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Inhibition of IL-4 Responses After T Cell Priming in the Context of LFA-1 Costimulation Is Not Reversed by Restimulation in the Presence of CD28 Costimulation

Scott A. Jenks and Jim Miller

Costimulation is one of several factors that influence the differentiation of CD4+ Th cell responses. Previously, we have shown that Ag presentation in the context of LFA-1 costimulation by fibroblasts transfected with class II and ICAM-1 (ProAd-ICAM) can drive naive CD4-positive T cells into cell cycle, but these T cells die by apoptosis 4–5 days after stimulation. In this report we show that the death of these cells can be prevented by the addition of exogenous IL-2 (20 U/ml) or by restimulation with Ag presented in the context of CD28 costimulation. Under these conditions, T cells go through extensive cell division and normal cell expansion. However, when T cells that have been primed by Ag presented in the context of LFA-1 costimulation are restimulated, they secrete IL-2 and IFN-γ, but little or no IL-4. The inability of ProAd-ICAM-primed T cells to produce IL-4 was restored by the addition of IL-4 to the priming culture. However, IL-4 responses were not restored by representation of Ag in the context of CD28 costimulation, even as early as 24 h after priming with Ag presented by ProAd-ICAM cells. These findings suggest that differential expression of B7-1 and ICAM-1 by APCs during the initiation of immune responses may alter the differentiation of Th populations. The Journal of Immunology, 2000, 164: 72–78.

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least in part through the induction of IL-4 production, because Th2 development in the presence of CD28 costimulation is blocked by the addition of anti-IL-4, and CD28 costimulation can be replaced by exogenous IL-4 (38). However, it is not clear whether proximal signaling events associated with CD28 costimulation or other CD28-dependent cytokines may also play a role in activation of Th2 responses. Members of the TNF-receptor family have also been associated with Th2 responses (40, 41). Most notably, costimulation through OX40 can increase the number of IL-4-producing cells and decrease the number of IFN-γ-producing cells, primarily through the up-regulation of IL-4 production (42). Thus, the environment in which T cells first encounter Ag, largely determined by the source and concentration of Ag and the specific APC involved, including the array of costimulatory molecules they express, can have a dramatic effect on the nature of T cell immune responses.

Several studies have also implicated LFA-1 in regulating Th2 cytokines. Both human (43) and murine (44) T cell clones derived by repeated TCR and LFA-1 stimulation produced Th1-type cytokines, but not Th2 cytokines. Blocking Abs to LFA-1 and ICAM increased IL-4 production by T cells stimulated with Ag presented by splenic dendritic cells (45). Finally, coexpression of ICAM-1 on insect cells transfected with class II and B7 inhibits IL-4 production by naive T cells (46). These results suggest that LFA-1 might negatively regulate IL-4 expression. We have recently found that costimulation through LFA-1 can drive naive T cells into the cell cycle (47, 48). However, 4–5 days after activation, these cells die of apoptosis. In this report we show that these cells can be rescued from cell death by the addition of exogenous IL-2 or by restimulation with Ag presented in the context of CD28 costimulation. Under these conditions, the T cells go through extensive cell division and expansion, but are unable to secrete IL-4 upon restimulation.

Materials and Methods

Cells

Cell lines derived from the fibrosarcoma 6132A-PRO (Pro) transfected with I-A<sup>d</sup> and ICAM-1 (ProAd-ICAM) or I-A<sup>a</sup> and B7-1 (ProAd-B7) have been previously described (47–49). All cell lines were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, 0.1 mM nonessential amino acids, 40 μg/ml gentamicin, and 50 μM 2-ME. G418 (200 μg/ml) and MXH (6 μg/ml mycophenolic acid, 250 μg/ml xanthine, and 15 μg/ml hypoxanthine) were included in the culture media during routine passage of the cells, but were removed at least 24 h before use in T cell assays. The continual expression of I-A<sup>d</sup>, ICAM-1, and B7-1 was verified by flow cytometric analysis. CD4-positive T cells were purified from lymph nodes of DO11.10 TCR transgenic mice by negative selection as previously described (47). Briefly, class II-positive cells (using mAbs M5/114 and 25-9-17) and CD8-positive cells (using mAb 2.43) were depleted by complement lysis (1/10 dilution of rabbit complement from Accurate Chemical, Westbury, NY) followed by magnetic separation of any remaining Ab-coated cells and B cells with a mixture of sheep anti-mouse IgG and sheep anti-rat IgG magnetic beads (Dynal, Oslo, Norway). The purity of the remaining CD4-positive population was routinely confirmed by the absence of [3H]thyminide incorporation following stimulation with Con A (Sigma, St. Louis, MO). T-depleted spleen cells were prepared by complement-mediated negative selection with anti-CD4 (GLK1.5) and anti-Thy-1 (3OH.12) as described above and irradiated with 2000 rad before use as APC.

T cell stimulation

Equal numbers of purified CD4-positive T cells and mitomycin C-treated ProAd-ICAM or ProAd-B7 cells were plated in flat-bottom 24-well plates in the presence of 2 μg/ml OVA<sub>23,33</sub>-peptide for various periods of time. In some experiments irradiated T-depleted spleen cells were used as APC at 10-fold the number of T cells. After activation, T cells were harvested by gentle resuspension followed by Ficoll purification to remove dead cells and debris. In some assays recombinant human IL-2 (Genzyme, Cambridge, MA), recombinant mouse IL-4 (R & D Systems, Minneapolis, MN), anti-mouse IL-4 mAb, or rat IgG1 isotype control mAb (Phar-Mingen, San Diego, CA) was added. Viable cell number was determined by trypan blue exclusion. To assay cytokine production, purified CD4<sup>+</sup> T cells were plated at 100,000 cells/well in a flat-bottom 96-well plate coated with either 1 μg/ml of anti-CD3 mAb 145-2C11 (gift from Dr. Bluestone) or control mAb (anti-CD18, M18/2 from PharMingen, San Diego, CA). After a 24-h stimulation, cultures were frozen and thawed, and supernatants were used for cytokine quantification by capture ELISA (PharMingen, San Diego, CA).

Analysis of cell division

Carboxy-fluorescein succinimidyl ester (CFSE)<sup>3</sup> labeling was performed as previously described (50). Purified CD4-positive T cells (1 × 10<sup>6</sup>/ml) from DO11.10 mice were washed once in PBS and then incubated with 2.5 μM carboxy-fluorescein diacetate succinimidyl ester ( Molecular Probes, Eugene, OR) in PBS for 5 min at 37°C followed by two washes in complete medium. T cells were then stimulated as described above. Cell division was assayed by harvest of the CFSE-stained T cells without washing and analysis by flow cyrometry. Cell viability was determined by propidium iodide exclusion and light scatter profiles. The proportion of viable cells in each division was analyzed using the ModFit LT program (Verity Software House, Tupsham, ME).

Results

T cells primed in the context of LFA-1 costimulation can be rescued from cell death

We have previously established a gene transfer system to assay the function of potential costimulatory molecules in T cell activation, using the fibroblast cell line, 6232A-PRO (Pro). This cell line lacks expression of accessory molecules both by flow cytometric analysis of known accessory molecules, including B7-1, B7-2, ICAM-1, ICAM-2, CD30L, CD70, OX40L, and 4–1BBL, and by functional analysis of class II-positive transfectants (47–49) (data not shown). However, given the growing awareness of the diversity of costimulatory molecules, we cannot exclude the possibility that unknown cell surface molecules or soluble factors expressed by the Pro cells are contributing to T cell activation in these studies. The Pro cells were transfected with MHC class II alone (ProAd), with class II and ICAM-1 (ProAd-ICAM) or, as a positive control, with class II and B7-1 (ProAd-B7). We have found that Ag presentation by either ProAd-ICAM or ProAd-B7 could drive freshly isolated CD4<sup>+</sup> lymph node T cells from DO11.10 TCR transgenic mice into the cell cycle (47, 48). However, while T cell priming with ProAd-B7 induced clonal expansion, priming by ProAd-ICAM resulted in cell death. Apoptotic cell death following Ag presentation by ProAd-ICAM is not evident until 3–5 days following T cell stimulation (47). This raised the question as to when these T cells are irretrievably committed to die. To determine whether naive T cells that were primed in the context of LFA-1 costimulation could be rescued from cell death, T cells were first activated by Ag presented by ProAd-ICAM and then 3 days later were restimulated with Ag presented by ProAd-B7 (Fig. 1). In the absence of restimulation, no viable cells were detected on day 7, whereas restimulation with Ag presented by ProAd-B7 resulted in substantial expansion. Overall, the level of expansion was similar to that seen in cells stimulated and restimulated with ProAd-B7, indicating that initial priming in the context of LFA-1 costimulation did not have a deleterious effect on T cell expansion. When ProAd-ICAM-stimulated cells were restimulated with ProAd-ICAM, this vigorous expansion was not seen, indicating that CD28 costimulation was necessary to allow for T cell expansion. Thus, as late as day 3 ProAd-ICAM-stimulated cells are not committed to apoptotic death, and death can be prevented by restimulation in the presence of CD28 costimulation.

Costimulation through CD28 could rescue T cells primed with LFA-1 costimulation through two possible mechanisms. First,

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3 Abbreviation used in this paper: CFSE, carboxy-fluorescein succinimidyl ester.
CD28 costimulation could induce the expression of intracellular survival factors (51–54). In support of this model we have previously shown that Ag presented by ProAd-B7, but not ProAd-ICAM, induces the expression of the antiapoptotic protein, Bcl-xL (47). Second, CD28 costimulation may increase the expression of one or more cytokines that have been implicated in T cell survival (55–58). We have found that naive T cells stimulated by ProAd-B7 secrete 50 U/ml of IL-2, whereas T cells stimulated with ProAd-ICAM secrete only 2 U/ml (47). If the levels of IL-2 are contributing to CD28-dependent T cell survival, inclusion of exogenous IL-2 should prevent the death of ProAd-ICAM-stimulated cells. We found this to be the case, and when culture conditions were optimized, addition of 20 U/ml of IL-2 was sufficient to provide sustained expansion of ProAd-ICAM-stimulated cells (Fig. 2). Expansion of T cell stimulated with Ag presented by ProAd-B7 cells was only modestly enhanced by exogenous IL-2 (Fig. 2). Although these results do not rule out CD28 costimulation having other antiapoptotic effects, they demonstrate that exogenous IL-2 alone is sufficient to prevent death after ProAd-ICAM stimulation.


T cells primed in the context of LFA-1 costimulation fail to express IL-4 after secondary stimulation

The ability of IL-2 to enhance the survival of ProAd-ICAM-stimulated cells allowed us to determine whether activation of T cells under different costimulatory conditions would modulate the differentiation of effector Th cell populations. To assess the cytokine potential of T cells primed under different conditions, we stimulated the naive DO11.10 T cells with Ag presented either by ProAd-B7 or by ProAd-ICAM in the presence of exogenous IL-2. On day 7, the T cells were harvested, and equal numbers of T cells were restimulated with plate-bound anti-CD3 to assay the cytokine expression of the activated T cells. Supernatants were removed at 24 h and assayed for the presence of IL-2, IL-4, or IFN-γ by capture ELISA (Fig. 3A). All three cytokines were detected in T cells that were primed by Ag presented by ProAd-B7. In contrast, restimulation of T cells primed by Ag presented by ProAd-ICAM resulted in the secretion of IL-2 and IFN-γ, but little or no IL-4.

The absence of IL-4 production was not the result of IL-2 causing Th1 skewing, because the addition of exogenous IL-2 to T cells stimulated with ProAd-B7 did not alter their ability to produce IL-4 (data not shown). CD4-positive T cells stimulated by ProAd-ICAM are not inherently incapable of producing IL-4, because addition of IL-4 to the priming cultures restored the ability to produce IL-4 upon restimulation (Fig. 3B). The ability of T cells primed in the context of CD28 costimulation was dependent on IL-4 production in the primary culture, because the addition of anti-IL-4 to cultures stimulated with Ag presented by ProAd-B7 inhibited the generation of IL-4 upon restimulation (Fig. 3B).

Taken together, these observations suggest that costimulation through LFA-1 may fail to induce IL-4 production during the initial priming of T cells. This failure to generate IL-4 results in the failure to generate IL-4-producing cells in secondary cultures.

It has recently been shown that cell division is required for the activation of IL-4 gene expression (59, 60). To be sure that the clonal expansion seen in T cells activated with ProAd-ICAM in the presence of exogenous IL-2 resulted in sufficient cell divisions to allow for IL-4 gene expression, we assayed cell division by staining with CFSE, a fluorescent dye that stably binds to cellular proteins (Fig. 4). During cell division the dye segregates equally into the two daughter cells, and each division can be visualized by flow cytometry as a halving of the mean fluorescence (50). On both day 3 (Fig. 4A) and day 7 (Fig. 4B) the percentage of cells in each generation was very similar in T cells primed with ProAd-B7 and T cells primed with ProAd-ICAM plus IL-2. Although we cannot exclude selective survival of a small population of IL-4-producing cells in the ProAd-B7-stimulated T cells, on a global level we cannot account for the failure of ProAd-ICAM cells to generate IL-4 responses to a failure to induce cell division.

Restimulation by Ag presented in the context of CD28 costimulation does not restore the ability of T cells primed by ProAd-ICAM to secrete IL-4

To determine whether exposure to Ag in the context of LFA-1 costimulation had any lasting effect on T cells, we assessed the cytokine potential of T cells primed with Ag presented by ProAd-ICAM and then restimulated with Ag in the context of ProAd-B7. Surprisingly, although restimulation in the context of CD28 costimulation was sufficient to induce T cell survival (Fig. 1), it does not restore the ability to generate IL-4 responses (Fig. 5A). We were initially concerned that in these experiments cytokine potential was determined only 4 days following restimulation with ProAd-B7. In contrast our control cells were stimulated with ProAd-B7 for 7 days. Thus, it was possible that the defect in IL-4 production was simply the result of insufficient culture time, rather than any inherent defect in the ProAd-ICAM-primed T cells. To address this concern, we assayed for cytokine production at 4 days following initial priming with ProAd-B7 cells and at 7 days after restimulating ProAd-ICAM-primed cells with ProAd-B7 (Fig. 5B). These experiments confirmed our initial results. Four days following initial ProAd-B7 stimulation is sufficient time to elaborate IL-4-producing cells, and T cells primed with ProAd-ICAM and restimulated with ProAd-B7 still expressed minimal IL-4 on day 10.

These results demonstrate that restimulation of ProAd-ICAM-primed T cells with Ag presented by ProAd-B7 did not restore secondary IL-4 responses. However, it remained possible that restimulation by conventional APC that express a full constellation of accessory molecules, including ICAM-1, B7-1, and B7-2, might have a different effect on the generation of IL-4 responses. To address this possibility, T cells were primed with Ag presented by ProAd-ICAM, and the T cells were restimulated on day 3 with Ag presented by ProAd-B7, as described above, or by Ag presented by T-depleted splenic APC (Fig. 6). Neither ProAd-B7 nor splenic APC was able to restore IL-4 responses. Thus, the inability of CD28 costimulation to rescue IL-4 responses was not secondary to an inherent defect in the ability of ProAd-B7 to activate T cells.

These results show that T cells that were initially primed in the context of LFA-1 costimulation were unable to secrete IL-4 even
if they re-encountered Ag presented in the context of CD28 co-stimulation. To address how quickly this effect of LFA-1 costimulation is bestowed upon T cells, we primed naive CD4+ T cells with Ag presented by ProAd-ICAM and then restimulated the T cells 1, 2, and 3 days later with Ag presented by ProAd-B7. At all time points restimulation with ProAd-B7 resulted in cell survival and expansion (data not shown). Strikingly, none of the restimulated cultures produced IL-4 (Fig. 7). Thus, even if T cells re-encounter Ag in the context of CD28 costimulation 24 h after initial exposure to ICAM-positive cells, they failed to express IL-4. These results suggest that T cells can be effectively skewed toward Th1-like responses by initial encounter with Ag presented by ICAM-positive, B7-negative APC.

**Discussion**

We have found that T cell priming in the context of LFA-1 costimulation does not elaborate the expression of IL-4 during

![FIGURE 5. Restimulation with ProAd-B7 does not restore IL-4 production. A, CD4-positive T cells (3 × 10^5) purified from DO11.10 TCR transgenic mice were cocultured with an equal number of ProAd-ICAM or ProAd-B7 cells in the presence of 2 μg/ml OVA peptide in a 24-well plate (day 0). T cells were harvested on day 3 and restimulated at 3 × 10^5 cells/well with 2 μg/ml OVA peptide presented by ProAd-ICAM or ProAd-B7 cells. T cells were reharvested on day 7, 1 × 10^5 T cells from each culture were restimulated with plate-bound anti-CD3 for 24 h, and supernatants were assayed for the presence of IL-4, IFN-γ, or IL-2 by capture ELISA. The difference in IL-2 production was not consistently observed between experiments. Representative data from one of four independent experiments are shown. *, No IL-4 was detected; the limit of detection in this assay was 0.078 ng/ml. B, CD4-positive T cells were stimulated as described in A and harvested on day 7 or day 10. In parallel, T cells were stimulated with Ag presented by ProAd-B7, and T cells were harvested on day 4. The harvested T cells were restimulated at 1 × 10^5 T cells/well with plate-bound anti-CD3 for 24 h, and supernatants were assayed for the presence of IL-4, IFN-γ; or IL-2 by capture ELISA. Only IL-4 levels from one of two representative independent experiments are shown. **, IL-4 was detected at 0.58 ng/ml.](http://www.jimmunol.org/)

![FIGURE 6. Restimulation of ProAd-ICAM-primed T cells with splenic APC does not restore IL-4 production. CD4-positive T cells (3 × 10^5) purified from DO11.10 TCR transgenic mice were stimulated with 2 μg/ml OVA peptide presented by ProAd-B7 or ProAd-ICAM (1° Stim). T cells were harvested on day 1, 2, or 3 and restimulated at 3 × 10^5 T cells/well with Ag presented by ProAd-B7 (2° Stim). T cells (1 × 10^5 T cells/well) were stimulated on day 7 with plate-bound anti-CD3 for 24 h, and supernatants were assayed for the presence of IL-2, IFN-γ, or IL-4 by capture ELISA. Only IL-4 is shown; all four conditions resulted in equivalent levels of IL-2 and IFN-γ. The relative increase in IL-4 levels seen by increasing the interval between Ag stimulation with ProAd-B7 may be due to a combination of inhibitory signals generated by rapid restimulation and hyperinduction signals generated by restimulation after longer intervals. Representative data from one of six independent experiments are shown. *, No IL-4 was detected; the limit of detection in this assay was 0.312 ng/ml.](http://www.jimmunol.org/)

![FIGURE 7. Restimulation with ProAd-B7 within 24 h is not sufficient to restore IL-4 production. CD4-positive T cells (3 × 10^5) purified from DO11.10 TCR transgenic mice were stimulated with 2 μg/ml OVA peptide presented by ProAd-B7 or ProAd-ICAM (1° Stim). T cells were harvested on day 1, 2, or 3 and restimulated at 3 × 10^5 T cells/well with Ag presented by ProAd-B7 (2° Stim). T cells (1 × 10^5 T cells/well) were stimulated on day 7 with plate-bound anti-CD3 for 24 h, and supernatants were assayed for the presence of IL-2, IFN-γ, or IL-4 by capture ELISA. Only IL-4 is shown; all four conditions resulted in equivalent levels of IL-2 and IFN-γ.](http://www.jimmunol.org/)

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restimulation of the T cells. Surprisingly, these cell exhibit this IL-4 defect even if they are restimulated with Ag presented by B7-positive cells 24 h after initial priming. Thus, even a short exposure to Ag presented by ICAM-positive, B7-negative APC can have a profound impact on the differentiation of T cell effector function.

Previous studies have indicated that CD28 costimulation during initial T cell priming was important for the development of Th2 responses (38). However, in the absence of CD28 costimulation, the majority of the responding T cells apoptose in culture (47, 53, 54), and it is difficult to distinguish a direct role for CD28 costimulation in IL-4 expression from an indirect role in T cell survival. Furthermore, it has recently been suggested that cell division is required to induce the epigenetic events that are necessary to activate IL-4 gene expression (59–61). In our studies we have been able to drive T cells activated by ProAd-ICAM in the presence of exogenous IL-2 to undergo equivalent cell division and expansion as cells activated by ProAd-B7. Nevertheless, T cells primed with Ag presented by ProAd-ICAM cells fail to secrete IL-4. These results indicate that T cell activation followed by cell division alone are not sufficient to activate IL-4 gene expression.

The most important factor that appears to be required for the elaboration of IL-4 responses is IL-4 itself (28, 29, 31, 34). One model for the interplay of CD28 and IL-4 is that CD28 costimulation is required to induce the initial IL-4 production by a minority population of cells, and this IL-4 then positively feeds back on the development of further IL-4 production. CD28 and/or IL-4 can provide two possible signals for the potentiation of secondary Th2 responses. First, they could impart an essential survival signal to the small number of T cells that are potentially precommitted to secreting IL-4. Both CD28 (47, 51–54) and IL-4 (56–58) can impart survival signals that can be mediated through Bcl family members. The small number of initial IL-4-producing cells may uniquely require one or more of these survival signals, and their selective death would be masked by the expansion of the majority, non-IL-4-producing cells. Second, CD28 and/or IL-4 signaling may be important for activation of the IL-4 gene locus. Several factors have been implicated in the activation of the IL-4 gene during Th2 cell differentiation, including maintenance of GATA-3 expression (62), changes in DNA methylation (59), and chromatin remodeling (61). CD28 and/or IL-4 signaling could impact one or more of these events.

We have found that this CD28 signal must be delivered within 24 h of initial T cell activation to up-regulate IL-4 expression. Two possible models might account for the rapid loss of CD28 responsiveness. First, Ag presentation by ProAd-ICAM may simply reflect activation in the absence of CD28 costimulation. Engagement of CD28 may be required early during priming to influence IL-4 production, and the absence of CD28 signaling leaves the cell hyporesponsive to subsequent triggering. Alternatively, costimulation through LFA-1 could transduce a dominant negative signal to down-modulate IL-4 expression that renders cells unable to respond to subsequent CD28 costimulation. The latter hypothesis is supported by a recent finding that ICAM-1 expression inhibits IL-4 even when B7 costimulation is present (45, 46).

Regardless of the mechanism, our results could provide insight to understanding Th skewing in vivo. Recently, it has been shown that Ag presentation by different populations of dendritic cells changes the production of IL-4 in vivo (63, 64). The influence of costimulation on this phenomenon has been discounted because the two populations express equivalent amounts of B7-1 and B7-2, but it is noteworthy that they differ in ICAM-1 expression (65). Differential expression of B7 and ICAM-1 could be influencing Th1 differentiation either in concert with, or possibly preceding, dendritic cell-derived cytokines. Furthermore, CD4-positive T cells may encounter ICAM-1 costimulation with little CD28 signaling when stimulated by unactivated APCs. For example, resting B cells express class II and ICAM-1, but only low levels of B7-2, and Ag presentation by resting B cells is ineffective and generally tolerogenic (66). Interestingly, resting B cells induce a phenotype similar to that of ProAd-ICAM cells after Ag presentation to naive CD4+ T cells. The responding T cells die in the absence of exogenous IL-2, and in the presence of IL-2 the surviving T cells secrete IL-2 and IFN-γ, but not IL-4 (67). Finally, inflammatory cytokines such as IFN-γ induce the expression of class II and ICAM-1, but not B7, in a variety of nonhemopoietic cells (68). We have shown that although Ag presentation by ICAM-positive, B7-negative APC is a tolerogenic signal, subsequent re-exposure to Ag presented by B7-positive APC can covert this tolerogenic response into a differentiated effector response. In vivo, T cells migrating into a site of inflammation may first encounter Ag presented by an immature or resting hemopoietic APC or by nonhemopoietic APC that have been induced to express class II and ICAM-1, but not B7. This signal would be sufficient for modest cell expansion. In the absence of a second stimulation, the initial activation would result in cell death and tolerance. However, if the T cells later encounter Ag presented by B7-expressing APCs or paracrine IL-2, the T cells would survive and expand. The result would be a population of Th1 cytokine-producing T cells that would shift the overall cytokine milieu, amplifying an inflammatory T cell response. This phenomenon may play an important role in the potentiation of antibacterial responses in tissue, in human transplantation where vascular endothelial cells express class II and ICAM-1, or in autoimmune disease. Recently, evidence for a role for presentation by nonhemopoietic cells has been demonstrated in one autoimmune model of nphritis, in which bone marrow chimeras of class II-deficient mice, in which class II is expressed only by hemopoietic cells, do not develop autoimmunity (69).

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