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Immunology

B cells are not essential for peripheral T-cell tolerance

(staphylococcal enterotoxin A/deletion)

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ABSTRACT **Some self-reactive T cells avoid thymic tolerance and become mature peripheral cells. Nevertheless, these cells do not usually attack their hosts because T cells can be inactivated or killed, even after they are mature, by various means. The details of these processes are not fully understood; however, a number of experiments have suggested that peripheral tolerance may be induced in mature mouse T cells by exposure to antigen on resting B cells, cells that can express antigen bound to major histocompatibility complex proteins but that lack critical costimulatory molecules such as B7-1 and B7-2. Conversely, previous experiments have indicated that mature T cells can be stimulated by exposure to antigen on cells such as dendritic cells, cells that are thought to express the essential costimulatory molecules. We tested this idea *in vivo* by using mice that lack B cells. Unexpectedly, T-cell tolerance and antigen-induced T-cell death occurred normally in mice free of B cells. On the other hand, antigen-specific T-cell expansion in the spleens of such mice was impaired. Finally, we have recently shown that T-cell death in mice can be prevented by exposure to antigen and an inflammatory agent such as bacterial lipopolysaccharide. This was also true in mice that lacked B cells. Overall, these data show that mature T cells can be tolerized and rescued from tolerance in the absence of B cells.**

It is well established that $CD4^+$ T cells can be eliminated in the thymus if their $\alpha\beta$ receptors (TCRs) react with self-antigenic peptides complexed with major histocompatibility complex class II molecules (1–3). Yet it is clear that many self-reactive T cells are present in the periphery of mouse, rats, and humans (4–6). Thus, removal of these autoreactive T cells is essential in order to preserve a nonautoimmune state. Failure to tolerize these self-reactive T cells can lead to progression of an autoimmune disease. Hence, peripheral tolerance mechanisms play a vital role in maintaining the balance that allows $CD4^+$ T cells to be activated and attack foreign invaders but not their own hosts. Exactly how the immune system performs this task is not fully understood.

A paradigm has emerged in recent years suggesting that T-cell stimulation in the absence of costimulation results in abortive activation (reviewed in ref. 7). For example, it is thought that T cells can be fully activated if, after their TCRs are engaged, a protein on their surface called CD28 is bound by its ligand, B7-1 or B7-2 (8–14). In the absence of CD28 costimulation, T cells have been shown, in some cases, to become anergic (15, 16). These anergic T cells by definition are unable to divide because of their lack of interleukin 2 production. In addition, several *in vitro* and *in vivo* studies have shown that antigen presented by dendritic cells, which constitutively express the costimulatory molecules B7-1 and B7-2, stimulates resting T cells very effectively (17–19). On the other hand, antigen presented by resting B cells does not stimulate naive T cells and, indeed, can lead to T-cell tolerance (20, 21). Taken together, these results suggest that resting B cells, the major population of class II^+, $B7^-$ cells *in vivo*, may be very important for induction of peripheral T-cell tolerance *in vivo* and that the absence of B cells might have a dramatic effect on the ease with which mature T cells can be tolerized.

To test this idea, in this study we examined the ability of antigen to tolerize mature T cells in mice that lacked B cells. We found that T cells in B-cell-deficient animals could be tolerized by exposure to antigen or could die in response to superantigen as readily as T cells in wild-type mice could. Both peptide-induced tolerance and superantigen-mediated T-cell death was blocked by coinjection of bacterial lipopolysaccharide (LPS). These results, in conjunction with previous reports, suggest that proinflammatory cytokines like tumor necrosis factor α (TNF-α) can provide survival signals to antigen-stimulated T cells, regardless of whether B cells or non-B cells present the antigen (22).

Finally, we set out to determine whether T-cell clonal expansion was normal in mice lacking B cells. T cells could not be primed with peptide antigens plus adjuvant quite as readily in mice lacking B cells as in normal animals. Likewise, $CD4^+$ T cells did not expand in response to superantigen in B-cell-deficient mice as well as they did in controls, although they were well activated as indicated by expression of the activation marker CD69. Collectively, these data show that B cells are not essential for induction of class II-restricted T-cell tolerance but can "help" antigen-specific $CD4^+$ T cells to expand *in vivo*.

MATERIALS AND METHODS

Mice. $k\mu MT^{-/-}$ and $k\mu MT^{+/-}$ animals, which were *H-2*^{k} homozygous and lacked viral superantigens capable of deleting variable region β-chain (V_β)3^+ T cells, were produced by three generations of selected brother/sister mating of the progeny of (B10.BR × $\mu MT^{-/-}$)F_1 mice. Spleens and lymph nodes from $k\mu MT^{-/-}$ mice contained few if any $B220^+$ cells (<3.0%). Controls were mice generated from a cross of F3 $\mu MT^{-/-}$ mice with B10.BR mice. In control mice ($k\mu MT^{+/-}$), >70% of splenocytes were $B220^+$ and the great majority of these cells were class II positive. The percentage of Mac-1/class II double-positive large cells in $k\mu MT^{+/-}$ mice was ≈1/4 that of $k\mu MT^{-/-}$ mice.

Immunization Protocol. Mice were immunized s.c. by injecting a 1:1 (vol/vol) ratio of pigeon cytochrome *c* (PCC) 88-104 (5 mg/ml) (Molecular Resource Center, National Jewish Center, Denver) in balanced salt solution (BSS) to complete Freund's adjuvant (CFA) in 50 μl. For *in vivo* stimulations with superantigens, staphylococcal enterotoxin A (SEA) (Toxin Technology, Sarasota, FL) was injected i.p. in 200 μl of BSS.

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Abbreviations: TCR, T-cell receptor; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α; V_β, variable region β chain; PCC, pigeon cytochrome *c*; SEA, staphylococcal enterotoxin A; APC, antigen presenting cell.

Stimulation Assays. To purify T cells, draining lymph nodes were teased through nylon mesh (Falcon) and single cell suspensions were placed over nylon wool columns as described (23). Routinely, >85% of the purified cells were TCR positive. Antigen presenting cells (APCs) were prepared from single cell suspensions from spleens of B10.BR mice (The Jackson Laboratory) as follows. Spleen cell suspensions were freed of red blood cells by lysing with ammonium chloride. The cells were then incubated with anti-thymocyte antiserum on ice for 30 min, washed, and then incubated with low tox-M rabbit complement (both reagents from Accurate Chemicals). T-depleted spleen cells (3×10^7 cells per ml) were treated with mitomycin C (25 μg/ml) (Sigma) or irradiated with 3000 rads. For stimulations, T cells were placed in wells of a 96-well plate (Falcon) and titered using 3-fold dilutions starting at $4-6 \times 10^5$ T cells. To each well, 5×10^5 APCs were added followed by the addition of peptide or SEB (Toxin Technology). The cells were harvested on a Harvester 96 Mach III M (Tomtec, Orange, CT) and the incorporation was determined on a Microbeta PLUS liquid scintillation counter (Wallac, Turku, Finland).

Flow Cytometry. Two-color flow cytometry was carried out as described (24). Briefly, biotinylated anti-$V_\beta 3$ (KJ25-607.7; ref. 25) was incubated with 10^6 T cells for 30 min at 37°C in staining buffer (Dulbecco's phosphate-buffered saline containing 2% fetal bovine serum and 0.1% sodium azide). After several washes, fluorescein-conjugated anti-CD4 (GK1.5; ref. 26) or anti-CD8 (53.6; ref. 27) antibody and streptavidin/phycoerythrin (PharMingen) were added to each sample and incubated for 30 min on ice. The washed samples were analyzed with a FACScan (Becton Dickinson) flow cytometer.

RESULTS

***In Vitro* Recall Responses to Peptide Antigen.** Recently, Kitamura *et al.* (28) produced mice that lack B cells ($\mu MT^{-/-}$ mice). To evaluate the role of B cells in induction of peripheral T-cell tolerance and T-cell priming, we crossed $\mu MT^{-/-}$ mice, which were originally obtained on a segregating (C57BL/6 × 129) *H-2*^{b} background strain, with B10.BR/SgSnJ (*H-2*^{k}) mice. The offspring were intercrossed and selected for homozygosity for *H-2*^{k} and $\mu MT^{-/-}$. These homozygotes lacked mammary tumor viruses encoding $V_\beta 3$-specific superantigens and contained no peripheral $B220^+$ cells ($k\mu MT^{-/-}$ mice). To test the ability of these animals to respond to antigen, they were primed with a conventional peptide antigen, PCC 88-104, in CFA in the base of the tail. T cells were then harvested from the draining nodes of these mice and titrated for their ability to respond *in vitro* to antigen. As shown in Fig. 1*a*, T cells from $k\mu MT^{-/-}$ mice responded about half as well to the peptide antigen as cells from their heterozygous littermates ($k\mu MT^{+/-}$) or control B10.BR mice. This was not because the T cells in these mice were intrinsically less responsive or that the control populations contained dividing non-T cells, since T cells from $k\mu MT^{-/-}$ mice and their littermate controls responded equally well to a stimulus that did not require *in vivo* priming (Fig. 1*b*). These results are similar to those generated earlier in μ-chain suppressed mice (29–34).

T-Cell Tolerance in $k\mu MT^{-/-}$ Mice. Next we asked whether peripheral tolerance mechanisms were defective. In our first attempt to address this issue, $k\mu MT^{-/-}$ and $k\mu MT^{+/-}$ mice were tolerized to the PCC peptide by i.v. injection with the soluble antigen. The animals were then challenged with anti-

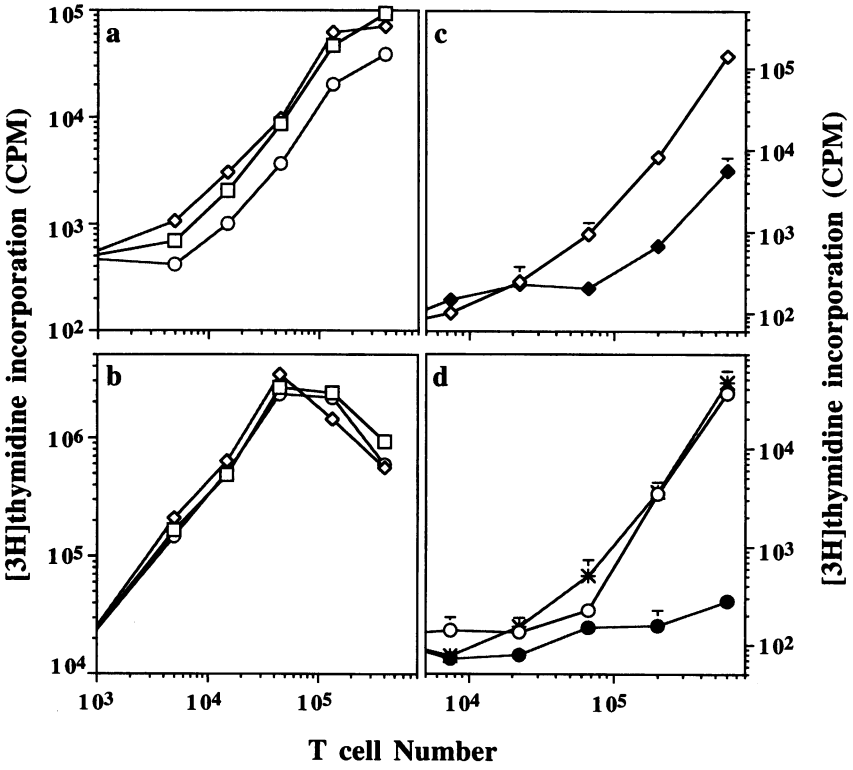

FIG. 1. Defective T-cell priming, efficient T-cell tolerance, and interference with tolerance in mice that lack B cells. (*a* and *b*) Three groups of mice were immunized s.c. in the base of the tail with 100 μg of PCC 88-104 emulsified in CFA. Nine days after immunization, draining lymph node T cells were purified and titrated into cultures containing T-cell-depleted B10.BR splenocytes as presenting cells. The cultures were stimulated for 72 h with 100 μg of PCC peptide per ml (*a*) or 10 μg of SEB per ml (*b*). (*c*) $k\mu MT^{+/-}$ mice were injected i.v. with 200 μg of soluble PCC peptide in balanced salt solution (BSS) on days 0 and 5 (◆) or were uninjected (◇). (*d*) $k\mu MT^{-/-}$ mice were injected i.v. with 200 μg of soluble PCC peptide alone (●), uninjected (○), or injected with 200 μg of soluble PCC peptide with 50 μg of LPS on day 0 and 5 days later (*). Five days after the last injection, all mice were immunized with PCC/CFA; 7 days later, the lymph node cells were set up in culture and stimulated with PCC and APCs as in *a*. During the last 8 h of culture 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine (Amersham) was added. Data are mean cpm ± SD from triplicate samples and are similar to two other experiments. T cells are from B10.BR (□), $k\mu MT^{+/-}$ (◇ and ◆), or $k\mu MT^{-/-}$ (○ and ●) mice.

gen in CFA and the ability of the 7-day-old T cells to respond to PCC peptide was evaluated *in vitro*. The results show that T cells from both kμMT$^{+/-}$ and kμMT$^{-/-}$ mice could be tolerized effectively [Fig. 1 *c* and *d*, compare open (untolerized) to solid (tolerized) symbols]. In addition, we assayed tolerance induction in day 5 T cells from both groups of mice and found that T cells from kμMT$^{-/-}$ mice were still easily tolerized as T cells from control B10.BR mice (data not shown). Nevertheless, these results show that antigen presented on cells other than B cells can induce class II-restricted peripheral T-cell tolerance.

Superantigen-Mediated T-Cell Priming and Deletion. Because we could not examine the T cells responding to peptide antigen in these mice directly, we could not tell whether tolerance induction in the kμMT$^{-/-}$ mice was due to T-cell death or inactivation. Likewise, we could not distinguish reduced clonal expansion from normal clonal expansion followed by partial tolerance induction in the experiments on T-cell priming. To distinguish these possibilities and others, the kμMT$^{-/-}$ mice were tested for their ability to respond to SEA, a superantigen that stimulates a readily identifiable subpopulation of T cells, those bearing V$_\beta$3 (35, 36).

SEA was injected into kμMT$^{-/-}$ and wild-type B10.BR mice. V$_\beta$3$^+$ T-cell deletion was observed in the spleens and lymph nodes of both types of mice 14 days after injection (Fig. 2 *a* and *b*). To determine whether T-cell proliferation could be observed in mice lacking B cells and also whether the kinetics of T-cell death were normal in such mice, we followed the activation state and percentages of T cells bearing V$_\beta$3 in the spleens and lymph nodes of the animals at various times after injection of SEA. T cells were well activated in the kμMT$^{-/-}$ animals 2 days after SEA treatment; 38% ± 3% of the V$_\beta$3$^+$ cells in the lymph nodes of wild-type mice and 55% ± 11% of the V$_\beta$3$^+$ cells in the lymph nodes of kμMT$^{-/-}$ animals bore the activation marker CD69. In wild-type mice, V$_\beta$3$^+$ cells expanded more efficiently in spleen than they did in lymph

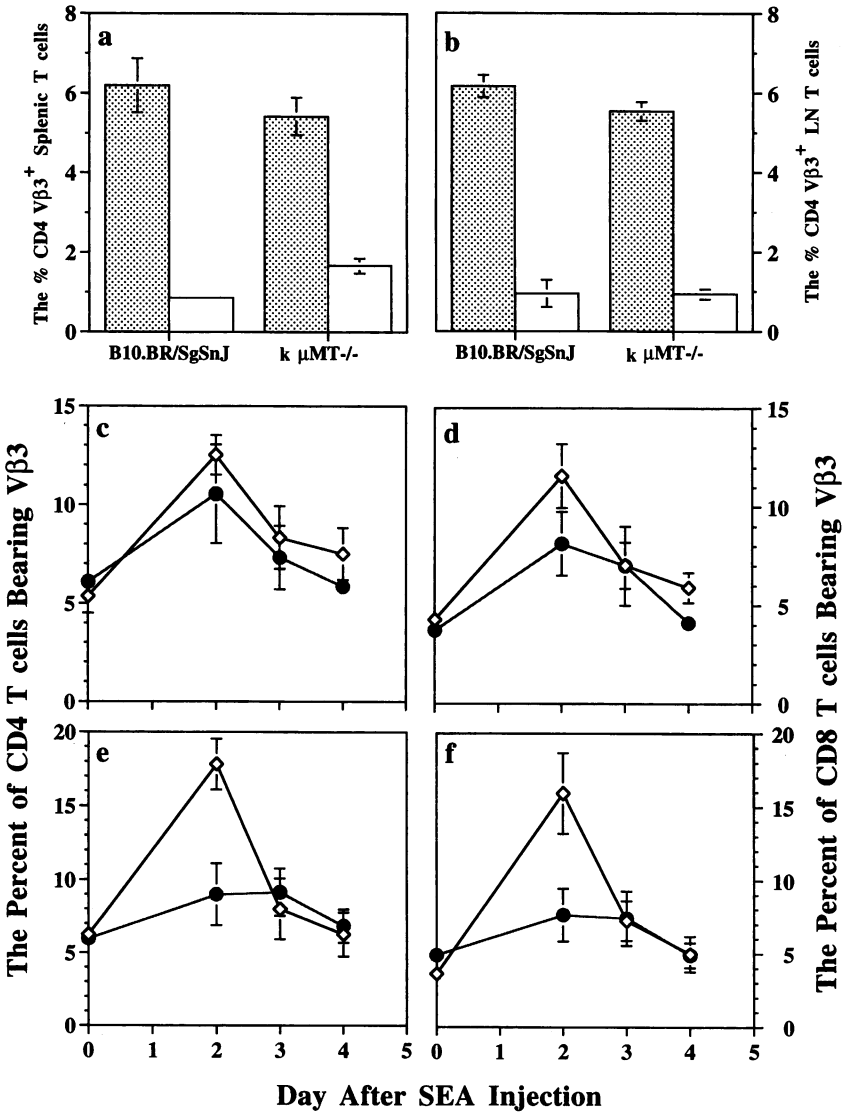

FIG. 2. SEA induces T-cell death in mice lacking B cells. Control B10.BR/SgSnJ and kμMT$^{-/-}$ mice were injected i.p. with 0.04 μg of SEA. Two weeks after injection, spleens and peripheral lymph nodes (inguinal, brachial, and axillary) were removed and single cell suspensions were generated. The percentage of CD4$^+$ splenocytes (*a*) or lymph node cells (*b*) from uninjected (stippled bars) or injected (open bars) mice bearing V$_\beta$3 was determined by flow cytometry. Each bar represents the mean percentage of CD4 V$_\beta$3 T cells from three mice ± SEM. Data from this experiment are comparable with those of two others. (*c–f*) kμMT$^{+/-}$ (◇) and kμMT$^{-/-}$ (●) mice were injected i.p. with 0.2 μg of SEA and at days 0 (uninjected), 2, 3, and 4 peripheral lymph nodes and spleens were processed as described. Lymph node (*c* and *d*) and spleen (*e* and *f*) populations were stained for CD4 cells (*c* and *e*) and for CD8 cells (*d* and *f*) expressing V$_\beta$3. Mean percentage of CD4 or CD8 T cells bearing V$_\beta$3 ± SEM is given. Results are from three experiments and each point contains data from five individual mice, except the day 0 points are from three mice.

node, as measured by percentages and numbers of cells 2 days after SEA administration (data not shown). In mice lacking B cells, $V_\beta 3$ cells expanded in both lymph nodes and spleens (Fig. 2 *c* and *d*, lymph nodes; Fig. 2 *e* and *f*, spleens). Overall, the T-cell proliferative responses in kμMT$^{-/-}$ lymph nodes were slightly reduced (Fig. 2 *c* and *d*) and in kμMT$^{-/-}$ spleens they were substantially reduced (Fig. 2 *e* and *f*) by comparison with those in normal controls. Clearly B-cell presentation contributes to T-cell proliferation in both organs, since T-cell division in response to SEA was reduced in both spleens and lymph nodes in kμMT$^{-/-}$ mice in spite of the fact that the $V_\beta 3$ T cells were well activated.

Four days after SEA injection, the numbers of $V_\beta 3$-bearing cells in the lymph nodes and spleens of both kμMT$^{+/-}$ and kμMT$^{-/-}$ mice had begun to decrease (Fig. 2 *c* and *d*, lymph nodes; Fig. 2 *e* and *f*, spleens). Thus, T cells can disappear after exposure to superantigen *in vivo*, even in the absence of B cells.

Effect of LPS on T-Cell Death and Peptide Tolerance. To test the idea that inflammatory cytokines can provide survival signals to antigen-stimulated T cells in the absence of B cells, we i.p. injected two groups of kμMT$^{-/-}$ mice with 0.04 μg of SEA. One day later, one group was injected i.v. with 40 μg of LPS. After 10 days, spleen cells were analyzed for the presence of CD4 T cells expressing $V_\beta 3$. As shown in Fig. 3, CD4 $V_\beta 3^+$ T cells were well protected from deletion by SEA when the mice were injected with LPS. To determine whether LPS could block peptide-induced tolerance, mice were injected i.v. with peptide in the presence or absence of LPS. As shown in Fig. 1*d*, the LPS treatment blocked tolerance induction to the peptide completely. Thus, B cells are not needed for LPS to function as an agent that can block T-cell death or T-cell tolerance.

DISCUSSION

In this report, we have shown that T cells from mice that lack B cells can be activated or tolerized. For example, in kμMT$^{-/-}$ mice T-cell primary responses to antigen were only slightly different from those of controls. T cells in kμMT$^{-/-}$ mice could be made tolerant by exposure to antigen very effectively, and peripheral T-cell death in response to superantigen occurred normally. However, a critical difference between B-cell-deficient and normal animals was observed in the response of superantigen-activated T cells *in vivo*. In mice that contain

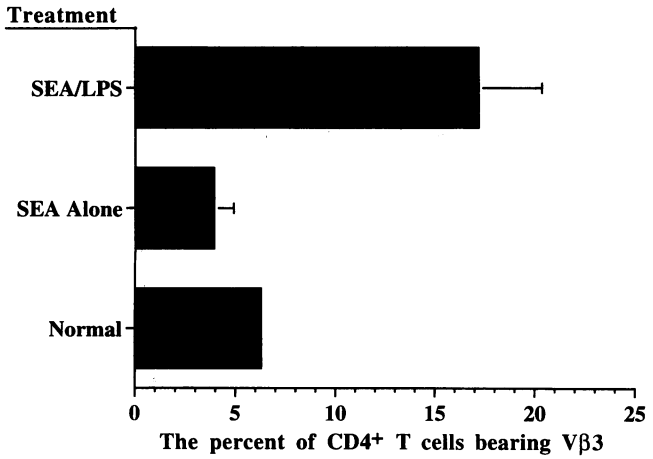

FIG. 3. Bacterial LPS prevents superantigen-induced T-cell death in mice lacking B cells. kμMT$^{-/-}$ mice were or were not (normal) injected i.p. with 0.04 μg of SEA and 1 day later were or were not injected i.v. with 40 μg of LPS. After 10 days, spleen cells were stained and analyzed for the presence of CD4 T cells expressing $V_\beta 3$. Each bar represents mean percentage of CD4 $V_\beta 3^+$ splenic T cells from three mice over two experiments. Data from lymph node cell populations were very similar (data not shown).

B cells, it was shown that superantigen-reactive T cells expanded, whereas clonal expansion of superantigen-reactive spleen T cells was not observed in kμMT$^{-/-}$ mice. This was not due to a limiting number of APCs presenting SEA, since a greater percentage of the $V_\beta 3$ T cells from the kμMT$^{-/-}$ by comparison with normal mice expressed the early activation marker CD69, strongly suggesting that these cells had received sufficient stimulus to become activated. One possible explanation is that B cells "help" activated T cells to stay alive, perhaps by blocking death signals directed toward antigen-activated T cells. Nevertheless, it is clear that LPS can overcome this effect and allow activated T cells to divide and survive whether B cells are present or not.

In the past, LPS has been shown to prevent T-cell tolerance *in vivo* (37). We have recently shown that this is not due to induction of B7-like molecules and is probably due to proinflammatory mediators, such as TNF-α, which can interfere with the induction of T-cell death in animals (22). The experiments in Fig. 1*d* and in Fig. 3 demonstrate that LPS can interfere with T-cell tolerance induction and/or superantigen-induced T-cell death in mice lacking B cells.

These results suggest that T-cell tolerance, as manifested both by the inability to respond to peptide challenge and by T-cell death, can occur in the absence of B cells. Tolerance in these animals was not due to deletion in the thymus since the failure to respond, or loss of target cells, occurred far too quickly to be explained by such a mechanism. Likewise, tolerance was probably not driven by V_β-specific CD8$^+$ T cells (38, 39), since superantigen-specific T cells started to disappear in SEA-challenged mice before such CD8$^+$ cells could be induced. Perhaps tolerance in these experiments is mediated by a program internal to the virgin T cell, triggered by contact with antigen, like that which operates in immature thymocytes. Alternatively, or in addition, T-cell death may be induced by proteins such as Fas (40, 41). Finally, it is possible that the disappearance of (super)antigen-stimulated T cells in these animals is due to excretion of the activated cells from lung and gut. Regardless of the mechanism, it is clear that tolerance can be prevented by exposure to an inflammatory agent, LPS. LPS has effects on many cell types including B cells and macrophages. However, it is the latter cells that secrete the proinflammatory cytokines, such as TNF-α, which have been implicated in T-cell rescue in response to LPS. Perhaps this is why it is easier to demonstrate T-cell tolerance after exposure to antigen on B cells than after exposure on other cells such as macrophages and dendritic cells.

Several years ago, it was well demonstrated that resting B cells could induce T-cell tolerance in a class I- and class II-restricted fashion (20, 21). In general, the authors concluded that resting B cells lacked critical costimulatory molecules and therefore when antigen was presented by these APCs T cells were rendered tolerant rather than activated. Paradoxically, however, Fuchs and Matzinger (20) also showed that activated B cells, but not professional APCs, which both express costimulatory molecules, could tolerize T cells in an antigen-specific fashion. Thus, it appears that the lack of costimulation may not be the only factor that drives tolerance. Consistent with this possibility are the data in this report showing that T cells can be tolerized in the absence of B cells, although it is has not been directly proven that presentation in these experiments has occurred in the presence of costimulation. Regardless, these data support the notion that APCs other than B cells can induce class II-restricted peripheral tolerance and, consequently, that the presence of costimulatory molecules is not the only parameter that controls the choice between life and death of a T cell.

It is well established that costimulation is necessary for T-cell activation (8, 14). This is probably the reason why dendritic cells, but not resting B cells, can fully activate naive T cells (42–46). Yet it is unclear whether costimulation is

enough to keep a T cell alive. We would like to suggest that contact with antigen on any cell is eventually tolerogenic for mature T cells but that different cells have differing abilities to produce the inflammatory cofactors that are essential survival signals to activated T cells *in vivo*. This notion may explain the long-standing association between inflammation and autoimmune disease.

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