CD4+ T Cell Activation and Tolerance Induction in B Cell Knockout Mice

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Summary

B cell knockout mice μMT/μMT were used to examine the requirement for B cell antigen (Ag) presentation in the establishment of CD4+ T cell tolerance. CD4+ T cells from μMT mice injected with exogenous protein Ag in adjuvant responded to in vitro challenge by transcription of cytokine mRNA, cytokine secretion, and proliferation. Peripheral tolerance could be established in μMT mice with a single dose of deaggregated protein. This tolerance was manifested by a loss of T cell proliferation and cytokine production (including both T helper cell type 1 [Th1]- and Th2-related cytokines), indicating that B cells are not required for the induction of peripheral T cell tolerance and suggesting that the dual zone tolerance theory is not applicable to all protein Ags and is not mediated through Ag presentation by B cells.

The role of B cells in Ag presentation to peripheral CD4+ T cell responses is controversial. Although B cells are the most abundant MHC class II–positive cell and have been shown in some studies to be involved in the activation of peripheral T cells, other investigators have argued against the notion that B cells are necessary as APCs for induction of T cell responses. Thus, results from studies have ranged from, first, the B cell as the crucial initiating APC in the lymph node (1), through second, the B cell as a necessary mediator of T cell clonal expansion and secondary proliferation (2–5), to third, normal priming and proliferation of T cells in the absence of B cells (6, 7). These studies all examined Ag-specific T cell activation in mice that had been rendered deficient in B cells, either by continuous injection of anti-μ Ab, by reconstitution of SCID mice with naive T cells, or, most recently, by targeted deletion of either the IgM heavy chain gene (8) or the J_H gene segment (5, 9). The role of B cells in the induction of peripheral T cell tolerance is less controversial. B cells can serve as APCs in the induction of Ag-specific tolerance in naive CD4+ and CD8+ T cells (10, 11) and in Ag-specific T cell clones (12). These reports led to speculation by investigators that tolerance induction in virgin T cells is a primary function of the B cell.

To reevaluate the role of B cells as APCs for activation and tolerization of CD4+ T cells, we used the B cell knockout mouse μMT/μMT (hereafter μMT), which was generated by introduction of a nonsense mutation into the transmembrane exon of the IgM heavy chain gene, resulting in a total deletion of B cells (13). The Ag used to investigate CD4+ T cell responses in these mice was human

γ-globulin (HGG),[1] which is a T-dependent Ag able to induce either tolerance or activation of T cells, depending on the injection procedure. HGG injected subcutaneously in adjuvant induces T cell proliferation and cytokine secretion as well as a significant anti-HGG Ag response, whereas a single injection of deaggregated HGG (DHGG) induces a long-lasting peripheral tolerance that affects both B cells and T cells (reviewed in 14 and 15). In the case of T cells, this tolerance results in a loss of proliferation and production of cytokines (both Th1- and Th2-related [16, 17]).

In this report, we show that CD4+ T cells from HGG-primed μMT mice can be activated to proliferate and to secrete both Th1- and Th2-related cytokines in response to HGG challenge in vitro. When μMT mice were injected with tolerogenic DHGG, peripheral tolerance was established at a level similar to that seen in the normal controls, as evidenced by decreased proliferation and cytokine secretion in response to HGG in vitro. Taken together, these data indicate that B cells are not necessary participants as APCs for the induction of peripheral T cell activation or tolerance.

Materials and Methods

Mice. μMT/μMT breeder stocks were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and were housed

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[1]*Abbreviations used in this paper:* AHGG, aggregated human γ-globulin; DC, dendritic cell; DHGG, deaggregated human γ-globulin; HGG, human γ-globulin; IF, immunofluorescence; PPD, purified protein derivative; SN, supernatant.

and bred in specific pathogen-free conditions at the Scripps Research Institute (La Jolla, CA). C57BL/6 mice were obtained from the Scripps Research Institute breeding colony and were housed and treated identically.

Antigens. HGG was purified from Cohn fraction II of human plasma (Hyland Laboratories, Glendale, CA) as described (18), purified protein derivative (PPD) was obtained from the Statens Seruminstitut (Copenhagen, Denmark), and KLH was obtained from Calbiochem Novabiochem (La Jolla, CA).

Immunization and Tolerance Induction. DHGG was prepared by ultracentrifugation of HGG as described (16). Mice were tolerized by a single injection of 1 mg DHGG i.p. in 1 ml saline. 7–10 d after tolerization, mice were injected with 200 μg HGG s.c. in CFA (Sigma Chemical Co., St. Louis, MO). Draining LNs were used 8–12 d after injection.

Cell Preparations and Culture. Draining LNs were removed 8–12 d after injection, and CD4$^+$ T cells were obtained by negative selection with an mAb cocktail and anti-Ig–conjugated magnetic beads (PerSeptive Diagnostics, Cambridge, MA) as described (16). CD4^{++} T cells were >95% pure after this procedure. APCs were generated by depleting splenocytes of T cells by treatment with anti–Thy 1.2 (Du Pont–New England Nuclear, Wilmington, DE) plus guinea pig complement (Cedar Lane Laboratories Ltd., Hornby, Ontario, Canada). For all assays, T cells were cultured at 10^6/ml with 1.5 × 10^6/ml irradiated (3,000 R) APCs in the presence of either HGG (1 mg/ml), PPD (25 μg/ml, Statens Seruminstitut), or no Ag as described (16). To generate culture supernatants (SNs) and RNA, cells were cultured in 24-well plates for 48–60 h with 1 mg/ml HGG, and SN was removed and RNA was isolated from the cells as described (19). For proliferation assays, quadruplicate cultures in 96-well plates were pulsed with 1 μCi/well [^{3}H]TdR (Amersham Corp., Arlington Heights, IL) for the last 18 h of culture and then were harvested onto glass filters. Tritium incorporation was measured by liquid scintillation spectroscopy.

Cytokine Analysis. The RNase protection assay for detection and quantitation of IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, TNF-α, and TNF-β mRNA was performed exactly as previously described (20). Message levels were quantitated by a series 400 PhosphoImager and ImageQuant Software (Molecular Dynamics, Sunnyvale, CA). Cytokine levels in culture SNs were measured by a two-site sandwich ELISA exactly as previously described (20, 21). Cytokine levels from unstimulated cultures were routinely below the level of detection.

Immunofluorescence Staining. PE-labeled anti-CD4 and fluorescein-labeled anti-CD44 (IM7.8.1), anti-CD45RB (C363.16A), and anti-CD62-L (MEL-14) were obtained from PharMingen (La Jolla, CA). Methods for two-color immunofluorescence (IF) staining have been described (22). Stained cells were analyzed by FACScan® (Becton Dickinson & Co., San Jose, CA).

Results

Initial analyses of the peripheral lymphoid organs of the μMT mice confirmed that there were no detectable surface Ig$^+$B220$^+$ dual-expressing cells in the spleen or LN (data not shown). We also examined the phenotype of the resident T cells by analyzing surface markers that are differentially expressed by naive and memory CD4$^+$ T cells. Splenic CD4$^+$ cells were stained for cell surface expression of CD44, CD45RB, or CD62L. CD44 increases and both CD45RB and CD62L decrease in expression during the shift from
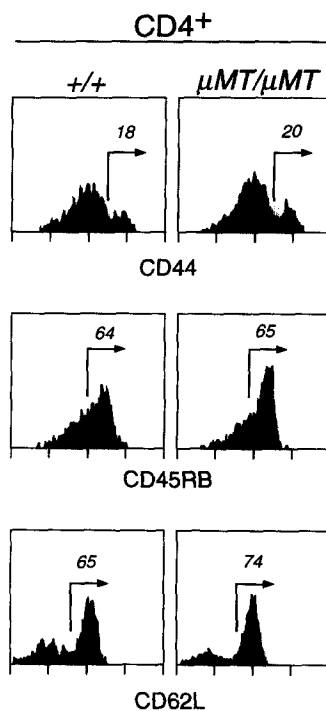

CD4+

Figure 1. Naive and memory CD4$^+$ T cells in normal and μMT mice. Pooled spleen cells from 2-mo-old μMT and C57BL/6 (+/+) mice were stained with PE-labeled anti-CD4 and FITC-labeled antibodies against either CD44, CD45RB, or CD62L. PE$^+$ events are displayed as fluorescent histograms, with markers placed to define "high" cell surface expression for each marker. The percentage of high cells is shown in each histogram.

naive to memory status (23–25). The majority of the CD4$^+$ spleen cells from 3-mo-old μMT mice were found in the CD44^{lo}, CD45RB^{hi}, and CD62L^{hi} populations (Fig. 1), the expected phenotypes of naive CD4$^+$ cells (21). The naive cells were present at levels similar to those seen in the age-matched control mice. The percentage of CD4$^+$ T cells that exhibited an activated/memory phenotype (CD44^{hi}, CD45RB^{lo}, and CD62L^{lo}) was also similar between the
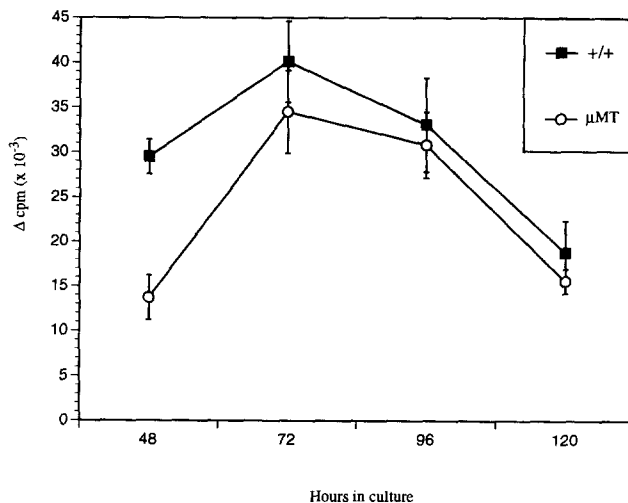

Hours in culture

Figure 2. HGG-specific proliferation by μMT and normal CD4$^+$ T cells. μMT T cells were able to proliferate and secrete cytokines in response to HGG and PPD. Since naive CD4$^+$ T cells secrete primarily IL-2 after initial stimulation (20), the appearance of IL-3, IL-4, IL-5, and IFN-γ in Ag-stimulated cultures from the μMT mice was an indication that the T cells had been primed in vivo. T cells were harvested from draining LNs of mice injected 10 d earlier with HGG–CFA; they were then cultured with HGG and T depleted, irradiated splenic APCs. Cultures were harvested at the times shown after being pulsed with 1 μCi of [^{3}H]TdR.

Table 1. *Cytokine Production by Ag-stimulated CD4+ T Cell Cultures*

T cells	Ag	IL-2	IL-3	IL-4	IL-5	IFN-γ
μMT	HGG	592	760	<5	12	3,085
+/+	HGG	301	350	<5	28	1,480
μMT	PPD	266	285	<5	<5	4,088
+/+	PPD	200	163	<5	8	1,800

CD4+ T cells from draining LNs of mice injected with HGG in CFA were cultured for 48 h with irradiated APCs and the indicated Ag. Culture supernatants were collected at 48 h. T cells cultured without Ag did not secrete detectable levels of cytokine. All cytokine levels are displayed in picograms per milliliter.

normal and the μMT mice. The presence of an activated/memory T cell population suggests that T cell priming to environmental Ag has occurred in both the normal and the knockout mice, despite the absence of B cell APC activity in the μMT.

Epstein et al. (8) have recently shown that μMT CD4+ T cells proliferate normally after in vivo priming and in vitro challenge with KLH. We have found that μMT CD4+ cells can proliferate in response to priming and subsequent challenge with HGG and PPD, as well as with KLH (data not shown). Both the kinetics and the magnitude of the μMT proliferative response to HGG were similar to those of the C57BL/6BL response (Fig. 2). Cytokine secretion by the μMT CD4+ T cells in response to both HGG and PPD was also similar to that of the C57BL/6 controls (Table 1). The cytokine responses were Ag specific, since cells cultured without Ag contained no detectable cytokines (not shown). A multiprobe RNase protection assay was used to examine cytokine mRNA transcription in Ag-stimulated T cell cultures. After 60 h of culture, mRNA for a variety of T cell–related cytokines, including
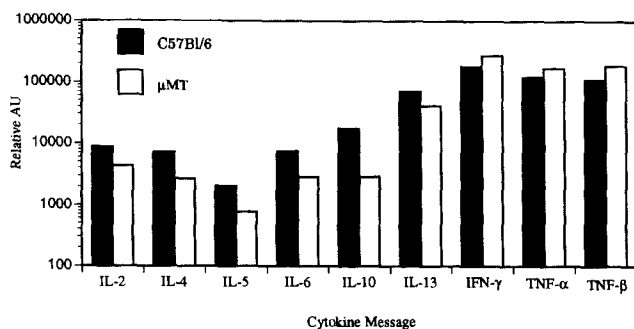

Figure 3. Expression of cytokine mRNA by Ag-stimulated CD4+ T cells from normal and μMT mice. CD4+ cells were purified from draining LNs 10 d after injection with HGG in CFA and then cultured in the presence of APCs and HGG (1 mg/ml). After 60 h, RNA was harvested from all cells. RNA originating from equal numbers of input cells (at time 0) was analyzed with the RNase protection assay. Absorbance units values were obtained with a Series 400 PhosphoImager. Cytokine message from cultures that were not given recall Ag (data not shown) was undetectable.

Table 2. *Proliferative Response from Tolerized or Primed LN CD4+ T Cells from Individual μMT and Normal Mice*

T cells	DHGG	[³H]TdR incorporation Δ cpm ± SD	
		HGG	PPD
μMT/μMT	−	14,123 ± 1,214	27,180 ± 1,374
μMT/μMT	−	21,965 ± 1,476	33,191 ± 1,889
+/+	−	12,447 ± 738	32,002 ± 1,560
+/+	−	19,755 ± 694	35,408 ± 2,206
+/+	−	20,823 ± 1,008	32,382 ± 1,458
μMT/μMT	+	1,957 ± 292	22,892 ± 661
μMT/μMT	+	3,792 ± 832	32,440 ± 2,557
+/+	+	3,476 ± 1,229	38,550 ± 1,396
+/+	+	3,939 ± 812	30,912 ± 2,316
+/+	+	4,992 ± 1,699	31,145 ± 1,488

Mice were tolerized by intraperitoneal injection of DHGG (1 mg per mouse). 7 d later, all animals were primed with HGG–CFA. Draining LNs were removed 12 d after priming, and purified CD4+ T cells were cultured with APCs and Ag for 96 h. [³H]TdR was added for the last 16 h of the culture.

IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, TNF-α, and TNF-β, was present (Fig. 3). The cytokine response of both normal and μMT T cells to Ag-specific stimulation at the level of message and protein secretion in both groups was diverse and included Th1-related (IL-2, IFN-γ) and Th2-related (IL-4, IL-5, IL-13) cytokines.

The induction of peripheral tolerance in μMT mice was examined by injection of DHGG and subsequent challenge with HGG–CFA. The experiment in Table 2 shows the response of individual animals to tolerance induction. Animals tolerized with DHGG and then challenged with HGG in CFA were unable to mount a proliferative response to HGG in vivo. The loss of proliferation to HGG was clearly Ag specific, since the proliferation to PPD was unaltered. The level of tolerance as measured by decreased proliferation appeared similar in both the normal and the μMT mice. There were no detectable cytokines in the culture SN from the tolerized mice (Table 3), and cytokine mRNA from tolerized T cells was not apparent with the RNase protection assay (data not shown), indicating that functional aspects of the CD4+ cells were also affected by the tolerizing regimen. Taken together, these experiments show that peripheral CD4+ T cell tolerance can be induced in μMT mice, indicating that APCs other than B cells have the ability to present Ag to CD4+ T cells in a tolerogenic fashion.

Discussion

In vivo tolerance to HGG is readily induced in CD4+ T cells of μMT mice as well as normal or near normal CD4+

T cells	DHGG	AHGG	IL-2	IL-3	IL-4	IL-5	IFN-γ
μMT	−	+	337	760	29	358	14,471
μMT	+	+	<20	<10	<20	<10	<100
+/+	−	+	101	1,004	53	293	58,903
+/+	+	+	<20	<10	<20	<10	<100

CD4⁺ T cells from tolerized (*DHGG*) or primed (*AHGG*) mice were cultured in the presence of HGG and APCs. Culture supernatants were collected after 48 h and examined for the presence of the cytokines shown by sandwich ELISA as in Table 1. T cells cultured without Ag did not secrete detectable levels of cytokine. All cytokine levels are displayed in picograms per milliliter. *AHGG*, aggregated human γ-globulin.

T cell responses to several T-dependent Ags. The fact that endogenous T cell priming has occurred in the μMT animals is evidenced by the appropriate surface expression of CD4⁺ T cell memory markers. When μMT mice were injected with HGG in CFA and then challenged in vitro, the kinetics and magnitude of the T cell proliferation were similar to those of normal controls, demonstrating that B cells are not necessary for the CD4⁺ T cell proliferative response to recall Ag. SN from these same cultures contained cytokines indicative of CD4⁺ priming in vivo. Peripheral T cell tolerance was examined both in terms of the subsequent T cell proliferation to HGG, which was greatly diminished, and by the absence of cytokine secretion. There was no evidence for selective tolerance of either Th1-like or Th2-like CD4⁺ cells; instead, the loss of cytokine transcription and secretion encompasses both subsets. Together, these data indicate that Ag presentation by B cells is not required to induce peripheral T cell tolerance or to mediate initial CD4⁺ T cell priming or proliferation to recall Ag.

The ability to induce a solid tolerant state in antigen-specific CD4⁺ T cells from μMT mice clearly shows that professional APCs (e.g., dendritic cells [DCs] and macrophages) are sufficient for the induction of peripheral tolerance. A single injection of DHGG induced T cell tolerance in the μMT mice. Furthermore, the level of tolerance achieved in the μMT CD4⁺ T cells was similar to that of normal mice in terms of Ag-specific proliferation and loss of cytokine production. The fact that peripheral tolerance to exogenous Ag can be induced in μMT mice does not conflict with work showing that Ag presentation by B cells can induce tolerance in naive T cells (10, 11). However, it does disallow the hypothesis that only B cell Ag presentation induces tolerance whereas all other APCs are immunogenic, an idea put forth to explain the phenomenon of low-zone/hi-zone tolerance (10, 11, 26). According to this theory, at extremely low doses of Ag, the specific B cells would preferentially pick up Ag through high affinity binding to surface Ig. Because resting B cells do not

express appropriate T cell costimulatory signals, any interaction with an Ag-specific virgin T cell would be tolerogenic. At an increased Ag concentration, uptake by other MHC class II–positive cells would occur. These cells, the so-called professional APCs, would present Ag to the virgin T cells in an immunogenic fashion. At high Ag concentrations, all B cells would take up Ag by pinocytosis. Since B cells far outnumber other MHC class II–positive cell types, the result would again be tolerance of the virgin T cells (10, 11, 26). This explanation presumes that first, only B cells are able to induce T cell tolerance; second, B cells are tolerogenic even in the presence of their specific Ag; and third, all non-B cell APCs are constitutive activators of virgin T cells. These assumptions provide a framework explaining the midrange, immunogenic dose of Ag and imply that a B cell–deficient animal would not be able to induce peripheral T cell tolerance to exogenous Ag. However, the fact that μMT T cells from mice given DHGG are tolerized rather than activated indicates that dual zone tolerance cannot be explained by the above hypothesis. In further support of the suggestion that peripheral T cell tolerance is not solely dependent on B cell Ag presentation is the work of Vella et al. (27), showing that tolerance to soluble peptide and to superantigen can be induced in B cell knockout mice. Actually, there is no evidence for two zones of tolerance to HGG over a wide range of tolerogenic doses (10 mg per mouse to 5 pg per mouse) (28). The experimental evidence for dual zone tolerance in an immunocompetent, adult animal is limited to one Ag: BSA (29), and both Dresser (30) and Thorbecke and Benacerraf (31) have speculated that the intermediate priming dose of BSA was actually due to low level contamination of the protein preparation by immunogenic aggregates. Since there is no experimental evidence that this intermediate priming zone exists for most Ags, and our results, along with those of Vella et al. (27), clearly show that resting B cells are not the only tolerogenic APC in vivo, the tolerogenic capacity of non-B cell APCs (e.g., macrophages and DCs) needs to be examined. In fact, Finkelman et al. have recently shown that Ags targeted to DCs can induce T cell tolerance in vivo (Finkelman, F.D., A. Lees, R. Birnbaum, W.C. Gause, and S.C. Morris, manuscript submitted for publication). It may well be that under the appropriate conditions, every APC is able to induce either a tolerogenic or an immunogenic T cell response. There is abundant evidence that peripheral T cells are tolerized in response to Ags presented by nontraditional APCs in the absence of appropriate costimulation (32–34).

In the HGG system, the immunological response depends primarily on the form of the Ag injected; i.e., DHGG induces tolerance, whereas aggregated HGG induces activation and T cell proliferation. It has been demonstrated that aggregated HGG is aggressively processed by the APCs, resulting in the secretion of cytokines (35–37). On the other hand, DHGG is not processed by APCs in a manner that results in the release of cytokines. The activation state of the initial APC plays a critical role in the subsequent T cell response, since the injection of either IL-1,

TNF-α, or generators of these cytokines interferes with the induction of tolerance to DHGG (36, 37). If, however, the APC presents Ag in the absence of any costimulatory signals, the result should be tolerance rather than immunity. This argues that every APC has the ability to present Ag in either a tolerogenic or an immunogenic fashion, depending on such factors as the form of the Ag (e.g., deaggregated vs. particulate), the route of Ag administration, and the presence of cytokines released by activated APCs.

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