CD4+ T cell responses in mice lacking MHC class II molecules specifically on B cells

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The role of B lymphocytes in initiating and maintaining a CD4+ T cell response has been examined using a variety of strategies, but remains controversial because of weaknesses inherent to each of the approaches. Here, we address this issue by measuring CD4+ T cell priming both in mutant mice devoid of B cells and in chimeric animals lacking major histocompatibility complex class II molecules specifically on B cells. We find that peptide and some protein antigens do not require B cells expressing class II molecules, nor B cells themselves, to efficiently prime. This could be demonstrated by the usual lymph node proliferation assay, a rather indirect *in vitro* measure of priming, and by a direct *ex vivo* assay of population expansion and activation marker expression. Interestingly, one protein antigen, conalbumin, could not prime in the absence of B cells, but could in the presence of B cells devoid of class II molecules. This finding constrains the possible mechanisms whereby B lymphocytes contribute to the initiation of a CD4+ T cell response, arguing against the importance of surface immunoglobulin-mediated antigen presentation by B cells.

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1 Introduction

B cells are one of the major cell types in the body expressing MHC class II molecules (reviewed in [1]). This property, coupled with an impressive capacity to take up antigen via their surface Ig receptors [2, 3], makes them ideal for presenting antigens to naive CD4+ T cells in order to initiate an MHC class II-restricted immune response. However, this potential conflicts with the demonstrated inefficiency of resting B cells to present antigen to naive T cells [4–8].

Several strategies have been used to determine whether B cells are required for initiating responses by CD4+ T cells *in vivo.* Studies employing anti-IgM-mediated elimination of B cells from birth found that they are required for initiating an effective CD4+ T cell response [9–16]. However, experiments relying on reconstitution of severe-combined immunodeficiency (SCID) mice with purified T cell populations gave the opposite result [17–21]. Another strategy was to examine MHC transgenic (tg) mice lacking class II molecules selectively on B cells; here, again, there seemed to be a requirement for class II-expressing B cells to generate a CD4+ T cell response [22]. Most recently, animals genetically deficient in B cell production, mice of either the μMT [23] or JHD [24] lines, have been used to address this issue. A range of results have been reported: CD4+ T cells could not be primed in the absence of B cells [25]; they could be primed with total, or at least a high degree of, effectiveness [26–29]; and whether they could be primed depended on the inciting antigen [30]. In addition, conflicting findings were reported on whether B cells are required for T cell help: they appeared to be needed for T cell-dependent class switching [25, 29, 31], but not for certain other help-mediated functions [26, 31]. The B cell-deficient mouse lines have also been used to explore the role of B cells in activation-induced cell death or tolerance induction [27, 28, 32], the maintenance of memory [33, 34], autoimmunity [35, 36] and allergy [37, 38].

At least some of the conflicting conclusions might result from drawbacks of the different strategies to produce B cell-deficient animals. The anti-IgM treatment protocol is very aggressive, beginning at birth and involving repetitive injections of large doses of antibody; such treatment

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could well compromise the function of other types of antigen-presenting cells (APC). Transfer of purified T cells into SCID mice risks co-transferring some B cells or multipotential lymphoid cell progenitors, and has the additional complication that the mature T cells transferred could change phenotype when introduced into an "empty" vessel. MHC tg mice with a compartmentalized deficiency in expression of class II molecules might have more complicated defects than first apparent – for example, lack of expression on a rare, but critical, APC. Employing mice with a genetic deficiency in B cell production would appear at first glance to be the cleanest approach, but it is possible that these animals harbor unsuspected immune system defects. For example, it might be that B cells, regardless of their Ig specificity, must be present for the development of normal spleen or lymph node architecture; very recent data indeed suggest that this might be true [39]. Conflicting conclusions also probably arise because of variations in the mode of assaying T cell priming, *i.e.* different antigens, differences in protocols for what is a rather indirect assay.

Here, we have employed a new strategy for assessing the role of antigen presentation by B cells in the priming of $CD4^+$ T cells, designed to overcome some of the problems of the previous approaches. Paired chimeras were constructed reconstituting lymphoid compartments in recombination activating gene (Rag)-deficient mice, by injecting them with bone marrow cells from either wild-type or MHC class II-deficient animals. Mice reconstituted with class II-deficient precursors should be able to generate a large repertoire of $CD4^+$ T cells through the class II molecules of the host thymic stroma and have class II-positive dendritic cells and macrophages, but have donor-derived B cells in good numbers entirely devoid of class II molecules. After reconstitution of their immune systems, the chimeras were immunized with various antigens, and the activity of $CD4^+$ T cells was evaluated in *in vitro* lymph node proliferation assays, by a direct *ex vivo* assay, or by measuring *in vivo* production of Ig isotypes. This strategy allowed us to compare animals differing only by the expression of MHC class II molecules on B cells. B cells were physically present, however, avoiding any artifacts resulting from their absence, and allowing us to directly measure antigen-specific Ig production.

2 Results

2.1 Generation of mice with B cells lacking MHC class II molecules

A bone marrow reconstitution protocol was used to generate mice with a normal immune system except that B cells were devoid of MHC class II molecules. Bone marrow cells from either C57BL/6 (B6) or MHC class II-deficient $A\beta^{0/0}$ [40] animals were depleted of mature T and B lymphocytes and injected into young $Rag^{0/0}$ animals. The resulting chimeras should either express class II molecules on all of the usual cell types (TB) or lack them specifically on B cells (TII^0). The TII^0 chimeras should express class II molecules normally on stromal cells in the thymus and periphery, and therefore harbor the usual complement of $CD4^+$ T lymphocytes, while non-B hematopoietic cells, such as macrophages and dendritic cells, should be a mixture of class II-positive and class II-negative. To control for reconstitution, we analyzed peripheral blood lymphocytes by flow cytometry.

To establish that lymphocyte differentiation was as expected in the chimeras, we examined thymus, spleen and lymph nodes at various times after the injection of bone marrow cells. As illustrated by the representative CD4/CD8 profiles in Fig. 1 panels A and B, T cells were present in the thymus and lymph nodes of mice 8 weeks after injection. The profiles in panel C demonstrate that the chimeras also harbored normal $B220^+IgM^+$ B cell compartments, and that the B cells in TB and TII^0 animals were all class II positive and negative, respectively.

2.2 *In vitro* assays of $CD4^+$ T cell priming

To test whether class II-positive B cells are required for antigen-specific priming of $CD4^+$ T cells, we immunized control and chimeric mice in the footpad with various peptide and protein antigens, removed the draining lymph nodes 9 days later, and restimulated enriched $CD4^+$ T cells with antigen and B6 APC. This protocol focuses on any potential differences in priming *in vivo* because the *in vitro* secondary responses are elicited under the same conditions – in particular, any antigen-specific (or other) B cells are removed, and equal numbers of class II-positive APC are added.

Data from several peptide antigens are pooled in Fig. 2. As expected, cells from the B6-positive controls responded, while those from $A\beta^{0/0}$-negative controls did not. Cells from the TII^0 chimeras responded as well as those from the TB chimeras, and those from the B6 controls. This was consistent with the normal response by $\mu MT^{0/0}$ cells, as expected from published data [30, 31]. These findings indicate that $CD4^+$ T cells can be effectively primed by peptide antigens in the absence of class II molecules on B cells, and even in the total absence of B cells.

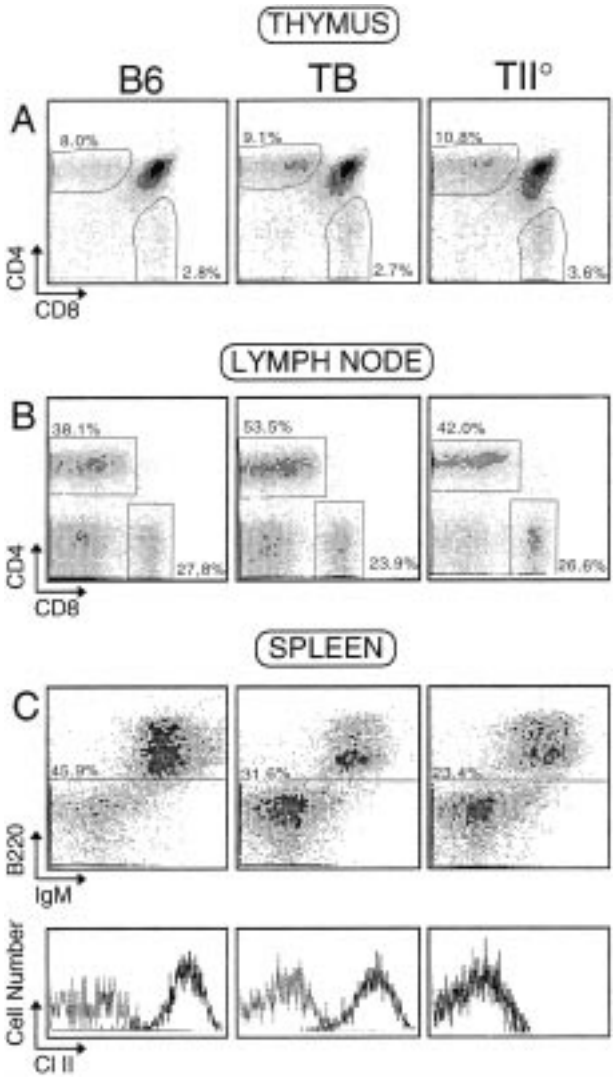

Figure 1. Lymphocyte compartments in reconstituted mice. (A) Representative CD4/CD8 profiles of thymocytes from reconstituted mice, 8 weeks after bone marrow transfer. B6, standard inbred B6 mice; TB, $Rag^{0/0}$ mouse reconstituted with B6 bone marrow; TII^0, $Rag^{0/0}$ mouse reconstituted with $A\beta^{0/0}$ bone marrow. (B) CD4/CD8 profile of lymph node lymphocytes from the same mice as in A. (C) B lymphocytes in spleen. Top panel, B220/IgM profiles of splenocytes. Lower panel: anti-class II staining on B220-positive cells from the upper panel. The dotted line represents control staining with an irrelevant antibody.

The different mice were also immunized with whole protein antigens in order to see whether a requirement for antigen processing would reveal any dependence on B cell presentation. As indicated in Fig. 3A, the TII^0 cells responded as well as those from TB or B6 mice, all clearly better than those from the $A\beta^{0/0}$ negative controls. The $\mu MT^{0/0}$ cells also responded normally, again as

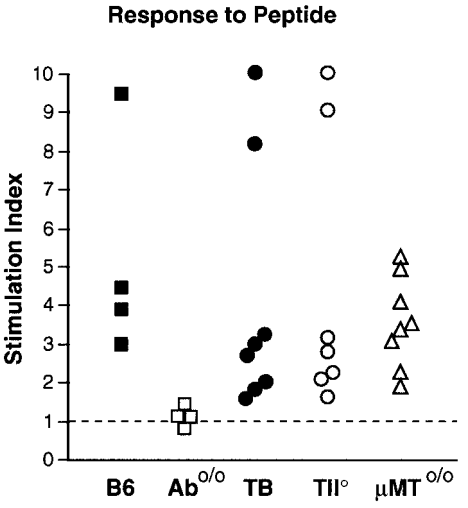

Figure 2. In vitro assay of priming to peptide antigens. Nine days after immunization of B6, μMT, $A\beta^{0/0}$, TB, and TII^0 mice (symbols as defined in Fig. 1), draining lymph nodes were removed, and purified lymph node $CD4^+$ T cells were cultured with 3×10^5 B6 spleen cells as APC, in the presence of graded concentrations of peptide. After 72 h, cultures were pulsed with [^{3}H]thymidine for 16 h, and incorporated thymidine was counted. Results are shown as stimulation index (at optimal peptide concentration counts incorporated in wells with peptide antigen divided by background counts in the absence of peptide). Each point represents an individual mouse, and the data shown are representative of several experiments using three different peptide antigens [Eα(52–68), Ova(323–339), and LCMVgp(61–80)].

expected from published data [26–30]. It is possible that, while $CD4^+$ T cells can be primed in $\mu MT^{0/0}$ and TII^0 mice in response to ovalbumin or other proteins, the absence of class II-positive B cells in these animals might alter the particular epitope favored. Ova (323–339) is the dominant epitope recognized in the anti-ovalbumin response in B6 mice. Therefore, we modified the assay, with Ova (323–339) peptide rather than with ovalbumin protein in the recall cultures. Again, there was an equal response by cells from the TII^0 and TB chimeras (Fig. 3A). These data show that it is possible to prime $CD4^+$ T cells with a foreign protein in the absence of class II molecules on B cells or of B cells, themselves.

However, a very different picture emerged when another protein antigen, conalbumin, was assayed. As shown in Fig. 3B, cells from $TBII^0$ mice responded just as well as TB and B6 cells; however, in agreement with previous data [30], cells from $\mu MT^{0/0}$ animals did not respond, just like $A\beta^{0/0}$ cells. So, in this case, priming of $CD4^+$ T cells by a protein antigen does not require class II molecules on B cells, but does need the physical presence of B cells.

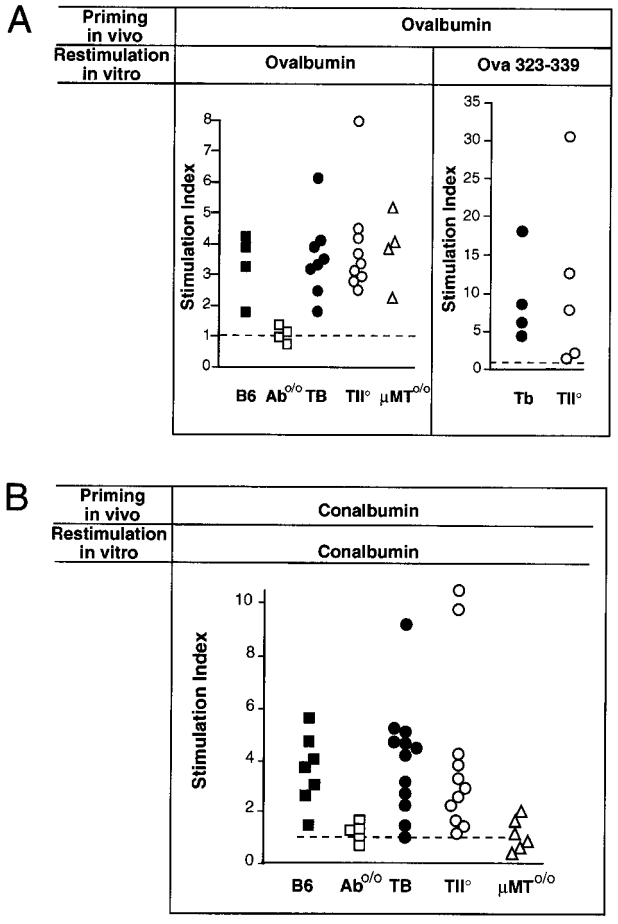

Figure 3. In vitro assay of priming to protein antigens. (A) Response to ovalbumin. Nine days after immunization of B6, μMT, Aβ$^{0/0}$, TB, and TII^{0} mice (symbols as above), draining lymph nodes were removed, and purified lymph node CD4^{+} T cells were stimulated with graded doses of ovalbumin (left panel) or the Ova 323–339 peptide. Each point represents the stimulation index ([^{3}H]thymidine incorporation at 72 h) for an individual mouse, pooled from several independent experiments. The results are somewhat variable but this appears to be a common occurrence with such assays and, among other possibilities, could reflect environmental variables. (B) Response to conalbumin, determined as in A.

2.3 An *ex vivo* assay of CD4^{+} T cell priming

Although our *in vitro* assay was designed to focus on CD4^{+} T cell priming, all aspects of the recall response being equalized, it is a rather indirect measure. We thought it important to have a more direct measure, and therefore devised an assay permitting *ex vivo* visualization of T cell activation and expansion. The same bone marrow reconstitution protocol was used, but bone marrow cells from Smarta TCR tg mice on a μMT$^{0/0}$ background were mixed with either the B6 or Aβ$^{0/0}$ cells before transfer into Rag$^{0/0}$ recipients. Smarta mice express a transgene-encoded receptor specific for LCMVgp (61–80), restricted by A^{b} and composed of Vα2 and Vβ8.3 variable segments [41]; the μMT$^{0/0}$ mutation ensures that the bone marrow cells from the TCR tg mice cannot give rise to class II-positive B cells. Thus, the resulting mixed chimeras should have class II molecules on all the usual cell types (TB/Sm) or on all but B cells (TII^{0}/Sm), and should harbor a population of Smarta-derived CD4^{+} T cells that can be directly monitored using anti-Vα2 and -Vβ8.3 mAb. Reconstituted mice were immunized with LCMVgp (61–80) in one footpad, and at different times after immunization, the draining popliteal and inguinal lymph nodes were removed, as were the contralateral (unimmunized) nodes. Antigen-specific CD4^{+} T cells were stained with anti-Vα2, -Vβ8 and -CD4 mAb and one of several mAb against different activation markers. The Smarta-derived CD4^{+} T cells in the contralateral lymph nodes served as an internal control for the degree of population expansion and levels of expression of activation markers. The Vα2^{+}Vβ8.3^{-}CD4^{+} T cells in each node, presumably unresponsive to the LCMVgp peptide, afforded a second internal control.

Fig. 4A shows cytofluorimetric profiles of the draining popliteal and control lymph nodes from either TB/Sm or TII^{0}/Sm mice. There was a similar two- to fourfold increase in the proportion of Smarta-derived CD4^{+} cells in the draining nodes from the two types of mice 5 days after antigen injection. This degree of increase was consistent in multiple experiments (Fig. 4A, bottom panels), regardless of the percentage of Smarta-derived CD4^{+} cells in the contralateral lymph nodes, and therefore of the proportion of Smarta bone marrow cells originally transferred (on average, 3.8- and 3.9-fold increases for TB/Sm and TII^{0}/Sm mice, respectively, $n = 6$). Profiles of activation markers on Smarta-derived CD4^{+} cells in the draining and control lymph nodes of TB/Sm and TII^{0}/Sm mice were compared as well. Many more cells displaying early (CD25^{+}, CD69^{+}) and late (CD44^{+}, CD62L^{lo}) activation markers were found in the draining lymph nodes in both TB/Sm and TII^{0}/Sm animals (Fig. 4A and data not shown). No differences in numbers of cells with activation markers or in the level of activation marker expression were seen in the two types of chimera. As expected, the Vα2^{+}Vβ8.3^{-}CD4^{+} cells were not activated following antigen injection (not shown).

We also compared the kinetics of the response by examining lymph node cells from mice 3, 5, and 9 days following immunization. At day 3, there were very few signs of CD4^{+} T cell activation in either the TB/Sm or TII^{0}/Sm animals. The data for day 5 were as discussed above; likewise, at day 9, there were no differences in expansion of

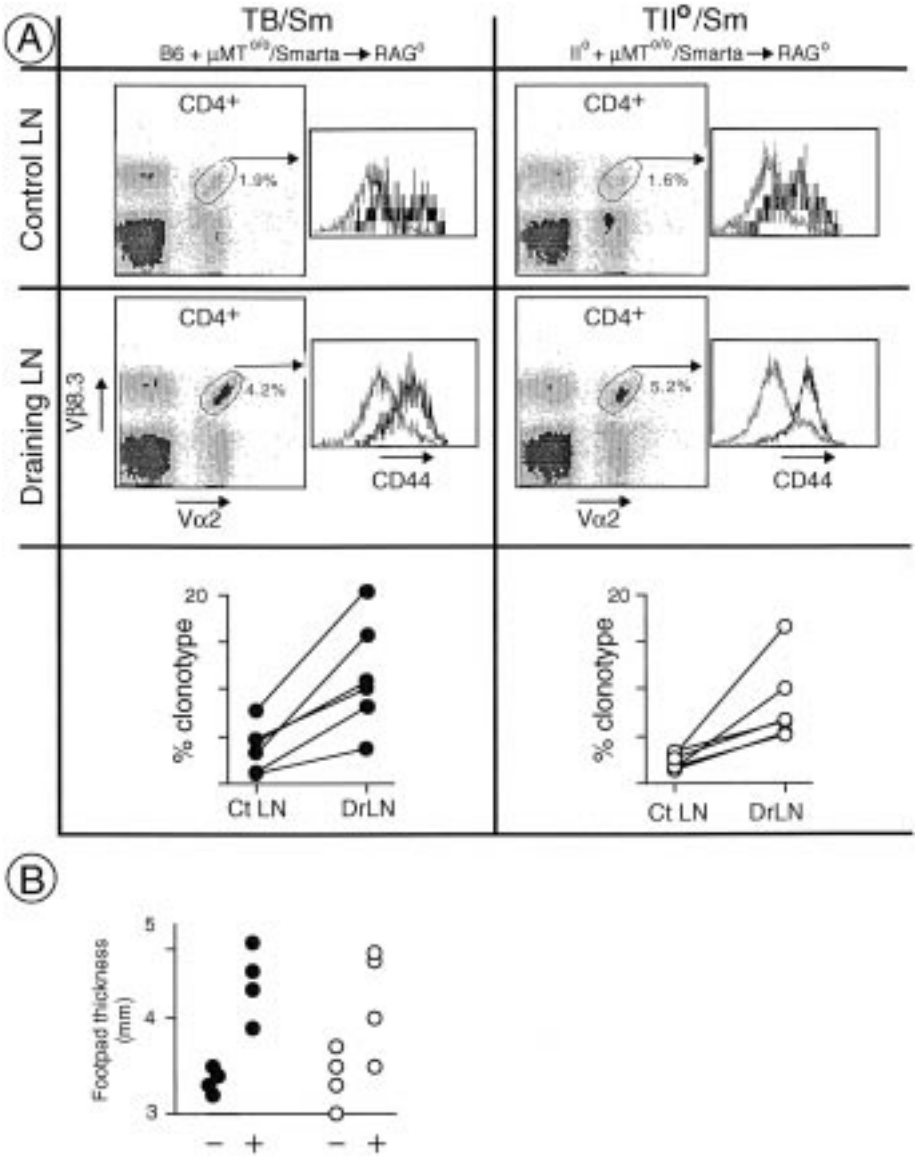

Figure 4. Ex vivo assay of priming. (A) Antigen-specific responses *in vivo.* Two parameter profiles of $CD4^+$ lymph node cells of TB/Sm and TII^0/Sm mixed chimeras immunized with the Smarta peptide. The top panels show the control (contralateral) lymph node, and the middle panels the immunized lymph node, both stained with anti-Vβ8.3 and -Vα2. The CD44 profiles are shown for the $CD4^+V\beta8.3^+V\alpha2^+$ cells. The dotted line on the histograms represents control staining in the absence of anti-CD44 antibody. The results of several such experiments are compiled in the bottom panels, where each point represents the percentage of $V\beta8.3^+V\alpha2^+$ among $CD4^+$ cells. (B) Footpad swelling in $B6^+$Smarta/μMT (solid circles) and TII^{0+}Smarta/μMT (open circles) mixed chimeras immunized with the Smarta peptide. Each point represents the footpad thickness of the unimmunized (–) and immunized (+) hind paws 5 days post immunization.

$CD4^+$ T cells in the two types of mice, nor in their activation markers (data not shown).

Finally, as an additional measure of priming, we quantitated footpad swelling (Fig. 4B). TB/Sm and TII^0/Sm chimeras showed similarly increased thickness in the injected, compared with non-injected, footpads.

2.4 B cell responses

We asked whether, in this context, an MHC class II-mediated T-B interaction was required for Ig class switching in a response to T-dependent antigens. Such a direct *in vivo* assay was not possible in previous studies on $\mu MT^{0/0}$ mice as they are devoid of B cells and there-

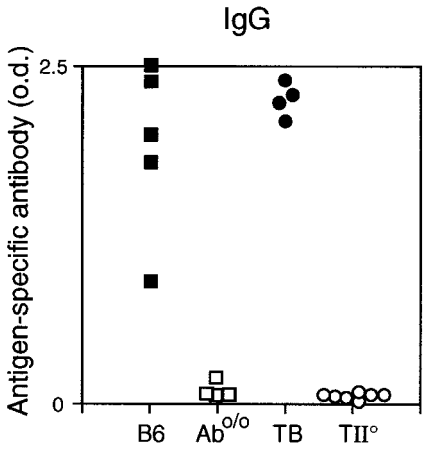

Figure 5. In vivo assay of T cell help. Antigen-specific serum IgG titers in the indicated mice, 2 weeks after immunization with protein antigen in CFA. Reactive IgG was determined by ELISA; each point represents an individual mouse. Data pooled from several experiments using ovalbumin and conalbumin as antigens (standardized on the basis of positive control sera).

fore Ig. Chimeric and control animals were immunized in the footpad with various protein antigens, blood samples taken 10 days later, and antigen-specific Ig quantitated by ELISA. As illustrated in Fig. 5, the TB mice, but not the TII^0 mice, made antigen-specific IgG at this time point. The kinetics of Ig production were evaluated by sampling blood 7, 14, 21, or 35 days after immunization (data not shown). The production of antigen-specific IgM followed the same kinetics in B6, TB, and TII^0 mice; the generation of antigen-specific IgG was similar for the B6 and TB mice, while the TII^0 animals did not produce any during the course of the experiment. Levels of total IgM and IgG in the serum were the same in TII^0 as in B6 and TB animals, indicating that B cells in TII^0 mice are capable of switching to IgG.

3 Discussion

Our data on CD4$^+$ T cell priming in mice devoid of B cells (μMT$^{0/0}$) agree with those previously reported by Constant et al. [30]: peptides and some protein antigens primed effectively, while other protein antigens did so only very inefficiently, or not at all. Most other studies on μMT$^{0/0}$ mice concluded that effective CD4$^+$ T cell priming can take place in the absence of B cells [26–29, 31]. These experiments missed the defect in priming by select protein antigens quite simply because they did not assay the right proteins. For example, several of the studies relied largely, or even solely, on measurements of priming by the commonly used antigen keyhole limpet hemocyanin (KLH). However, we have found that, not only can KLH prime effectively in μMT$^{0/0}$ mice, it can also do so in A$\beta^{0/0}$ animals devoid of class II molecules on all APC (SW, CB, DM unpublished results). This brings into question whether KLH is a T-dependent, class II-restricted antigen at all, at least at the level of priming and, therefore, whether its ability to prime in μMT$^{0/0}$ animals has any relevance to the issue at hand.

The data on CD4$^+$ T cell priming in animals devoid of MHC class II molecules selectively on B cells (TII^0) made several new points. First, it was possible to exploit more direct assays of T cell priming and helper activity. By spiking the inoculate with bone marrow cells from Smarta TCR tg mice, we generated mixed chimeras that allowed us to monitor T cell expansion and activation marker expression directly *ex vivo,* side-stepping several pitfalls of the standard lymph node proliferation test. TB/Sm and TII^0/Sm mixed chimeras showed no difference in CD4$^+$ T cell priming when evaluated by the new *ex vivo* assay. Furthermore, the fact that TII^0 animals actually contain B cells (and produce Ig) permitted us to quantitate T helper activity directly *in vivo* simply by measuring antigen-specific production of Ig isotypes, avoiding *in vitro* tests and the more complicated hapten/carrier transfer tests. According to the direct *in vivo* assay, TII^0 mice were incapable of antigen-specific Ig class switching, even when priming by the same antigen had been effective. This result is in keeping with recent reports showing that priming in the presence of B cells may be required to elicit full helper activity [25, 31], but also with classical experiments which demonstrated the need for MHC restriction in T-B interactions [42–44].

Secondly, our data on priming in TII^0, in comparison with μMT$^{0/0}$, mice imposes constraints on what the role of B cells can be. In agreement with Constant et al. [30], we found that conalbumin was not able to prime CD4$^+$ T cells in μMT$^{0/0}$ animals. Constant et al. favored the explanation that B cells exert their influence by acting as APC, their Ig receptors rendering them particularly effective at presenting the corresponding antigen, as has already been established ([2, 3] reviewed in [45]). However, at least two other explanations are also viable. It is possible that B cell-derived serum Ig, rather than surface Ig receptors, is the key, as it has been demonstrated that Ig/antigen complexes form *in vivo,* can be taken up very efficiently by Fc receptors on non-B APC like dendritic cells and macrophages, and can be presented extremely well [45–48]. In fact, Fc receptor-facilitated presentation can be as effective as Ig-mediated presentation by B cells, in both cases being 10^3- to 10^4-fold enhanced [45]. It is also possible that B cells influence T cell priming independently of Ig, perhaps by secreting cytokines, chemokines or other mediators that influence the micro-

environment wherein the T cell response is initiated. For example, it has been shown quite recently that $\mu MT^{0/0}$ mice have a defect in splenic architecture, attributed to a need for B cell-derived lymphotoxin in order for the network of follicular dendritic cells to develop correctly [39]. The fact that conalbumin cannot prime in $\mu MT^{0/0}$ mice but can in TII^0 animals argues for the second and third explanations over the first. TII^0 animals have serum Ig and presumably B cell-derived mediators, but B cells cannot act as APC because MHC class II molecules are absent. Furthermore, the observation that priming by some proteins but not by others or by peptides is affected in $\mu MT^{0/0}$ mice appears to favor the second explanation over the third. It is easier to rationalize antigen specificity when Ig rather than cytokines, chemokines, etc. are involved. Thus, the data are most readily explained if some protein antigens can only be effectively presented after being complexed with serum Ig and picked up and internalized with the help of Fc receptors. This requirement could be a feature of certain proteins or of particular epitopes within a protein.

Finally, our results highlight the care that needs to be exercised in choosing a system for evaluating the efficiency of $CD4^+$ T cell priming. A variety of peptide and protein antigens must be assayed, and it needs to be rigorously established that the antigens are actually eliciting a T-dependent, class II-restricted response.

4 Materials and methods

4.1 Generation of chimeric mice

Bone marrow was harvested from B6 or MHC class II-deficient ($A\beta^{0/0}$) [40] donor mice. The $A\beta^{0/0}$ mice used as donors were extensively backcrossed to B6 (>10 generations). Mature T cells were removed by treatment with anti-CD4 and -CD8 mAb, followed by complement killing (Lo-Tox, Cedarlane). Surface Ig-positive B cells were removed by panning on plastic plates coated with affinity-purified polyclonal rabbit anti-mouse Ig. Recipients were B and T cell deficient because of a mutation in the Rag-1 gene [49]. They were irradiated 16 h prior to transfer (500 rad from a ^{60}Co source), and received the equivalent of 2×10^6–5×10^6 pre-depletion bone marrow cells, injected i.v. Reconstituted mice were housed under filter tops in a conventional mouse facility. To construct mixed chimeras, we utilized bone marrow from mice of the Smarta TCR tg line, which recognizes the peptide 61–80 from the glycoprotein (gp) of the LCMV virus presented by the A^b molecule [41] and also carried the $\mu MT^{0/0}$ mutation; bone marrow from these donors was mixed with either B6 or $A\beta^{0/0}$ bone marrow (in a proportion of of 1 to 50 %). Eight to ten weeks after reconstitution, blood T cells were analyzed to choose animals having a low but detectable contribution from the Smarta tg donor. These were immunized in one footpad with the LCMVgp peptide. After 3, 5, or 8 days, the draining popliteal, inguinal, and contralateral inguinal and axillary lymph nodes were remoced. Single-cell suspensions were made and stained with anti-Vβ8.3 followed by Texas Red-anti-rat Texas Red, PE-anti-CD4, FITC-anti-Vα2, and one of the following: biotin-anti-CD40, biotin-anti-CD69, biotin-anti-CD44, biotin-anti-CD25, or biotin-anti-CD62L followed by streptavidin-Cy5. Live cells were gated on forward-scatter versus side-scatter profiles. Fluorescence signals were treated by four-decade logarithmic amplification.

4.2 Flow cytometry

Mice were killed and cell suspensions from thymus, spleen and lymph nodes prepared in Dulbecco's modified Eagle medium (DMEM), 5 % fetal calf serum (FCS). Cells were stained with the following reagents; PE-anti-CD4 (Caltag, San Francisco, CA), FITC-anti-CD8 (Caltag), FITC-anti-CD45RA [50] PE-anti-IgM (Jackson Immunoresearch, West Grove PA). All antibodies were used in saturating amounts. Flow cytometry was performed on either a Coulter Elite, or Profile flow cytometer, and data stored as list mode for analysis. Live cells were gated on forward-scatter versus side-scatter profiles. Fluorescence signals were treated by four-decade logarithmic amplification.

4.3 Proteins and peptides

Conalbumin and ovalbumin were purchased from Sigma. The sequences of the peptides used are: Eα(52–68), ASFEAQGALANIAVDK [51]; Ova(323–339), ISQAVHAAHAE-INEAGR [52]; and LCMVgp(61–80), GLNGPDIYKGVYQFKS-VEFD [41].

4.4 Immunization and lymph node proliferation assays

Mice were immunized in the footpads with protein (100 μg) or peptide (50 μg) emulsified 1:1 in CFA. Nine days later, the draining popliteal lymph nodes were removed. $CD4^+$ T cells were enriched from single-cell suspensions by incubating with anti-CD8, -MHC class II, -Mac 1 and -B220 mAb, followed by anti-rat Ig-coated Dynabeads (Dynal, Oslo). $CD4^+$ T cells (10^5–2×10^5) were restimulated *in vitro* with graded amounts of antigen and 2×10^5 irradiated B6 spleen cells as APC. After 72 h, [^{3}H]thymidine was added, the cells harvested after another 12 h, and incorporation was measured using a gas-phase counter Packard matrix 9600 counter.

4.5 Serum Ig assays

Mice were immunized as above with antigen in CFA. Ten days later, they were bled from the tail vein, and total and antigen-specific Ig titers determined by ELISA, as described [40].

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