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Fas Ligand Is Required for Resolution of Granulomatous Experimental Autoimmune Thyroiditis

Yongzhong Wei,* Kemin Chen,* Gordon C. Sharp,* and Helen Braley-Mullen2*†‡

We previously suggested that CD8+ T cells promoted resolution of granulomatous experimental autoimmune thyroiditis (G-EAT) at least in part through regulation of Fas ligand (FasL) expression on thyroid epithelial cells. To directly evaluate the role of the Fas pathway in G-EAT resolution, Fas- and FasL-deficient mice on the NOD.H-2h4 background were used as recipients of activated G-EAT effector cells. When MTg-primed wild-type (WT) donor splenocytes were activated and transferred to WT recipients, thyroid lesions reached maximal severity on day 20 and resolved on day 50. Fas, FasL, and FLIP were up-regulated, and many apoptotic inflammatory cells were detected in recipient thyroids on day 20. Fas was predominantly expressed by inflammatory cells, and FasL and FLIP were mainly expressed by thyroid epithelial cells. After depletion of CD8+ T cells, G-EAT resolution was delayed, FLIP and FasL were predominantly expressed by inflammatory cells, and few inflammatory cells were apoptotic. When WT donor splenocytes were transferred to gld recipients, disease severity on day 20 was similar to that in WT recipients, but resolution was delayed. As in CD8-depleted WT recipients, there were few apoptotic inflammatory cells, and FLIP and FasL were expressed primarily by inflammatory cells. These results indicated that the expression of functional FasL in recipient mice was critical for G-EAT resolution. WT cells induced minimal disease in lpr recipients. This was presumably because donor cells were eliminated by the increased FasL on lpr recipient cells, because donor cells were not eliminated, and the mice developed G-EAT if lpr recipients were given anti-FasL mAb.

Granulomatous experimental autoimmune thyroiditis (G-EAT) is a CD4+ T cell-mediated autoimmune disease inducible in genetically susceptible strains of mice by immunization with mouse thyroglobulin (MTg) and adjuvant or by adoptive transfer of spleen cells from MTg-primed donors activated in vitro with MTg and IL-12 (1–6). G-EAT is characterized by proliferation of thyroid epithelial cells (TEC), granuloma formation, and infiltration of the thyroid by T lymphocytes, histiocytes, multinucleated giant cells, and variable numbers of neutrophils (1, 4, 6). G-EAT lesions reach maximal severity 19–21 days after cell transfer, and when disease is moderately severe (3–4+), thyroid inflammation resolves almost completely by days 35–50 (1, 4, 6). CD4+ T cells are the primary effector cells for G-EAT and depletion of CD4+ T cells in recipient mice can prevent or reverse development of G-EAT (1, 4). Depletion of recipient CD8+ T cells has no effect on G-EAT development, but inhibits G-EAT resolution, indicating that spontaneous resolution of G-EAT requires CD8+ T cells (3–5).

Fas ligand induces apoptosis of Fas-expressing cells through formation of a death-inducing signaling complex and initiation of a signaling cascade of caspases. FLIP can block activation of caspase-8 and is an important inhibitor of the initial upstream steps of Fas-mediated apoptosis (7). Apoptotic signaling through Fas and Fas ligand plays a critical role in the maintenance of peripheral lymphocyte homeostasis and termination of immune responses (7, 8). The Fas/Fas ligand (FasL) pathway plays an important role in several autoimmune diseases, including Hashimoto’s thyroiditis, Grave’s disease (9–11), multiple sclerosis (12), insulin-dependent diabetes (13), experimental allergic encephalomyelitis (EAE) (14, 15), type 1 diabetes in NOD mice (16–19), and EAT (9, 20–23). However, the mechanisms by which the Fas/FasL pathway regulates autoimmune diseases remains controversial, and additional studies are required to fully elucidate its role in regulating autoimmune inflammatory responses. Recovery from EAE is associated with apoptotic elimination of encephalitogenic T cells through the Fas pathway, and FasL also contributes to initiation of EAE (14, 15, 24). Fas-deficient NOD-lpr/lpr mice have a reduced incidence of diabetes after transfer of diabeticogenic T cells (16, 17, 25), and β cell destruction correlates with Fas expression on β cells (26, 27). NOD mice heterozygous for the gld mutation have reduced functional FasL expression on T cells, but no lymphadenopathy, and they do not develop diabetes (16), and administration of anti-FasL Ab prevents diabetes induced by cyclophosphamide (19). Although these results clearly demonstrate a significant role for the Fas/FasL pathway in autoimmune diabetes, other results suggest that FasL is not an effector molecule in β cell death and autoimmune diabetes (28–30).

Previous studies from our laboratory suggested that the Fas pathway plays a critical role in G-EAT resolution, but is not required for initial damage of thyroids by activated effector cells (5, 22). To directly determine whether a functional Fas pathway is critical for G-EAT resolution, we performed a series of adoptive transfers of splenocytes using wild-type (WT), Fas-deficient, and FasL-deficient mice on the NOD.H-2h4 background. Resolution was delayed when WT splenocytes were transferred to gld recipients, indicating that the expression of functional FasL in recipient mice is critical for G-EAT resolution.

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†Abbreviations used in this paper: G-EAT, granulomatous experimental autoimmune thyroiditis; EAE, experimental allergic encephalomyelitis; FasL, Fas ligand; MTg, mouse thyroglobulin; TEC, thyroid epithelial cell; WT, wild type.

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Materials and Methods

Mice

NOD.H-2b4 WT mice (31) were generated in our breeding colony at University of Missouri. NOD-lpr and NOD-gld male mice, obtained from Dr. Y. Wang (Alexion Pharmaceuticals, Cheshire, CT) (16), were crossed with NOD.H-2b4 females, and the F1 mice were crossed to generate F2 mice. The F2 progeny were selected for homozygosity at H-2Kk by flow cytometry analysis of peripheral blood, and H-2Kk mice were selected for expression of the lpr or gld phenotypes by PCR analysis of tail DNA. H-2Kk mice heterozygous for the lpr and gld mutations were bred and maintained under specific pathogen-free conditions, and the homozygous lpr and gld offspring were selected and used for the studies reported in this study. WT control mice were +/- or +/- littermates of the lpr and gld mice. NOD.H-2b4 Thy1a+ mice were generated by breeding NOD.Thy1a mice (The Jackson Laboratory, Bar Harbor, ME) with NOD.H-2b4 (Thy1b) females. F2 progeny were selected for homozygosity at H-2Kk and for the expression of Thy1a and the absence of Thy1b by flow cytometry of peripheral blood. Both male and female mice were used for these experiments. Donor mice were generally 8–10 wk old at the time of immunization, and recipients were 6–7 wk old. All mice were bred under specific pathogen-free conditions in accordance with University of Missouri institutional guidelines for animal care.

Induction of G-EAT

Donor mice (NOD.H-2b4 or NOD.H-2b4-Thy1a) were injected i.v. twice at 10-day intervals with 150 µg of MTg and 15 µg of LPS (Escherichia coli 011:B4; Sigma-Aldrich, St. Louis, MO). Seven days later, donor spleen cells were activated in vitro with 25 µg/ml MTg and 5 ng/ml IL-12 (1, 4). Cells were harvested after 72 h and washed twice, and 3.5 × 10^6 cells were transferred i.v. to 500-rad irradiated syngeneic recipients. Recipient thyroids were examined 19–21 days (peak of disease) or 35–50 days (resolution) after cell transfer (1, 4, 22).

Depletion of CD8+ T cells

In all experiments, donor mice were given anti-CD8 mAb (ATCC HB129; American Type Culture Collection, Manassas, VA) 4 days after the second immunization with MTg and LPS. In some experiments recipient mice were given anti-CD8 mAb 1 day after cell transfer and then every 10 days until termination of the experiment (3). CD8+ T cell depletion in both donors and recipients was determined to be complete by flow cytometry. Depletion of recipient CD8+ T cells inhibits G-EAT resolution, resulting in chronic inflammation that persists for 2–3 mo, whereas depletion of donor CD8+ T cells has no effect on G-EAT development in recipient mice (3, 6, 22, 23).

Anti-FasL treatment

Recipient lpr mice were given the anti-FasL mAb MFL1 or hamster Ig as a control previously described (22).

Evaluation of thyroiditis

Thyroids were collected, fixed in formalin, sectioned, and stained with H&E as previously described (4). Thyroids were scored quantitatively for EAT severity (the extent of thyroid follicle destruction) using a scale of 1+ to 5+, as described previously (1, 4, 5). The 1+ thyroiditis is defined as an infiltrate of at least 125 cells in one or several foci, 2+ is 10–20 foci of cellular infiltration involving up to 25% of the gland, 3+ indicates that 25–50% of the gland is infiltrated, 4+ indicates that >50% of the gland is destroyed by infiltrating inflammatory cells, and 5+ indicates virtually complete destruction of the thyroid with few or no remaining follicles.

Flow cytometry

For typing of lpr, gld, and Thy1a+ mice, peripheral blood was analyzed for expression of the NOD.H-2b4 H-2Kk allele and absence of the NOD.H-2b4-H-2Kk allele using PE-conjugated 16-1-11N (anti-H-2k, Caltag Laboratories, Burlingame, CA) and SF1.1 (anti-K+, BD Pharmingen, San Diego, CA) and for expression of Thy1a and absence of Thy1b using PE-conjugated anti-Thy1.1 and anti-Thy1.2 (BD Pharmingen). In experiments involving transfer of Thy1a+ WT donor cells into Thy1b+ WT, lpr, or gld recipients, recipient spleen cells were dual-stained and examined for expression of CD4+ T cells expressing Thy1a or Thy1b using FITC-conjugated anti-CD4 (BD Pharmingen) and PE-conjugated anti-Thy1a or anti-Thy1b. Spleen cells were also examined for expression of B220+Thy1b+ cells using FITC-conjugated B220 (Caltag Laboratories) and PE-conjugated anti-Thy1b. Cells were examined using a FACScan (BD Biosciences).

Immunohistochemistry

Immunohistochemical staining for Fas and FasL was described previously (22). Briefly, formalin-fixed, paraffin-embedded tissue sections were deparaffinized and incubated with affinity-purified rabbit polyclonal anti-Fas (M20; 1/200) or anti-FasL (N20; 1/400; Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min. After incubation with a secondary biotinylated goat anti-rabbit Ab (1/500; Jackson Immunoresearch Laboratories, West Grove, PA), immunoreactivity was demonstrated using the avidin-biotin complex peroxidase system (Vector ABC peroxidase kit; Vector Laboratories, Burlingame, CA) with 3,3-diaminobenzidine tetrahydrochloride. Immunohistochemical staining for FLIP used cryostat sections fixed in acetone for 10 min at 4°C. After treatment for 30 min with 0.1% saponin in 1% BSA, slides were washed and incubated 30 min with rabbit anti-FLIP (H150; 1/200; Santa Cruz Biotechnology) Goat anti-rabbit (1/500; Jackson Immunoresearch Laboratories) IgG was used as secondary Ab, with Vector VIP (very intense purple; Vector Laboratories) as the chromogen. Slides were counterstained with hematoxylin. Primary Ab was replaced with an equal amount of normal rabbit IgG (negative control for Fas, FasL, and FLIP).

RT-PCR amplification

Total RNA was isolated from spleens using TRIzol (Invitrogen Life Technologies, Gaithersburg, MD). Total mRNA was converted to cDNA by murine leukemia virus reverse transcriptase (PerkinElmer/Cetus, Branchburg, NJ) and oligo(dT)-16 primers. To determine the relative initial amounts of target cDNA, each cDNA sample was serially diluted 1/5, 1/25, and 1/125, and each dilution was amplified with primers. Hypoxanthine phosphoribosyltransferase was used as a housekeeping gene to verify that the same amount of RNA was amplified. The Fas and FasL gene primers were described previously (5). To compare relative levels of mRNA transcripts between different groups, samples were reverse transcribed and amplified at the same time using aliquots of reagents from the same master mix. PCR was performed as previously described (5). PCR products were separated by electrophoresis in 2% agarose gels and visualized by UV light after ethidium bromide staining. Densitometric analysis was performed using an IS-1000 Digital Imaging System (Life Sciences, St. Louis, MO). Samples within the linear relationship between input cDNA and final PCR products (usually 1/25 cDNA dilution) were collected, and the densitometric units for each cytokine band were normalized to those for the corresponding hypoxanthine phosphoribosyltransferase band.

Western blot

Spleens were homogenized using lysis buffer (50 mM HEPES, 250 mM NaCl, 5 mM EDTA, and 0.1% Triton). After centrifugation at 15,000 rpm for 10 min at 4°C, supernatants were collected and electrophoresed by adding 40 µg of protein to a 10% SDS-PAGE gel. Samples were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were incubated overnight at 4°C in TBS containing 5% nonfat dry milk and 0.05% Tween 20 and probed 2 h at room temperature with anti-FasL (1/5000). After washing, membranes were incubated with a peroxidase-conjugated goat anti-rabbit IgG (1/5000) for 1 h at room temperature, and the reaction was detected with a chemiluminescence detection kit (Pierce, Rockford, IL) (23). For normalization of signals, the membranes were stripped by incubating at 60°C for 30 min in stripping buffer (62.5 mM Tris-HCl (pH 6.8), 100 mM 2-ME, and 2% SDS), washed in TBS-T, and reprobed with 1/2000 anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA) primary Ab and 1/2000 HRP-conjugated anti-rabbit IgG as secondary Ab. Bands were scanned and quantitated using Quantity One-4.1 software (Quality One, Huntington Station, NY). Each lane in the figures represents protein extracts pooled from three spleen fragments (each representing ~10% of the whole spleen) from normal mice or mice with the severity scores specified in the figure legends.

Determination of apoptosis

Apoptosis was determined using TUNEL (Intergen, Purchase, NY), following the manufacturer’s instructions with some modification (22). Briefly, sections were deparaffinized and dehydrated, and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in PBS for 30 min. Sections were incubated at room temperature for 10 min with equilibration buffer, followed by 1-h incubation at 37°C with TdT enzyme (or reaction buffer negative controls) diluted in reaction buffer in a humidity chamber. The TdT reaction was stopped with stop/wash buffer, and sections were washed with PBS before 30-min incubation with antidigoxigenin conjugated with HRP. After washing, color development was

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obtained by incubating the sections with 0.05% 3,3-diaminobenzidine tetrahydrochloride. Slides were counterstained with hematoxylin. Positive reactions were characterized by the brown color of the nuclear or perinuclear region of the cell.

Statistical analysis

All experiments were repeated at least three times. Statistical analysis was performed using unpaired two-tailed Student’s t test. A value of $p < 0.05$ was considered significant and is designated by asterisks in the figure legends.

Results

WT spleen cells transfer severe G-EAT to both WT and gld recipients, but G-EAT spontaneously resolves in WT, but not gld, recipients

We previously showed that anti-FasL treatment of recipient mice inhibited resolution of G-EAT, but had no effect on the development of thyroid lesions (22), suggesting that the Fas/FasL pathway is important for G-EAT resolution, but is not required for thyroid damage by activated effector cells. To directly examine the role of Fas and FasL in G-EAT, MTg-sensitized splenocytes from WT mice were activated in vitro and transferred to WT and gld recipients. WT donor splenocytes transferred to WT recipients induced G-EAT that reached maximal severity 20 days after cell transfer. Thyroid lesions diminished (resolved) by 50 days in most WT recipients (Fig. 1). FasL-deficient (gld) recipients of the same WT donor cells developed G-EAT with similar severity scores on day 20 (Fig. 1). However, G-EAT resolution was inhibited in gld recipients, and most mice still had severity scores of 3–4/H11001 20 (Fig. 1). However, G-EAT resolution was inhibited in gld recipients of WT donor cells 20 days after cell transfer (Fig. 2). Although some TEC in gld recipients of WT cells expressed FasL, FasL was primarily expressed by inflammatory cells in thyroids of gld recipients (Fig. 2I). This suggests that up-regulation of FasL by TEC may be important for clearing effector CD4+ T cells by apoptosis, thus promoting G-EAT resolution, whereas expression of FasL primarily by inflammatory cells results in chronic inflammation.

FLIP can inhibit apoptosis in the Fas, TNF receptor, and TRAIL pathways through its ability to block activation of caspase-8 (7). FLIP was predominantly expressed by TEC in WT recipients of WT donor cells 20 days after cell transfer (Fig. 2J), and this could be important in protecting the FasL+ TEC from apoptosis. In anti-CD8-treated WT recipients and in gld recipients of WT donor cells, G-EAT resolution was inhibited, and FLIP was primarily expressed by inflammatory cells (Fig. 2, K and L). This could play a role in protecting inflammatory cells from apoptosis so that they persist, resulting in chronic inflammation.

WT donor cells do not survive after transfer to lpr recipients

The results presented to date suggest that expression of functional FasL and FLIP by TEC in recipient mice is important for G-EAT resolution. To determine whether Fas expression by recipient cells also played a role in G-EAT, WT donor cells were transferred to Fas-deficient (lpr) NOD.H-2h4 recipients. Minimal disease developed in lpr recipients 20 days after cell transfer (Fig. 3), and this diminished completely by day 50 (data not shown). The fact that WT donor cells did not induce G-EAT in lpr recipients could indicate that lpr recipients are resistant to G-EAT or that donor WT cells were depleted or eliminated before they could express their pathogenic potential. Other studies (data not shown) suggest that
lpr mice are not inherently resistant to development of EAT, because lpr (and gld) mice immunized with MTg and LPS developed mild EAT (average 1+/H11001 severity) that was only slightly less severe than that developing in similarly immunized WT mice (average 1–2+/H11001 severity). To determine whether the lack of development of G-EAT in lpr recipients of WT donor cells could be explained by poor survival of donor cells in lpr recipients, splenocytes from Thy1a+/NOD.H-2h4 mice were activated with MTg and IL-12 and transferred to WT, lpr, or gld Thy1.2+/H11001 recipients. Thyroids were removed 20 days later to assess disease severity, and spleen cells were examined for expression of Thy1a (donor) vs Thy1b (recipient) by flow cytometry. As shown in Table I, 28–44% of the CD4+/H11001 T cells detected in WT and gld recipients were Thy1a+/H11001 cells derived from the donors. In contrast, very few Thy1a+/H11001 donor CD4+/H11001 T cells were detected in most lpr recipients on days 20–21 (Table I) or as early as 7 days after cell transfer (data not shown), suggesting that WT spleen cells survived poorly in lpr recipients.

Others have shown that lpr cells overexpress FasL (16, 32, 33), which could result in killing of lymphocytes from WT mice. To determine whether FasL was up-regulated in NOD.H-2h4/lpr mice, FasL expression was compared in thyroids and spleens of lpr

Table I. Survival of donor Thy1.1+ WT CD4+ T cells in WT, lpr, and gld, recipient spleens

<table>
<thead>
<tr>
<th>Recipients</th>
<th>CD4+/Thy1.1+</th>
<th>CD4+/Thy1.2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>32, 32, 33</td>
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</tr>
<tr>
<td>lpr</td>
<td>0.3, 0.7, 0.4</td>
<td>92, 91, 88</td>
</tr>
<tr>
<td>gld</td>
<td>28, 31, 44</td>
<td>65, 59, 52</td>
</tr>
<tr>
<td>WT</td>
<td>33, 37, 40, 43, 44</td>
<td>66, 62, 59, 56, 55</td>
</tr>
<tr>
<td>lpr</td>
<td>0.9, 2, 2, 15, 27</td>
<td>99, 97, 97, 84, 72</td>
</tr>
<tr>
<td>lpr anti-FasLb</td>
<td>13, 16, 38, 40, 46</td>
<td>87, 83, 62, 60, 53</td>
</tr>
<tr>
<td>gld</td>
<td>28, 43, 43, 48</td>
<td>71, 56, 57, 52</td>
</tr>
</tbody>
</table>

*Results are expressed as the percentage of recipient CD4+ splenic T cells expressing Thy1.1 (donor) or Thy1.2 (recipient) 20 (lines 1–3) or 21 (lines 4–7) days after cell transfer. Values represent the percentages of cells for individual mice tested in each group.

bThe lpr recipients received 0.5 mg of anti-FasL (MFL1) every 3 days beginning on the day of cell transfer.
and WT recipients. Consistent with previous reports (16, 33), FasL mRNA (Fig. 4A) and protein (Fig. 4B, lane 1 vs lane 3) were clearly increased in spleens of normal nonimmunized lpr compared with WT mice. FasL protein was also higher on spleen cells of lpr recipients of WT donor cells compared with WT recipients of WT cells (Fig. 4B, lane 2 vs lane 4; p < 0.04 for digitalized images of triplicate Western blots). FasL was expressed by TEC of nonimmunized lpr mice (Fig. 4C), but not by TEC of WT mice (Fig. 4D). These results suggest that elevated levels of FasL in lpr recipients may have induced apoptosis of WT donor spleen cells so that only minimal G-EAT developed.

To directly determine whether increased FasL in lpr recipients could explain the poor survival of WT cells and their inability to induce G-EAT in lpr recipients, MTg-sensitized and activated Thy1a+ donor cells were transferred to WT, lpr, and gld recipients, and one group of lpr recipients was treated with the anti-FasL mAb MFL1 to block Fas/FasL interactions. As shown in Table I, many Thy1a+ donor cells were detected in WT and gld recipients, but many fewer donor Thy1a+ cells were detected in most untreated lpr recipients. Treatment of lpr recipients with anti-FasL resulted in increased survival of Thy1a+ donor cells, suggesting that increased FasL expression could explain the poor survival of WT cells in lpr recipients. G-EAT severity scores in anti-FasL-treated lpr recipients were comparable (2–4+ severity) to those induced by the same cells in WT (Fig. 3) and gld (data not shown) recipients. The necessity to treat lpr recipients with anti-FasL to achieve survival of WT donor cells did not allow us to determine whether G-EAT would resolve normally in lpr recipients, because anti-FasL inhibits G-EAT resolution (22). FasL (Fig. 4, I and J) and FLIP (data not shown) were mainly expressed by TEC in thyroids of untreated lpr recipients and also in thyroids of untreated WT recipients whose lesions resolved (Figs. 4H and 2J). As expected, spleens of anti-FasL-treated or untreated lpr recipients expressed minimal Fas (not shown). Fas expressed by inflammatory cells in thyroids of anti-FasL-treated lpr recipients of WT donor cells (Fig. 4G) was presumably contributed by WT donor cells, because Fas was undetectable in thyroids of untreated lpr recipients in which few donor cells survived (Fig. 4F).

**Discussion**

In this study we asked whether FasL-mediated apoptosis of MTg-specific T cells contributed to resolution of G-EAT in NOD.H-2b4 mice. Although FasL-deficient gld recipients of WT donor cells developed G-EAT similar in incidence and severity to that developing in WT recipients of the same donor cells, G-EAT resolution was inhibited in gld recipients compared with WT recipients (Fig. 1). This observation together with our previous study in which G-EAT resolution was prevented in CBA/J mice given anti-FasL mAb (22) demonstrate that FasL expression by the recipient (presumably the thyroid) is critical for G-EAT resolution. Fas and FasL proteins were not detected in thyroids of nonimmunized WT NOD.H-2b4 mice (Fig. 4, A, B, and D). However, both proteins
were up-regulated 20 days after cell transfer when disease severity was maximal (Fig. 2, D and G) and declined when lesions resolved (data not shown). Many apoptotic inflammatory cells were detected on day 20 in thyroids of WT recipients of WT donor cells (Fig. 2A), and FasL and FLIP were expressed primarily by TEC (Fig. 2, D and G). Anti-CD8 treatment of WT recipients inhibited G-EAT resolution (Fig. 1), and few apoptotic inflammatory cells were detected (Fig. 2B). This could be due to decreased FasL expression by TEC (Fig. 2H) and/or increased FLIP expression by inflammatory cells (Fig. 2K). G-EAT resolution was also inhibited in FasL-deficient gld recipients (Fig. 1). Their thyroids had few apoptotic inflammatory cells (Fig. 2C), and FLIP was expressed mainly by inflammatory cells (Fig. 2L). Expression of FLIP by inflammatory cells in thyroids of gld and anti-CD8-treated WT recipients could function to inhibit apoptosis of effector CD8+ T cells, leading to chronic inflammation. In contrast, expression of FLIP by TEC in WT recipients of WT donor cells in which G-EAT lesions resolved (Fig. 2J) could protect TEC from apoptosis.

The results with gld mice directly demonstrate that FasL expression by recipient mice is important for G-EAT resolution. Because FasL is up-regulated on TEC of recipients when thyroid lesions resolve and is expressed primarily by inflammatory cells when lesions persist, e.g., in gld and anti-CD8-treated WT mice (Fig. 2), the thyroids of recipient mice clearly need to express functional FasL for resolution to occur. However, CD8+ T cells, shown to be derived primarily from recipient mice, are critical for G-EAT resolution (3, 5, 22), and they function, at least in part, to promote up-regulation of FasL on TEC (22). Therefore, these results do not rule out the possibility that expression of functional FasL by recipient CD8+ T cells is essential for induction of FasL on TEC. Studies with bone marrow chimeric mice are in progress to determine whether functional FasL expression by recipient thyroids, recipient CD8+ T cells, or both is most critical for resolution of G-EAT. The finding that expression of FasL by tissue cells such as TEC can contribute to resolution of inflammation is consistent with results in other models. For example, FasL expression by cells in the CNS is important for spontaneous remission in EAE (14, 15), and expression of FasL by nonlymphoid tissues or in immunologically privileged sites such as the eye can promote the survival of these tissues after injury or inflammation (34–37). A critical role for FasL expression by nonlymphoid cells in termination of immune responses to foreign Ags has also been demonstrated (38). In our studies depletion of CD8+ T cells in WT recipients inhibited G-EAT resolution, and FasL expression in thyroids was reduced. FasL, induced on TEC by the infiltrating T cells, e.g., CD8+ T cells, might function to induce apoptosis of effector cells and resolve the disease. The precise mechanisms by which CD8+ T cells regulate FasL expression by TEC are unknown. CD8+ T cells may produce inflammatory cytokines such as TNF-α, which was shown to enhance FasL mRNA expression in intestinal epithelium (39). TNF-α is expressed in thyroids of mice with G-EAT, and neutralization of TNF-α promotes G-EAT resolution (our unpublished observations). Studies are in progress to determine whether TNF-α regulates FasL expression by TEC.

FLIP is a well-defined inhibitor of Fas receptor signaling (7). Overexpression of FLIP inhibits Fas-mediated apoptosis of T lymphocytes (40) and can exacerbate autoimmune diseases such as EAE and multiple sclerosis (12, 41, 42). FLIP was strongly expressed by inflammatory cells in thyroids of gld recipients and anti-CD8-treated WT recipients in which G-EAT resolution was inhibited (Fig. 2, K and L), whereas FLIP was expressed by TEC in WT mice whose lesions resolved (Fig. 2J). This suggests that increased FLIP and decreased FasL expression by inflammatory cells might block apoptosis of effector CD4+ T cells, resulting in chronic inflammation. In contrast, high FLIP expression on TEC that also express FasL, observed in WT recipients with early resolution, might protect the TEC from apoptosis.

MTg-primed WT spleen cells transferred minimal G-EAT to lpr recipients (Fig. 3). Others have shown that lpr mice highly express FasL mRNA (16, 32, 33), and in our studies FasL expression was stronger in thyroids of lpr recipients than in WT recipients with similar disease severity (not shown). Spleens and thyroids of nonimmunized lpr mice expressed FasL (Fig. 4, A–C), whereas FasL was undetectable in spleens and thyroids of nonimmunized WT mice (Fig. 4, A–C). The elevated FasL, apparently contributed to the early depletion of WT donor cells in lpr recipients because administration of anti-FasL Ab resulted in increased survival of donor cells (Table I) and development of G-EAT in lpr recipients (Fig. 3). Transgenic mice that overexpress FasL on TEC are resistant to EAT due to killing of infiltrating inflammatory cells by FasL-expressing thyrocytes (20). The high levels of FasL in Fas-deficient lpr mice were shown to eliminate transferred T cells in other models (28, 33), and T cells from WT NOD donors were selectively eliminated by FasL-expressing, double-negative host cells in NOD-lpr recipients after adoptive transfer, presumably accounting for their failure to infiltrate host islets and induce diabetes (16). These results are consistent with those reported in this study and suggest that increased FasL expression by lymphoid or nonlymphoid tissues can protect such tissues from damage and limit the duration of immune responses by inducing apoptosis of infiltrating Fas+ cells (35, 36, 38, 39).

In summary, our results indicate that apoptosis involving the Fas-FasL pathway is critically involved in G-EAT resolution. It is likely that Fas-FasL interactions lead to the deletion of disease-initiating, autoreactive T cells in thyroids when FasL is expressed by thyrocytes. Coexpression of the antiapoptotic molecule FLIP by FasL-expressing TEC apparently promotes resolution by protecting FasL-expressing TEC from apoptosis. These findings are important in understanding the mechanisms of resolution of inflammation in autoimmune diseases and may be helpful in designing new protocols for the treatment of autoimmune disease.

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