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A Critical Role for B Cells in the Development of Memory CD4 Cells

Phyllis-Jean Linton,* Judith Harbertson, † and Linda M. Bradley 3†

Activated B cells express high levels of class II MHC and costimulatory molecules and are nearly as effective as dendritic cells in their APC ability. Yet, their importance as APC in vivo is controversial and their role, if any, in the development of CD4 memory is unknown. We compared responses of CD4 cells from normal and B cell-deficient mice to keyhole limpet hemocyanin over 6 mo and observed diminished IL-2 production by cells primed in the absence of B cells. This was due to lower frequencies of Ag-responsive cells and not to decreased levels of IL-2 secretion per cell. The absence of B cells did not affect the survival of memory CD4 cells since frequencies remained stable. Despite normal dendritic cell function, multiple immunizations of B cell-deficient mice did not restore frequencies of memory cells. However, the transfer of B cells restored memory cell development. Ag presentation was not essential since B cells activated in vitro with irrelevant Ag also restored frequencies of memory cells. The results provide unequivocal evidence that B cells play a critical role in regulating clonal expansion of CD4 cells and, as such, are requisite for the optimal priming of memory in the CD4 population. The Journal of Immunology, 2000, 165: 5558–5565.

It has long been considered that B cells have an important, although not obligatory, role as APC for primary CD4 responses. In vitro studies have clearly shown that activated B cells are nearly as effective as dendritic cells (DC) in their capacity to activate naive CD4 cells (3). Although primary CD4 cell responses can develop in mice that genetically lack B cells (4–9), depletion of B cells from normal mice can reduce the magnitude of the response, implying that B cells may contribute to the level of T cell priming (10–13). Recent reports confirm that Ag-bearing B cells induce naive CD4 cell proliferation in vivo (14, 15), and studies of the progressive migration of subsets of cells within lymphoid tissue during the CD4 response to Ag indirectly support a hypothesis that precisely regulated interactions with APC influence the magnitude of the response (14, 16–18).

DC that localize in the T cell zones are the primary APC for the initial expansion of naive CD4 cells (19). Naive B cells are also transiently found in the T cell zone as they migrate to the follicles or B cell areas (16, 18). After Ag exposure, B cells expand in the T cell zone where CD4 memory cells appear to primarily arise (20) and the response then proceeds to the follicle (18). Either engagement of the B cell receptor (BCR) by Ag (21, 22) or ligation of CD40 by CD40 ligand (CD154) expressed on activated T cells (23) can rapidly elicit expression of B7-2 (CD86) on B cells which would enable them to provide costimulation for naive CD4 cells. Thus, although CD4 cells may first encounter Ag presented on DC, shortly after a response is underway, B cells could become competent APC. As Ag-specific B cells proliferate, a bias toward usage of B cells as APC by the expanding T cell population in the face of limiting numbers of DC would likely occur, providing a means to sustain further proliferation of both T and B cells engaged in the response.

Since the extent of CD4 cell expansion during a primary response might impact the development of a persistent memory population, we asked whether B cells have an essential role for the development of memory CD4 cells using B cell-deficient mice. Our results indicate that although CD4 cell priming occurred in the

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Abbreviations used in this paper: DC, dendritic cells; BCR, B cell receptor; KLH, keyhole limpet hemocyanin; LDA, limiting dilution analysis; NP, N-phenyl acetyl.

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absence of B cells, there were significantly lower frequencies of both primary and memory Ag-specific cells. The deficiency was unaffected by boosting with Ag under conditions that favor CD4 cell priming via DC (24, 25), but was completely reconstituted when B cells were provided. Moreover, B cells activated in vitro with IL-4 and either cognate or noncognate Ag could also restore CD4 priming in B cell-deficient mice. The results provide direct evidence that B cells are necessary for the optimal development of memory in the CD4 population by regulating the magnitude of the primary CD4 cell response independently of Ag presentation.

Materials and Methods

Mice

C57BL/6 mice were bred at The Scripps Research Institute. C57BL/6 Ig̅6-μMT (Ig μ chain knockout) mice (26) were purchased from The Jackson Laboratory (Bar Harbor, ME).

Abs and Ags

mAb for the depletion of T and B cells were generated and purified as previously described (27). These included anti-Thy 1.2 (HO13.14, F7D5), -CD8 (3.155), -CD4 (RL172.4), -B220 (RA36B2), -J11d, anti-class II (CA-4.A12, anti-i-Aββ), and mouse anti-κ rat chain (MAR.18), FITC-antimouse κ, -anti-i-Aβ, -B7-2, -CD40, -ICAM-1, -LFA-1, and PE-anti-B220, -CD4, and -Thy 1.1 were produced from PharMingen (La Jolla, CA). Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem (La Jolla, CA) and OVA was obtained from Sigma (St. Louis, MO). For pulsing of B cells, KLH and OVA were conjugated with 4-hydroxy-3-nitrophenyl acetyl (NP; Cambridge Research Biochemicals, Valley Stream, NY) (28).

B cell and DC isolation and immunizations

To reconstitute B cell-deficient mice, λ light chain-bearing B cells were isolated from a preparation of negatively enriched splenic B cells (Stem Cell Technologies, Vancouver, Canada) by sorting for κ B220+ cells with a FACSC Vantage flow cytometer (Becton Dickinson, Mountain View, CA). These cells were pulsed by overnight culture with NP-KLH or NP-OVA at 100 μg/ml in RPMI 1640 media (Irvine Scientific, Santa Ana, CA) containing 7% FCS (HyClone, Logan, UT), 200 μg/ml penicillin, 200 U/ml streptomycin, 4 mM l-glutamine, 10 mM HEPES, and 5 × 10−7 M 2-ME, and supplemented with 10 ng/ml IL-4. Two × 105 freshly isolated or Ag pulsed, 1′ B cells were injected i.v. into recipient mice that were either unprimed or immunized 1 day earlier by i.p. injection of 100 μg KLH precipitated with alum and combined with Bordetella pertussis vaccine organisms as described previously (29). Splenic DC were isolated by magnetic separation from C57BL/6 or μMT mice using negative selection with a cocktail of mAb from Stem Cell Technologies that included anti-CD3, -CD4, -CD8, -B220, -Gr-1, and -TER mAb according to the manufacturer’s instructions. There was <1% B cell contamination in DC preparations from C57BL/6 mice. DC were pulsed by overnight culture with 100 μg/ml KLH or NP-KLH, and 2–5 × 104 were injected into recipient mice.

In vitro responses of CD4 cells

CD4 cells were enriched from spleens at various times after immunization by cytotoxic depletion with anti-CD8 mAb and panning on 150-μm plates (Fisher Scientific, Pittsburgh, PA) coated with polyclonal goat anti-mouse IgG (H + L specific; Fisher Biotech). The resulting populations were 70% CD4+ cells. For in vitro restimulation of CD4 cells, APC were spleen cells from normal C57BL/6 mice that were pulsed overnight with 100 μg/ml KLH in the presence of 10 μg/ml dextran sulfate and 5 μg/ml LPS (Difco, Detroit, MI) to induce activation, followed by treatment with 25 μg/ml mitomycin C (Sigma) as described previously (28). Viable CD4 cells enriched from lymphoid tissues of KLH-primed mice were quantitated by flow cytometry using PE-anti-CD4 and cultured at the concentrations indicated in the text in triplicate in 250 μl in 96-well flat-bottom plates (Costar, Cambridge, MA) with 2 × 105 KLH-pulsed APC. Limiting dilution analysis (LDA) was performed as previously described (30, 31) by plating serial 1.5-fold dilutions of viable CD4 cells in 36-well replicates with KLH-pulsed APC. To evaluate IL-2 production, culture supernatants were harvested at 36 h and 25-μl volumes from individual wells were tested for the presence of IL-2 by measuring proliferation of the NK-3 cell line (32) as previously described (31). The bioassay is specific for IL-2 when IL-4 is blocked by anti-IL-4 mAb (11B11). IL-2 was quantitated by comparison of test supernatants to standard curves generated with recombinant cytokines (R&D Systems, Minneapolis, MN). The sensitivity of detection for IL-2 is 0.2 pg/ml. For LDA, wells giving cpm values of >3 SDs above the mean of values obtained from 36 wells containing APC only were considered positive. Frequencies were calculated using maximum likelihood analysis (33).

Results

Reduced CD4 cell priming in the absence of B cells

Despite convincing evidence that activated B cells are competent APC for naïve T cells both in vitro and in vivo, the importance of the APC function of B cells in vivo has been widely debated in part because of conflicting data from a number of different models. In particular, studies of B cell-deficient mice, μMT mice that have a targeted deletion of the μ region (26) or mice in which the JH region (JHD) (34) of the IgM locus is disrupted, suggest both that B cells are completely unnecessary as APC for primary CD4 cell responses, as measured by proliferation and cytokine production to protein (4, 5) and viral (9) Ag, and that B cells are necessary as APC for protein (35) but not peptide Ag (36) and for responses to some parasites (37). To assess the requirement of B cells in the development of memory CD4 cells in vivo, we initially compared CD4 responses to KLH in μMT mice vs normal C57BL/6 mice over a 3-mo time period.

As shown in Fig. 1, CD4 cells from the spleens of both μMT and control mice responded to i.p. immunization with KLH and adjuvant as measured by the secretion of IL-2, the principle cytoxin produced in response to restimulation with Ag-pulsed APC in this model (28, 29). As previously reported, the absence of B cells did not prevent CD4 cell priming (4, 5, 21). IL-2 secretion at 5 days represents the peak response of primary effectors (28), whereas that at 3 mo after priming reflects the Ag recall response of resting memory CD4 cells (29). However, when we compared
levels of cytokine production by CD4 cells from control and μMT mice, we found that CD4 cells primed in B cell-deficient mice consistently produced lower levels of IL-2 than did CD4 cells from C57BL/6 controls. The results suggest that B cells can play a role in priming of CD4 cells.

Potential explanations, which alone or in combination could account for the observed differences in IL-2 production in B cell-deficient mice, include decreased expansion of CD4 cells in the absence of B cells and/or a lower level of cytokine production per cell. Therefore, we determined whether limited expansion of IL-2-producing T cells resulted in reduced IL-2 levels in cultures of CD4 cells primed in the absence of B cells. We analyzed the frequencies of KLH-specific cells from groups of C57BL/6 and μMT mice over a 6-mo period after priming with KLH by LDA using a highly sensitive bioassay for this cytokine (30, 31). This assay allows the detection of KLH-specific CD4 cells after a brief culture period of 36 h to stimulate cytokine secretion. The results of the frequency analysis of KLH-specific CD4 cells from individual mice shown in Fig. 2 revealed that indeed, 5- to 40-fold fewer Ag-specific CD4 cells producing IL-2 were generated in the absence of B cells. Moreover, in C57BL/6 mice, frequencies similar to those found at the peak of the primary response were observed in the memory population during the 6 mo of observation. A gradual initial reduction in the number of Ag-specific CD4 cells was evident in μMT mice, but frequencies then stabilized. Before immunization, frequencies of IL-2-producing KLH-responsive cells were <1/300,000–1/600,000, indicating that significant expansion occurred in the CD4 population as a result of priming with KLH.

To determine whether a lower production of IL-2 might also contribute to diminished cytokine levels in cultures of CD4 cells from KLH-primed μMT mice, the amount of IL-2 per cell was determined at limiting dilution, where it was estimated that ≤1 cell/well was plated. As shown in Fig. 3, CD4 cells from KLH-primed μMT mice produced similar levels of IL-2 per cell compared with cells from C57BL/6 controls. The average IL-2 secretion/KLH-specific CD4 cell from normal C57BL/6-primed mice \((n = 5)\) >30 days after priming was 1.36 ± 0.18 pg, whereas the average IL-2 amount from a single cell from matched μMT mice \((n = 5)\) was 1.66 ± 0.47 pg. The results indicate that differences in frequency were the primary defect observed in the CD4 population of μMT mice; although we cannot rule out the possibility that IL-2 production may be altered in the μMT model without any effect on the expansion of total KLH-specific CD4 cells.

The results are consistent with studies of normal mice depleted of B cells by anti-μ Abs, which have suggested that reduced CD4 cell responses develop in the absence of B cells (10–13). Although a 2–3-fold reduction in IL-2 production was observed in bulk cultures of CD4 T cells from μMT mice, a 5–40-fold reduction in the frequency of IL-2-producing CD4 T cells was found by LDA. Among the many mechanisms that may contribute to such differences may be the consumption of IL-2 by cells in culture, the presence of cytokines or cell interactions that may inhibit or promote IL-2 production (e.g., the production of IFNγ), or simply the limitations of the LDA in detecting all IL-2-secreting cells.

To determine whether the reduced expansion of CD4 cells in μMT mice could be overcome by multiple immunizations that would result in repeated exposure of CD4 cells to Ag presented by DC, μMT and control mice were primed with KLH on three occasions at weekly intervals before resting for 30 days and evaluation of the frequencies of Ag-specific cells. The results in Fig. 4 show that the number of Ag-specific CD4 cells in either μMT or C57BL/6 mice was not significantly affected by boosting with Ag in adjuvant either once (data not shown) or twice.
comparable priming of CD4 cells to KLH was seen at 5 days after immunization as measured by IL-2 production from bulk cultures. In addition, we found that the spleens of μMT mice contained similar numbers of DC to C57BL/6 mice (data not shown). Therefore, DC from μMT mice are not only present in comparable numbers but also possess a comparable capacity to present Ag.

B cells are required APC to reconstitute CD4 memory

The foregoing results point to a defect in the initial expansion of CD4 cells resulting in diminished levels of CD4 memory in the absence of B cells that could not be attributed to defective DC. To directly assess the role of B cells as APC, we studied the effects of B cell reconstitution of μMT mice on the CD4 response to KLH. We took advantage of the well-characterized response of IgH<sup>−</sup> mice to the nitrophenyl (NP) hapten in the B cell population bearing the λ light chain of Ig (39). Enrichment of λ<sup>−</sup> B cells from these mice results in the presence of a high FACS sorted frequency of normal, naïve B cells that bind NP (40). We transferred λ-bearing B cells into μMT mice immunized with NP-KLH and determined the frequency of memory CD4 cells 30 days later. Consistent with our previous findings, the frequency of memory CD4 cells generated in μMT mice was ~30-fold lower than that in C57BL/6 mice (Fig. 6). As predicted, reconstitution of μMT mice with B cells enriched for Ag recognition restored the frequency of memory CD4 cells generated, thus verifying the need for B cells for the optimal expansion of memory CD4 cells.

To further explore the role of B cells in the priming of memory precursors, we primed mice with KLH and adjuvant 1 day before injecting B cells. We chose this model since it approximates a physiological situation where we envision that initial priming of naïve CD4 cells occurs via DC (25) and activated B cells assume a greater role in cell-cell interactions as the response progresses. λ<sup>−</sup> B cells were used as a source of Ag-presenting B cells since a high frequency of these cells bind to the hapten NP (39, 40). These B cells were pulsed by overnight culture with KLH and adjuvant, thus verifying the need for B cells for the optimal expansion of memory CD4 cells.

Normal DC function in B cell-deficient mice

Previous studies have shown that CD4 cell function in μMT mice is normal (4, 12). Although priming of μMT mice was successful, albeit at reduced levels compared with control mice, we determined whether the Ag-presenting function of DC was impaired in B cell-deficient mice. This was especially relevant since JHD mice, which are rendered B cell deficient by disruption of the JH region of the IgM locus (34), do have impaired APC/costimulation function that contributes to lower levels of CD4 priming (35). Therefore, to rule out a similar condition in μMT mice, we isolated DC from the spleens of C57BL/6 or μMT mice by magnetic separation, pulsed them with KLH, and used them to prime normal mice. Under such circumstances, T cell priming has been shown to be directly induced by Ag-bearing donor DC in the absence of Ag transfer to host APC (38), which we have confirmed in our model (data not shown). The results in Fig. 5 demonstrate that irrespective of whether DC were derived from C57BL/6 or μMT donors,
NP-KLH-pulsed IL-4-treated \( \lambda^+ \) B cells. On day 5 (primary effector) or \( >30 \) days (memory) after KLH priming, responses of CD4 cells from \( \mu \)MT mice that did not receive B cells were compared with those from \( \mu \)MT mice that were reconstituted with B cells and to C57BL/6 controls.

The results shown in Fig. 8 indicate that provision of Ag-pulsed activated B cells to \( \mu \)MT mice shortly after immunization with KLH completely reconstituted the frequencies found in the primary response as well as the memory response to levels found in C57BL/6 controls. The results clearly demonstrate that a B cell deficit can alone account for diminished priming of CD4 cells to KLH, and this results in a reduced frequency of CD4 cells that persist as memory cells. Moreover, not only does this system of adoptive transfer reinforce the findings shown in Fig. 6 but it also enables the elucidation of the nature of the T-B interaction. Although not shown, we were unable to achieve a similar level of restoration with freshly isolated B cells presumably because few B cells in the \( \lambda^+ \) population would have the capacity to specifically take up KLH and become activated in vivo. \( \lambda^+ \) B cells cultured with rIL-4 alone but not NP-KLH were only minimally able to restore the response of \( \mu \)MT mice to KLH (data not shown), suggesting that BCR-mediated Ag uptake plays an important role in activating B cells.

We could detect \( \lambda^- \) B cells in the spleens of \( \mu \)MT mice that had received either resting or activated populations by histological and flow cytometric analysis, and homing of B cells to the spleen was comparable to that seen in C57BL/6 mice (data not shown). By fluorescent dye analysis (42), we found that transferred, Ag-pulsed/activated \( \lambda^- \) B cells divided shortly after injection into either \( \mu \)MT or control mice. However, B cells were no longer detected in the spleen by 14 days, suggesting that rejection had occurred, as reported in other studies (35, 43), or that the transferred B cells either did not survive for an extended period or did not persist in the spleen once the primary CD4 response subsided. Thus, the requirement for B cells for optimal priming of CD4 cells and generation of a persisting high frequency of memory cells is critical at the early stages of the response.

The mechanism by which B cells enhance memory CD4 cell expansion can be envisioned in several ways. B cells can efficiently activate T cells through their ability to capture Ag via the BCR and present peptide in the context of MHC class II molecules to the T cell. Alternatively, activated B cells may interact with T cells through other molecules, such as costimulatory molecules and their ligands, e.g., B7-2/CD28, CD40/CD40 ligand, and/or through other yet to be defined molecules. In the former scenario, T-B interaction would necessitate cognate Ag interaction. However, in the latter case, T-B interaction minimally requires that B cells be activated; thus the requisite for T-B interaction through linked (cognate) recognition of Ag is eliminated. The Ag noncognate interaction between T cells and activated B cells may not be unique to B cells in that other cells, such as DC, may function in a similar capacity.

To test these mechanisms, groups of \( \mu \)MT or control mice were immunized with KLH in adjuvant and 1 day later \( \lambda^- \) B cells pulsed with NP-OVA (noncognate) or NP-KLH (cognate) in the presence of IL-4 were transferred. One month after the transfer of B cells, the frequencies of KLH-specific CD4 cells were quantitated. As shown in Fig. 9, B cells that were pulsed with NP-OVA were as effective as those pulsed with NP-KLH in increasing the frequency of memory CD4 cells generated in \( \mu \)MT mice. Based on this, one might predict that the additional transfer of Ag-pulsed DC to the \( \mu \)MT mice would also be effective in increasing the frequency of memory CD4 cells. As shown in Fig. 9, this is also the case. Thus, at the very minimum, further expansion of primed T cells does not require Ag cognate interactions.

**Discussion**

The introduction of foreign Ag and presentation of processed peptides by class II MHC molecules on APC in the presence of optimal costimulation initiates the activation of naive CD4 cells that is followed by Ag-driven T cell proliferation, differentiation to cytokine-secreting effectors, and selection of memory CD4 cells. Although DC are the predominant APC population to prime naive T cells (19), B cells can also clearly initiate in vivo responses and induce CD4 cell expansion (14, 15). Consequently, it has been difficult to delineate the requirement for B cells as APC. In this study, we have addressed two key issues regarding memory in the CD4 population: the role of APC in the generation of memory CD4 cells and the need for expansion in the primary CD4 response to achieve a high frequency of persistent memory cells. Our results provide direct evidence that B cells can have an essential role in vivo to amplify the CD4 response to Ag and that the size of the memory CD4 pool is determined by the degree of B cell-dependent T cell expansion that occurs in the primary response.
The T-B cell interactions that support CD4 cell expansion need not be linked via recognition of cognate Ag; thus other activated APC have the capacity to serve this function. However, the anatomical location of different APC could preclude them from participating in the response in this role. After Ag stimulation in vivo, activated B cells are present in larger numbers in lymphoid organs than are DC or macrophages, making them the choice candidate for interaction with primed T cells. Moreover, recent studies indicate that DC mature after encounter with inflammatory stimuli and diminish in their ability to function as APC (19). DC also appear to exit lymphoid tissue within 48 h after priming (44), suggesting that the capacity of DC to sustain a CD4 response in situ is limited. Our results provide direct evidence that B cells can be essential for optimal priming of CD4 cells in vivo and that the frequencies of primed CD4 cells generated as a consequence of initial immunization are reflected in a stable pool of long-term memory cells.

Although some reports have failed to detect differences in CD4 priming in C57BL/6 and μMT mice, many in vitro studies suggest that B cells are necessary in addition to DC as APC for optimal priming and responses of CD4 cells (35, 45–47). Moreover, early in vivo studies showed reduced CD4 cell responses develop when B cells are depleted from normal mice by treatment with anti-IgM Ab (10–13). More recently, analyses of CD4 responses in B cell-deficient mice have in many instances shown diminished CD28-dependent responses to foreign proteins (35, 36) and pathogens (37). In our model, we show that a much lower response in B cells is not necessarily dependent on TCR engagement via peptide/MHC class II.

The results of our study demonstrate that, as has been shown for CD8 cells (53), the initial clonal burst size in the primary response determines the extent of CD4 memory that develops and is then maintained. In our model, an optimal primary effector response translates into a maximal level of memory. Similar results have been reported for CD4 cells after viral immunization (9, 54). The results imply that for CD4 cells conditions that most effectively promote development of primary effector populations in vivo correspond to those that are best for the generation of memory. Because of the close correspondence in frequency, it is most likely that memory CD4 cells develop directly from effector cells rather
than exclusively from a separate population of precursors, as suggested in recent studies of CD8 cells (55). However, unlike CD4 cells, CD8 cells expand enormously after viral infection and then decline dramatically as the response contracts (2, 56, 57), suggesting that expansion and survival of CD4 and CD8 cells are regulated differently.

Our results showing that memory CD4 frequencies are determined during the primary response to Ag add to an increasing number of characteristics of T cell memory that are fielded as primary effector cells. Recent studies indicate that Ag-driven selection of CD4 cells with high-affinity TCRs that comprise the persistent memory population is completed within the first few days of the primary response (20). Likewise, strongly polarizing conditions to generate Th1 or Th2 primary effecter CD4 cells by 3 or 4 days in culture result in similarly polarized CD4 memory cells after adoptive transfer (58). These findings underscore the critical importance of designing vaccines to induce optimal priming of appropriate CD4 effecter cells in response to initial immunization with Ag. Our data suggest that Ag targeted exclusively to DC as a vaccine strategy may not induce the optimal development of CD4 memory. Protocols that promote priming of B cells to provide costimulation for responding primary CD4 cells will achieve a much higher overall level of memory in the CD4 population. We conclude from our study that in vivo B cells provide a significant contribution to CD4 memory by promoting the generation of high frequencies of Ag-specific cells, a hallmark of immunological memory.

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