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TLR and BCR signals to B cells differentially programme primary and memory Th1 responses to *Salmonella enterica*[1]

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Abstract

Protective Th1 responses to *Salmonella enterica* do not develop in the absence of B cells. Using chimeric mice, we dissect the early (innate) and late (cognate) contributions of B cells to Th programming. B cell intrinsic MyD88 signaling is required for primary effector Th1 development, while antigen-specific BCR-mediated antigen presentation is necessary for the development of memory Th1 populations. Programming of the primary T cell response is BCR/B cell MHC II-independent but requires MyD88-dependent secretion of cytokines by B cells. Chimeras in which B cells lack IFN-γ or IL-6 genes make impaired Th1 or Th17 responses to *Salmonella*.

INTRODUCTION

The main function of B cells is to make antibodies that provide protection against potential pathogens. Sometimes, due to a failure of tolerance, B cells also make antibodies that bind self-antigens and so cause autoimmune disease. For this reason B cell depletion therapy is increasingly used in the rheumatology clinic (1). In some patients the treatment is effective despite unchanged autoantibody titers (2), highlighting antibody-independent functions of B cells, including antigen presentation and cytokine secretion (3-5).

B cells present antigen very effectively only when they take up antigen via their B cell receptor (BCR) (6), however, their exact role as antigen presenting cells (APC) *in vivo* remains unclear (7-12). B cells begin to contribute as APC during the primary response (8, 11, 13), however, their major impact is thought to be in the re-activation of CD4 memory T cells (14-18). Cytokine production by B cells extends their sphere of influence to the development of lymphoid tissue microenvironments (19) and regulation of T cell responses (20-22). For instance, the secretion of IL-10 by B cells modulates T cell function (23, 24) to bring about resolution of inflammation in autoimmune disease models (25-27). Regulatory B cell activity has also been seen in infection models protecting against acute Th1 (24, 28) and chronic Th2 (29-31) inflammation.

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In influencing T cell responses, B cells usually show a propensity to drive Th2 responses (32-35). Lund and colleagues (4) demonstrated that B cells can be polarized in their cytokine production in the same way as CD4 T cells, to become Be1 (36) and Be2 cells (37). More recently, cytokine production by B cells was shown to be necessary for the development of protective antibody-mediated Th2 immunity to *H. polygyrus* infection (38). As the Be2 cells were required to express IL-4R (38), this seemed to involve cross-talk between T cells and B cells rather than any exogenous pathogen-associated signal. Like other APC, B cells express a broad range of toll-like receptors (TLR) that upon ligation stimulate the secretion of cytokines (39). The direct stimulation by TLR ligands of cytokine production by B cells offers a means to influence at an early stage the differentiation of Th subsets.

To address this issue and to investigate differential roles of TCR and BCR in the programming potential of B cells we chose an infection model in which both TLRs and BCR would be stimulated as the disease progressed and one that generates a strong Th1 response. *Salmonella enterica* serovar Typhimurium is a bacterium that resides within the phagosomal compartment of macrophages (40). The infection has a transient extracellular phase, during systemic dissemination (41). To combat the infection, macrophages must be activated by inflammatory cytokines (42, 43). Thus, Th1 CD4 responses are crucial for the clearance of the bacteria and for protective immunity upon re-infection (44, 45). B cells have two roles, first as producers of antibodies (46, 47) and second, an antibody-independent role (48, 49). Thus, B cell deficient mice can clear infection, however, the Th1 response they develop is transient and they are not protected from re-infection with a virulent strain (50).

In this study, we find that B cells contribute to the early phase of T cell programming via a MyD88-dependent mechanism and are required in a BCR-dependent process for the development of the memory T cell response. Thus, TLR activation of B cells optimises the generation of the primary Th1 response, a process that does not require antigen presentation but relies on B cell cytokine secretion. For the development of Th1 memory cells and hence protective immunity to *Salmonella*, BCR recognition and B cell antigen presentation are an absolute requirement.

MATERIALS AND METHODS

Mice and generation of bone marrow chimeras

C57BL/6, μMT, MyD88$^{-/-}$, BALB/c, J_HD and MD4 mice were aged 6-10 weeks old at the start of experimental regimes. Mixed bone marrow chimeric mice were generated as described previously (51). Briefly, host B cell-deficient mice (μMT) were lightly irradiated with 8Gy of gamma-radiation. Mice were then reconstituted with 2×10^6 mixed-inoculum bone marrow cells (80% B cell-deficient marrow (μMT) and 20% knock-out or WT marrow). To control for a possible deficiency of 20% in other hematopoietic cells we made chimeras in which 20% of all the hematopoietic lineages are derived from gene-deficient bone marrow (by transferring 80% wild-type bone marrow + 20% gene-deficient bone marrow into irradiated recipients). Results from these chimeras and further validation of the chimera model is provided in Supplemental Fig. 1). MyD88$^{-/-}$ mice (52) and TRIF−/− (53) were provided by S. Akira (Hyogo College of Medicine, Japan). BCR transgenic MD4 strain (54). J_HD (BALB/c B cell-deficient (55) and μMT (C57/BL6 B cell-deficient (56) IFNγ$^{-/-}$ (57), IL-6$^{-/-}$ (58), I-Aβ$^{-/-}$ (59) mice have been bred in-house long-term and have been backcrossed to C57Bl/6 or BALB/c (MD4 and JhD) in excess of 10 generations. Experiments were covered by a Project Licence granted by the UK Home. This licence was approved locally by the University of Edinburgh Ethical Review Committee.

Salmonella enterica serovar Typhimurium infection

The *aro*A attenuated strain of *S. enterica* serovar Typhimurium, SL3261, was used for all infections (60). Bacteria were grown as stationary-phase overnight cultures in Luria-Bertani (LB) broth. Animals were injected intravenously (i.v.) with 1×10^6 CFU.

Preparation of bacterial antigens

Bacterial antigen (C5SENaOH) was prepared as described previously (61). Briefly, overnight stationary-phase cultures of the C5 strain were washed twice in PBS containing 5mM EDTA. The resulting suspension was sonicated on ice and cellular debris removed by centrifugation at 13,000g. The supernatant was alkali-treated with 5M NaOH for 3 h at 37°C and then neutralised with HCl. The NaOH-treated antigen was then 0.22um sterile filtered and stored at −80°C. Protein content was calculated using a standard Bradford assay. Heat-killed bacteria (HKB) were prepared by pelleting cells from snap-frozen stock vials of SL3261, re-suspending in complete Iscove's modied Dulbecco's media (IMDM) then heating to 80°C for 30 mins.

Preparation and sorting of $CD4^{+ve}$ and $CD19^{+ve}$ lymphocytes

$CD4^{+ve}$ and $CD19^{+ve}$ lymphocytes for use in all *in vitro* re-stimulation assays were isolated from mice using standard magnetic sorting techniques. Following manual disruption and lysis of red blood cells, splenocytes were positively sorted using anti-CD19 or anti-CD4 microbeads and LS columns according to manufacturer's instructions (Miltenyi-Biotech). CD4 T cells and B cells were greater than 98% pure following 2-column passes.

In vitro re-stimulation assays

Highly purified B cells from SL3261 infected mice were cultured (2×10^6 cells/ml) for 5 days with stimuli including; HKB SL3261, *Salmonella* LPS (1μg/ml - InVivogen), PMA and ionomycin (10ng/ml and 1μg/ml – Sigma). Supernatants were harvested at day 5. T cell re-stimulation assays were set up by co-culturing highly purified CD4 T cells with irradiated (30Gy) wild-type, naïve splenocytes at a 1:1 ratio (2×10^6 cells/ml). Supernatants were harvested at 24 hours for IL-2 and 72 hours for IFNγ, IL-10, and IL-17 detection.

Cytokine ELISA

Paired antibody sets for IFNγ, IL-2, IL-6, IL-10 and IL-17 (BD Bioscience) were used for capture and detection in ELISA assays Cytokine concentrations were determined by extrapolation from a standard curve. Minimum levels of detection were; IL-2 = 0.2ng/ml, IL-6 = 0.3ng/ml, IL-10 = 0.1ng/ml, IL-17 = 0.1ng/ml, and IFNγ = 0.4ng/ml.

RESULTS

BCR and TLR signals are necessary for cytokine secretion by B cells in *Salmonella* infected mice

We wished to investigate the relative importance of BCR and TLR stimulation in the B cell cytokine response. To address the antigen specificity of the bacteria-elicited cytokine response, we infected anti-HEL BCR (MD4) transgenic mice with *S. enterica* and then re-stimulated purified B cells from these mice *in vitro* with HKB. We found that cytokine secretion was substantially impaired in B cells unable to respond to the bacteria through their BCR (Figure 1A). Some IL-6 and IL-10 was still secreted by the MD4 B cells, indicating only a partial dependence on BCR signals. However, B cells from *S. enterica*-infected MD4 mice made no detectable IFNγ, indicating a total dependence on BCR stimulation for IFNγ secretion (Figure 1A). To confirm that B cells from MD4 mice could not respond via any endogenous BCR re-arrangements (receptor editing) we measured anti-

Salmonella antibody responses in these mice. Antibody was undetectable up to 51 days post-infection (Fig. 1B). Experiments have been repeated with MD4 mice crossed onto a JhD background (endogenous BCR re-arrangements not possible), with identical results.

To address the TLR-dependency of B cell cytokine production during *S enterica* infection we used MyD88-deficient mice. Thus, MyD88-deficient mice were infected with *S enterica* and splenic B cells taken for *in vitro* re-stimulation with HKB at 7 days. IL-6, IL-10 and IFNγ secretion was negligible, illustrating total dependence upon TLR stimulation of B cells for secretion of these cytokines by B cells (Fig. 1A). Normal BCR reactivity in MyD88$^{-/-}$ mice was confirmed by measuring antibody responses during infection; these mice showed titres of anti-*Salmonella* total immunoglobulins roughly equivalent to wild-type controls (Fig. 1B). It was not possible to assign cytokine production to particular B cell subsets, as during *Salmonella* infection classical subset distinctions cannot be made (see Supplemental Fig. 2).

MyD88 signals to B cells enhance primary development of CD4 Th1 and IL-10 responses, but are dispensable for memory development

To study the role of MyD88 signalling in B cells as APC in the development of CD4 Th1 responses during *S enterica* infection, we constructed bone marrow chimeric mice in which the B cells lacked MyD88. Thus, transfer of bone marrow cell inocula from MyD88−/− mice and from B cell-deficient (μMT) mice in a 20:80 ratio into irradiated μMT recipients meant that after reconstitution the B cell compartment was wholly MyD88-deficient while the other hematopoietic populations were mainly wild-type with respect to MyD88. Further validation of this chimera model is provided in Supplemental Fig. 1. CD4 T cells were isolated from the spleens of MyD88$^{B-/-}$ and WT chimeras at the peak of primary infection (day 7) and also after mice had cleared the infection (8 weeks). These T cells were re-stimulated *in vitro* with an *S. enterica* antigen preparation. The B cells in these chimeras showed equivalent expression of activation markers (Supplemental Fig. 2B). Figure 2A shows IL-2, IFNγ and IL-10 production by CD4$^+$ T cells at the peak of primary infection (day 7). IFNγ and IL-10 production are significantly impaired in the primary responses, with T cell derived IL-10 being particularly dependent upon B cell MyD88 expression (both $P<0.0001$). IL-2 production from T cells was below the limit of detection during the primary response in MyD88$^{B-/-}$ and WT chimeric mice. Thus, MyD88-dependent (TLR) stimulation of B cells *in vivo* is necessary for optimal Th1 differentiation of CD4$^+$ T cells and also for the generation of IL-10 producing T cells during *S. enterica* infection. MyD88 also acts as a signaling adapter for IL-1 and IL-18, however, we feel that the effect on Th1 response development shown here is TLR-mediated, as we see a similar impairment of the IFN-γ response in TRIF−/− knockout mice (Supplemental Fig. 3).

At week 8 post-infection when bacteria had been cleared we harvested CD4$^+$ T cells from spleens of infected chimeras to test the memory response. This revealed robust and equivalent cytokine secretion in both groups of chimeras. Thus, the memory IL-2, IFNγ and IL-10 responses were similar in MyD88$^{B-/-}$ and WT chimeric mice. Despite the significant reduction in early cytokine production the development of Th1 and IL-10-producing memory T cells is not impaired. Indeed, the late/memory Th1 cell development was sufficiently strong to resolve the infection. Bacterial burdens in the spleen at day 7 were similar in WT, μMT and MyD88$^{B-/-}$ mice. MyD88$^{-/-}$ mice showed increased bacterial burden at day 7, and took longer to resolve the infection, however, all types of mice clear bacteria at a roughly similar rate (Figure 2B).

Development of memory but not primary CD4 Th1 and IL-10-producing T cells is dependent on antigen-specific BCR signals to B cells

MD4 transgenic mice expressing a HEL-specific BCR were used to investigate the requirement for BCR signals to B cells in their capacity to influence primary and memory T cell responses during *S. enterica* infection. $CD4^+$ T cells were isolated from MD4 spleens at the peak of primary infection (day 7) and re-stimulated *in vitro* with *S. enterica* antigen. Figure 3A shows IL-2, IFNγ and IL-10 secretion by T cells from MD4 mice. At day 7, T cells from MD4 mice showed largely normal cytokine production, with a small but significant reduction in IFNγ ($P<0.05$). IL-10 secretion by T cells from MD4 mice was equivalent to those from WT mice. Antigen specific IL-2 secretion by T cells isolated from *S. enterica*-infected mice was below the limit of detection in both WT and MD4 mice.

A second cohort of animals was left to clear the infection, in order to quantify the development of cytokine-secreting memory T cells. T cells from week 7 post-infection were re-stimulated as previously. Figure 3A shows that at week 7 there is no antigen-specific Th1 response from these T cells (no detectable IL-2 or IFNγ; $P<0.001$) and it is clear that generation of memory Th1 cells is seriously impaired in mice which cannot respond through the BCR. Interestingly, IL-10 secretion was still apparent in T cells isolated from MD4 mice 7 weeks post immunisation, though was partially impaired ($P<0.05$). Thus, stimulation of B cells through the BCR is necessary for generation of Th1 memory in *Salmonella* infection. As MD4 mice clear bacteria less efficiently (Figure 3B) than wild type mice we also carried out experiments in which the mice were treated with antibiotic from the peak of infection for 10 days. Supplemental Fig. 4 shows that similar levels of antibiotic-mediated clearance of bacteria in MD4 and wild-type mice gave exactly the same result: the Th1 memory response failed to develop in MD4 mice. The frequency of activated ($CD44^+$) cells within the CD4 T cell population are similar in MD4, uMT and WT mice, indicating there is no failure of activation or survival (Supplemental Figure 5).

Memory T cell responses require antigen presentation by B cells whereas priming does not

Our demonstration that primary T cell responses were influenced by B cells in an antigen non-specific manner while memory T cell responses were dependent upon BCR suggests antigen presentation by B cells is dispensable at early stages but crucial later on. To investigate this further we generated mixed bone marrow chimeras in which B cells could not present antigen via MHC class II (MHC-$II^{B-/-}$). Primary T cell responses to *Salmonella* in these mice, as in BCR-irrelevant mice, were found to be largely normal (Figure 4) and were sufficient to allow bacterial clearance. In contrast, at week 8 post-infection memory Th1 responses (IL-2 and IFNγ secretion) were both highly dependent upon B cell presentation (Fig 4). The same is not true of the memory IL-10 response, which is unaffected in MHC-$II^{B-/-}$ chimeras. To test if this failure of Th1 memory development influenced the protection, we challenged the mice, 12 weeks later, with a virulent *Salmonella* strain and found that they survived less well than controls (Supplemental Fig. 6).

Effect of B cell cytokine deficiency on the development of T cell responses

To see if there was a direct effect of B cell-derived cytokines on the polarization of T cell responses we constructed bone marrow chimeras in which the B cell compartment was deficient in IL-6 or IFNγ. In IL-$6^{B-/-}$ chimeras we noted no effect on CD4 T cell derived IL-2, IL-10 (not shown) or IFNγ responses (Figure 5A), but did note a significant decrease in the production of IL-17 during *Salmonella* infection (Figure 5A),. In IFN$\gamma^{B-/-}$ chimeras the CD4 T cell derived IL-2 (not shown) and IL-10 (Figure 5B) responses were slightly lower than in WT chimeras, but this was not statistically significant. The IL-17 response was not altered. However, the CD4 T cell-derived IFNγ response to *Salmonella* was

significantly impaired in the mice in which B cells could not secrete IFNγ (Figure 5B). In summary, B cell-derived IL-6 and IFNγ supported development of the Th17 and the Th1 responses respectively. T cell cytokine response was not completely absent in the cytokine chimera, so we conclude that B cell derived cytokines play an important but supporting role.

DISCUSSION

In this study we have identified two distinct and functionally separable phases of the B cell contribution to T cell differentiation during a response to an ongoing *Salmonella* infection. The first, early phase is dependent on B cell-intrinsic MyD88 signals, most likely as a result of TLR ligation. This phase is almost wholly independent of BCR-mediated antigen uptake or presentation to CD4 T cells via MHC class II on the B cells. B cell cytokine production is largely MyD88-dependent and we propose that the early influence of B cells on the developing CD4 T cell response is mediated by cytokines produced in response to TLR ligation. In support of this we show that when B cells are incapable of making certain cytokines (eg. IFNγ or IL-6), this has measurable effects on the development of polarized effector T cell subsets (eg. Th1 and Th17). We do not know if the B cell-derived cytokines act directly on activated T cells or on other cell types that influence T cell differentiation. The second phase of B cell influence is during the development of CD4 T cell memory and is dependent on recognition and uptake of antigens by BCR and their subsequent presentation by MHC class II. In the absence of this cognate phase of B-T cell interaction, the protective Th1 memory cell response fails to develop.

Evidence of the importance of B cells in the development of CD4 T cell memory has accumulated over several years in studies using protein antigens and TCR transgenic models (13, 17, 62). The data presented here along with other recent studies (14-16, 18, 63), shows, *during infection,* the failure to develop T cell memory in the absence of antibody-independent B cell function. We identify the expression on B cells of both an antigen-specific BCR and MHC class II as crucial for memory T cell development, presumably to enable antigen uptake and presentation. It is equally clear that these “cognate” molecules on B cells are dispensable for the generation of the primary CD4 T cell response. This means that other APC (eg. DC) are competent and sufficient to drive the primary response, strongly suggesting that, as the response to *Salmonella* antigens proceeds, the participating APC changes from the DC, initially, to B cells later on. It is not simply that B cells present antigen upon secondary exposure, but rather perform a “follow-on” APC function as DC presentation falls away. In support of this, the primary IFNγ response we observe in MD4 (BCR restricted) mice at day 7 is already reduced compared to control mice and this response declines in these mice over the next 2-3 weeks. Antibody seems to play no significant role in enhancing Ag-presentation during the primary response, as provision of anti-*Salmonella* antibodies to the MD4 mice did not alter the magnitude or the longevity of their primary response. As we did not identify individual antigen-specific memory T cells in this study, we can say little about memory T cell survival. Our definition, of memory here is a functional one in which we detect enhanced responsiveness to antigen recall. Thus, we do not know if the memory we see is mediated by bona fide memory T cells or long-lived effectors, but given that systemic CD4 T cell memory is not apparent in some mice (eg. MD4, MHC II$^{B-/-}$ chimeras), we feel this demonstrates that that functionally either or both cell types are missing. Future studies using Salmonella that expressing a peptide epitope detectable using MHC II tetramers will address events at a cellular level.

In previous work from this lab (13), we showed that antigen presentation by B cells affected the T cell response as early as day 3-4 post-immunization, somewhat earlier than we indicate here. The two studies differ in antigen delivery, here we follow the primary response to bacterial infection, and this may lead to enhanced APC activation in comparison to protein

immunization with adjuvant. More importantly, Crawford et al (13) used a TCR transgenic model in which the frequency of naïve antigen-specific T cells was artificially increased: Under these conditions it is possible that the APC capacity of endogenous DC was insufficient to allow optimal activation of the transferred transgenic T cells and that the "follow-on" APC function of B cells was apparent earlier. Taken together, these data indicate that *in vivo* the contribution of DC is time-limited and cannot lead to optimal memory cell development. Ahmed and colleagues (18) also saw rapid decline (within 3 weeks) of LCMV-specific CD4 T cell response in B cell-deficient mice and concluded that B cell involvement is in the establishment and maintenance phase of CD4 T cell memory; we would agree.

The majority of published studies that have addressed the influence of B cells on T cell differentiation have noted their involvement in Th2 responses (32-35, 38). Here we show a clear contribution of B cells to the development of an effective Th1 (and Th17) response to *Salmonella* infection. As discussed above, for Th1 memory this relates to continued APC function. However, in the primary response B cells are to a large extent dispensable as APC (MD4, BCR-restricted mice and MHC II$^{B-/-}$ chimeras exhibit relatively normal primary responses). Despite this, the presence of B cells does have a measurable effect on the development of polarised Th subsets. In MyD88$^{B-/-}$ chimeras the primary IFNγ and IL-10 T cell responses are significantly reduced, indicating that TLR-induced polyclonal B cell activation during the infection elicits mediators that can act on differentiating T cells. This conclusion is supported by our recent study of antibody responses in MyD88B−/− chimeras in which the IFNγ-dependent IgG2c responses were most severely impaired (64). We investigated the cytokines, known to be secreted by B cells after TLR activation (39). In IFNγ$^{B-/-}$ chimeras, infected with *Salmonella* the primary CD4 T cell IFNγ response was lower than in control chimeras. Recent data suggests that Th1 commitment and T-bet expression occurs in a biphasic fashion, the early phase involving TCR and IFNγ signals and the later consolidating phase requiring IL-12 (65). Our data suggest that TLR-activated B cells are a likely source of this early IFNγ that optimises Th1 commitment. The fact that the T cell IFNγ response in the IFNγ$^{B-/-}$ chimeras is only partially inhibited may be because IL-12 has an overriding effect (65) or that there are other cellular sources of IFNγ. It has also been shown that *Salmonella* can induce early innate activation of CD4 T cells that then make IFNγ (66); whether the innate activation and IFNγ secretion by B cells influences this T cell response is not known. In IL-6$^{B-/-}$ chimeras, the T cell IL-17 response to *Salmonella* was reduced, which we think indicates a significant contribution by B cells of the IL-6 required for Th17 development (67). TNFα expression by B cells has been shown to augment Th1 effector responses in *Toxoplasma gondii* infection, but we have not yet tested this in appropriate *Salmonella*-infected chimeras. An obvious candidate Th1 polarising cytokine is IL-12, however, mouse B cells make very little IL-12p40 (39) and chimeras in which B cells lack IL-12, exhibit completely normal primary and memory Th1 responses to *Salmonella* (not shown). We show here a direct effect of B cell cytokines on the development of effector T cell responses during infection. The worry that the use of B cell depletion therapy in autoimmune patients might have consequences for infection is only exacerbated by these data, as B cells, in addition to antibody production, also support the differentiation of inflammatory, protective Th1 and Th17 responses.

The impairment of the primary T cell cytokine responses to *Salmonella* in MyD88$^{B-/-}$ chimeras indicates the importance of an innate component of the B cell response. The adaptive B cell response including APC function becomes absolutely crucial later (7-14 days) for sustaining the effector T cell response and enabling memory T cell formation. It will be intriguing to discover where these two distinct phases of the B cell dependent T cell response occur within lymphoid tissues. We conclude that a complete and rounded CD4 T cell response is dependent on B cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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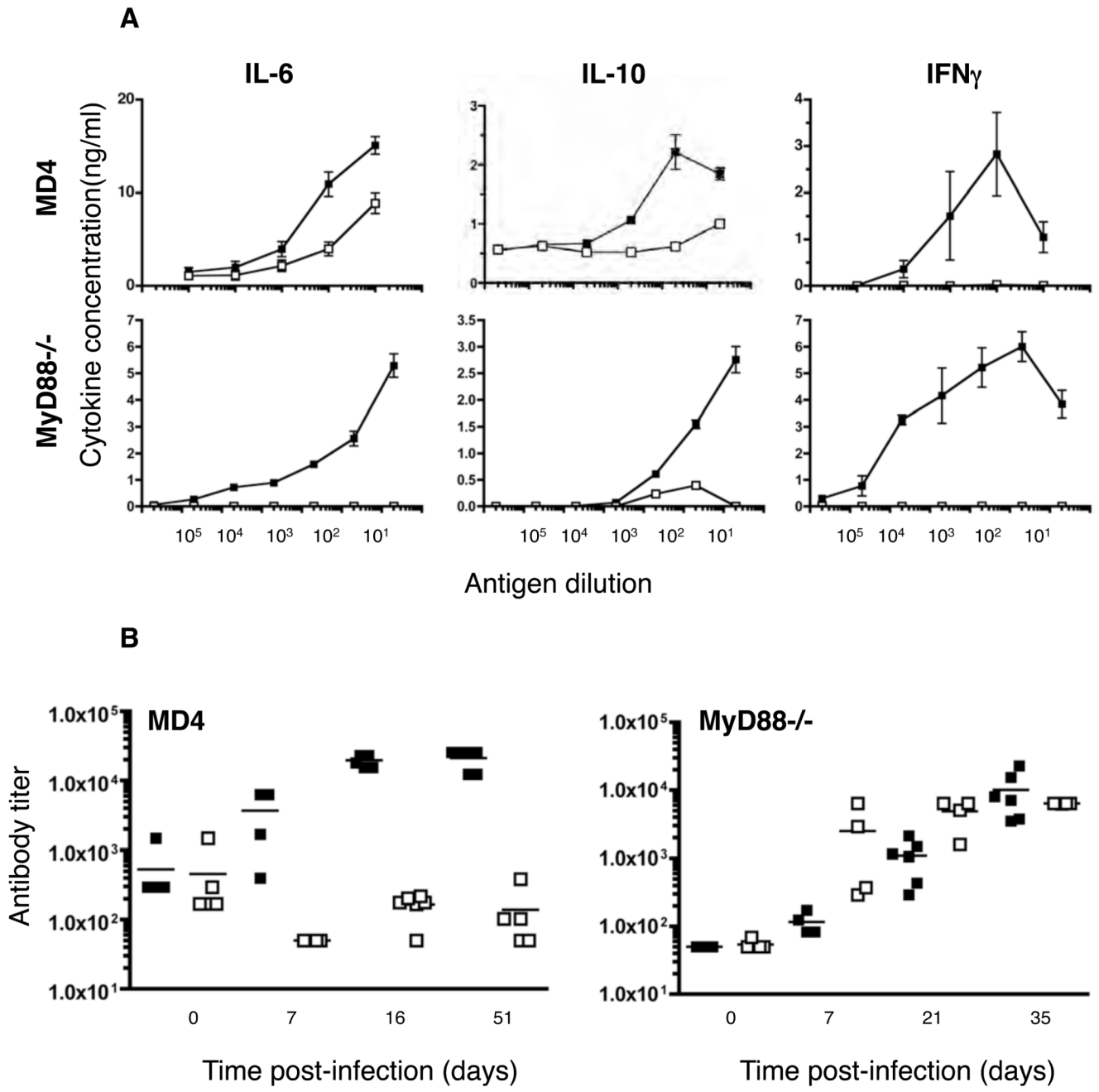

Figure 1. Cytokine production is dependent upon both TLR and BCR reactivity

A. CD19^{+} B cells from *Salmonella*-infected MyD88$^{-/-}$ and MD4 mice were isolated from spleens at day 7 of infection and cultured for 5 days with a serial dilution of HKB as indicated on the X-axis. In each case black symbols represent wild-type controls and open symbols knock-out or transgenic mice. Data points represent mean values of triplicate cultures carried out on pooled B cells from groups of 4 mice and error bars represent SEM. Data is representative of 3 independent experiments. Naïve WT CD19^{+} B cells when stimulated with HKB maximally secreted 1-4ng/ml IL-6, 1-2ng/ml IL-10 and no IFNγ.

B. Anti-*Salmonella* antibody titres in MD4 (left) and MyD88$^{-/-}$ (right) mice were determined by ELISA on sera from 4 groups of mice bled at time points indicated (n=4-6).

Black symbols represent wild-type controls and open symbols represent transgenic or knock-out mice. Each data point represents an individual mouse and bars represent mean for the group. Data is representative of 3 separate experiments.

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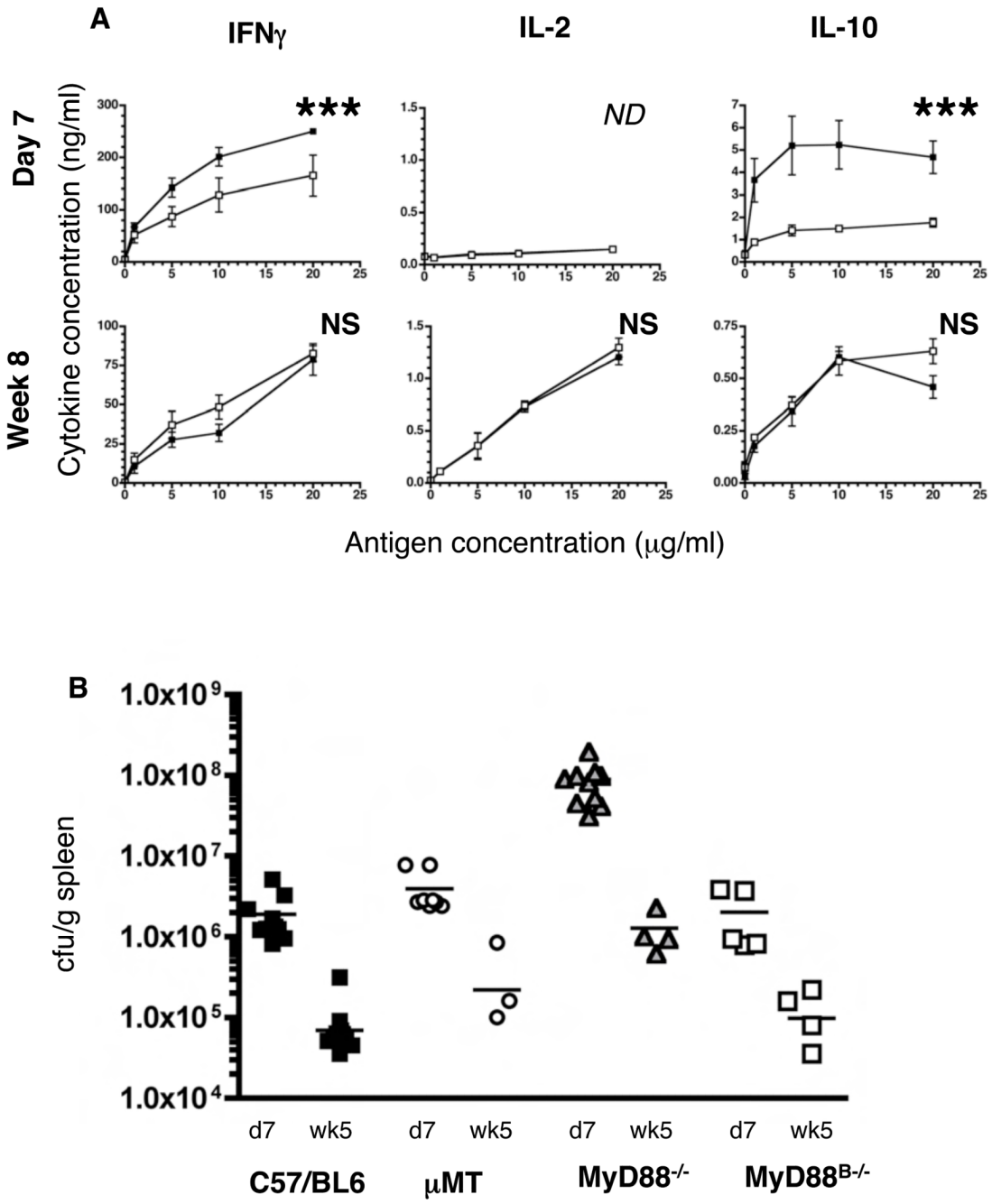

Figure 2. Primary Th1 responses require B cell TLR reactivity

A. CD4^{+} T cells from *Salmonella*-infected MyD88$^{B-/-}$ bone marrow chimeric mice were sorted by magnetic separation at day 7 (top) and week 8 (bottom) and cultured for 3 days with C5SENaOH at a range of concentrations as indicated on the X-axis. In each case black symbols represent wild-type bone marrow chimeras and open symbols MyD88$^{B-/-}$ chimeras (*ND* = not detected). Data presented is the mean of duplicate cultures on groups of 5 animals (n=5) and is representative of 3 separate experiments. Error bars indicate SEM. Asterisks indicate statistically significant impaired cytokine production as determined by 2-way ANOVA (*P<0.05, **P<0.01, ***P<0.001 and NS = not significant).

B. Bacterial load in spleens from infected mice were calculated at day 7 and week 5. Each symbol represents CFU from an individual mice and the line represents the mean for each group. Presented data is representative of 3 independent experiments.

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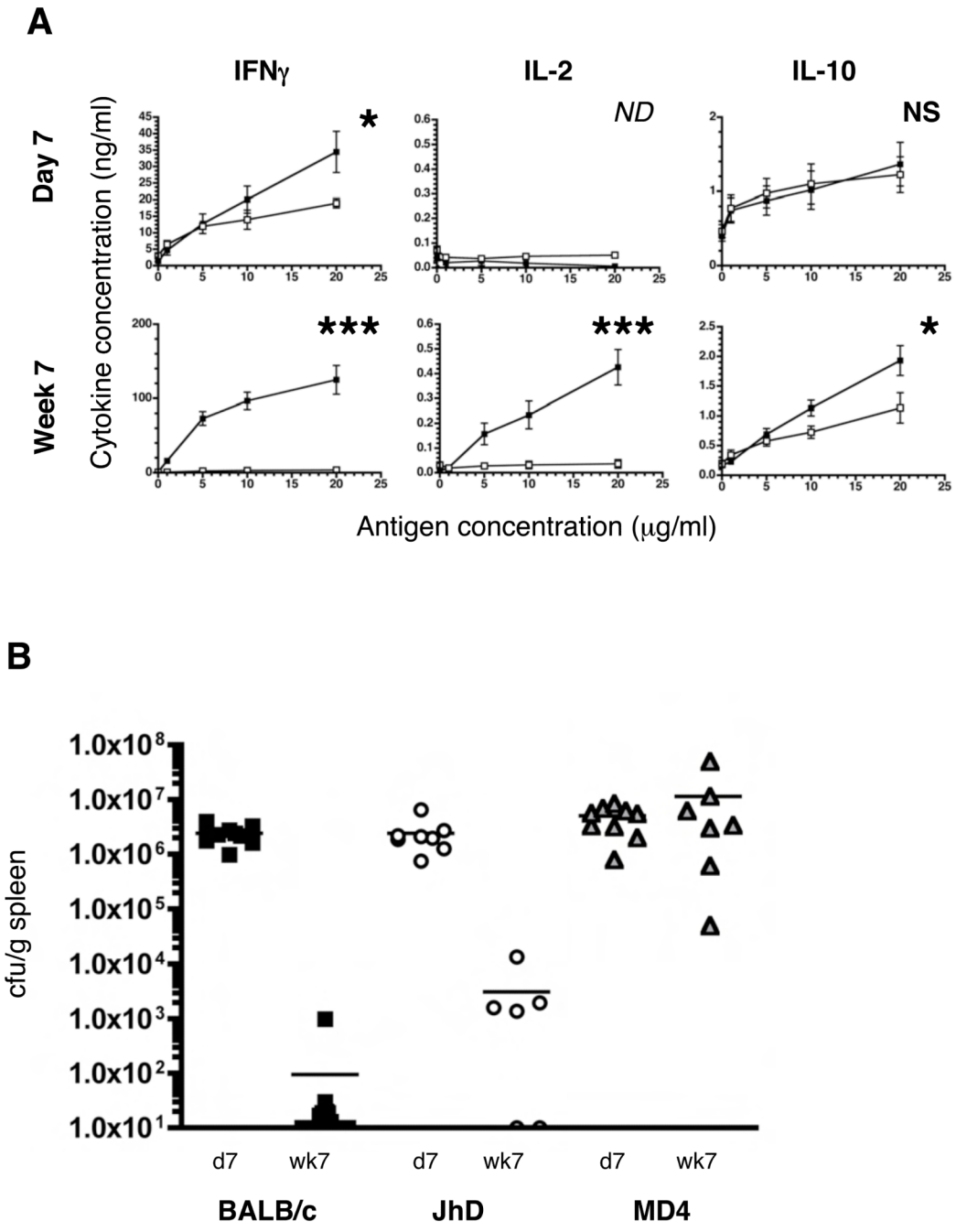

Figure 3. Development of memory Th1 responses requires BCR reactivity

$CD4^+$ T cells from *Salmonella*-infected MD4 were sorted by magnetic separation at day 7 (top) and week 7 (bottom) and cultured for 3 days with C5SENaOH at a range of concentrations as indicated on the X-axis. In each case black symbols represent BALB/c and open symbols MD4 transgenic mice (*ND* = not detected). Data presented is the mean of duplicate cultures on groups of 5 animals (n=5) and is representative of 3 separate experiments. Error bars indicate SEM. Asterisks indicate statistically significant impaired cytokine production as determined by 2-way ANOVA (*P<0.05, **P<0.01, ***P<0.001 and NS = not significant). As a result of the failure to clear bacteria, memory T cell responses in MD4 mice were tested at week 7 and not week 12.

B. Bacterial load in spleens from infected mice were calculated at day 7 and week 7. Each symbol represents CFU from an individual mice and the line represents the mean for each group. Presented data is representative of 3 independent experiments.

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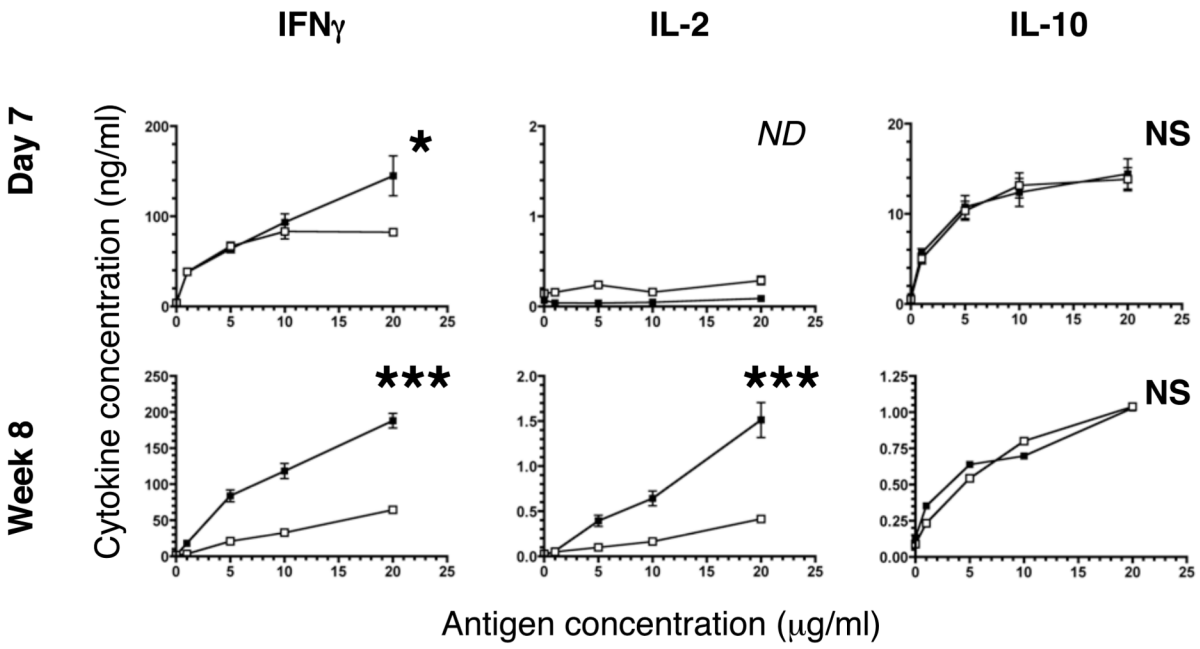

Figure 4. Antigen presentation by B is dispensable for the primary T cell response but is necessary for the memory T cell response

$CD4^+$ T cells from *Salmonella*-infected MHC-$II^{B-/-}$ chimeras were sorted by magnetic separation at day 7 (top) and week 8 (bottom) and cultured for 3 days with C5SENaOH at a range of concentrations as indicated on the X-axis. In each case black symbols represent WT chimeras and open symbols MHC-$II^{B-/-}$ chimeras (*ND* = not detected). Data presented is the mean of duplicate cultures on groups of 5 animals (n=5) and is representative of 4 separate experiments. Error bars indicate SEM. Asterisks indicate statistically significant impaired cytokine production as determined by 2-way ANOVA (*P<0.05, **P<0.01, ***P<0.001 and NS = not significant).

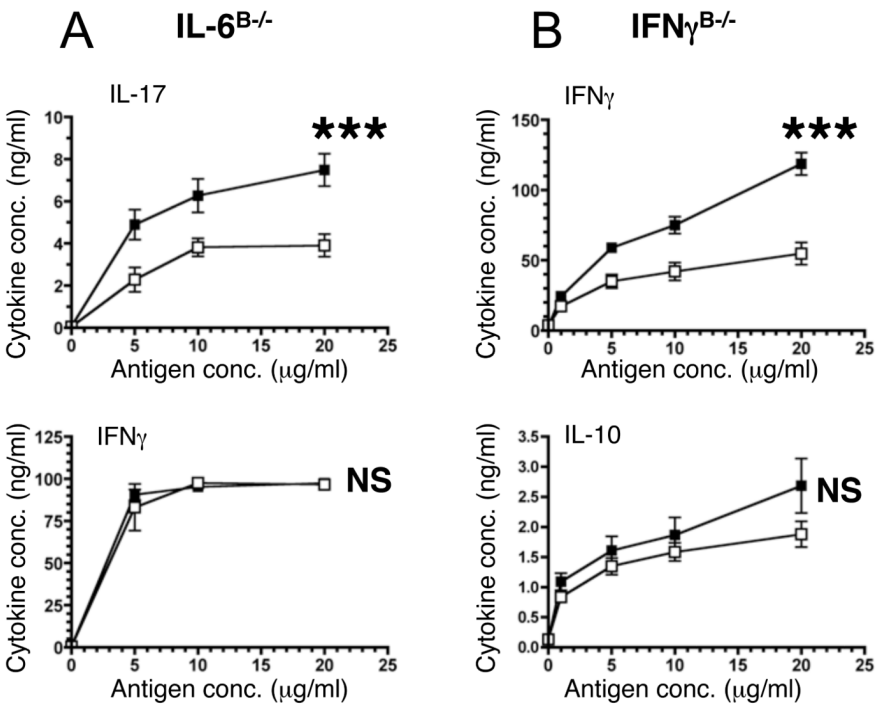

Figure 5. Cytokine production by B cells influences the primary effector T cell response

A. IL-17 and IFNγ responses of $CD4^+$ T cells from spleens of *Salmonella*-infected IL-$6^{B-/-}$ chimeras, re-stimulated *in vitro* (see legend to Figure 4) at day 56.

B. IFNγ and IL-10 responses of $CD4^+$ T cells from spleens of *Salmonella*-infected IFN$\gamma^{B-/-}$ chimeras, re-stimulated *in vitro* at day 56 (see legend to Figure 4).

Statistical analysis of **A** and **B** above was by 2-way ANOVA (*P<0.05, **P<0.01, ***P<0.001 and NS = not significant). Error bars indicate SEM. Data shown in **A** were representative of 8 independent experiments and in **B** of 6 independent experiments. In all panels, open symbols represent cytokine$^{B-/-}$ chimeric mice and black symbols wild-type control chimeras. All the cytokine chimeras cleared bacteria normally.