fig. 19*A*-*B***).

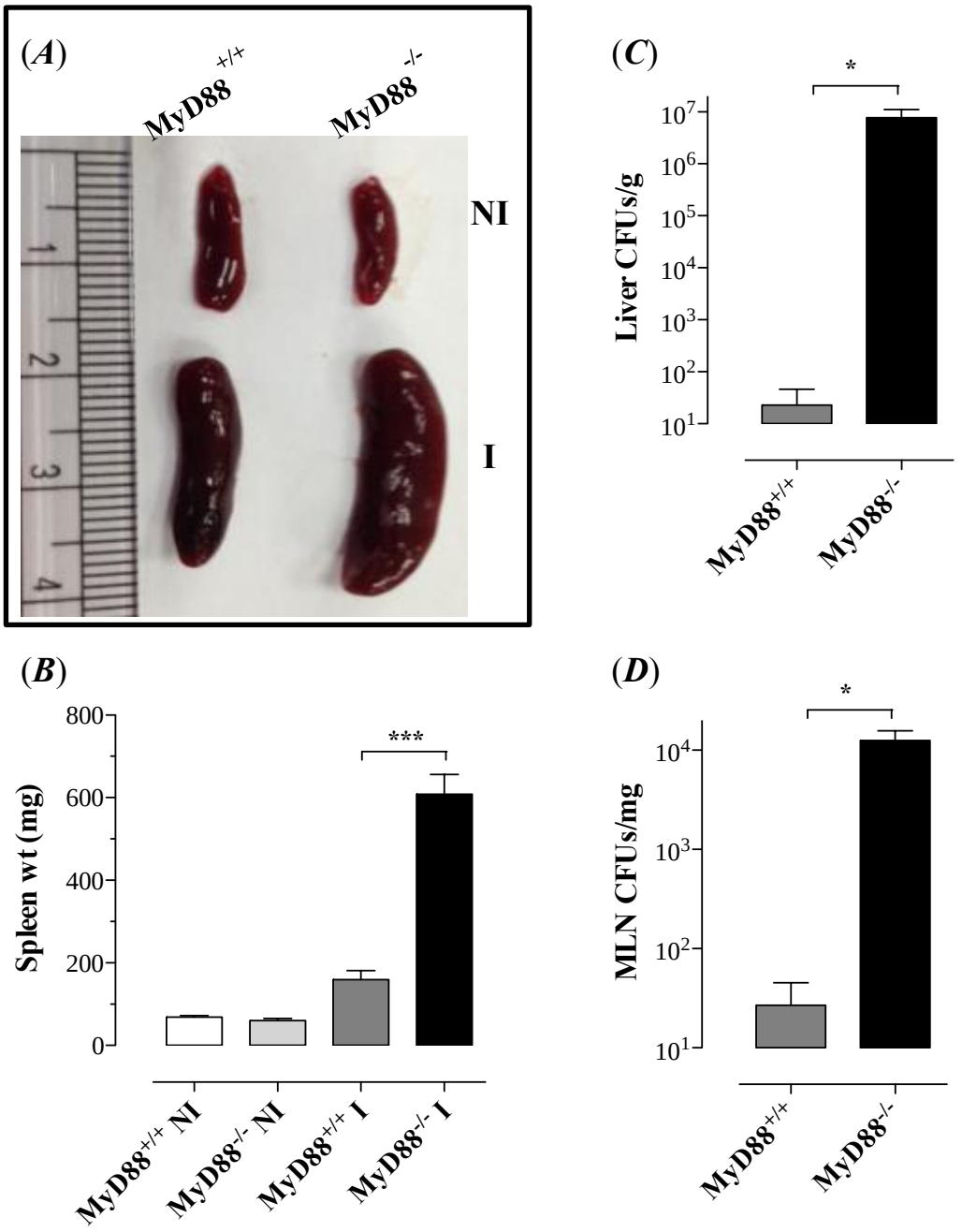

Fig. 19. Splenomegaly elevated bacterial organ loads in infected MyD88$^{-/-}$ mice. Mice infected with BRD509 (3000 CFUs/mouse) were sacrificed at day 21. Significantly enlarged spleens are observed, particularly in infected MyD88$^{-/-}$ mice (panels ***A-B***). Spleens of non-infected (NI) groups are shown for comparison (***A-B***). Bacterial loads in liver (C) and MLNs (D) were also determined. This data is a representative of 3 independent experiments (*, $p<0.05$, ***, $p<0.001$).

Flowcytometric analysis was carried out on spleen cell populations from non-infected as well as infected animals using a panel of monoclonal antibodies (mAbs) specific to each major cell population as well as a series of activation markers for each cell subpopulation. Based on staining with a combination of mAbs to CD11b and Gr-1 cell surface molecules, three $CD11b^{+}$ myeloid subpopulations could be discerned, $Gr\text{-}1^{H}$, $Gr\text{-}1^{Int}$ and $Gr\text{-}1^{L}$ which represent granulocytes, activated macrophages and monocytes, respectively (**Fig. 20**). In the spleens of non-infected C57BL/6 mice, the mean percentages of each of these populations were 2.0 ± 0.5, 3.2 ± 0.7, and 6.2 ± 1.3, respectively (**Fig. 20*A* *and* *Fig. 21A***). By day 21 post-infection with BRD509 strain, a time by which the infection has been largely resolved in wild-type mice, the respective percentages were 4.4 ± 0.4, 6 ± 0.7, and 5.2 ± 0.5 (**Fig. 20*C* *and Fig. 21A***), representing about 2-fold increase in percentage of $Gr\text{-}1^{H}$ and $Gr\text{-}1^{Int}$ myeloid cell populations. The cellular changes observed in the spleens of $MyD88^{-/-}$ mice were more profound (**Fig. 20*B, D* *and Fig. 21A***); the percentages of $Gr\text{-}1^{H}$, $Gr\text{-}1^{Int}$ and $Gr\text{-}1^{L}$ cells increased from 0.7 ± 0.2, 1.8 ± 0.1, and 4.7 ± 0.4 in non-infected mice to 3.7 ± 0.5, 14.2 ± 2.2, and 4.5 ± 0.3 in infected animals, respectively. These results represent ~5-fold and ~8-fold increase in $Gr\text{-}1^{H}$ and $Gr\text{-}1^{Int}$ myeloid cells, largely as a consequence of the persistent infection in $MyD88^{-/-}$ mice. No significant differences were observed in the $CD11b^{+}Gr\text{-}1^{low}$ myeloid subpopulation in either mouse strain (**Fig. 21*A*)**.

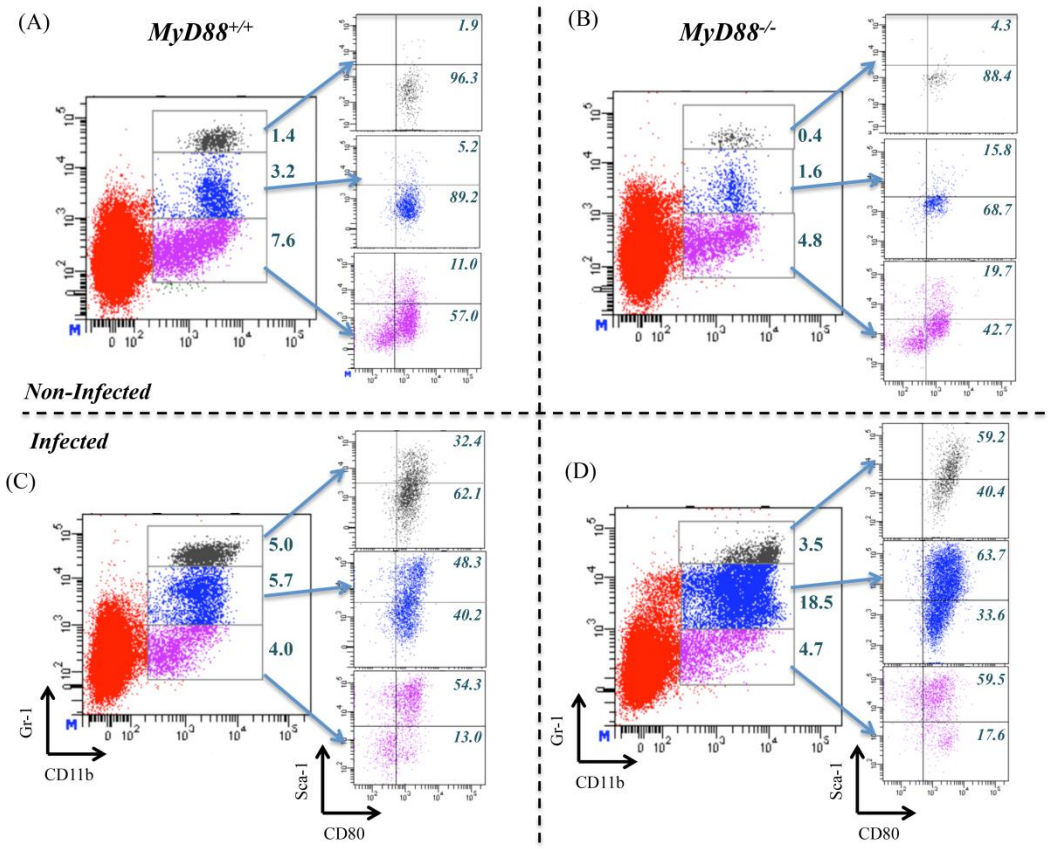

Fig. 20. Flow cytometric analysis of inflammatory myeloid cell influx and activation status in the spleen. Splenocytes were prepared from non-infected (***A-B***) or *Salmonella*-infected (***C-D***) MyD88$^{+/+}$ (***A, C***) or MyD88$^{-/-}$ (***B, D***) mice and analyzed for CD11b and Gr-1 positivity. Analysis was done on day 21 post-infection with the BRD509 strain. Based on level of expression of Gr-1 protein, three distinct CD11b^{+} myeloid populations could be discerned in each experimental mouse group, CD11b^{+}Gr-1^{high} (top panel), CD11b^{+}Gr-1^{Inter} (middle panel), and CD11b^{+}Gr-1$^{-/low}$ (bottom panel). Each of these sub-populations was further analyzed for the expression of CD80 and Sca-1 activation markers, as indicated. Cell percentages are shown in each quadrant. Results of individual mice are shown and are representative of four independent experiments.

The activation status of the various populations of myeloid cells was also assessed by co-staining with antibodies to Sca-1 and CD80 proteins. *Salmonella* infection induced a dramatic upregulation of Sca-1 expression on all myeloid cell populations in both mouse strains. Within the infected groups, the level of Sca-1 expression was higher in MyD88$^{-/-}$ than in MyD88$^{+/+}$ mice (**Fig. 21*B***). This suggests a robust IFNγ-driven up regulation following infection. Interestingly in the Gr-1^{L} monocytic populations, the MyD88$^{-/-}$ controls exhibited a higher level of Sca-1 expression than the wild-type control group (**Fig. 21*B***). Regarding CD80 expression, the majority of myeloid cells in non-infected mice showed substantial levels of CD80 (**Fig. 21*C***) and there was no significant alteration in the expression of this costimulatory molecule in any of the myeloid populations after infection in either of the two mouse strains (**Fig. 20*C*-*D* and 21*C***). Thus, by day 21 post inoculation, persistence of *Salmonella* infection in MyD88$^{-/-}$ mice correlated with increased expansion of activated splenic macrophages and granulocytes which was more apparent than in MyD88$^{+/+}$ counterparts in which the infection had largely been resolved by that time.

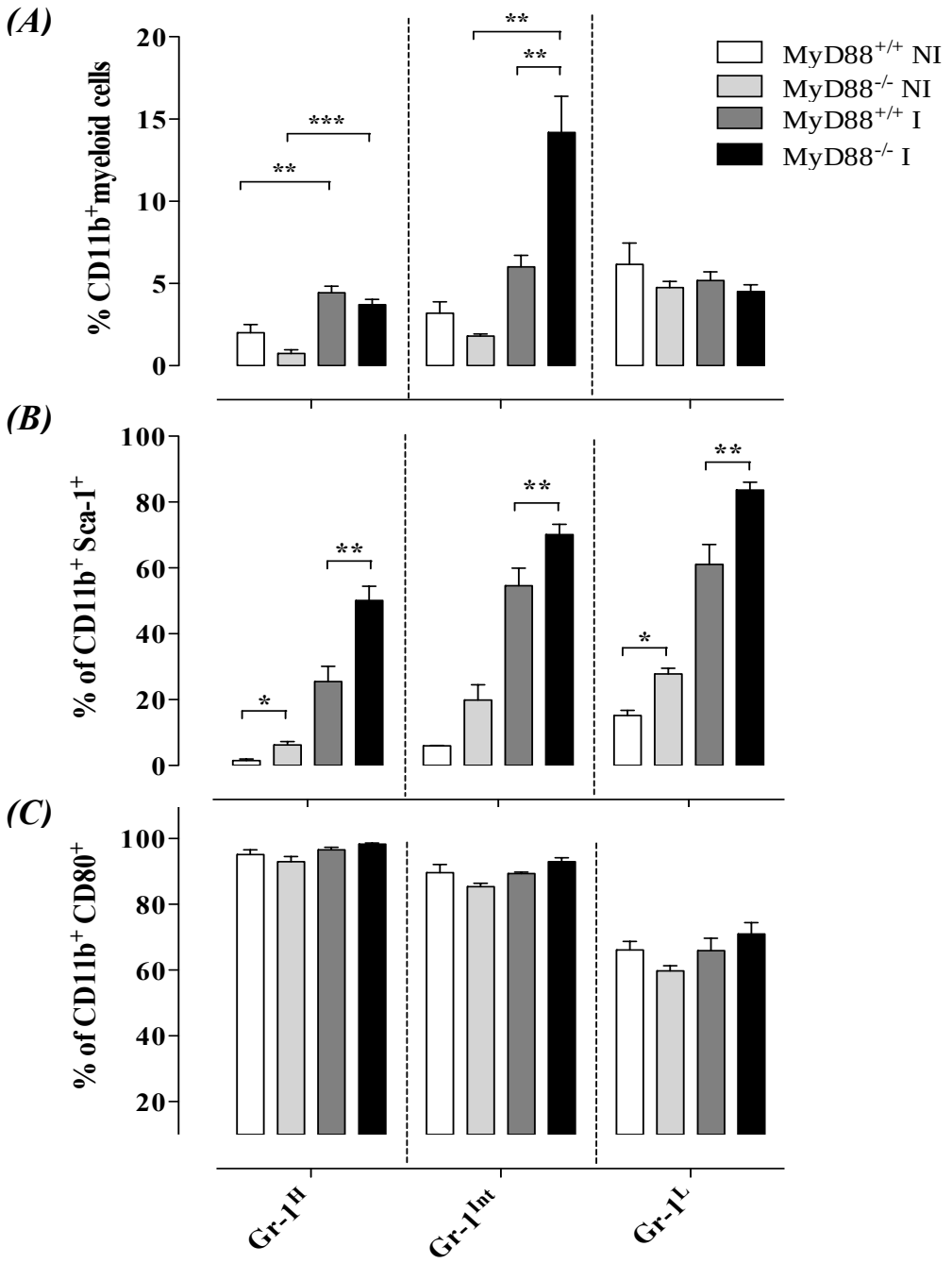

Fig. 21. Quantification of alterations in splenic myeloid cell sub-populations and their activation status following infection. (***A***). Percentages of $CD11b^+$ myeloid cells expressing either high, intermediate or low levels of Gr-1 protein in non-infected or BRD509-infected $MyD88^{+/+}$ or $MyD88^{-/-}$ mice. (***B-C***) Alterations in expression of Sca-1 (***B***) or CD80 (***C***) proteins in the different myeloid subpopulations. Graphs are compiled from 4 independent experiments (*, $p<0.05$, **, $p<0.01$).

4.9. Enhanced lymphocyte activation in the spleens of *Salmonella*-infected MyD88$^{-/-}$ mice

We further assessed the splenic population of B lymphocytes (**Fig. 22*A*)** and their activation status after infection at day 21. In saline-treated MyD88$^{-/-}$ mice, CD19^{+} B lymphocytes constituted ~55% of the splenic population which was slightly higher than those observed in the wild-type strain (~49.3%). After *Salmonella* infection, percentage of B lymphocytes decreased to 37% in MyD88$^{-/-}$ mice compared to 44% in MyD88$^{+/+}$ mice (**Fig. 22*B*)**.

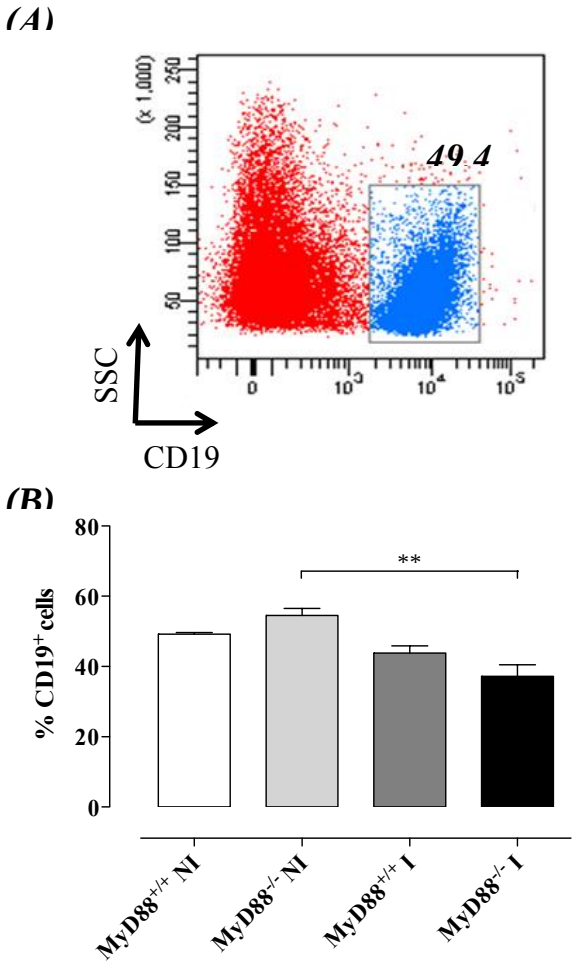

Fig. 22. Flow cytometric analysis of splenic CD19^{+} B lymphocytes in various experimental animal groups. (***A***) representative dot blot of gated CD19^{+} cells. (***B***) Percentage of CD19^{+} B cells in infected and non-infected groups. Analysis was done on day 21 post-infection with the BRD509 strain. Data are compiled from 4 independent experiments (**, p<0.01).

The activation status of B cells was determined by evaluating the expression of Sca-1, CD80 and CD86 markers. Representative dot plots of Sca-1 and CD80 staining are shown in **Fig. 23** and quantitative analysis for each marker is shown in **Fig. 24A-C**. The majority of B cells from both mouse strains (51% and 75% in MyD88$^{+/+}$ and MyD88$^{-/-}$, respectively) were positive for Sca-1 in normal animals. After infection, the percent positive cells increased to 92% and 99%, respectively (**Fig. 23*C-D*** and **Fig. 24*A***). In contrast, CD80 expression was not induced upon infection on B cells of either mouse strain (**Fig. 24*B*)**. However, the percentage of B cells expressing CD86 increased by 2-fold and 3-fold in infected MyD88$^{+/+}$ and MyD88$^{-/-}$ mice, respectively (**Fig. 24*C***). Thus, B lymphocytes of MyD88$^{-/-}$ mice appear to be at a heightened level of activation, especially after *Salmonella* infection.

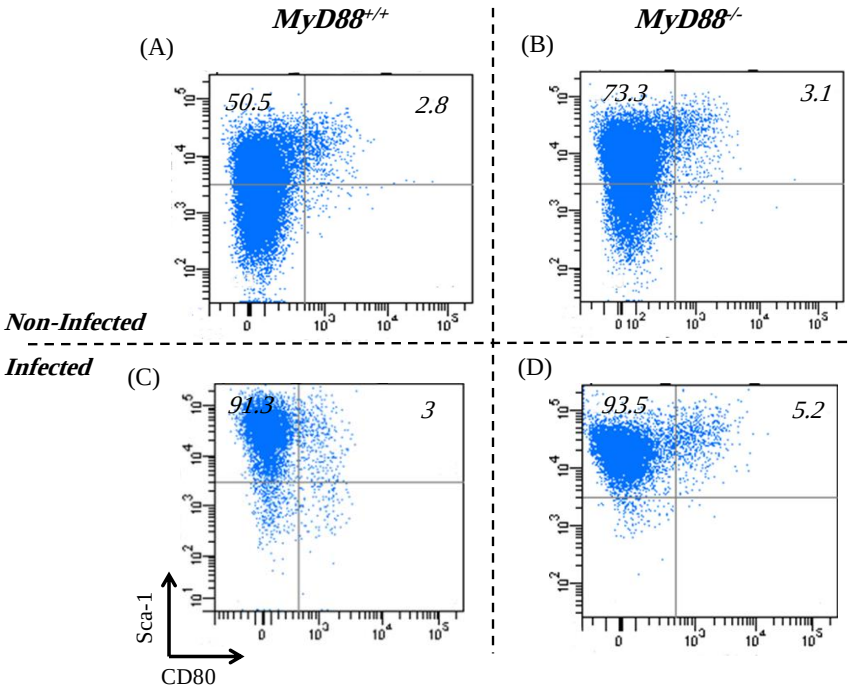

Fig. 23. Increased B lymphocyte activation in the spleens of MyD88$^{-/-}$ mice following infection with *Salmonella*. Dot plots depicting the level of expression of Sca-1 and CD80 proteins on gated CD19^{+} B cells in non-infected (***A-B***) and *Salmonella* infected (***C-D***) MyD88$^{+/+}$ (***A, C***) or MyD88$^{-/-}$ (***B, D***) mice. Numbers within quadrants represent percentage of positive cells in that population. Analysis was done on day 21 post-infection with the BRD509 strain. Results of individual mice are shown and are representative of four independent experiments.

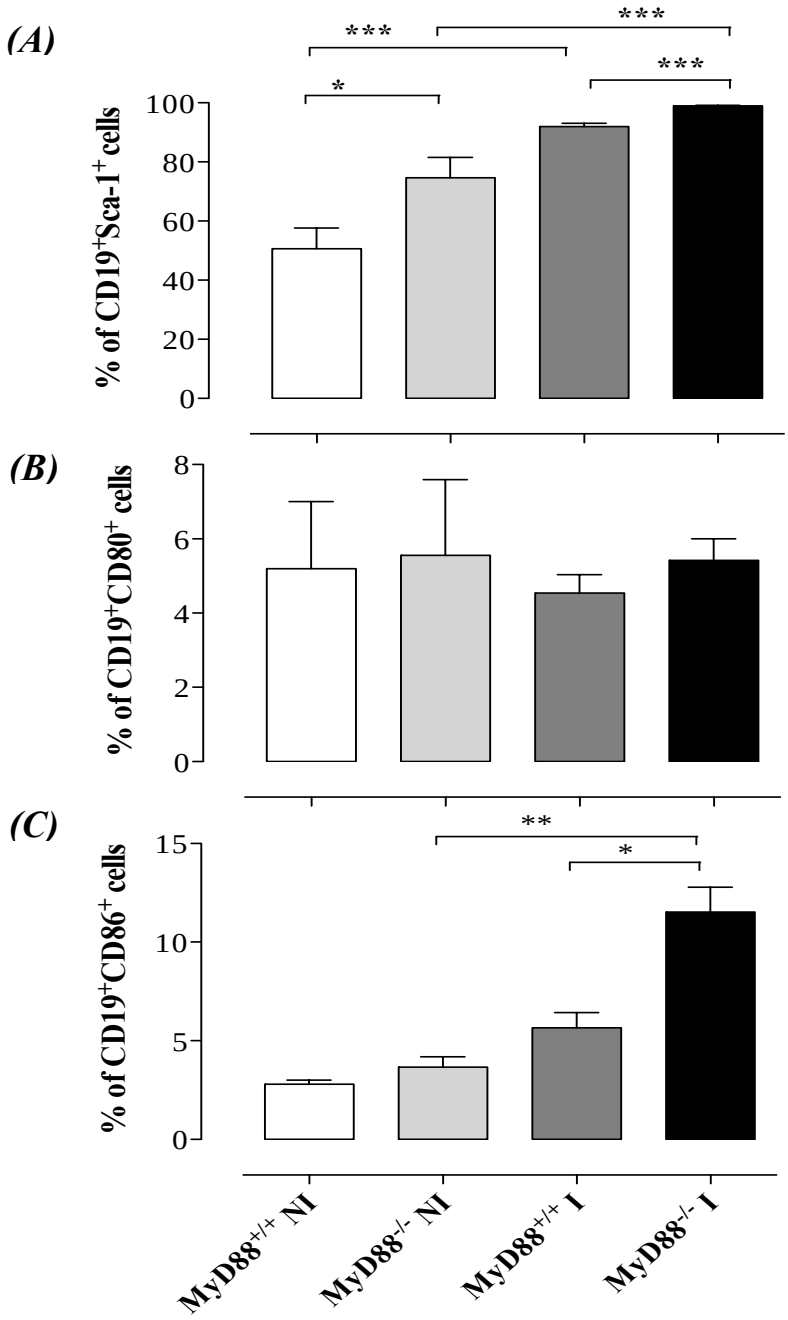

Fig. 24. Changes in expression level of activation markers on B lymphocytes. Percentages of $CD19^+$ lymphocytes expressing Sca-1 (***A***), CD80 (***B***) or CD86 (***C***) proteins in non-infected or BRD509-infected $MyD88^{+/+}$ or $MyD88^{-/-}$ mice. . Analysis was done on day 21 post-infection with the BRD509 strain. Graphs are compiled from 4 independent experiments (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$).

The splenic $CD3^+$ T cell populations, their subsets and activation status were also evaluated at day 21 post-infection. A representative dot plot of the gated $CD3^+$ T cells **(Fig. 25*A*)** and their $CD4^+$ and $CD8^+$ subsets **(Fig. 25*C*)** is shown. The $CD3^+$ T cells constituted up to 33% of the splenic populations in wild-type mice which did not change significantly on infection **(Fig. 25*B*).** However, infected $MyD88^{-/-}$ mice were observed to have significantly lower percentages (~26%) of $CD3^+$ T cells **(Fig. 25*B*).** On evaluating the T cell subsets, the decrease of $CD3^+$ T cells in the infected $MyD88^{-/-}$ mice constituted mainly a reduction in the percentage of $CD8^+$ T cells and not $CD4^+$ T cells **(Fig. 25*D*)**.

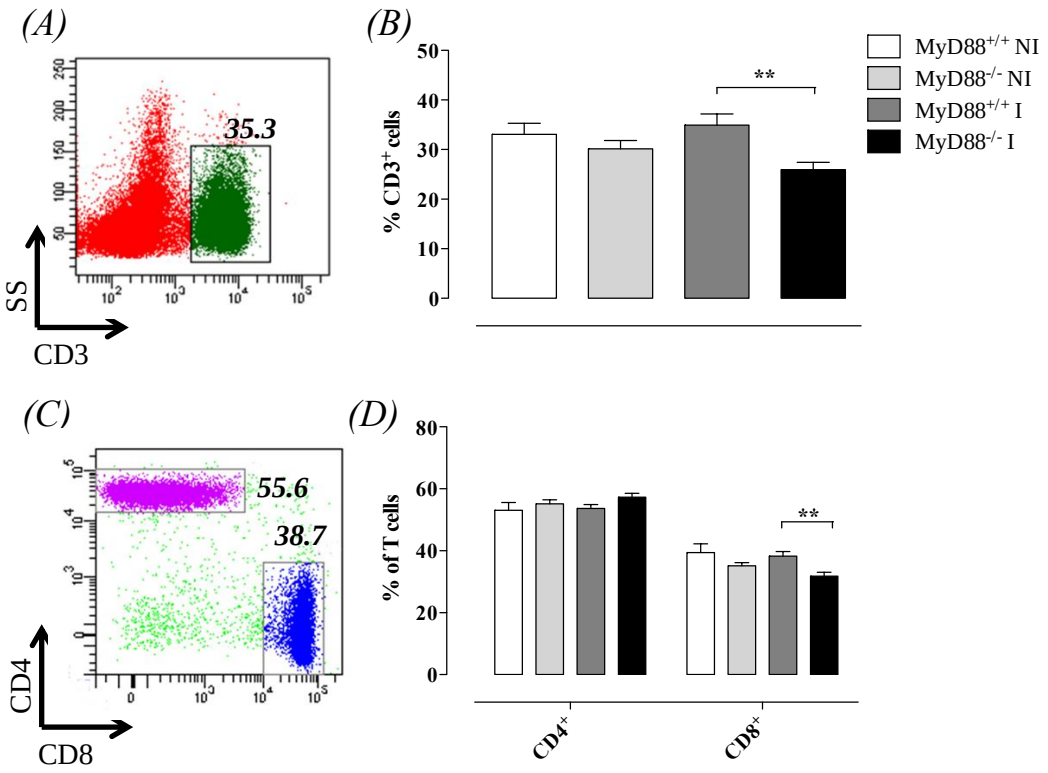

Fig. 25. Changes in splenic T lymphocyte populations following infection. Representative dot plot of gated $CD3^+$ T lymphocytes (***A***) and CD4 and CD8 subpopulations (***C***) within gated $CD3^+$ cells. (***B***). Changes in the percent of $CD3^+$ lymphoid cells in the whole spleen. (***D***) Changes in the ratios of $CD4^+$ and $CD8^+$ cells within the T cell populations. Analysis was done on day 21 post-infection with the BRD509 strain. Graphs are compiled from 4 independent experiments for (B) and (D) (**, $p<0.01$).

Using appropriate gates, the expression of activation markers CD25 and Sca-1 was studied on $CD4^+$ and $CD8^+$ T cells (**Fig. 26 and 27**). Similar to previous observations with B cells, the great majority of $CD4^+$ T cells in uninfected spleens (76% and 85% in $MyD88^{+/+}$ and $MyD88^{-/-}$ mice, respectively) were positive for Sca-1 antigen (**Fig. 26*A*-*B***). These percentages were further increased to >94% in infected mice. A much more limited expression of CD25 was observed on $CD4^+$ T cells (**Fig. 26*A, C***). In non-infected mice, only 7-8% of $CD4^+$ T cells were positive for CD25 (**Fig. 26*C***). The only significant alteration to this ratio was observed in infected $MyD88^{-/-}$ mice where the percentage of positive $CD4^+$ $CD25^+$ T cells increased to ~12% (**Fig. 26*C***). For $CD8^+$ T cells, the percentage positive for Sca-1 antigen was 45% and 59% in uninfected $MyD88^{+/+}$ and $MyD88^{-/-}$ mice, respectively, which was increased to 84% and 98% in the corresponding infected groups (**Fig. 27*A*-*B***). As for CD25 expression, the only significant alteration was the increase in ratio of positive cells to 5.4% in infected $MyD88^{-/-}$ mice, most likely a response to an ongoing active infection (**Fig. 27*C***).

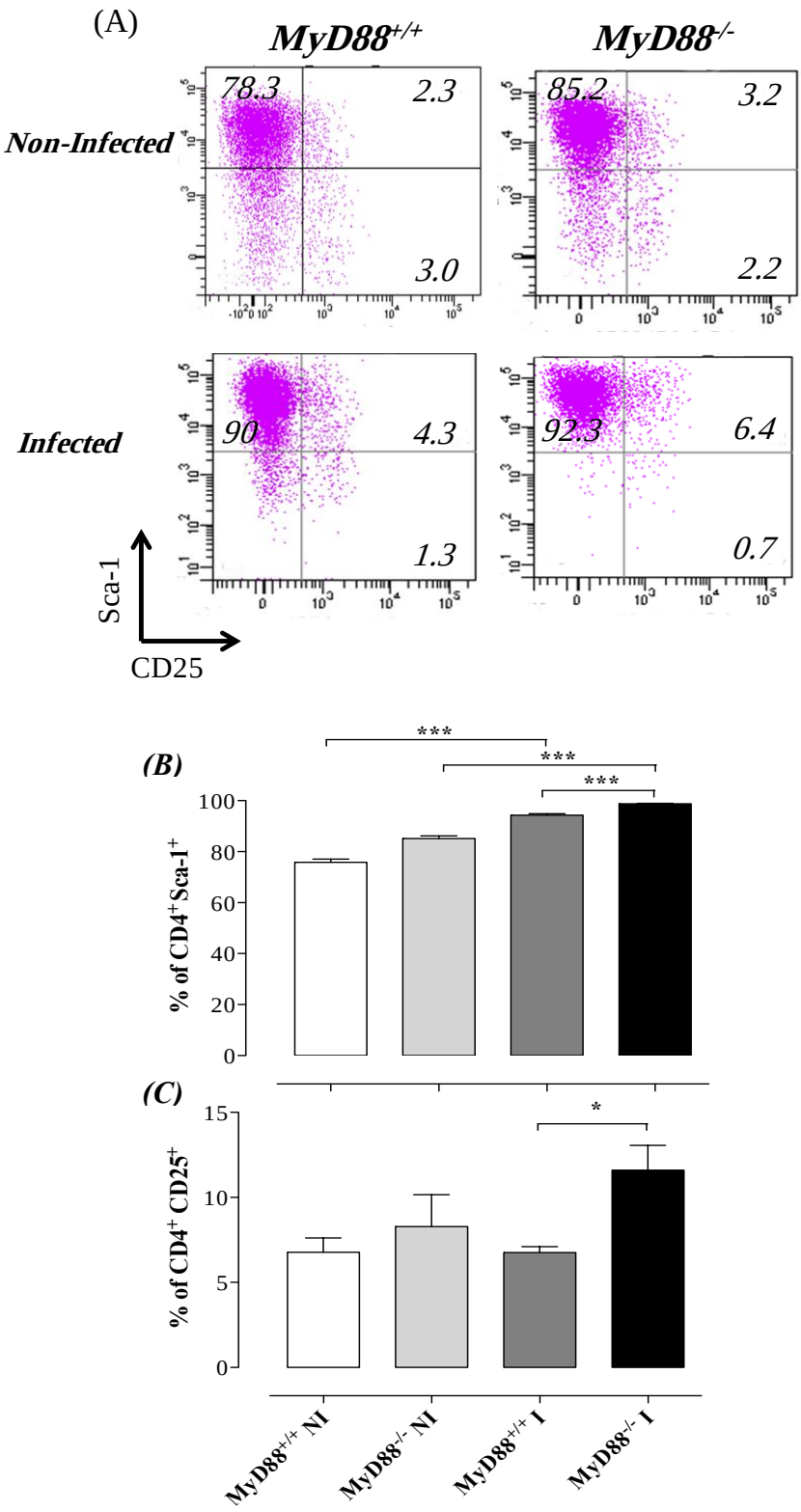

Fig. 26. Increased CD4$^+$ T cell activation in the spleens of MyD88$^{-/-}$ mice following infection with *Salmonella*. (***A***) Expression of Sca-1 and CD25 proteins on gated CD4$^+$ T cells. Changes in the percent of cells positive for Sca-1 (***B***) or CD25 (***C***) are shown. Analysis was done on day 21 post-infection with the BRD509 strain (A) Results of individual mice are shown and are representative of four independent experiments. (B-C) Data are compiled from 4 independent experiments (*, p<0.05, ***, p<0.001).

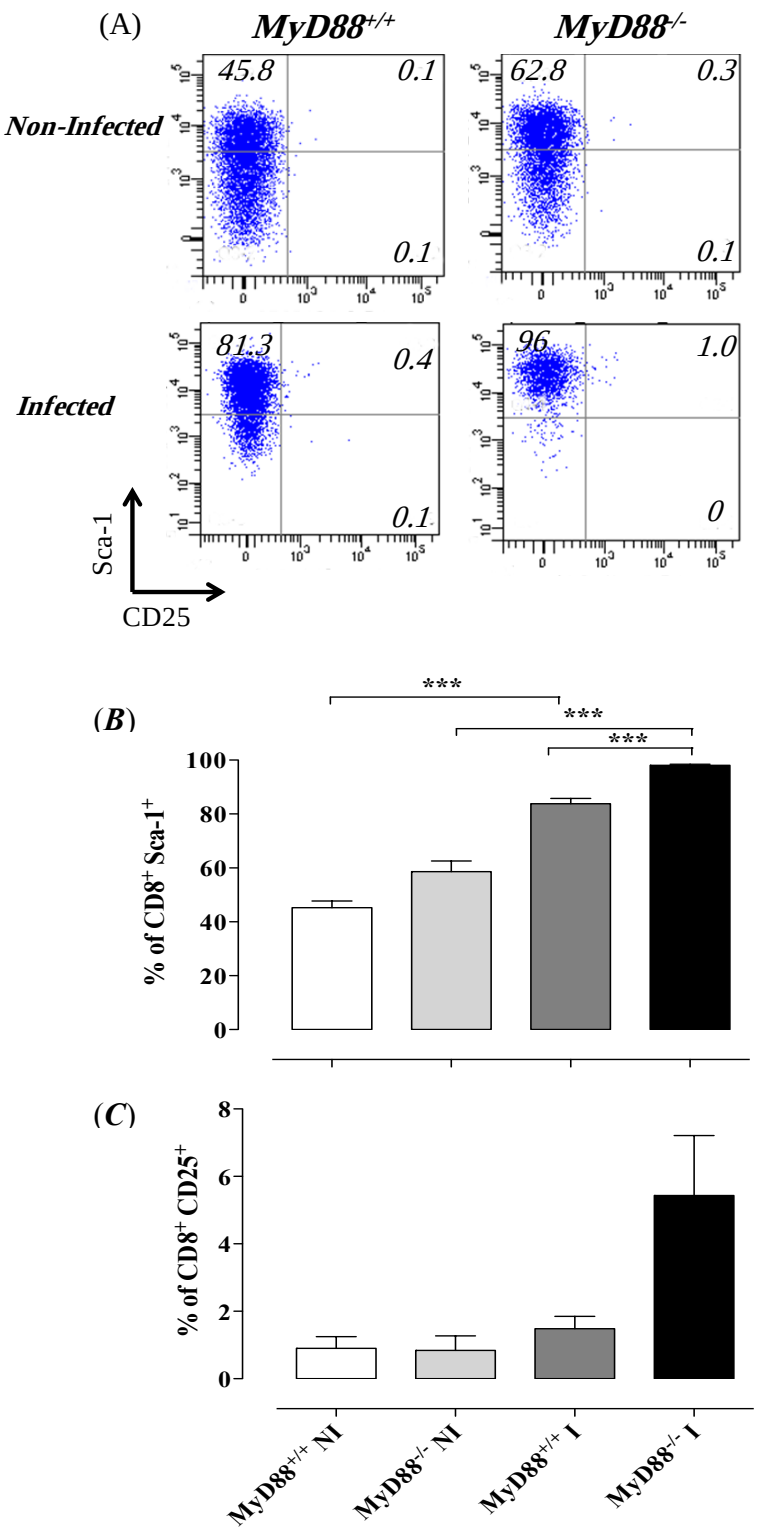

Fig. 27. Increased CD8$^+$ T cell activation in the spleens of MyD88$^{-/-}$ mice following infection with *Salmonella*. (***A***) Expression of Sca-1 and CD25 proteins on gated CD8$^+$ T cells. Changes in the percent of cells positive for Sca-1 (***B***) or CD25 (***C***) are shown. Analysis was done on day 21 post-infection with the BRD509 strain. (A) Results of individual mice are shown and are representative of four independent experiments. (B-C) Data are compiled from 4 independent experiments (***, p<0.001).

The absolute numbers of the myeloid and lymphoid cells were also determined per spleen. The most noticeable changes in cell number were observed in the $CD11b^{+}$ myeloid cell populations. Compared to non-infected spleens, there were 3-fold and 6-fold increase in these cells in $MyD88^{+/+}$ and $MyD88^{-/-}$ mice, respectively (**Fig. *28A***). Correlating with the chronicity of infection in $MyD88^{-/-}$ mice, we observed a 40% increase in splenic myeloid cell number compared to infected $MyD88^{+/+}$ counterparts (**Fig. *28A***). In general, infected spleens also had increased lymphocyte numbers but to a much lesser extent than the myeloid population. The increases in lymphocyte populations averaged 30-40% above those in non-infected spleens for B lymphocytes for both mouse strains (**Fig. *28B***). For T lymphocytes, the increases in absolute cell number were 44% and 112% in infected $MyD88^{+/+}$ and $MyD88^{-/-}$ mice, respectively (**Fig. *28C***). Therefore, the changes in absolute cell number of myeloid cells are in agreement with the observed cell percentages calculated from the flow cytometry data. However, for the B and T lymphocyte populations, the changes in absolute cell numbers following infection were not as dramatic as for myeloid cells. These findings confirm the predominance of myeloid cells in the cellular influx into the spleen, as a result of *Salmonella* infection.

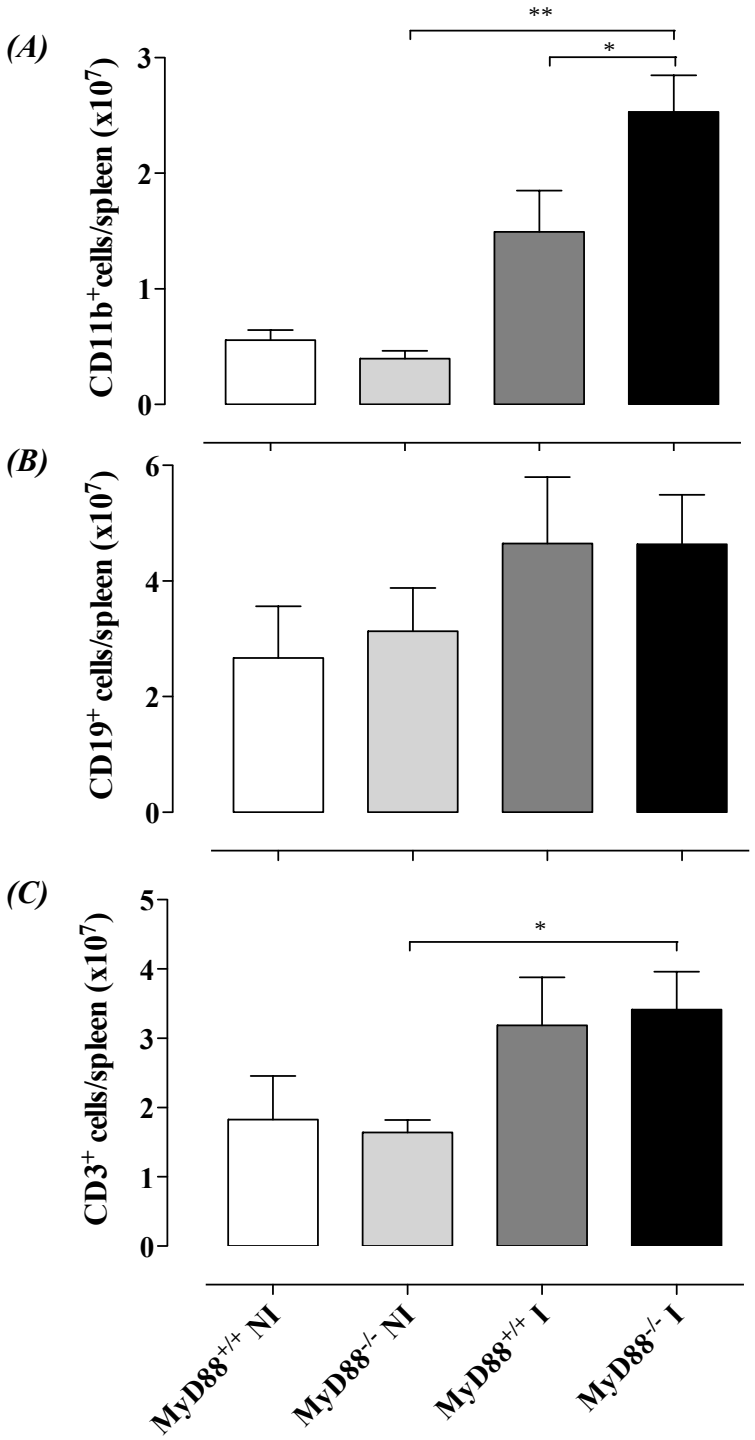

Fig. 28. Changes in spleen cellularity expressed in terms of absolute cell counts. Absolute counts of $CD11b^+$ myeloid cells (***A***), $CD19^+$ B lymphocytes (***B***) and $CD3^+$ T lymphocytes (***C***) per spleen. Analysis was done on day 21 post-infection with the BRD509 strain. Data are compiled from 3 independent experiments (*, $p<0.05$, **, $p<0.01$).

4.10. Dysregulated T helper cells responses in MyD88$^{-/-}$ mice after *Salmonella* infection

Given the hyper antibody responsiveness and the dysregulated isotypes observed in MyD88$^{-/-}$ mice, and the fact that these responses are T cell-dependent, we compared gene expression profiles of purified splenic CD4^{+} T (**Fig. 29*A***) cells isolated from the different experimental groups at day 21 post-infection. The transcriptional level of IFN-γ was significantly elevated (27-32 fold) upon infection with *Salmonella* in both MyD88$^{+/+}$ and MyD88$^{-/-}$ mice (**Fig. 29*B***). This was correlated with increased expression of t-bet, the transcriptional factor responsible for Th1 differentiation (**Fig. 29*C***). Moreover, CD4^{+} T cells from infected MyD88$^{-/-}$, but not wild-type, mice expressed significant levels of IL-4 and IL-21, signature cytokines of Th2 and T_{FH} cells, respectively. The significant level of constitutive IL-4 expression in uninfected MyD88$^{-/-}$ mice was noteworthy which further increased upon *Salmonella* infection to a level that was 10-fold higher than infected MyD88$^{+/+}$ mice (**Fig. 29*B***). Another intriguing difference in cytokine transcriptional activity was observed with IL-10 which was expressed at low levels in CD4^{+} T cells of infected wild-type mice (~2.5-fold over background) but was significantly elevated (~14-fold) in infected MyD88$^{-/-}$ mice (**Fig. 29*B***). Finally, no evidence for IL-17 or TGF-β production by CD4^{+} T cells of any of the groups was observed. Of all of the transcription factors analyzed, only t-bet was expressed at high levels by both MyD88$^{+/+}$ and MyD88$^{-/-}$ CD4^{+} T cells (**Fig. 29*C***). No evidence for expression of GATA-3, RoR γ, or Bcl-6 was seen. Curiously, significant levels of Foxp3 were expressed by CD4^{+} T cells of non-infected MyD88$^{-/-}$ mice, but this was curtailed following infection (**Fig. 29*C***). This

suggests a reduced expansion of the Treg cells in the absence of MyD88 as a result of a persistent *Salmonella* infection.

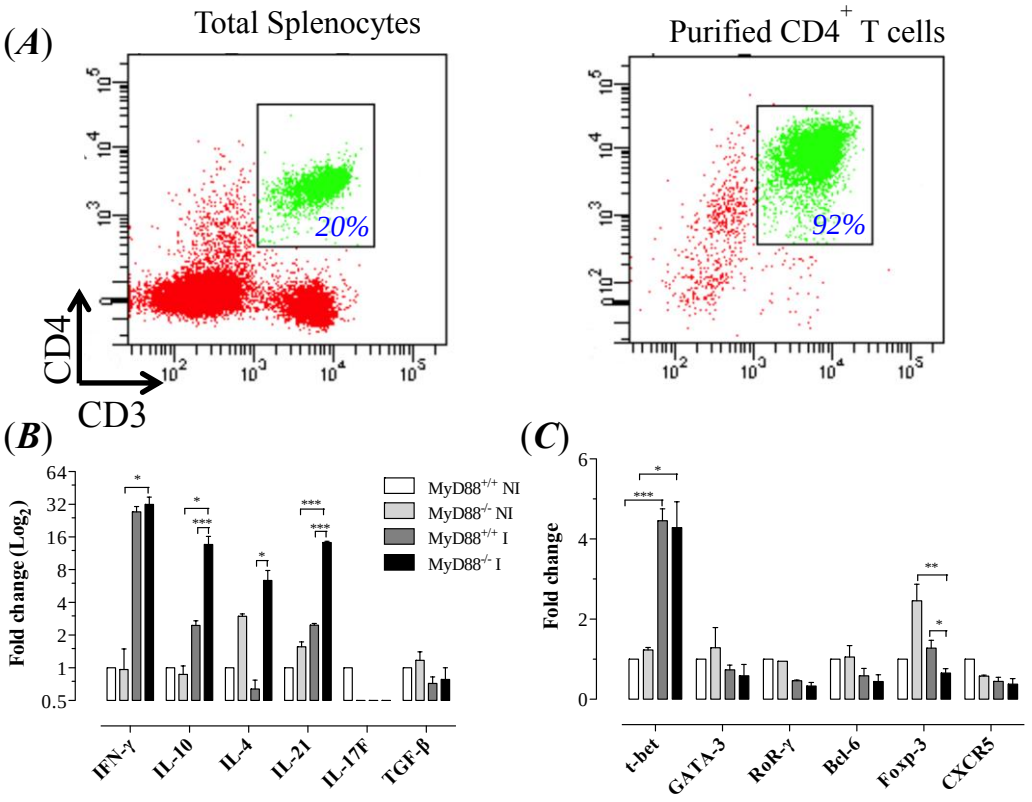

Fig. 29. Gene expression profiles in purified CD4$^+$ T lymphocytes. (***A***). Representative dot plots of splenic CD4$^+$ T cells before and after purification. Gene expression levels of key cytokines (***B***) and transcription factors (***C***) in CD4$^+$ T cells of different experimental groups were analyzed by quantitative real-time PCR. Analysis was done on CD4$^+$ T cells purified from non-infected or *Salmonella*-infected mice (~3000 CFUs/mouse) at day 21. Alterations in gene expression are depicted as fold-change compared to non-infected WT mice. Data are compiled from 2-3 independent experiments (*, p<0.05, **, p<0.01, ***, p<0.001).

Given the increased infiltration of inflammatory myeloid cells into the spleens of MyD88$^{-/-}$ mice at day 21 of infection, the capacity of purified myeloid cells (**Fig. 30*A***) to produce inflammatory mediators was assessed. The highest transcriptional activity observed was for iNOS, which was expressed at >1000-fold higher levels in both MyD88$^{+/+}$ and MyD88$^{-/-}$ myeloid cells compared to uninfected controls (**Fig. 30*B***). Equivalent levels of induction of IL-6 and IL-12p35 were also evident in both mouse strains. Interestingly, a small but significant level of expression was observed for BAFF, a cytokine associated with polyclonal B cell responses and autoimmune diseases, in MyD88$^{-/-}$ mice (**Fig. 30*A***). Consistently, this induction was absent in infected wild-type mice.

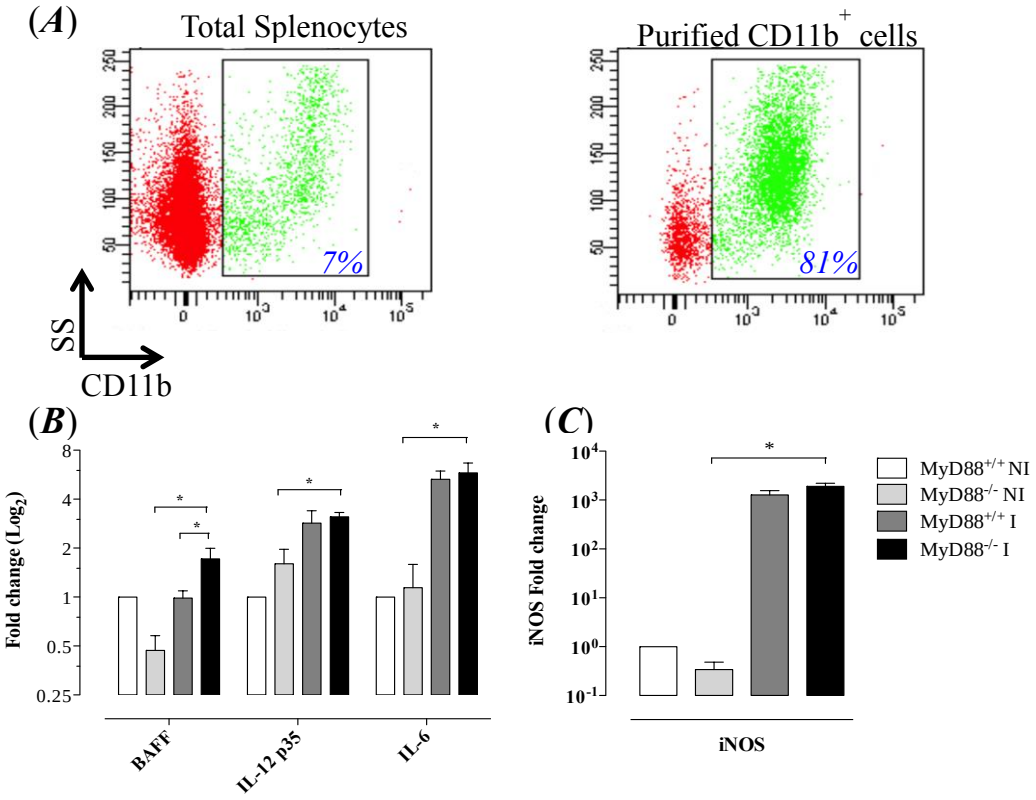

Fig. 30. Gene expression profiles in purified CD11b^{+} myeloid cells. (***A***). Representative dot plots of splenic CD11b^{+} cells before and after purification. Gene expression levels of BAFF, IL-12p35 and IL-6 (***B***) and iNOS (***C***) in myeloid cells of different experimental groups were analyzed by quantitative real-time PCR. Analysis was done on myeloid cells purified from non-infected or *Salmonella*-infected mice (~3000 CFUs/mouse) at day 21. Alterations in gene expression are depicted as fold-change compared to non-infected WT group (*, p<0.05).

In order to validate cytokine production by $CD4^+$ T cells, purified cells were stimulated with a combination of plate-bound anti-CD3/CD28 mAbs for 24 hours after which culture supernatants were collected and assayed for IFN-γ, IL-4 and IL-10 content. In confirmation of gene expression data, $CD4^+$ T cells of infected $MyD88^{+/+}$ or $MyD88^{-/-}$ mice secreted abundant levels of IFN-γ reaching up to 14.3 ng/ml and 11.5 ng/ml, respectively (**Fig. 31*A***). In sharp contrast, IL-4 secretion was detected only from $MyD88^{-/-}$ $CD4^+$ T cells, which is in agreement with the gene expression findings (**Fig. 31*C***). Interestingly, $CD4^+$ T cells from uninfected $MyD88^{-/-}$, but not $MyD88^{+/+}$, mice could also secrete IL-4 upon induction via the TCR, suggesting an endogenous predisposition to Th2 responses in these mice (**Fig. 31*C***). Moreover, high levels of IL-10 (mean of 2174 pg/ml) were uniquely observed in *Salmonella*-infected $MyD88^{-/-}$ mice (**Fig. 31*B***). Overall, the data suggest the prevalence of $CD4^+$ T cells producing both the proinflammatory cytokine IFN-γ and/or anti-inflammatory cytokines IL-4 and IL-10 in $MyD88^{-/-}$ mice, following infection with BRD509. This contrasts with wild-type mice that exhibit predominantly Th1-mediated immune responses against *Salmonella* infection.

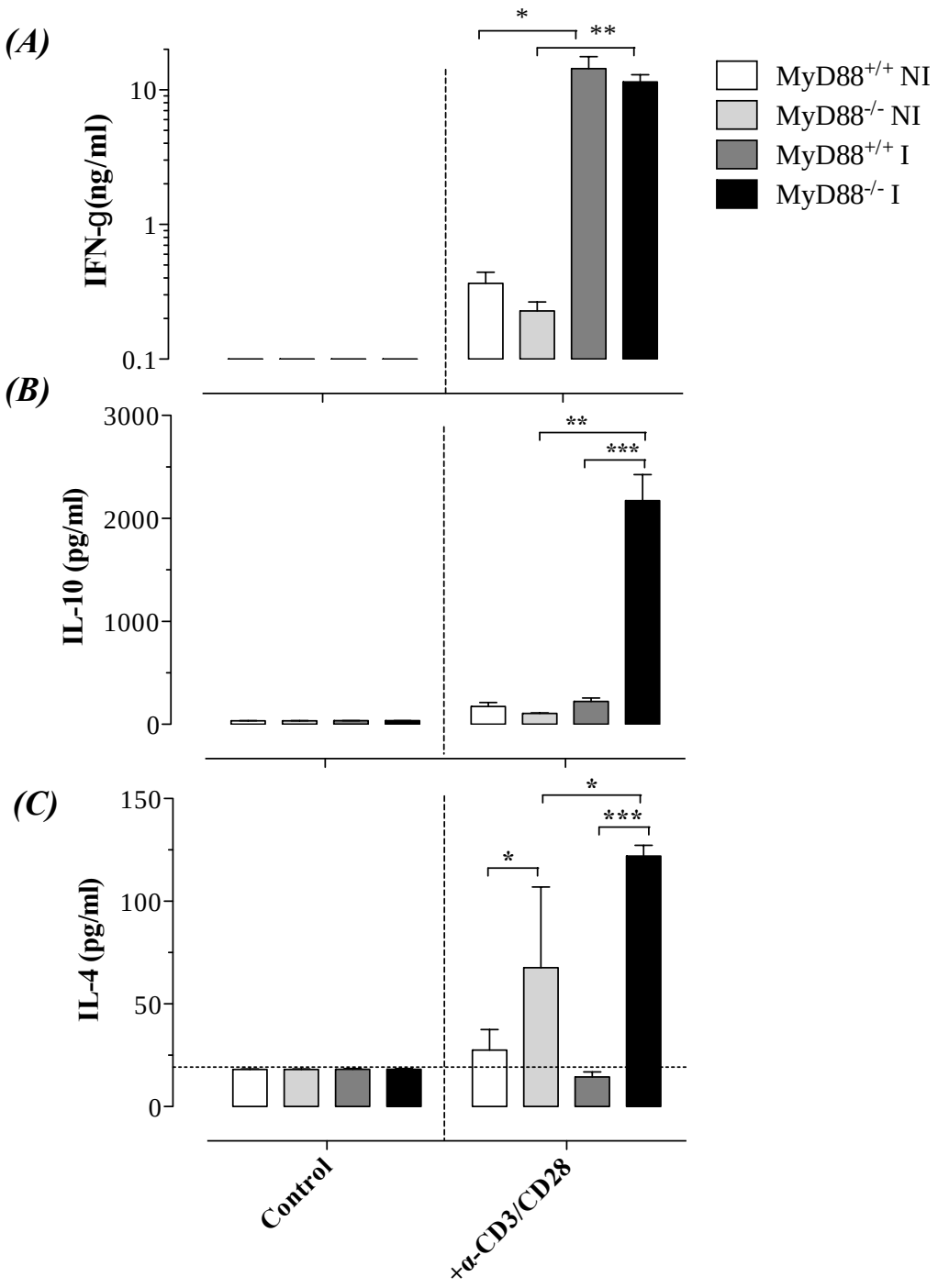

Fig. 31. Evidence of IL-4 and IL-10 production by purified CD4$^+$ T cells in infected MyD88$^{-/-}$ mice. Secretion of IFN-γ (***A***), IL-10 (***B***) and IL-4 (***C***) by purified CD4$^+$ T cells after stimulation with plate-bound anti-CD3/CD28 antibodies. CD4$^+$ T cells were purified from non-infected or *Salmonella*-infected spleens of WT and MyD88$^{-/-}$ mice at day 21 post-infection. Data are compiled from 2 independent experiments (*, p<0.05, **, p<0.01, ***, p<0.001).

An important question that was still unanswered by the above data was whether the proinflammatory (IFN-γ) and anti-inflammatory cytokines (IL-4 and IL-10) were produced by the same or distinct populations of $CD4^+$ T cells. To address this issue, we carried out intracellular cytokine analysis by flowcytometry. Highly purified $CD4^+$ T cells from infected or uninfected mice were stimulated overnight with anti-CD3/CD28 antibodies and assessed for the intracellular levels of IFN-γ, IL-4 and IL-10 cytokines. No evidence for any IL-10 production was observed when $CD4^+$ T cells from any of the 4 experimental groups of animals were cultured without TCR stimulation (**Fig. 32**; Control groups). Similarly, less than 1% of $CD4^+$ T cells in unstimulated cultures were positive for IFN-γ. The same was also true for IL-4 production (**Fig. 33**). After TCR ligation, the percentage of $CD4^+$ T cells from uninfected mice that were positive for IFN-γ was ~2.3% and 0.7% for $MyD88^{+/+}$ and $MyD88^{-/-}$ mice, respectively (**Figs. 33**). After infection, the percentage of cells positive for IFN-γ increased dramatically to 21% and 30%, respectively. We focused our attention on defining populations of $CD4^+$ T cells that co-expressed both IFN-γ and IL-4 or IFN-γ and IL-10. In infected $MyD88^{-/-}$ mice, approximately 4.2% of $CD4^+$ T cells were IFN-γ^+IL-10^+ (**Fig. 32** and **Fig. 34*B***). Importantly, there was no evidence for equivalent cells in infected $MyD88^{+/+}$ mice. In terms of IL-4/IFN-γ co-expressers, approximately 2% of $CD4^+$ T cells from infected $MyD88^{-/-}$ mice were positive, which was significantly different from the case in infected wild-type mice (**Fig. 33** and **Fig. 34*C***).

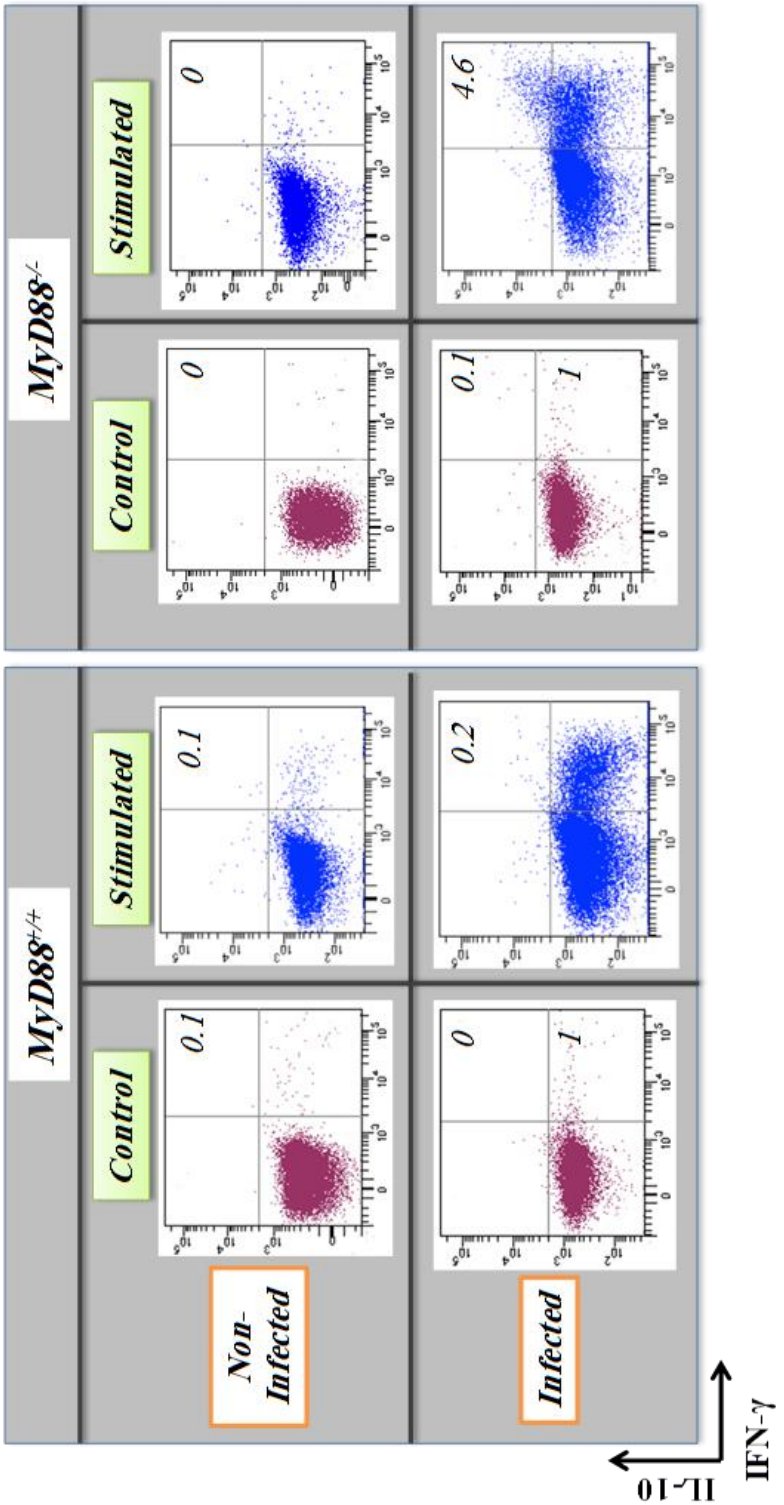

Fig. 32. Co-production of IFN-γ and IL-10 by CD4$^+$ T cells of MyD88$^{-/-}$ mice following *Salmonella* infection. Cytokine secretion by purified T cells was detected by intracellular staining for IFN-γ and IL-10. Purified CD4$^+$ T cells were cultured with or without overnight stimulation with plate bound anti-CD3/CD28 antibodies and analyzed. CD4$^+$ T cells were purified from non-infected or *Salmonella*-infected spleens of WT and MyD88$^{-/-}$ mice at day 21 post-infection Percent of various cell populations is shown in each quadrant. The data are representative of three independent experiments.

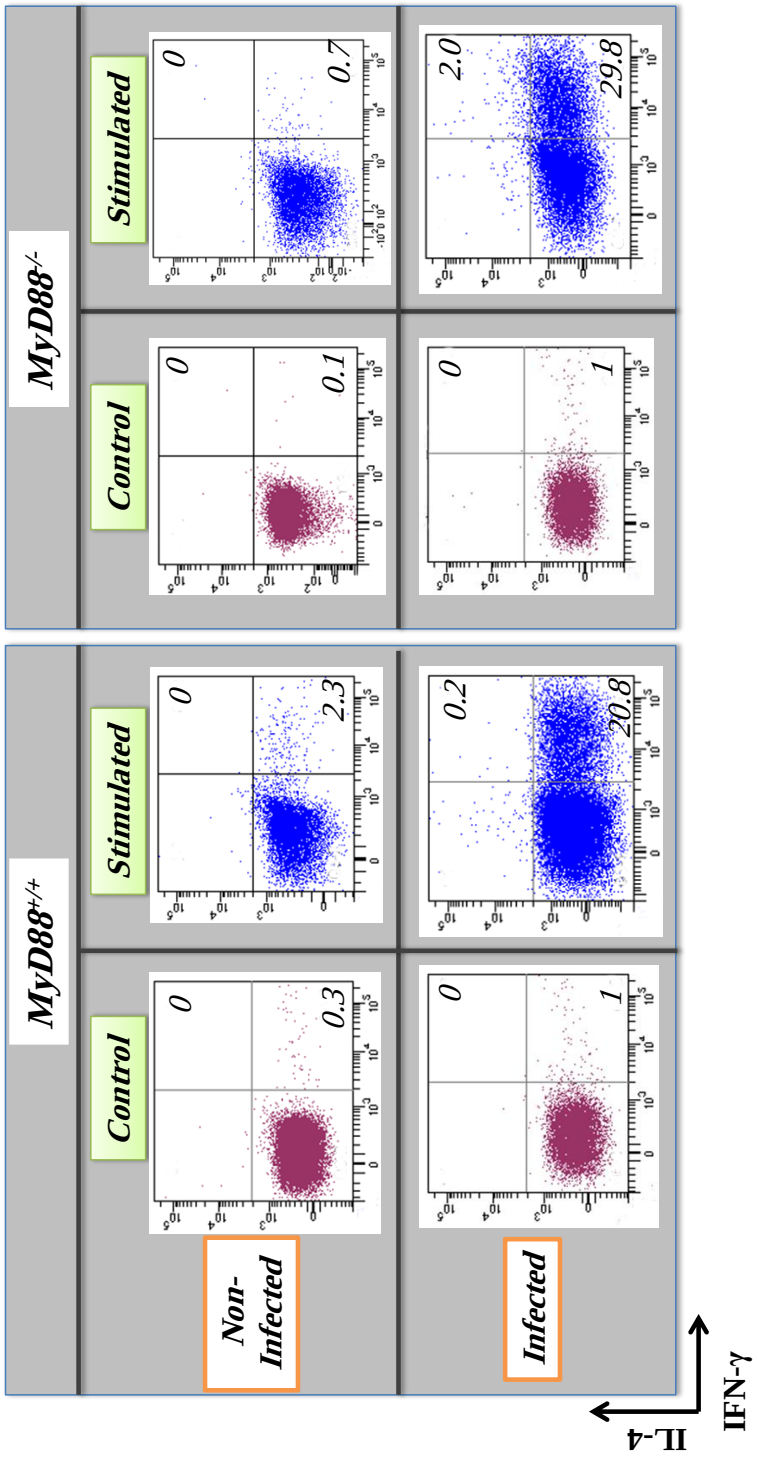

Fig. 33. Co-production of IFN-γ and IL-4 by CD4$^+$ T cells of MyD88$^{-/-}$ mice following *Salmonella* infection. Cytokine secretion by purified T cells was detected by intracellular staining for IFN-γ and IL-4. Purified CD4$^+$ T cells were cultured with or without overnight stimulation with plate bound anti-CD3/CD28 antibodies and analyzed. CD4$^+$ T cells were purified from non-infected or *Salmonella*-infected spleens of WT and MyD88$^{-/-}$ mice at day 21 post-infection Percent of various cell populations is shown in each quadrant. The data are representative of three independent experiments.

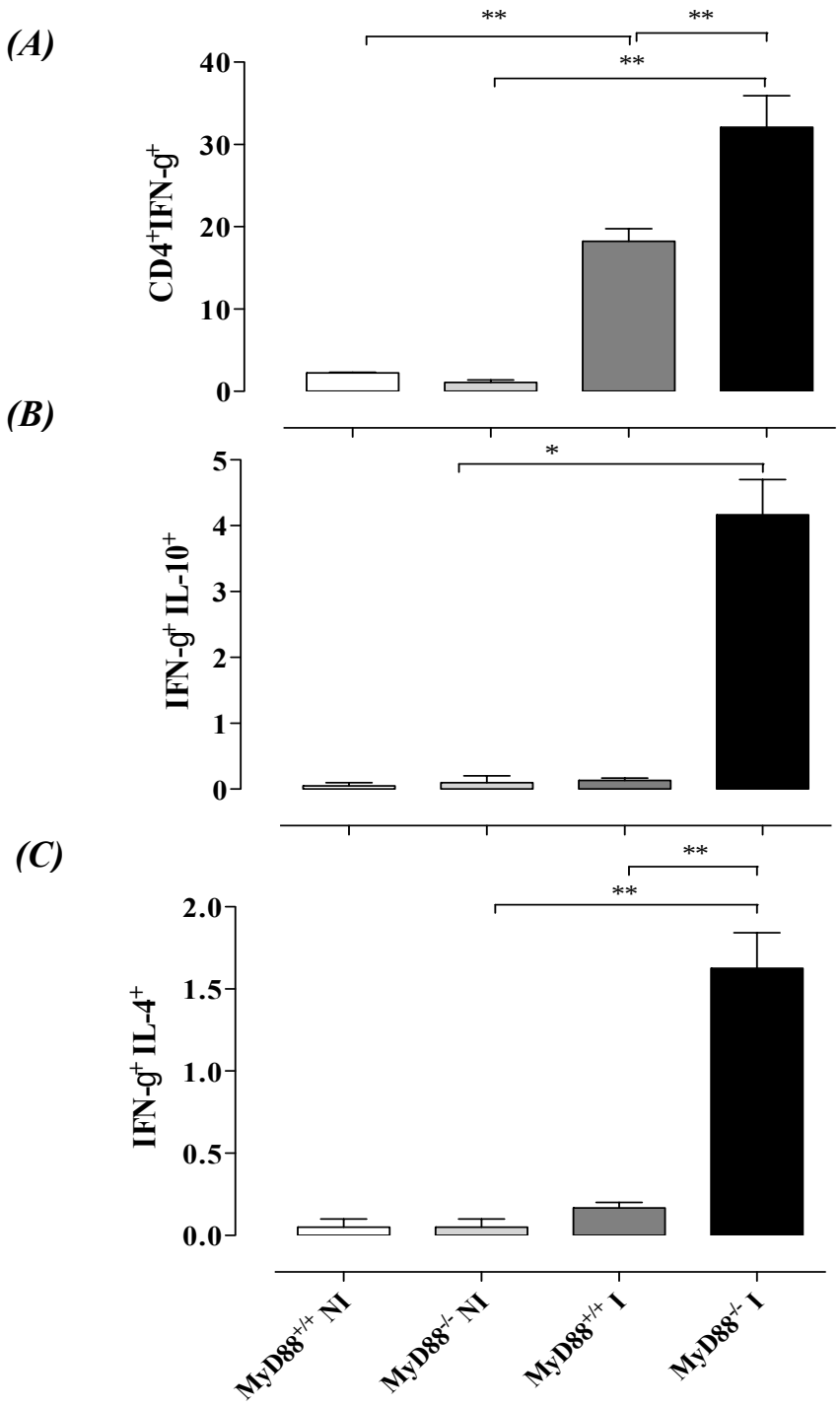

Fig. 34. Graphical representation of intracellular cytokine data. (***A***) Percent of IFN-γ-expressing $CD4^+$ T cells. Percent of IFN-γ^+ IL-10^+ (***B***) and IFN-γ^+ IL-4^+ double positive (***C***) cells in different experimental groups. Data points depict the mean ± SEM of 3 (infected) or 2 (non-infected) mice per group. (**, p<0.01)

To validate the presence of cytokine co-expressers among the peripheral $CD4^+$ T cell pool in infected $MyD88^{-/-}$ mice, we carried out a similar analysis in a non-related mouse strain that was deficient in CD154 protein. These mice are hypersusceptible to *Salmonella* infection because of a defect in T-cell-dependent macrophage activation and antibody responses (al-Ramadi et al., 2006). These mice were infected with a dose of about 0.5×10^6 CFUs/mouse, sacrificed three weeks later and splenic $CD4^+$ T cells were purified and analyzed by intracellular staining following $CD4^+$ T cell stimulation, as described earlier. A substantial percentage (17-18%) of stimulated $CD4^+$ T cells was positive for IFN-γ as opposed to cells from non-infected mice that exhibited only 1.6% positivity. Importantly, however, there was no evidence of any IFN-γ/IL-10 or IFN-γ/IL-4 co-expressers among the infected spleens in these mice (**Fig. 35**). This suggests that the observed phenomenon in $MyD88^{-/-}$ mice is associated with the dysfunction of the adaptor molecule at the level of innate immune response to the intracellular pathogen and hence, possibly the induction of other compensatory pathways that lead to the expansion of these specialized $CD4^+$ T cells.

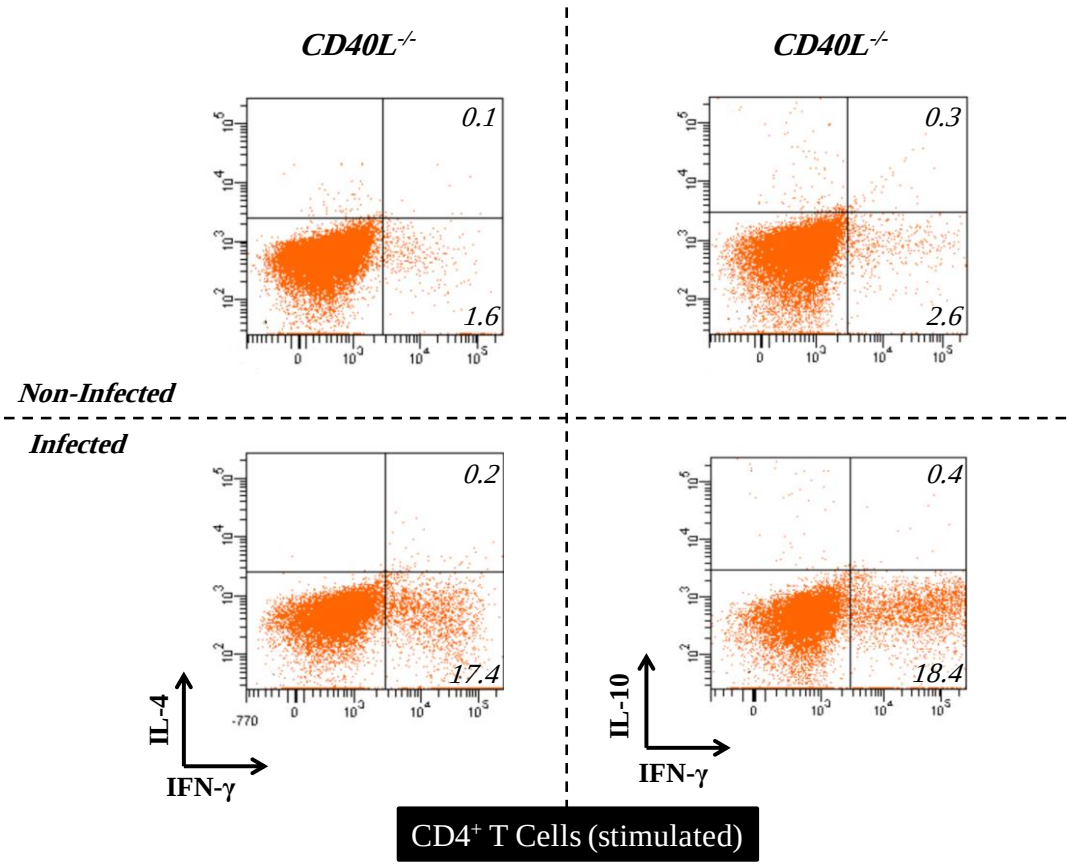

Fig. 35. No evidence for co-production of IFN-γ /IL-4 or IFN-γ /IL-10 by $CD4^+$ T cells from *Salmonella* infected $CD154^{-/-}$ ($CD40L^{-/-}$) mice. $CD40L^{-/-}$ mice were infected with 0.5 x 10^6 CFUs/mouse of BRD509 and sacrificed 3 weeks later. The expression of IFN-γ, IL-10 and IL-4 in purified splenic $CD4^+$ T cells was determined by intracellular cytokine staining following overnight stimulation with plate bound anti-CD3/CD28 antibodies.

5. DISCUSSION

We and others have previously demonstrated the critical importance of the TLR-MyD88 pathway in defense against microbial infections (Sukhumavasi et al., 2008); (Talbot et al., 2009); (Weiss et al., 2004); (Issac et al., 2013). In our laboratory, mice lacking the MyD88 signaling pathway were shown to be hypersusceptible even to low doses of an attenuated, double auxotrophic, strain of Salmonella enterica serovar Typhimurium (S. Typhimurium), as a result of defective clearance of the pathogen. This is mainly due to the delayed recruitment of inflammatory cells and defective production of inflammatory cytokines that are critical for controlling early phase of infection (Issac et al., 2013). In the case of the facultative intracellular pathogen, S. Typhimurium, susceptibility to infection was accompanied by the peculiar phenomenon of hypergammaglobulinemia (Issac et al., 2013). This study is aimed at defining the cellular and molecular parameters that predispose to dysregulated immunoglobulin synthesis and, by extension, autoantibody production.

In the present study, the aberrant antibody responses developed within the context of a strong $CD4^+$ T cell-generated IFN-γ response combined with a background of Th2-biased immune responsiveness that was exhibited by $MyD88^{-/-}$ mice. Interestingly, small but significant levels of IFN-γ/IL-4 and IFN- γ/IL-10 co-producing $CD4^+$ T cells were uniquely observed in *Salmonella*-infected $MyD88^{-/-}$ mice. The dysregulated antibody responses were largely driven by *Salmonella* infection in a time and dose-dependent fashion and culminated in production of antibodies reactive to several types of ubiquitous self-antigens. Given the defect in B lymphocyte central tolerance observed in infants born with a deficiency in MyD88 (Isnardi et al., 2008), it is plausible that a similar phenomenon exists in

MyD88-deficient mice, leading to the production of potentially self-reactive B cell clones. As a result of delayed clearance of bacteria, persistent *Salmonella* infection could lead to the activation of idle autoreactive B cells in MyD88$^{-/-}$ mice. This could also lead to the expansion of distinct populations of IFN-γ^+ T cells and extrafollicular CD4$^+$ T helper cells that possibly activate aberrant B cell responses and autoantibody production.

5.1. MyD88 signaling is crucial for early phase control of bacterial growth

The BRD509 strain of *S.*Typhimurium has a slow growth rate due to the inability to synthesize essential aromatic compounds. In the early phase of infection, the survival and growth of *Salmonella* in C57BL/6 mouse is determined by the induction of SPI-2 encoded virulence factors (Cirillo et al., 1998); (Pfeifer et al., 1999). The innate immune response that involves the bactericidal effector function as a result of activation of the host macrophages, plays a vital role in the control of the proliferation of the pathogen. Although TLRs have a critical role in protection against bacterial infections, there is data that suggests that *S.* Typhimurium can exploit the TLR signaling in macrophages for enhanced intracellular replication and survival. One study demonstrated that treatment of macrophages with various TLR agonists, like LPS, poly (I:C), peptidoglycan or CpG, increased bacterial uptake and enhanced *Salmonella* survival (Wong et al., 2009). In another study, *Salmonella* bacteria were shown to exploit recognition by TLR9 on macrophages to enhance their virulence. This was shown to be mediated through TLR-dependent acidification of SCV which in turn, upregulates several T3SS genes necessary for bacterial survival, replication and virulence (Arpaia et al., 2011a). In a third report, concomitant infection with an attenuated strain of *Salmonella* was found to aggravate infection with a virulent strain of *Salmonella*.

This is mediated by TLR4-induced production of IL-10 triggered by the attenuated bacteria which in turn, exacerbates infection by the virulent strain (Foster et al., 2008).

Neutrophils play a critical role as the first line of innate immune defense against *Salmonella* (Conlan, 1997). In response to *Salmonella* infection in the early phase, several anti-microbial defense mechanisms are activated, which mainly involves the recruitment of neutrophils and other phagocytic cells to the site of infection as well as induction of their microbicidal effector functions (O'Brien et al., 1980); (Royle et al., 2003). TLRs promote the bactericidal activity of macrophages against *Salmonella* through increased cytotoxic activity and TNF-α production (Weiss et al., 2004). In our model, the delayed recruitment of inflammatory cells, consisting mainly of granulocytes, at the site of infection is apparent at 24 hours. Though we have not assessed the exact role of MyD88 in inflammatory cell recruitment, other studies have highlighted its critical function in chemokine expression that coordinate early phagocyte response (Bandow et al., 2012) (Rydstrom and Wick, 2009). MyD88/TLR4-dependent chemokine expression of CCL2, CXCL9 and CXCL2 is crucial for neutrophil and monocyte recruitment following oral infection with *Salmonella* (Rydstrom and Wick, 2009). By 48 hours, the bacteria start to mobilize to the target organs in $MyD88^{+/+}$ mice, leading to a further reduction in polymorphonuclear cells by 72 hours. In contrast, bacterial CFUs and neutrophil recruitment continue to expand in $MyD88^{-/-}$ mice at 72 hours post-infection in the peritoneal cavity. As early as 48 hours post-infection, $MyD88^{-/-}$ mice display defect in phagocytic cell recruitment and proinflammatory cytokine production, such as IL-6, IL-12 as well as antimicrobial effectors like nitric oxide (Fig. 3C-E) (Issac et al., 2013). These defects may well

contribute to MyD88$^{-/-}$ mice being susceptible to infection. Even up to 10 days post-infection, the accumulation of these cells was more reduced in MyD88$^{-/-}$ mice than TLR2/4$^{-/-}$ mice (Seibert et al., 2010). Ko and colleagues (Ko et al., 2011) in their findings have shown accumulation of inflammatory myeloid cells at 4 weeks post oral administration of recombinant attenuated *S.* Typhimurium (RASV) strain with increased levels of inflammatory cytokines, resulting in systemic inflammation in MyD88$^{-/-}$ mice. The substantial delay in the recruitment and activation of phagocytic cells in MyD88$^{-/-}$ mice could be a major contributor to the increased host mortality observed in our study (Fig. 20, 21). Despite this delay, the fact that phagocytic cell infiltration occurs indicates the initiation of other signaling pathways as a substitute for MyD88.

TLR signaling has also proven to be critical in Gram-positive intracellular infections like *L. monocytogenes* where bacterial titers were higher in the organs of TLR2$^{-/-}$ and MyD88$^{-/-}$ mice than control mice. There is decreased activation of TLR-deficient macrophages and dendritic cells on *L.monocytogenes* infection leading to substantially reduced production of TNF-α, IL-12 p40 and nitric oxide (Torres et al., 2004). Studies with intracellular facultative Gram-negative bacterial infections of *S.* Typhimurium (Talbot et al., 2009) and *Ehrilichia muris* (Koh et al., 2010) in MyD88-deficient mice display enhanced bacterial numbers in the target organs and blood. Neutrophil recruitment is MyD88-dependent in early phase infections even with other intracellular pathogens (Sukhumavasi et al., 2008); (Koh et al., 2010); (Lebeis et al., 2007). By 21 days post-infection with *S.* Typhimurium, the percentage of myeloid cells was substantially higher in infected MyD88$^{-/-}$ spleens (Fig. 21A) which leads to lower percentages of lymphoid cells (Fig. 22B, 24B). The total splenic myeloid cells recovered were significantly

higher when compared to those of the wild-type infected mice. However we did not observe any dramatic changes in the absolute numbers of lymphocyte populations. In contrast, Ko and coworkers reported substantial increase in lymphoid and myeloid populations in MyD88$^{-/-}$ spleens compared to those of wild-type mice at 4 weeks post oral infection with *Salmonella* (Ko et al., 2011). In addition to the increased myeloid cell numbers in the spleen of *Salmonella* infected MyD88$^{-/-}$ mice, the increased spleen weights in these mice could be contributed by the enhanced erythropoiesis in the spleen to the growing *Salmonella* infection (Jackson et al., 2010).

The production of IL-12, IL-6 and nitric oxide by myeloid cells of mice lacking MyD88 at late stage of *Salmonella* infection suggests the induction of these cytokines in a MyD88-independent manner, possibly driven through TLR4-triggered TRIF pathway. Yamamoto et al (2003) demonstrated that LPS-induced secretion of IL-6, IL-12 and TNF-α was abrogated in macrophages from TRIF$^{-/-}$ mice. Furthermore, proliferative responses of splenocytes to LPS were severely impaired in these mice (Yamamoto et al., 2003). IFN-α and IFN-β production through the TRIF mediated pathway leads to nitric oxide release in response to *Salmonella* LPS (Zughaier et al., 2005). Moreover TLR-independent intracellular PRRs, such as NLRs, are involved in the production of inflammatory cytokines and development of adaptive response to Gram-positive (Fritz et al., 2007) and Gram-negative bacteria (Geddes et al., 2010). Nod1 and Nod2 provide effective antibacterial inflammatory responses (IL-1β, keratinocyte derived chemokine) and induce generation of enteric Th17 responses to *Salmonella* infection (Geddes et al., 2010); (Geddes et al., 2011). Peptidoglycan recognition by Nod1 directs Th1, Th2 and Th17 responses (Fritz et al., 2007). Another MyD88 independent

mechanism is complement activation which on sensing LPS of Gram-negative bacteria generates anaphylatoxins that induce inflammatory responses (Winter et al., 2010).

5.2. TLR signaling in IFN-γ production and T cell mediated immune response

Control of *Salmonella* infection requires strong T cell responses involving both $CD4^+$ and $CD8^+$ T lymphocytes (al-Ramadi et al., 2006); (Hess et al., 1996); (Mittrucker and Kaufmann, 2000). The $CD4^+$ Th1 response in *Salmonella* infected mice is characterized by inflammatory cytokine secretion, particularly IFN-γ (Pie et al., 1997); (Kaufmann, 1993); (Mastroeni, 2002). Deficiencies in IL-12 and IL-12-dependent production of Th1 cytokines, such as IFN-γ and TNF-α, caused profound susceptibility to *Salmonella* infection in mice (Jouanguy et al., 1999); (Tite et al., 1991) and predisposition to Salmonellosis in humans (de Jong et al., 1998); (Altare et al., 1998). During the early stage of infection, NK cells are the major source of IFN-γ which is necessary for macrophage activation and effective protection to *Salmonella* (Ramarathinam et al., 1993). In infected MyD88-deficient mice, the IL-12 response was severely impaired in the acute phase between 2-48 hours of *Salmonella* infection (Fig. 3D) (Issac et al., 2013). Despite the observed defect in IL-12 production at 48 hours post-infection, comparable levels are detected at three weeks post-infection in wild-type and $MyD88^{-/-}$ mice. The production of IL-12 also correlates with IFN-γ production. The Th1 dependent induction of IFN-γ at three weeks post-infection was at substantially high levels, comparable to those of immunized $MyD88^{+/+}$ mice (Fig. 34A). In experimental models of other intracellular pathogens, the early acute IFN-γ release from splenocytes was MyD88 dependent (Naiki et al., 2005) but the Th1-induced cytokine response eventually developed with time in $MyD88^{-/-}$ mice

(Sukhumavasi et al., 2008). A robust *Salmonella*-specific T cell memory response developed in MyD88$^{-/-}$ mice following infection with a sublethal dose of BRD509 strain. However, memory T cells failed to provide effective protection to immunized MyD88$^{-/-}$ mice as they remained susceptible to virulent challenge. The overall mortality was much higher when compared to the immunized wild-type mice. This lack of effective protection in the absence of MyD88 further highlights the critical role of phagocytes in the control of *Salmonella* bacterial proliferation (Issac et al., 2013). A recent study demonstrated that neutrophils are able to produce IFN-γ in response to intracellular pathogens, including *Salmonella*, in mice with TLR11-deficiency. In this report, IFN-γ production by neutrophils was regulated by TNF-α and IL-1β (Sturge et al., 2013). MyD88 is a crucial component of the signaling pathway of IL-1R and IL-18R. IL-1β and IL-18 are potent activators of the acute phase of anti-bacterial host defense (Adachi et al., 1998a) through activation of NK cells (Chaix et al., 2008); (Raupach et al., 2006) and development of Th1 responses (Takeda et al., 1998). IL-18 was shown to be required for essential clearance of bacteria and to be involved in the secretion of IFN-γ by CD4^{+} T cells of *Salmonella* infected mice (Srinivasan et al., 2007). Despite the evidence of Th1 responses, the increased susceptibility of MyD88$^{-/-}$ mice to *Salmonella* infection could also be explained by the lack of IL-1β and IL-18 signaling as a result of the loss of MyD88.

5.3. TLR signaling in B cell mediated adaptive immune response to infection

The role of TLRs in the induction of antibody responses is controversial. Antigen-specific antibody responses have been reported to depend on TLR activation on B cells (Chandrashekhar and Ruslan, 2005). However, there are

several other studies that have highlighted the importance of signaling through TLRs for the control of the pathogen but not for the induction of adaptive immune responses (Gavin et al., 2006); (Ko et al., 2009). B cells express most TLRs (Dasari et al., 2005); (Rawlings et al., 2012) and notably TLR engagement promotes B cell proliferation, survival and differentiation to long lived antibody secreting plasma cells (Gerondakis et al., 2007); (Gray et al., 2007); (Boeglin et al., 2011);(Ruprecht and Lanzavecchia, 2006). TLR4 promotes B lymphocyte maturation while TLR2 arrests the process (Hayashi et al., 2005). Some TLRs have also been suggested to have a role in the early stages of B cell development (Applequist et al., 2002). TLR signaling has proven to be essential for generation of antibody secreting plasma cells in both the spleen and bone marrow in MyD88$^{-/-}$ mice after influenza VLP vaccination (Kang et al., 2011). The expression of TLRs varies during B cell development and increased antibody production has been demonstrated on TLR activation of terminally differentiated plasma cells in humans. In our study, the loss of MyD88 did not affect B cell numbers in naïve mice (Fig. 22B) which is in agreement with other studies (Adachi et al., 1998b); (Kawai et al., 1999). In an experimental system utilizing mice with MyD88 deficiency restricted to the B cell compartment (MyD88$^{B-/-}$), *Salmonella*-immunized animals possess fewer and smaller germinal centers (GCs) than those of wild-type mice, thus suggesting that GC B cell differentiation is also dependent on TLR signaling (Barr et al., 2009). Direct TLR-induced activation of human B cells also leads to expression of a large number of cytokines (Agrawal and Gupta, 2011); (Jagannathan et al., 2009). On the other hand, at 4 weeks post oral administration of RASV, GC formation was increased in MyD88$^{-/-}$ mice when compared to wild-type mice (Ko et al., 2011). Thus, B cell-specific loss of

MyD88 affects their activation in GCs, while total MyD88 deficiency seems to not adversely affect B cells in GCs, suggesting that MyD88 functions in different capacities depending on the cell type.

One of the main findings in our study is the observed phenomenon of hypergammaglobulinemia. Wild-type mice in response to *Salmonella* infection, typically exhibit humoral immune response skewed by Th1 type cytokines. Following infection with attenuated *Salmonella*, antibodies of IgG2c and IgG3 are the predominant isotypes being produced in C57BL/6 mice (al-Ramadi et al., 2006); (Issac et al., 2013) or isotypes of IgG2a and IgG3 in BALB/C mice (Harrison et al., 1997). In addition to the cytokine-induced Ig isotype switching, interaction between TLR9 expressed on B cells and pathogen ligands has been shown to induce IgG2a class switching. Interaction of pathogen with ligands can directly activate B cells, possibly explaining as to why they generally induce strong IgG2a responses (Jegerlehner et al., 2007). In contrast to wild-type mice, MyD88$^{-/-}$ mice irrespective of the route of *Salmonella* infection, in response to *Salmonella* infection developed aberrant humoral immune response with anti-*Salmonella* antibodies of all isotypes being produced at high levels, establishing a state of hypergammaglobulinemia (Fig. 4). Infection with *B.* Burgdorferi bacteria induced IgM hypergammagobulinemia along with IgG1 and IgE activated responses in the absence of MyD88 (Woods et al., 2008). Interestingly, uninfected MyD88 deficient mice exhibited high levels of total IgG1 antibody subclass in their serum which were largely unchanged after infection (Fig. 7C). The clear dominance of Th2-biased adaptive immune response is confirmed by high levels of IL-4 in the MyD88$^{-/-}$ mice. In hygienic conditions, the immune response is predisposed to a Th2 response which shifts to a Th1 response on exposure to a

pathogen. In the absence of MyD88 the Th2 profile remains intact. The predominant Th2 profile could be associated with the inability to respond to pathogens in the absence of TLR signaling. The lack of MyD88 undermines the exposure to pathogen naturally acquired through air or water, maintaining the de facto Th2 response. Moreover, the influence of these responses by commensal bacteria can be evaluated by continuous treatment with antibiotics from birth in both $MyD88^{+/+}$ and $MyD88^{-/-}$ mice for up to 8-12 weeks. Following this, $CD4^{+}$ T cells can be purified and levels of IL-4 can be determined. This will clarify any role of the commensals in driving the de facto Th2 responses. Several animal models of infection have highlighted the development of Th2 responses due to MyD88 deficiency (Chen et al., 2010); (Debus et al., 2003); (Liu et al., 2004); (Ko et al., 2009). Ko and colleagues reported that signaling through MyD88 is dispensable for the induction of LPS-specific B cell responses. Moreover they demonstrate decreased IgG2a:IgG1 antibody responses (Ko et al., 2009). Similarly, in another study, IgG1 response to T-cell dependent antigens took place independently of MyD88 signaling along with loss of IgG2c in mice with MyD88-deficient B cells infected with *Salmonella* (Barr et al., 2009). Our data are consistent with these findings of a Th2 induced IgG immune response (Issac et al., 2013). However these levels were inevident in $MyD88^{-/-}$ mice that possessed reduced isotype switched IgG antibodies on immunization with influenza viral particles (VLP), as a result of defective secretion of antibodies from splenic B cells (Kang et al., 2011). Interestingly, DCs have been claimed to support Th2 cell development in the absence of TLR signaling (Kaisho et al., 2002). This was not the case in another study where MyD88 signaling in $Gr\text{-}1^{+}CD11b^{+}$ myeloid cells facilitated Th2 polarized B cell responses in a polymicrobial sepsis model (Delano

et al., 2007). Overall, a controversial role of MyD88 signaling in controlling B cells responses is portrayed.

Even infection with cytokine expressing strains of attenuated *Salmonella* failed to influence the trend of dysregulated antibody titers following infection in MyD88$^{-/-}$ mice (Fig. 17). Interestingly, this phenomenon of uncompromised antibody response was not observed in MyD88$^{-/-}$ mice infected with other Gram-negative bacteria like *A. baumanii* or *E. coli* or Gram-positive bacteria like *GpB Streptococcus* or even with heat-killed BRD509 (Fig. 15C-E, Fig. 15). Though an exaggerated humoral immune response was generated to *Salmonella* infection in the MyD88$^{-/-}$ mouse strain, this failed to provide effective protection to control the bacterial growth, further underlining the crucial role of the innate immune cells in the early phase of infection. In certain infections like *B. burgdorferi*, the antibody response to the pathogen appeared to control the bacterial burden substantially in MyD88$^{-/-}$ mice (Liu et al., 2004). The antibody responses mounted in the absence of MyD88 could be through compensatory alternative sensory pathways being activated in response to the pathogens. One such mechanism is through NLRs particularly Nod1 and Nod2 that potentiate the generation of antigen-specific adaptive immune responses to bacterial ligands. This involves antigen specific immunoglobulin production and induction of optimal T cell response of Th1 or Th2 pathway (Fritz et al., 2007); (Kobayashi et al., 2005).

Stimulation of purified CD4^{+} T cells from spleen or lymph node after infection, produced prominent IFN-γ levels in wild-type mice and IL-4 levels in MyD88-deficient mice (Koh et al., 2010); (Muraille et al., 2003). These observations were conflicting in our study following *Salmonella* infection where we confirm the production of strong IFN-γ levels in MyD88$^{-/-}$ mice similar to

those in the wild-type mice (Fig. 31A). The increased presence of IFN-γ on infection contributed to increased expression of Sca-1 activation markers in the myeloid and lymphocyte populations in the spleen (Fig. 21B, 24A, 26A, 27A). Furthermore, an aberrant induction of IL-4 was also observed in MyD88$^{-/-}$ mice after infection with attenuated *Salmonella* (Fig. 29A). These levels observed at the RNA level were further confirmed with intracellular FACS staining and cytokine levels obtained after *ex vivo* restimulation of CD4^{+} T cells (Fig. 31B, 34B). The induction of increased amounts of both IFN-γ and IL-4 in MyD88$^{-/-}$ mice coincides with the presence of prominent levels of cytokine-driven antibody isotypes after infection with *Salmonella*. Interestingly, Neves and colleagues reported stronger NK and T cell responses to *Salmonella* in mice with B cell-specific MyD88 deficiency (MyD88$^{B-/-}$) than those in wild-type animals (Neves et al., 2010). The stronger anti-*Salmonella* response was correlated with reduced production of IL-10 by the activated B cells in MyD88$^{B-/-}$ mice, demonstrating a potentially important regulatory role for B cell-specific IL-10 synthesis.

5.4. Breakdown of self-tolerance as result of loss of MyD88

MyD88/IRAK deficiency in humans was first identified in 2003 (von Bernuth et al., 2012). The adaptor molecule is crucial for infancy survival and early adulthood. Similar to MyD88$^{-/-}$ mice, patients deficient in MyD88/IRAK-4 had increased susceptibility to infections with invasive pyogenic Gram-positive and Gram-negative bacteria (von Bernuth et al., 2008); (von Bernuth et al., 2012). The signaling proteins are more crucial during childhood. With age, infections in patients that lack these signaling molecules are rarer, probably due to adaptive immune responses from B and T lymphocytes (Picard et al., 2010). MyD88 is vital for the negative selection of autoreactive immature B cells in the bone

marrow. MyD88 and IRAK-4 deficient patients exhibited increased ratios of autoreactive B cells and high frequency of HEp-2 reactive clones (Isnardi et al., 2008). This strongly implies a disruption of the B cell activation pathway and a breakdown of central tolerance in the absence of MyD88 signaling. However, no ANA or other polyreactive antibodies were detectable in sera collected from these patients within 3 months after birth (Isnardi et al., 2008). This could suggest either of two possibilities; one that TLR7/9 signaling that enhances costimulatory signal on BCR recognition of immune complexes gets thwarted and second that certain infections can breakdown self-tolerance as a result of loss of MyD88. As explained earlier, various experimental models have proved that infections can lead to autoimmunity (Tardieu et al., 1984); (Wucherpfennig, 2001); (Fairweather et al., 2001). Our findings show B cell hyper activation in mice with defective MyD88 signaling caused an aberrant and dysfunctional humoral immune response of all isotypes against the *Salmonella* antigens with high affinity to autoantigens such as dsDNA, thyroglobulin and other nuclear antigens (Fig. 10, 11). These antibodies also formed immune complexes in the glomerular membrane of the kidney (Fig. 12). Similar findings have been reported by Ko et al (Ko et al., 2011). In our study, infection with other Gram-negative pathogens though harboured higher bacterial counts in the MyD88$^{-/-}$ spleen, failed to mount any phenomenon of hypergammaglobulinemia with autoantibody production (Fig. 14). This suggests that specific properties of *Salmonella* organisms such as their mode of survival and replication in the intracellular compartments of the macrophages may be responsible for the observed auto reactivity in MyD88-deficient mice. The chronic infection of *Salmonella* in MyD88-deficient mice could probably disrupt the anergic state of the autoreactive B cells, leading to the observed production of

antibodies to nuclear antigens. In one study, administration of poly(I-C) highlighted the role of MyD88 independent signalling in the development of autoimmunity in lupus prone mice deficient in MyD88. This has been associated with increased levels of IFN-α jointly with IL-6 and IL-12 (Sadanaga et al., 2007) Contrary to this is the involvement of MyD88 in the pathogenesis of autoimmunity in various autoimmune mouse models. In MRL/lpr mice, MyD88 signaling in B cells mainly contributed to ANA and RF formation (Teichmann et al., 2013). Loss of MyD88 in MRL/lpr mice reduced immunoglobulin serum levels and anti-dsDNA antibody titres with reduced inflammatory lesions thus improving the survival of the mice (Sadanaga et al., 2007). Viral and bacterial infections have been known to drive polyclonal B cell activation, hyperglobulinemia and possible reactivity to self-antigens from chronic infections (Soulas et al., 2005); (Jellison et al., 2007). This non-specific B cell activation can be through direct activation by B cell mitogens or peptide derived interaction with $CD4^+$ T cell irrespective of the specificity of BCR (Stevenson and Doherty, 1999); (Hunziker et al., 2003). Furthermore, MyD88 expression in RF B cells is required for their initial activation and expansion in responses to IgG2a anti-chromatin (Herlands et al., 2008). With the activation of autoreactive B cells in the primary step of autoimmunity, the role of TLR signals in mediating these responses has been comprehended.

As mentioned previously, elevated levels of BAFF have been associated with increased survival of autoreactive B cells in the periphery (Lesley et al., 2004); (Mackay et al., 2003); (Thien et al., 2004). Though BAFF has no role in the elimination of high affinity self-reactive B cells in the bone marrow, peripheral deletion of autoreactive B cells has been found to be rescued by increased

expression of BAFF protein and hence, permit their entry to forbidden microenvironments of the spleen. Furthermore, the rescued self-reactive B cells that partially expressed CD86 were adequate for its response to antigenic stimulation leading to autoantibody production (Thien et al., 2004). IFN-α has been well established in the pathogenesis of SLE (Ronnblom and Alm, 2001); (Barrat et al., 2005); (Pascual et al., 2003) and IFN-α/β has been known to upregulate the expression of BAFF in autoimmune mouse models (Jacob et al., 2011) as well as in humans (Krumbholz et al., 2008); (Litinskiy et al., 2002); (Yao et al., 2009). Other than exercising MyD88 as the adaptor molecule, TLR-4 can signal through TRIF for the induction of Type1 IFN genes (Yamamoto et al., 2003). Thus we suggest if this pathway could be triggered as a compensatory route in the absence of MyD88, due to enhanced bacterial burden. In our results, evaluation of BAFF expression revealed increased levels of the B cell activating factor being expressed as a result of loss of MyD88 in the myeloid cells of the spleen by 3 weeks post-infection in the mouse strain (Fig. 30A). Ko and colleagues in their findings have shown increased levels of expression of BAFF and APRIL in the spleen of $MyD88^{-/-}$ mice at two days, following oral administration of RASV strain (Ko et al., 2009). In one study, the presence of undigested mammalian DNA has also been implicated in the activation of Type I IFN genes (Okabe et al., 2005). Nickerson and colleagues in their study of the role of TLR7/9 in regulating autoimmunity, observed dermatitis and hypergamaglobulinemia even in MyD88 deficient animals, further suggesting possible contributions of MyD88 independent components such as PRRs, that recognise nucleic acids like RIG-I or MDA or other nucleic acid sensory pathways (Nickerson et al., 2010).

IL-17 has been known to be involved in the pathogenesis of various inflammatory autoimmune diseases (Zhu and A Qian, 2012), one of which is its synergistic effect with BAFF in promoting B cell survival in SLE (Doreau et al., 2009). However, there was no apparent induction of IL-17 or its regulatory protein in MyD88 deficient mice, to chronic *Salmonella* infection in our observations (Fig. 29) excluding its role in the observed autoimmune response as a result of self-tolerance breakdown. Furthermore, the reduction in the expression of Foxp-3 in $CD4^+$ T cells of infected $MyD88^{-/-}$ mice when compared to $MyD88^{+/+}$ mice suggests dependency of Tregs on MyD88 signaling in response to infection (Fig. 29B). This further implies a decreased Treg mediated suppression of immune responses, particularly T cell expansion to infection.

A distinct population of B helper T cells first identified in tonsils (Breitfeld et al., 2000) is localized in the B cell zone of lymphoid organs and has been associated with various autoimmune diseases in humans (Simpson et al., 2009); (Ma and Deenick, 2013). IL-6 and IL-21 proinflammatory cytokines along with costimulatory molecule stimulation are involved in the differentiation of this specialized subset of T cells called T_{FH} cells. PD-1 and CXCR5 are upregulated in T_{FH} cells, the latter of which is crucial for its migration to the B cell follicle (Park et al., 2014). These cells are regulated by the transcription factor B cell lymphoma-6 (bcl-6) and secrete IL-4 and IL-21 that promotes B cell somatic hyper mutation, affinity maturation, Ig isotype switch and plasma cell differentiation. IL-21 directly acts on B cells in germinal centers (Zotos et al., 2010) to regulate Bcl-6 expression for affinity maturation of B cells (Linterman et al., 2010). IL-21 deficient mice immunized with T-dependent antigen showed defect in affinity maturation of plasma cells and formed abnormal germinal

centers. The abnormal GC development however, was not associated with unaffected T_{FH} cells suggesting mainly the prerequisite of IL-21 in GC reactions and not T_{FH} cell development (Zotos et al., 2010). Although Tfh cell formation is IL-21 independent, the maintenance of this T cell subset is impaired in the absence of the cytokine (Linterman et al., 2010). T_{FH} cells facilitate the survival and differentiation of self-reactive B cells, eventually leading to autoantibody production and organ damage (Zhang et al., 2013). The dysregulation of T_{FH} and extrafollicular Th cells has been linked to chronic autoimmunity in several autoimmune murine models (Odegard et al., 2008); (Park et al., 2014). Despite the fact that Bcl-6 suppresses differentiation of other T helper cells, increased levels of IFN-γ have been reported to promote accumulation of T_{FH} cells in sanroque mouse model (Lee et al., 2012). Three weeks after infection with attenuated *Salmonella* in MyD88$^{-/-}$ mice, a significant expression of IL-21 was observed, but with no evidence of Bcl-6 in splenic CD4^{+} T cells (Fig. 29). Lack of Bcl-6 expression could possibly reflect a late stage of T_{FH} cell differentiation (Ma and Deenick, 2013). One study has also shown a downregulation of Bcl-6 expression in some murine T_{FH} cells (Kitano et al., 2011). The extrafollicular T cell zone in the spleen was found to be a region of spontaneous autoantigen responses. Extrafollicular T helper cells promote the formation of somatic mutated autoantibodies in MRL/lpr mice in the extrafollicular sites of B cell proliferation (Odegard et al., 2008). Low transcriptional levels of CXCR5 in the splenic CD4^{+} T cells of MyD88$^{-/-}$ mice (Fig. 29) suggest these to be extrafollicular T helper cells that contribute to antibody class switched isotypes and autoantibody production through IL-21 (Odegard et al., 2008). There was a dramatic reduction in the accumulation of B and T cells in the spleens of IL-21R$^{-/-}$ MRL/lpr mice along

with decreased B cell activation and autoantibody production (Rankin et al., 2012). In support of our findings of increased populations of $CD11b^{+}Gr\text{-}1^{+}$ myeloid cells with enhanced activation in the spleens of $MyD88^{-/-}$ mice following infection (Fig. 28A) (Fig. 21), are the results of Ko and colleagues (Ko et al., 2011). They suggest that the increased infiltration, expansion and activation of myeloid cells in the spleen could modulate proliferation of T_{FH} like cells that include T_{FH} cells and extrafollicular helper T cells, resulting in hyper IgG responses in chronic *Salmonella* infection. Though IL-6 production from inflammatory myeloid cells was involved in T_{FH} cell differentiation in $MyD88^{-/-}$ mice (Ko et al., 2011), in our results with intraperitoneal infection at day 21, the cytokine was produced at significantly high levels in both the wild-type and $MyD88^{-/-}$ mice (Fig. 30). This suggests involvement of other candidate cytokines in T_{FH} cell development. Alternatively, in a study with lupus mice the frequency of T_{FH} cells as well as IFN-γ producing $CD4^{+}$ T cells were substantially reduced in MyD88 deficient B cell and DCs (Teichmann et al., 2013). Expansion of IFN-γ secreting $CD4^{+}$ T cells and IL-21 producing extrafollicular T helper cells is one important feature in MRL/lpr mice (Rankin et al., 2012); (Odegard et al., 2008). An intriguing observation in our results is the generation of a population of $CD4^{+}$ T cells on infection with *Salmonella* in $MyD88^{-/-}$ mice that produce both IFN-γ and IL-4. These double positive cells were not observed in the infected spleens of wild-type mice and the non-infected $MyD88^{-/-}$ mice (Fig. 33, 34C). A study with intramacrophage parasitic infection, recovered $CD4^{+}$ T cells that are capable of producing both IFN-γ and IL-4 to the infection in the draining lymph nodes (Reiner et al., 1994) which were later confirmed to be T_{FH} cells (Reinhardt et al., 2009). In our study, T_{FH} like cells seem to be generated as a result of persistent

infection of *Salmonella*. In one report, persistent viral infection was shown to induce the differentiation of Th cells to T_{FH} cell responses (Fahey et al., 2011). In our study, the enhanced induction of IFN-γ was confirmed by the increased level of its master regulator, T bet. However, this was not the case with GATA-3 that was thought to regulate the observed IL-4 T cell responses (Fig. 29). Migration of T helper cells to the extra follicular sites and expression of CXCR4 are largely dependent on ICOS costimulation. $ICOS^{-/-}$ MRL/lpr mice developed reduced class switched antibodies and autoantibody titers (Zeller et al., 2006); (Odegard et al., 2008). The levels of expression of CXCR4, ICOS and PD-1 in the $CD4^{+}$T cells are yet to be assessed through PCR and intracellular staining in order to obtain further understanding of the accumulation of T_{FH} cells and extrafollicular T helper cells and their respective contribution to the hyper antibody responses. This further highlights therapeutic approaches of targeting T helper 1 (Th1) cells and T_{FH} cells in treating antibody mediated diseases.

Infection with GIDIL2/GIDIFN leads to hyper IgG responses in $MyD88^{-/-}$ mice but failed to induce any significant autoantibody production, as observed with BRD509 infected $MyD88^{-/-}$ mice (Fig. 17, 18). This could be related to the persistence and chronicity of infection with BRD509 in the defective mouse strain. The IFN-γ and IL-2 expressing strains of *Salmonella* are cleared more effectively in the wild-type mice (Al-Ojali et al., 2012a); (Al-Ojali et al., 2012b); (al-Ramadi et al., 2004); (al-Ramadi et al., 2002). IL-2 expression in the bacteria improves NK cell cytotoxicity and IFN-γ production and, hence, increases the synthesis of anti-*Salmonella* effector molecules such as NO (al-Ramadi et al., 2003). On the other hand, several studies have also highlighted the role of IL-2 in enhancing the suppressive activity and expansion of Treg cells (de la Rosa et al.,

2004); (Thornton et al., 2004). The delivery of IFN-γ by *Salmonella* strain could further promote Th1 cell mediated responses thereby downregulating T_{FH} /Th2 cell expansion and function.

During chronic infections, $CD4^+$ T cells that secrete both IFN-γ and IL-10 have been identified which are absent during normal conditions. There is evidence indicating the suppressive role of these double positive cells in controlling immune responses through IL-10 with IFN-γ help (Flores-García et al., 2011); (Liu et al., 2009). These functionally distinct $CD4^+$ T cells identified in an infection with intracellular pathogen *T. gondii* display both Th1 effector function as well as regulatory activity. The IL-10^+IFN-γ^+ $CD4^+$ T lymphocytes when cultured with the parasite infected IL-10 deficient macrophages were able to control the intracellular *T. gondii* proliferation, effectively highlighting its effector function. However, the IL-12 production from infected macrophages was significantly reduced in the presence of these double positive cells (Jankovic et al., 2007). In one study involving a murine model of *L. major*, secretion of IL-10 by Th1 cells prevented the effective elimination of the pathogen contributing to chronic infection (Anderson et al., 2007). Conditions that drive a highly polarized Th1 response were found to induce the appearance of these apparent regulatory cells (Jankovic et al., 2007). In our infection model, IL-10^+IFN-γ^+ $CD4^+$ T cells were found to be induced only in $MyD88^{-/-}$ mice after infection and not in wild-type mice or $MyD88^{-/-}$ uninfected mice (Fig. 32, 34B). Also, there was no evidence for IL-10^+ or IL-4^+ cells in $CD154^{-/-}$ mice. The presence of a chronic *Salmonella* infection in $MyD88^{-/-}$ mice due to their inability to effectively clear the infection could probably generate these double producer cells. These cells could also contribute to the prolonged persistence of the bacteria. We have only

observed the presence of this distinct population of cells but their regulatory role to the persistent infection is yet to be elucidated.

Overall, our data demonstrate the crucial role of MyD88 mediated TLR signaling in regulating immune response against *S.* Typhimurium and its importance in maintaining self-tolerance in B lymphocytes.

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