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B Cell Intrinsic MyD88 Signals Drive IFN-γ Production from T Cells and Control Switching to IgG2c

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The question of whether Ab responses to T-dependent Ags require B cell intrinsic signaling via the main TLR adaptor (MyD88) has become embroiled in confusion. In part this may be related to the methods used to analyze B cell intrinsic signaling. We have used a mixed bone marrow chimera model to generate mice in which the B cell compartment is completely deficient in MyD88 expression, while the other hematopoietic lineages are largely normal. These mice were immunized with T-dependent Ags or infected with Salmonella. We found that the Ag-specific IgG2c primary response was absolutely dependent on MyD88 signaling to B cells, while other Ig classes were not (IgG1 and IgG3) or much less so (IgG2b, IgA). The MyD88−/−/H11546 chimeric mice exhibited an impairment of development of IFN-γ effector T cells, a likely contributory factor in the lack of IgG2c. We also found that B cell intrinsic MyD88 signals are required for the production of natural Abs. The data emphasize the nonredundant role of B cells as programmers of T cell differentiation in vivo. The Journal of Immunology, 2009, 183: 1005–1012.

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3 Abbreviations used in this paper: TI, T independent; TD, T dependent; WT, wild type; DNP, dinitrophenylated; GC, germinal center; KLH, keyhole limpet hemocyanin.

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Ags and TLR ligands. The consistent finding was that Ab responses in the IgG2a/c subclass were profoundly impaired when B cells could not signal via MyD88. With some Ags (hapten-carrier conjugates or bacteria), the response impairment was broader including other isotypes, but the most profound defect always involved the IgG2a/c response. The likely reason for this lies in defects of T cell IFN-γ production in these chimeras.

Materials and Methods

Mice and generation of bone marrow chimeras

Mice were bred and maintained in specific pathogen-free conditions at the School of Biological Sciences Animal Facility at the University of Edinburgh and the Department of Veterinary Medicine, University of Cambridge. C57BL/6, μMT, and MyD88−/− mice ages 6–10 wk old at the start of immunization regimens were used throughout. Mixed bone marrow chimeric mice were generated as described previously (14). Briefly, host B cell-deficient mice were lightly irradiated with 8 Gy of gamma radiation from a sealed source. Mice were then reconstituted with 2 × 10^9 CD90-depleted mixed inoculum bone marrow cells. The bone marrow inocula were composed of 80% B cell-deficient marrow (μMT) and 20% knockout or wild-type (WT) marrow (MyD88−/−, IFN-γ−/−, or C57BL/6). Thus, all B cells in these mice were arose from the knockout (or WT) portion of the Ag) in PBS at 4°C. Plates were then blocked for 2 h with 1% BSA in PBS. Mice were bled at 100 μl i.p. on the day of immunization, and a further 300 μl were injected via the i.v. route with 200 μl sterile filtered and stored at −80°C. Bacteria were grown as stationary-phase overnight cultures in Luria-Bertani broth (Difco Laboratories). Animals were used for all infections (19). Bacteria were grown as stationary-phase preparation was via the i.v. route with −1 × 10^9 CFU diluted in sterile PBS. Mock infection control animals were injected i.v. with 200 μl of sterile PBS. Viable counts in each infective dose were determined by pour plating on Luria-Bertani agar plates. Mice were bled before infection and at weekly intervals following infection until termination of the experiment (~100 days). Salmonella Ag for T cell restimulations was prepared as described previously (20). Briefly, overnight stationary-phase bacterial cultures were washed twice in PBS containing 5 mM EDTA. The resulting suspension was sonicated on ice and cellular debris were removed by centrifugation at 13,000 × g. The supernatant was alkali treated with NaOH for 3 h at 37°C and then neutralized with HCl. The NaOH-treated Ag was then 0.22-μm sterile filtered and stored at −80°C. Protein content was calculated using a standard Bradford assay. The Ag for ELISA was a preparation of sonicated SL3261 (20).

Serum Ab response

To quantify Ag-specific serum Ab titers, plates were coated overnight with relevant Ag at 10 μg/ml (OVA, DNP-BSA, PE, LPS, or SL3261 sonicate Ag) in PBS at 4°C. Plates were then blocked for 2 h with 1% BSA in PBS at room temperature (RT). Following washing, serum was then applied to the plate and serial diluting dilutions were performed. Plates were incubated for another 2 h at RT, then washed. Alkaline phosphatase-labeled isotype-specific detection Abs (Southern Biotechnology Associates) were then added and the plates were incubated for another 1 h at RT. Following a final wash, plates were developed with p-nitrophenylphosphate substrate and absorbance at 405 nm was determined. Serum Ab titer was calculated by extrapolating dilution which gave the same OD as ½ maximum OD of a standard serum sample.

Preparation and sorting of CD4-positive and CD19-positive lymphocytes

CD4-positive and CD19-positive lymphocytes were isolated from SL3261 or mock-infected mice using standard magnetic sorting techniques. Following manual disruption in complete medium (IMDM plus 5% FCS and penicillin/streptomycin) and lysis of RBC, splenocytes were labeled with anti-CD19 or anti-CD4 microbeads for B and T cell sorting, respectively. After incubation, cells were washed and sorted over two consecutive LS columns in accordance with the manufacturer’s instructions (Miltenyi Biotech). Passing cells over a second column lead to purities in excess of 99% (data not shown). These highly purified B and T cells were then used in subsequent in vitro restimulation assays.

In vitro restimulation assays

T cell in vitro restimulation assays were set up by coculturing purified CD4-positive T cells from SL3261-infected chimeric mice along with gamma-irradiated (30 Gy) WT, naive spleenocytes at a 1:1 ratio (final total cell density of 2 × 10^6 cells/ml). Supernatants were harvested for quantification of IFN-γ after 72 h.

Cytokine ELISA

IFN-γ levels in supernatants from T cell stimulation cultures were quantified by standard capture ELISA techniques using a commercially available paired Ab set (BD Pharmingen). Briefly, Nunc Maxisorp plates (Fisher Scientific) were coated overnight at 4°C with capture mAb at 5 μg/ml in PBS. Plates were then blocked for 2 h at RT with 1% BSA. Supernatants were incubated on plates for 2 h at RT. Following washing, biotinylated capture mAbs were added and incubated for 1 h at RT. Streptavidin-alkaline phosphatase was then added and following incubation at RT and washing, p-nitrophenylphosphate substrate was applied. IFN-γ concentration was determined by extrapolation from the standard curve. Minimum level of detection for IFN-γ was 0.8 ng/ml.

Immunohistochemistry and quantitative histology

Spleens were frozen in OCT-embedding medium (BDH) in cryomoulds (BDH) on dry ice and stored at −80°C. Tissue sections (5 μm) were cut, dried, and fixed with acetone. Sections were stained with Texas Red-labeled polyclonal goat anti-mouse IgM (Southern Biotechnology Associates) and FITC-conjugated peanut agglutinin (Vector Laboratories) to visualize the germinal centers (GC). Slides were viewed on an Olympus BX50 microscope under reflected light fluorescence and images were captured using Openlab imaging software (Improvision). The number of GC per unit area of spleen tissue was enumerated and the total CD19+ B cell compartment was calculated using Velocity software (Improvision).

Results

To address the importance of B cell-intrinsic MyD88 signaling in Ab responses, we constructed bone marrow chimeric mice in which the whole B cell compartment lacked the capacity to express the MyD88 gene. To do this, recipient B cell-deficient (μMT) mice were irradiated and then reconstituted with a mixture of bone marrow: 80% from μMT mice and 20% from MyD88+/− mice. In these chimeras, the only source of B cells is from the MyD88−/− bone marrow. The μMT bone marrow gives rise to all other hematopoietic lineages and these are MyD88 sufficient. All immunizations described below are conducted on these chimeric mice, along with control chimeras in which the MyD88−/− bone marrow inoculum is replaced with WT bone marrow cells.

The role of B cell MyD88 signaling in Ab responses to T-dependent protein Ags

Protein Ags were emulsified in IFA along with LPS and immunizations were done via the s.c. route. The Ab response of the
B cell-intrinsic MyD88 signals are required for IgM and IgG2c responses to TD Ags. Mice were immunized s.c. with 100 μg of OVA (A) or DNP-OVA (B) emulsified in IFA with 10 μg of LPS. To measure the response to TI-1 Ag, mice were immunized with 10 μg of LPS in IFA (C). Animals were boosted at day 30 with a second dose of 100 μg of OVA or DNP-OVA (A and B) or 10 μg of LPS. Ag-specific Ab titers were measured by ELISA for each Ab isotype at the time points indicated. In each case, filled symbols represent WT control chimeras and open symbols MyD88<sup>−/−</sup> cell chimeras. Values indicate mean titers for groups of six animals and error bars represent SEM. Two-way ANOVA analysis was performed to determine significance of variation for the primary and boosted response. Values of p are given above each graph. Data presented are representative of three independent experiments.
MyD88<sup>B<sup>−/−</sup></sup> chimeric mice to OVA is shown in Fig. 1A. The total Ag-specific response is significantly reduced (total Ig) following primary immunization, although in some subclasses (IgG1, IgG2b, and IgG3), there is no difference in the Ag-specific response. The most striking deficiencies was seen in the IgG2c subclass, and IgG3), there is no difference in the Ag-specific response with immunization, although in some subclasses (IgG1, IgG3, but neither type of chimera made detectable IgM or IgG2c responses.

The Ab response to Ags physically linked to TLR ligands

To see what effect the physical linkage of TD Ags with TLR ligands (e.g., LPS) would have on the B cell MyD88 dependence of Ag-specific Ab response, we chose to infect the chimeric mice with Salmonella typhimurium. Although there is a TI component of the IgM response to Salmonella, the switched isotype response is largely TD (21). It is clear that in this circumstance the Ab responses in all classes except IgG1 and IgG3 were affected by the lack of MyD88 in B cells (Fig. 1B). There is minimal response to Salmonella proteins in the IgG1 and IgG3 subclasses during infection, although, interestingly, in the MyD88<sup>B<sup>−/−</sup></sup> chimeras there is an enhanced IgG1 response (Fig. 2). Again, the most profound lack was in the IgG2c subclass, the onset of this response was delayed by 10–15 days, and the peak levels never reached those seen in the WT chimeras. The same was true of the serum IgA response.

MyD88<sup>B<sup>−/−</sup></sup> responses to OVA, where the primary response in the MyD88<sup>B<sup>−/−</sup></sup> mice was nonexistent (nota bene: C57BL/6 mice do not carry the IgG2a isotype, but IgG2c instead). The IgM response is difficult to interpret as again baseline levels of DNP-specific IgM was lower in MyD88<sup>B<sup>−/−</sup></sup> mice made anti-OVA Ab in both IgG2c and IgM classes, although still these were significantly reduced compared with responses in control chimeras. We also analyzed the response to the DNP hapten on an OVA carrier. In this case, the total Ig response was reduced (Fig. 1B), while the IgG1, IgG3, and IgA were not (we did see a statistically significant boost in the IgG3 response in MyD88<sup>B<sup>−/−</sup></sup> mice). Anti-DNP responses in the IgG2b subclass were quite dramatically reduced and the IgG2c primary response was again absent (Fig. 1B). The IgM response is difficult to interpret as again baseline levels of DNP-specific IgM was lower in MyD88<sup>B<sup>−/−</sup></sup> vs WT chimeras. None of the reduced/absent responses recovered completely after boosting on day 30. Fig. 1C shows that there was no Ab response in the MyD88<sup>B<sup>−/−</sup></sup> chimeras when they were immunized with LPS (TI type 1). We have not shown the data from immunizations with protein Ag in IFA or alum in the absence of LPS. In this case, both MyD88<sup>B<sup>−/−</sup></sup> and control chimeras made equivalent levels of Ag-specific IgG1, IgG2b, and IgG3, but neither type of chimera made detectable IgM or IgG2c responses.

The Ab response to Ags physically linked to TLR ligands

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To see what effect the physical linkage of TD Ags with TLR ligands would have on the B cell MyD88 dependence of Ag-specific Ab response, we chose to infect the chimeric mice with Salmonella typhimurium. Although there is a TI component of the IgM response to Salmonella, the switched isotype response is largely TD (21). It is clear that in this circumstance the Ab responses in all classes except IgG1 and IgG3 were affected by the lack of MyD88 in B cells (Fig. 2). There is minimal response to Salmonella proteins in the IgG1 and IgG3 subclasses during infection, although, interestingly, in the MyD88<sup>B<sup>−/−</sup></sup> chimeras there is an enhanced IgG1 response (Fig. 2). Again, the most profound lack was in the IgG2c subclass, the onset of this response was delayed by 10–15 days, and the peak levels never reached those seen in the WT chimeras. The same was true of the serum IgA response.
As indicated above, baseline levels of Ag-specific IgM Abs were reduced in the MyD88−/− chimeras. In fact, as with the MyD88−/− mice themselves (2, 4), the MyD88−/− chimeras exhibit lower levels of serum Ig in all classes and subclasses (IgM and IgG2c are shown in Fig. 3), suggesting that MyD88 signaling in B cells drives the secretion of natural serum Abs. These natural Abs have been implicated in the enhancement of Ag uptake by APC and the priming of the immune response (22–24). We wondered whether replenishment of the natural Abs in the MyD88−/− chimeras would influence the Ag-specific response upon immunization. To test this, WT and MyD88−/− chimeras were immunized with DNP-OVA (plus LPS in IFA) and half of the mice were given i.v. injections of normal C57BL/6 mouse serum on days −1, 0, and +2. Fig. 3B shows that the serum transfer significantly increased the levels of serum Ig over the first 10 days. IgM and IgG2c levels were also increased after serum transfer but were still subnormal (data not shown). Despite this reconstitution of serum natural Abs, the specific Ab response to DNP in the MyD88−/− mice was not augmented (Fig. 3C). MyD88-deficient B cells can secrete IgM and IgG2c upon stimulation

To determine whether there was an inherent defect in Ab production in MyD88-deficient B cells, we cultured CD19-positive cells from MyD88−/− and WT mice either with CpG (a MyD88-dependent stimulus) or with activated OT-II (OVA-specific TCR-transgenic) CD4 T cells plus OVA peptide (a MyD88-independent stimulus). After 5 days, the Ab produced was measured and Fig. 4 shows that, although the MyD88-negative B cells made no Ab in response to CpG, they made as much Ab as WT B cells when cultured with OT-II T cells. Importantly, the IgM and the IgG2c levels were equivalent.

**B cell clonal expansion and germinal centers in MyD88−/− chimeras**

To determine whether MyD88-transduced signals influenced clonal expansion of B cells during an in vivo response, we measured the frequency of Ag-specific B cells 20 days after primary immunization (in the blood) and 20 days after secondary immunization. Fig. 5A shows that the frequency of PE-specific circulating...
B cells in the blood 20 days after immunization is similar in MyD88<sup>−/−</sup> and control chimeras. Similarly, the number of PE-specific B cells in spleen and lymph node 20 days after boosting is equivalent in both groups of chimeras (Fig. 5B). We would predict that the frequency of IgG2c-switched PE-specific B cells will be equivalent in both groups of chimeras (Fig. 5B) or MyD88<sup>−/−</sup> chimeras. We found that IgG2c (and IgG2b) secretion in T cell restimulation assays at early time points during Salmonella infection (day 7) was significantly lower.

**Discussion**

The strong conclusion after analyzing the responses to a variety of TD Ags is that MyD88-dependent activation of B cells is absolutely required to make primary responses in the IgG2c class. Another strong conclusion is that the IgG1 response to most TD Ags is independent of MyD88 signals to B cells. IgM responses seemed also to be impaired, but this was difficult to draw a strong conclusion on given the lower baseline levels (at day 0) of Ag-specific IgM in the MyD88<sup>−/−</sup> compared with WT chimeras in anti-hapten-protein responses (this was not apparent in the anti-Salmonella). Also, we never observed any MyD88 dependence of the IgG3 Ag-specific response; however, the responses in this subclass were often low and sometimes absent, making conclusions difficult to reach. In several instances, we observed clearly reduced IgG2b Ag-specific responses. Thus, the main impairment in the IgG subclasses is in responses that are associated with Th1 differentiation. Ligation of certain TLRs (e.g., TLR9) has been reported to induce directly the expression of Th-bet in B cells, which, in turn, drives IgG2a/c switch recombination and concomitant inhibition of IgG1 and IgE switching (28). This study did not detect the same effect with LPS and, therefore, this may not be the basis for our observation in immunizations with LPS as part of the adjuvant. We (27) and others (29) have shown that B cells make IFN-γ following TLR stimulation and the B cell-intrinsic role of TLR9 in directing IgG2a/c switching has been noted (30, 31); therefore, a test of autocrine IFN-γ secretion to induce the IgG2c switch seemed appropriate. However, the construction of chimeric mice in which B cells lacked the gene for IFN-γ had no significant effect on the Ag-specific IgG2c response to DNP-OVA or Salmonella in two of three experiments performed. Thus, the lack of IgG2c (and IgG2b) in MyD88<sup>−/−</sup> chimeras is not the result of impaired B cell IFN-γ production.

The main reason for the lack of IgG2c (and IgG2b) is a suboptimal primary T cell response in MyD88<sup>−/−</sup> chimeras. We found that IFN-γ production in T cell restimulation assays at early time points during Salmonella infection (day 7) was significantly lower.
in the MyD88−/− chimeras than in controls. Thus, the primary Th1 response, generating IFN-γ-secreting effector cells, seems to be dependent on the activation of B cells via MyD88. Whether, in this context, MyD88 signals impinge upon the APC function of B cells or on their production of mediators that are involved in aiding or even programming Th differentiation is not entirely clear; however, our preliminary data suggest the latter (D. Gray and T. A. Barr, unpublished data). The original characterization of the adaptive immune response in MyD88-deficient mice showed a failure in the development of Th1 effectors and a complete loss of any IgG2a Ab response (2). What we see when B cells alone do not express MyD88 is almost as profound: no Ag-specific IgG2c response. However, we did detect some IgG2c (and IgM) responses after boosting, albeit at reduced levels compared with control chimeras. Whether MyD88 knockouts can make IgG2a/c after boosting was not tested (by us or by Schnare et al.; (2)). Taken together, our data are a strong reiteration of the crucial role that B cells play as APC and programmers of Th differentiation (32). Interestingly, if the deficiency in MyD88 is restricted instead to DC, the effect on Ab responses is similar (IgG2b is reduced and IgG2c is almost absent) (33); thus, the development of an optimal Th1 cell response requires both MyD88-dependent activation of DC and B cells. The simplest scenario is that after the early phase of DC response (Fig. 2), a feature noted in the original description of the MyD88 knockouts (2), why, in contrast to IgG2c, is the IgG1 response independent of MyD88 signals to B cells? Driven by the Th2 response that is also relatively independent of MyD88 signals (2), this may not be surprising. Also, switching to IgG1 requires fewer cell divisions and is detected earlier than IgG2a/c (34) and therefore may be less dependent on sustained Th support.

The reduction in the magnitude of the GC response in immunized MyD88−/− chimeras fits with the notion that some aspect of T-B cell cooperation is impaired. Interestingly, even though the GC reaction is reduced in scale, this has not affected the frequency of Ag-specific B cells that develop in the primary response (day 20) or memory B cells detectable after boosting in the secondary response. Presumably, the primary clonal expansion occurs before the GC reaction and therefore is independent of it; however, we can only speculate on why memory cell numbers are unaffected by a reduced GC reaction. One possibility is that GC have the potential to generate more Ag-specific memory cells than are normally selected or survive in the long-lived pool. Thus, in the MyD88−/− chimeras, because there are fewer GC B cells, there is less competition for antigenic selection, which in turn is less stringent, allowing more to exit the GC. Alternatively, if the size of memory cell clones is set by homeostatic mechanisms in the periphery (outside the GC), then even a 50% reduction in GC output may be compensated by post-GC expansion. The fact that the GC in MyD88−/− chimeras are smaller and fewer may indicate a role for MyD88 signals in the GC itself and, in fact, Meyer-Bahlberg et al. (9) recently showed enhanced TLR responsiveness in GC B cells. Whether or how TLR signaling contributes to the GC remains to be discovered. TLR signaling within the GC, however, need not be the explanation for our observation; a simple defect in T cell differentiation, as demonstrated here, could lead to this phenotype. TLRs have also been proposed to mediate survival of memory B cells (35). The survival of normal numbers of MyD88-negative PE-binding memory B cells over 50 days certainly indicates no role for TLR-mediated survival signals over this time; however, a much longer period of observation would be needed to test this hypothesis properly. Given the rather restricted impairment in IgG responses in our MyD88−/− chimeras, we agree with Richard et al. (36) that TLR stimulation of memory B cells is unlikely to be a major driver of differentiation to plasma cells in vivo (35).

The total serum levels of IgM and all of the IgG subclasses are reduced in MyD88-deficient mice (2, 4). Much of this circulating Ab, especially in the IgM and IgG3 classes, is what comprises the natural Ab pool and this is largely derived from the B1 population of B cells (37–39). Other authors have shown that human transitional B cells from cord blood make natural Abs as a result of TLR ligation (40) or that TLRs stimulate B1 cells directly to become plasma cells (41). However, the data presented here show clearly that the production of serum natural Abs is almost wholly dependent on B cell-intrinsic MyD88-transduced signals and is not due to any contributory defect in another cell lineage. Thus, the MyD88−/− chimeras exhibit 10-fold lower levels of "background" serum IgM compared with control chimeras. They also show much (50-fold) lower serum IgG2c levels; much of this Ab represents the history of previous TD Ag-specific responses, which as our results indicate are both MyD88 and IFN-γ dependent. Because natural Abs have been implicated in priming of adaptive responses (22, 23), this raised the possibility that the impaired T cell priming and Ag-specific Ab responses might result from severely reduced natural Ab levels. Although it is difficult to reconstitute all of the subclasses to WT levels by serum transfer, to the best of our ability we are confident that replenishing the serum natural Ab levels does not reconstitute the adaptive Ab response.

How do these data fit with the other studies? Pasare and Medzhitov (4) show a loss of IgM and IgG2a/c when B cells are MyD88 deficient, but also show impairment of IgG1 and IgG3, which we do not. The same group showed normal IgG1 responses in MyD88 knockout mice in a previous publication (2). A very recent study measured Ab responses to Salmonella Ags and found impaired IgM in TLR4−/− mice and lower IgG2c in TLR2−/− mice (42). Gavin et al. (6) analyzed TD Ab responses in MyD88/TRIF double knockout mice that are unable to signal via TLRs. They concluded that TLR signaling made no significant contribution to TD Ab responses. Interestingly, they did see a small reduction in IgG2b and IgG2c when they immunized with Ribi adjuvant (containing a TLR4 ligand). They immunized their mice with haptenated keyhole limpet hemocyanin (KLH) as an Ag. KLH is a very large (multisubunit) molecule that can carry large numbers of DNP haptens and this may allow extensive BCR cross-linking. KLH is also highly glycosylated and derived from an invertebrate, it may well be that the molecule itself activates a known or unknown pattern recognition pathway in a way analogous to the action of alum via the Nalp3 pathway (7, 8).

In conclusion, we think that the present study goes some way in clarifying a confused picture in relation the role of B cell-intrinsic MyD88 activation as part of the TD Ab response. It impinges particularly on the primary IgM and IgG2c responses and at least the latter is the result of an impairment of the helper Th1 cell response. This emphasizes the importance of B cells in vivo in programming Th differentiation, a process that cannot be left to dendritic cells alone. Whether this role is as APC or as producers of programming mediators warrants further investigation.

Disclosures
The authors have no financial conflict of interest.

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