Tolerant B Lymphocytes Acquire Resistance to Fas-mediated Apoptosis after Treatment with Interleukin 4 but Not after Treatment with Specific Antigen Unless a Surface Immunoglobulin Threshold Is Exceeded

By Linda C. Foote,* Ann Marshak-Rothstein,*‡ and Thomas L. Rothstein*§

From the *Department of Microbiology, the ‡Department of Pathology, and the §Department of Medicine; and the §Evans Memorial Department of Clinical Research, Boston University Medical Center, Boston, Massachusetts 02118

Summary

Susceptibility to Fas-mediated apoptosis in nontolerant B cells is regulated in a receptor-specific fashion. To explore the regulation of Fas killing in tolerant, autoreactive B cells, mice doubly transgenic for hen egg lysozyme (HEL)-specific B cell receptors and soluble HEL were examined. Engagement of CD40 led to enhanced Fas expression and acquisition of sensitivity to Fas-mediated apoptosis in tolerant B cells, similar to that observed in nontolerant, receptor transgenic B cells. Engagement of surface immunoglobulin by specific (HEL) antigen failed to induce Fas resistance in tolerant B cells, in contrast to its effect on nontolerant B cells; however, cross-linking of biotinylated HEL with streptavidin induced similar levels of Fas resistance in tolerant and nontolerant B cells, which approximated the degree of Fas resistance produced by anti-Ig. Unlike surface Ig (sIg) engagement, physiological engagement of IL-4 receptors produced similar levels of Fas resistance in tolerant and nontolerant B cells. Thus, tolerant B cells differ from nontolerant B cells in the diminished capacity of surface immunoglobulin engagement to produce Fas resistance; however, tolerant B cells can be induced to become resistant to Fas-mediated apoptosis by IL-4 or by higher order cross-linking of sIg receptors.

B cells can be targets for cytotoxicity mediated by CD4+ Th1 effector cells that express Fas ligand and induce apoptosis in a Fas (CD95)-dependent fashion (1–7). Susceptibility to Fas-mediated apoptosis is dependent on the state of B cell activation and is regulated in a receptor-specific fashion. CD40 engagement by soluble, chimeric CD40 ligand (CD40L) induces expression of cell surface Fas and renders primary B cells highly sensitive to Fas killing (8–10). In contrast, antigen receptor cross-linking actively opposes Th1 cell-mediated cytotoxicity (Th1-CMC), as shown by the observation that B cells treated with CD40L in conjunction with anti-IgM, or in conjunction with specific antigen, are resistant to Th1-CMC (8, 11, 12). In addition to antigen receptor engagement, B cells are also rendered Fas-resistant by IL-4 receptor engagement (13). Both anti-IgM and IL-4 induce protection against Fas killing that is not associated with a loss or alteration of Fas expression, and that is intrinsic to the B cell target itself (11, 13). Despite these similarities, the signaling pathways engaged by anti-IgM and IL-4 to produce Fas resistance are distinct as shown by several criteria, and suboptimal doses of these reagents act in synergy to bring about maximal levels of Fas resistance (11, 13, 14).

Autoreactive B cells express receptors with specificity for self-antigen, and several mechanisms provide for their exclusion from the immune repertoire. High affinity autoreactive B cells undergo clonal deletion or receptor editing in the bone marrow (15–18). Many of the remaining clones that recognize autoantigens migrate to the periphery where they exist in a state of tolerance characterized by impaired signaling via the antigen receptor, normal signaling via CD40 and IL-4R, and shortened life span (19–21). Several lines of evidence suggest that Fas (CD95) plays a role in regulating the disposition of activated, tolerant, autoreactive B cells: (a) Fas-deficient lpr/lpr mice and Fas knockout mice express excessively high titers of autoantibodies (22, 23); (b) the recovery of tolerant B cells after adoptive transfer is improved if the B cells are Fas-deficient or if the

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1Abbreviations used in this paper: BCR, B cell antigen receptor; CD40L, soluble, chimeric CD40 ligand; DTg, double transgenic (anti-HEL/HEL); HEL, hen egg lysozyme; rIL-4, recombinant mouse interleukin 4; sHEL, soluble hen egg lysozyme; sIg, surface Ig; STg, single transgenic (anti-HEL); Th1-CMC, Th1 cell-mediated cytotoxicity.
cotransferred T cells are deficient in Fas ligand (24, 25); and (d) transgenic mice that overexpress IL-4 produce autoantibodies (13).

To determine the potential role of inducible Fas resistance in determining the fate of tolerant B cells, we carried out experiments using anti–hen egg lysozyme (HEL)/HEL double transgenic (DTg) mice. We sought to determine whether the rules for receptor-specific induction of Fas resistance that we previously identified in nontolerant B cells would apply to tolerant B cells as well. This is particularly relevant with respect to the antigen receptor, where it is important to delineate the capacity of self-antigen to protect autoreactive B cells against Fas-mediated apoptosis.

Materials and Methods

Animals. Male BALB/cByJ and C57BL/6J mice at 8–14 wk of age were obtained from The Jackson Laboratory (Bar Harbor, ME). MD4 × M.L5 anti-HEL/soluble HEL (sHEL) DTg and MD4 anti-HEL single transgenic (STg) breeders were kindly provided by Dr. Michael C. Carroll (Harvard Medical School, Boston, MA) and Dr. Christopher C. Goodnow (Howard Hughes Medical Institute and Stanford University School of Medicine, Stanford, CA). DTg and STg mice were bred at Boston University Medical Center and offspring were screened as described below. DTg and STg mice were studied at 8–14 wk of age.

Genotyping. The mice were genotyped by PCR carried out on tail-digest DNA (26). In brief, tail DNA was analyzed by two separate PCR reactions. The first reaction, to screen for the presence of endogenous B cell antigen receptor (BCR) and anti-HEL transgenic BCR, included three oligonucleotide primers: Igα 5′ ACCACAGACCAGCAGGAGA 3′, shared by both the endogenous and transgenic BCR; Igα 5′ GCCAAGATTCTACACAGCAGGAGA 3′, unique to the endogenous BCR; and Igα 5′ CTCGGAGCTTAGCAAGGAT 3′, unique to the transgenic BCR. The second PCR reaction used an oligonucleotide primer pair to screen for the presence of HEL: HEL 5′ GAGCACTGCATCATCAGGAGA 3′, and HEL 5′ TCTGGTGACCCCTGGAGGAT 3′. After 32 cycles of amplification, the PCR products were size fractionated on 8% native polyacrylamide gels and visualized by ultraviolet transillumination after ethidium bromide staining. DTg anti-HEL/sHEL mice yield PCR products of 264 bp (endogenous Igα), 430 bp (Igα transgene), and 160 bp (lysozyme transgene).

Cell Purification. Splenic B cells were purified by depletion of T cells with antibody (13-4 anti-Thy 1.2, GK1.5 anti-CD4, and 3.1.55 anti-CD8), plus rabbit complement, and dead cells were removed by sedimentation on Lymphocyte M (Cedarlane Labs., Ltd., Hornby, Ontario, Canada), as previously described (13). Viable cells were resuspended in RPMI 1640 medium (Bio–Whittaker, Walkersville, MD) supplemented with 5% heat-inactivated FCS (Sigma Chemical Co., St. Louis, MO), 50 μM 2-ME, 2 mM l-glutamine, 10 mM HEPES, pH 7.2, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cell-mediated Cytotoxicity. B cells (2 × 10⁹) were cultured in 1.2 ml of supplemented RPMI 1640 in the wells of 24-well tissue culture plates (Costar Corp., Cambridge, MA) for a total of 16 h at 37°C and 6% CO₂. B cells were stimulated with CD40L–CD8α fusion protein (CD40L) cross-linked with CD8α-specific antibody (as 1:10 and 1:80 dilutions of diazoylated supernatant, respectively) to induce susceptibility to Fas-mediated apoptosis, as previously described, or with CD40L–CD8α/anti-CD8 in combination with sHEL, F(ab)₂ fragments of goat anti–mouse IgM antibody (anti-IgM), dextran-conjugated anti–mouse IgD antibody (anti-IgD), or recombinant mouse IL-4 (rIL-4). B cells were also treated with CD40L–CD8α/anti-CD8 in combination with biotinylated sHEL in the presence or absence of streptavidin (SA). B cells were then tested as targets for Fas-mediated apoptosis in standard lectin-dependent 4-h ⁵¹Cr-release assays, as previously described (11), using AE7 CD4⁺ Th1 effector cells.

Flow Cytometric Analysis. B cells stimulated as described above were examined for Fas expression by staining with PE-conjugated Jo-2 anti-Fas Ab or anti-CD28 isotype-matched control Ab (PharMingen, San Diego, CA) as previously described (11, 13). Unstimulated DTg and STg B cells were examined for bound HEL by staining with biotin-conjugated HyHEL9 anti-HEL monoclonal antibody, followed by PE-conjugated SA secondary antibody (PharMingen). B cells were stained with HyHEL9 in the presence of 0.1% azide before and after incubation with sHEL (Sigma Chemical Co.) at 100 ng/ml for 1 h at 4°C. Cells were then washed and analyzed by flow cytometry on a FACScan® instrument (Becton-Dickinson, San Jose, CA).

Results

Several previous reports suggest that Fas-mediated apoptosis may play a role in regulating autoreactive B cells, in which case inducible Fas resistance might be expected to influence this process (13, 22–25). To directly study the capacity of tolerant B cells to acquire resistance to Fas-mediated apoptosis in a receptor-specific fashion, mice expressing transgenes for B cell receptors that recognize HEL (anti-HEL), with or without transgenes for sHEL, were examined. B cells from DTg mice develop in the continual presence of soluble autoantigen and are rendered tolerant. B cells from STg mice are not exposed to cognate antigen during development and thus are not tolerized (30, 31).

Primary B cells from DTg and STg mice were stimulated for 16 h with CD40L to assess relative levels of Fas sensitivity. As shown in Fig. 1 A, CD40L treatment resulted in substantial upregulation of Fas expression, which was similar for B cells from DTg and STg mice. B cells were then tested as targets for Fas-mediated apoptosis in lectin-dependent 4-h ⁵¹Cr-release assays using Fas−-bearing AE7 CD4⁺ Th1 effector cells, as previously described (11). As shown in Fig. 1 B, CD40L-stimulated B cells from both DTg and
STg mice were susceptible to Th1-CMC at a level that approximated the specific lysis of CD40L-stimulated wild-type C57BL/6J B cells. In 10 separate experiments, the specific lysis of DTg B cells was 43.9 ± 3.4%, indicating little difference between the two populations.

To examine the capacity of surface Ig (sIg) to mediate Fas resistance in tolerant B cells, cytotoxicity assays were carried out following concurrent treatment of B cell targets with CD40L plus soluble HEL for 16 h as indicated. B cells were then radiolabeled and tested for susceptibility to Fas-dependent lysis in standard 4-h lectin-dependent 51Cr-release assays using AE7 CD4+ Th1 effector cells. Results for E/T cell ratios of 9:1 are presented. For each condition, the mean percentage of specific lysis of triplicate assays is displayed, along with a line indicating the SEM. One of three comparable experiments is shown.

In direct contrast, HEL had no effect on the Fas sensitivity of wild-type B cells, in keeping with the expectation that normal mice would have no or few B cells with chicken lysozyme specificity. Thus, engagement of sIg by specific antigen did not alter the susceptibility to Fas-mediated apoptosis of tolerant B cells, yet induced a high level of Fas resistance in nontolerant, HEL-specific B cells.

Cytotoxicity assays were also carried out to examine the effect of cross-linking B cell receptors with antibodies. Primary B cells from wild-type, STg, and DTg mice were treated with CD40L alone or were treated with the combination of CD40L and anti-IgD. Results for E/T cell ratios of 9:1 are presented as described above.
Fas Resistance in Tolerant B Cells

The addition of anti-IgM to CD40L produced similar levels of resistance to Th1-CMC in all three B cell populations. Further, STg and DTg B cell populations differed little in the levels of Fas resistance produced by anti-IgD (Fig. 2B). Thus, although sIg engagement by specific antigen failed to induce Fas resistance in tolerant B cells, sIg cross-linking by either anti-IgM or anti-IgD did so. These results indicate that the variant effect of sHEL on tolerant and nontolerant B cells cannot be explained by the different levels of sIgM and sIgD expressed.

In the anti-HEL/sHEL system, tolerant B cells develop in the presence of specific antigen, raising the possibility that constitutive occupation of transgenic BCR by HEL might block additional antigen binding (31). To address this issue, the degree of BCR occupancy was evaluated by flow cytometric analysis after staining of B cells with HyHEL9. DTg and STg B cells were examined before and after addition of sHEL in vitro. As shown in Fig. 3, addition of HEL to the STg BCR produced a marked increase in HEL binding detected by HyHEL9. Addition of HEL to the DTg BCR also produced an increase in HEL binding although the increment was not as great as that observed with STg B cells, because DTg B cells contain some bound HEL at baseline, derived from endogenous sources. Thus, DTg BCR can be effectively engaged by added sHEL, albeit to a lesser extent than the STg BCR. The identification of unoccupied receptors in DTg B cells suggests that some mechanism other than simple receptor blockade is responsible for deficiencies in sIg signaling unless there is an incremental threshold required for induction of Fas resistance.

The disparity between sHEL and anti-Ig in the induction of Fas resistance in tolerant B cells could relate to the relative degree of sIg cross-linking produced by these agents. To address this possibility, the outcome of cross-linking sHEL bound to the DTg BCR was evaluated. DTg B cells were treated with CD40L alone or with CD40L in conjunction with biotin-conjugated sHEL in the presence or absence of SA for 16 h, and then tested as targets for Fas-dependent lysis in standard 4-h lectin-dependent 51Cr-release assays using AE7 CD4+ Th1 effector cells. Results for E/T cell ratios of 9:1 are presented. For each condition, the mean percentage of specific lysis of triplicate assays is displayed, along with a line indicating the SEM. One of three comparable experiments is shown.
similar levels of resistance to Th1-CMC both in tolerant B cells from DTg mice and nontolerant B cells from STg mice. This is consistent with previous reports that IL-4R signaling in tolerant B cells is unimpaired (19, 20). In neither case could IL-4–mediated Fas resistance be attributed to a change in the level of Fas expression (compare with Fig. 1A). Thus, physiologic engagement of IL-4R in tolerant B cells produces Fas resistance, whereas BCR engagement by specific antigen does not.

Discussion

Our results delineate the regulation of Fas-mediated apoptosis in tolerant B cells. In several respects tolerant B cells behave like nontolerant B cells: susceptibility to Fas killing is produced by CD40 engagement and is opposed by higher order cross-linking of surface immunoglobulin and by engagement of IL-4 receptors by physiological ligand. However, tolerant B cells differed markedly from nontolerant B cells in the response to BCR triggering by cognate antigen, in that Fas-resistance is not induced in tolerant B cells but is induced in nontolerant B cells. These results show for the first time that specific antigen fails to elicit Fas-resistance in tolerant B cells, but that tolerant B cells may be protected against Fas killing by higher order BCR engagement and by engagement of IL-4R.

Tolerant B cells can be lost through a Fas-dependent mechanism (references 24 and 25 and our results), and Fas deficiency is associated with substantial autoactive antibody formation (22, 23), suggesting that interference with the susceptibility of tolerant B cells to Fas-mediated apoptosis may play a role in autoimmunity. This work suggests two mechanisms by which tolerant autoreactive B cells may escape Fas-dependent deletion. One involves high order cross-linking of surface immunoglobulin such as might be produced by immune complexes or by elevated levels of multivalent antigen. The other involves Th2-derived IL-4. Notably, sepsis may result in conditions fostering Fas-resistance in tolerant B cells in view of the association between overwhelming microbial infection, circulating microbial antigen, and elevated levels of cytokines (32). Considering the observation that autoantibodies in some cases recognize microbial antigens (33–35), it may be hypothesized that Fas resistance in tolerant B cells could be responsible for returning autoreactive B cells to the immunocompetent pool, resulting in the production of antimicrobial antibodies that cross-react with self, at the risk of initiating autoimmunity. This would be consistent with our earlier finding that IL-4 overexpressing transgenic mice express serological autoactivity (13).

Tolerant B cells were relatively restricted in the nature of sIg cross-linking capable of inducing Fas resistance, in contrast to nontolerant B cells, which were more permissive. This suggests that a threshold exists in the level of BCR stimulation required to complete signaling for Fas resistance, and that the threshold is higher for tolerant as opposed to nontolerant B cells. Consistent with this, we found that B7.2 (CD86) expression, although effectively induced in tolerant B cells by anti-IgM, was poorly stimulated by sHEL, whereas both agents induced B7.2 in nontolerant B cells (data not shown).

It is worth noting that induction of Fas resistance by soluble antigen in nontolerant, BCR transgenic B cells extends our earlier observation that Fas resistance is induced in normal, wild-type B cells by cognate antigen that is membrane bound (8), and provides a direct explanation for the improved recovery of nontolerant HEL-specific B cells after adoptive transfer with HEL and HEL-specific T cells (24, 25).

In sum, these results indicate that tolerant B cells are relatively insulated from self-antigen–induced modulation of the Fas death pathway, but, through the influence of IL-4 or super-threshold levels of BCR stimulation, may acquire Fas resistance and thereby contribute to autoantibody formation.

This work was supported by United States Public Health Service grant AI-40181 awarded by the National Institutes of Health.
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