Cell Research (2000),**10**, 245-266

REVIEW

Inducible resistance to Fas-mediated apoptosis in B cells

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ABSTRACT

Apoptosis produced in B cells through Fas (APO-1, CD95) triggering is regulated by signals derived from other surface receptors: CD40 engagement produces upregulation of Fas expression and marked susceptibility to Fas-induced cell death, whereas antigen receptor engagement, or IL-4R engagement, inhibits Fas killing and in so doing induces a state of Fas-resistance, even in otherwise sensitive, CD40-stimulated targets. Surface immunoglobulin and IL-4R utilize at least partially distinct pathways to produce Fas-resistance that differentially depend on PKC and STAT6, respectively. Further, surface immunoglobulin signaling for inducible Fas-resistance bypasses Btk, requires NF-κB, and entails new macromolecular synthesis. Terminal effectors of B cell Fas-resistance include the known anti-apoptotic gene products, Bcl-xL and FLIP, and a novel anti-apoptotic gene that encodes FAIM (Fas Apoptosis Inhibitory Molecule). faim was identified by differential display and exists in two alter-

Abbreviations: AICD, activation-induced cell death; ALPS, autoimmune lymphoproliferation syndrome; CARD, caspase recruitment domain; CK, casein kinase; CMC, cell-mediated cytotoxicity; DISC, death-inducing signaling complex; EST, expressed sequence tag; FADD, Fas-associating protein with death domain; FAIM, Fas apoptosis inhibitory molecule; FISH, fluorescence in situ hybridization; FLICE, FADD-like ICE; FLIP, FLICE inhibitory protein; HEL, hen egg lysozyme; ICE, interleukin-1 β-converting enzyme; IFN, interferon; KO, knock-out; LPS, lipopolysaccharide; PARP, poly-ADP ribose polymerase; PCR, polymerase chain reaction; PH, pleckstrin homology; PKC, protein kinase C; PMA, phorbol myristate acetate; RT, reverse transcription; TNFR, tumor necrosis factor receptor

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natively spliced forms; faim-S is broadly expressed, but faim-L expression is tissue-specific. The FAIM sequence is highly evolutionarily conserved, suggesting an important role for this molecule throughout phylogeny. Inducible resistance to Fas killing is hypothesized to protect foreign antigen-specific B cells during potentially hazardous interactions with FasL-bearing T cells, whereas autoreactive B cells fail to become Fas-resistant and are deleted via Fas-dependent cytotoxicity. Inadvertent or aberrant acquisition of Fas-resistance may permit autoreactive B cells to escape Fas deletion, and malignant lymphocytes to impede anti-tumor immunity.

key words: *Apoptosis, Fas, B lymphocytes, FAIM, FLIP, Bcl-*x_L*, surface immunoglobulin, IL-4R, CD40, autoreactivity.*

INRODUCTION

Fas/APO-1 (CD95) is a death receptor of the TNF receptor (TNFR) family, and shares with death receptors TNFR1, DR3 (TRAMP, Wsl, APO-3), DR4 (TRAIL-R1) and DR5 (TRAIL-R2) extracellular cysteine-rich pseudorepeats, intracellular C-terminal death domains, and reliance on FADD as a critical early mediator of signaling for cell death (although FADD-dependence has been questioned for DR4)[1-7]. The cognate ligand for Fas (FasL) is an activation antigen expressed by CD4+ Th1 effector cells and CD8 cytotoxic T lymphocytes, (although its activity in the latter is frequently masked by the more substantial destructive power of the perforin-granzyme system), as well as natural killer cells[8-13]. The congenital loss of Fas (as in lpr/lpr mice and Fas null mice, and in human Autoimmune Lymphoproliferation Syndrome) or of its ligand (as in gld/gld mice) is accompanied by severely elevated levels of serum autoantibodies associated with frank autoimmunity[14-24]; for this reason Fas is thought to play a key role in the regulation of B cells, particularly in the regulation of autoreactive B cells. These observations are supported and refined by additional experiments, carried out over the last dozen years, that more clearly document abnormal B cell function in Fas-deficient animals and distinguish B cell defects from abnormalities in the T cell population. Thus, in both mixed radiation chimeras and tetraparental chimeras, autoimmune immunoglobulin was derived solely from lpr B cells and not from co-existing phenotypically normal B cells that were simultaneously exposed to abnormal lpr T cells [25-27]. Moreover, autoantibody production was present and undiminished when Fas deficiency was restricted to B cells through production of transgenic MRL- lpr mice

that express Fas within the thymocyte and peripheral T cell populations by use of the murine lck promoter, in comparison to unmanipulated MRL-lpr mice[28]. These results indicate that a block in the Fas death pathway leads to dysregulation of B cells that express self-recognizing specificities, suggesting that under normal circumstance Fas functions to delete such autoreactive B cells.

In lpr and Fas null B cells, Fas-induced cell death is blocked congenitally by loss of surface Fas expression. However, this is a somewhat artificial, certainly non-physiological, way of thwarting Fas killing. In view of the very severe immune dyscrasias attendant to genetic interference with Fas signaling for cell death, the question arose as to whether Fas-mediated cell death is ever blocked during normal immune cell activity, because if that would happen, it might contribute to autoimmune B cell activity. More generally, it was unclear to what extent the outcome of Fas signaling is modulated by other influences, particularly receptor-specific ones. Although it was known that B cell activation, as with lipopolysaccharide, was associated with Fas expression and sensitivity to Fas signaling for cell death that greatly exceed the low levels present in naive B cells[29-33], we pursued this issue further, by evaluating the effect of triggering certain surface receptors in primary B cells. Early on it was found that B cell stimulation through CD40 for 1-3 d markedly upregulated both surface Fas expression, and sensitivity to Fas-mediated apoptosis. In direct contrast, B cell stimulation through surface immunoglobulin (sIg) failed to enhance susceptibility to Fas killing at all. Thus, these two mitogenic receptor- mediated signals differ markedly in their capacity to increase the sensitivity of B cell targets to Fas-mediated apoptosis[34].

We then evaluated whether the failure of sIg engagement to induce Fas-sensitivity resulted from a passive process, due to inadequate signaling for this outcome, or a dominant process, due to active suppression of cytotoxicity. To address this issue, primary B cells were stimulated with CD40L in combination with, or sequentially with, anti-Ig, and then examined for susceptibility to apoptosis in the form of FasL-dependent Th1-cell mediated cytotoxicity (Th1-CMC). B cells stimulated by CD40L plus anti-Ig expressed increased levels of surface Fas comparable in magnitude to the elevated levels present in B cells stimulated by CD40L alone; however, the susceptibility of dual-stimulated B cells to Fas-mediated apoptosis was much reduced in comparison to B cells stimulated only by CD40L[34], [35]. Whereas the percent specific lysis produced during a 4 h ^{51}Cr release assay of B cells stimulated by CD40L alone typically ranged from 40-60 % at the highest Th1 effector: B cell target ratio tested (9:1), the corresponding percent specific lysis for B cells stimulated by both CD40L and anti-Ig was only 0-15%. Considering a large number of experiments, the mean level of protection against Fas killing produced by sIg signaling amounted to more than 20-fold, meaning that a greater than 20-fold higher Th1 effector: B cell target ratio was needed to produce comparable levels of apoptosis in B cells stimulated by the combination of CD40L plus anti-Ig as in B cells stimulated by CD40L alone[35]. Thus, anti-Ig-induced sIg crosslinking established a state of resistance to Fas-mediated apoptosis, or Fas-resistance. Moreover, sIg

engagement by specific antigen similarly produced Fas-resistance, in experiments in which B cells were obtained from transgenic animals expressing either B cell receptors that recognize H-2K^{k,b}, or B cell receptors that recognize HEL, demonstrating that sIg-induced Fas-resistance is a physiologic process and is not dependent on the use of crosslinking anti-Ig antibody[34],[36].

The opposing effects of CD40 and sIg engagement on Fas signaling for cell death raised the question of whether engagement of other surface receptors might similarly alter susceptibility to Fas-mediated apoptosis. To address this issue, various ligands were tested in conjunction with CD40L stimulation prior to evaluating sensitivity to Th1 cell-mediated cytotoxicity. Through this effort IL-4, alone among the cytokines examined, was found to produce Fas-resistance[37]. As with anti-Ig, IL-4 produced Fas-resistance when added either concurrently, or sequentially, with CD40L, and did so without any change in the elevated level of Fas expression produced by CD40 signaling; however, IL-4 in optimal doses was less effective than anti-Ig, providing a level of protection against Fas-mediated apoptosis that averaged about 10-fold[37].

These results with anti-Ig and IL-4 indicate that in normal B cells, Fas-mediated apoptosis can be suppressed through inducible receptor-triggered mechanisms completely distinct from genetic alteration of Fas. This paradigm is supported by subsequent reports of in vitro experiments in the human system, and of in vivo experiments in the murine system[38-41]. Thus, the outcome of B cell stimulation with respect to Fas signaling is regulated on the basis of particular environmental cues as interpreted by specific receptor binding.

In the experiments described above, Fas-mediated apoptosis was initiated by Th1 cell membrane-bound FasL, and Fas-resistance was observed in B cells stimulated by CD40L plus anti-Ig, or by CD40L plus IL-4. Similar results were obtained with recombinant, soluble FasL[35]. Surprisingly though, a different outcome appeared when Fas killing was triggered by soluble Jo-2 monoclonal anti-Fas antibody, in which case the level of protection against Fas-mediated apoptosis was much reduced in comparison to that which was apparent with Th1 effector cells, to the point that Fas-resistance was not always evident. The failure to observe Fas-resistance in antibody-treated B cells suggests that Jo-2 produces a super-physiological stimulus that overwhelms receptor-driven Fas-resistance. The reason for this might be that B cell FcR crosslinking of whole anti-Fas antibody produces more efficient Fas engagement, inasmuch as Jo-2-induced Fas killing was blocked by 2.4G2 anti-FcR antibody, or it might be that the anti-Fas antibody binds an epitope distinct from that engaged by FasL, inasmuch as soluble FasL did not block the ability of Jo-2 to produce apoptosis in Fas-resistant B cells treated with CD40L plus anti-Ig[35] (and unpublished results). Regardless of the mechanism, however, the results with Jo-2 indicate that sIg- and IL-4R-induced protection against Fas-mediated apoptosis is relative rather than absolute, and can be overcome by especially strong apoptotic signals derived from Fas, including those produced by particularly high Th1 effector cell: B cell target cell ratios.

Receptor signaling for Fas-resistance

Although the outcomes of engaging sIg and IL-4R in terms of inducible resistance to Fas-mediated apoptosis are similar, the pathways utilized to bring this about differ, as evidenced by the reliance of Fas-resistance induction on specific signaling mediators. Thus, inhibition of PKC by cell-permeant H7, or depletion of PKC by prolonged exposure to PMA, blocked induction of Fas-resistance by anti-Ig but had no effect on IL-4R-induced Fas-resistance[35]. Conversely, the loss of STAT6 (in B cells obtained from STAT6-deficient mice) blocked induction of Fas-resistance by IL-4 but had no effect on sIg-induced Fas-resistance[42]. (Of interest, sIg engagement was recently shown to trigger STAT6 activation[43], which raised the possibility that STAT6 might form the basis of a common pathway leading both from sIg and from IL-4R to inducible Fas-resistance; however, the results discussed above negate this possibility). Further, the relative speed with which Fas-resistance was established also differed for anti-Ig and IL-4. Whereas anti-Ig acted rapidly to produce Fas-resistance that was apparent at 3 h, and maximal after 12 h, IL-4-induced Fas-resistance developed much more slowly and only reached a maximum after 24 h of exposure[35],[37]. These results taken together indicate that sIg and IL-4 receptors utilize at least partially non-overlapping intracellular signaling pathways to bring about Fas-resistance.

The timecourse experiments were carried out by stimulating B cells with CD40L for a total of 48 h, and adding anti-Ig or IL-4 at various times before the end of the culture period. Because B cell treatment with CD40L for 24 h was sufficient to induce Fas-sensitivity, the capacity of anti-Ig and IL-4 to produce maximal Fas-resistance when added 24 h before the end of 48 h culture periods with CD40L means that these reagents do not simply prevent the development of Fas-sensitivity in response to CD40 engagement but actually reverse susceptibility to Fas-mediated apoptosis that has already been established.

Additional work on metabolic pathways has focused on sIg-induced Fas-resistance. In view of the PKC requirement for Fas-resistance produced by anti-Ig, the capacity of PKC-activating phorbol ester treatment to induce Fas-resistance was examined. Phorbol ester produced only partial protection against Fas-mediated apoptosis, whereas the combination of a phorbol ester plus a calcium ionophore brought about virtually complete Fas-resistance, the level of which was as high or higher than that associated with anti-Ig[35]. This recapitulates results with cell cycle progression as an endpoint, for which only the combination of a phorbol ester and a calcium ionophore, which together mimic the effects of sIg-triggered PLC-mediated second messengers, produces S phase entry[44],[45]. Thus, Fas-resistance can be established in the absence of specific receptor engagement by appropriate stimulation of intracellular mediators.

Btk is an indispensable intermediary in the sIg pathway leading to cell cycle progression, as evidenced by the complete failure of S phase entry in B cells from xid mice, in which Btk is mutated, or in B cells from Btk knock-out mice, in which Btk is absent

[46-48]. In view of the importance of Btk for sIg-induced proliferation, the role of Btk in sIg signaling for Fas-resistance was examined using B cells from both kinds of mice. With xid B cells, the levels of Fas-sensitivity produced by CD40L stimulation, and of Fas-resistance induced by anti-Ig, were indistinguishable from results obtained with wild-type control B cells. With Btk knock-out B cells, CD40L stimulation produced Fas-sensitivity that was minimally increased (by 20-30 %) over that observed with control B cells; however, there was no difference between B cells obtained from Btk knock-out and from wild-type control mice in the degree of Fas-resistance produced by anti-Ig[49]. The unexpected minor increase in Fas-sensitivity in Btk knock-out B cells may relate to the report that Btk binds Fas and interferes with Fas signaling for cell death, although the modulation of Fas-sensitivity observed here in murine B cells was much less impressive than that reported for chicken DT40 B cells[50]. The xid and Btk knock-out results together indicate that Btk plays no role in sIg signaling for Fas-resistance. Thus, the signaling pathway that leads from sIg to Fas-resistance differs completely from the route leading from sIg to S phase entry, in that Btk is absolutely required for the latter but not at all for the former.

The finding that Fas-resistance develops over a period of hours following anti-Ig treatment suggested that new gene expression might play a key role. To examine this possibility, B cells were treated with inhibitors of macromolecular synthesis along with anti-Ig during the last 6 h of 48 h cultures with CD40L (longer periods of exposure being precluded by drug toxicity); the addition of either cycloheximide or actinomycin D completely blocked the induction of Fas-resistance by anti-Ig[35],[51]. Thus, Fas-resistance induced by sIg engagement appears to depend on new gene expression and new protein synthesis. These results also indicate that the mechanism responsible for sIg-induced Fas-resistance in primary B cells differs from that reported for the A20 B cell line, which is not interrupted by cycloheximide[52].

The dependence of sIg-induced Fas-resistance on new gene expression implies, in turn, a role for factors that transcriptionally activate anti-apoptotic genes. A candidate factor might be NF-κB, a transactivating complex that is developmentally regulated and is induced as a result of sIg engagement, inasmuch as NF-κB has been reported to regulate the expression of some anti-apoptotic genes, and to influence the Fas-sensitivity of T cell lines[53-61]. To evaluate the role of NF-κB, inhibitors of nuclear NF-κB translocation were added along with anti-Ig during the last 12 h of 48 h cultures with CD40L; the proteasome inhibitor lactacystin[62],[63] and the antioxidant pyrrolidinedithiocarbamate (PDTC) [64] each completely blocked sIg-induced Fas-resistance, while having no effect on the ability of CD40L alone to upregulate Fas expression and to produce Fas-sensitivity. As an important corollary, lactacystin and PDTC also inhibited the activation of NF-κB that was produced when anti-Ig was added to CD40L-stimulated B cells. These results with two different inhibitors, operating through two different mechanisms of action, indicate that sIg signaling for Fas-resistance requires NF-κB activation and is thus NF-κB-dependent. Notably, PMA induces

vigorous nuclear translocation of NF-κB in mature B cells but only partial resistance to Fas-mediated apoptosis[35],[53], suggesting that either NF-κB induction is a necessary but not sufficient condition for Fas-resistance, or that Fas-resistance depends on the induction of sIg-specific κB-binding complexes.

Recently, two groups reported that sIg-induced NF-κB activation, like sIg-induced proliferation, is Btk-dependent[65], [66]. In contrast, the experiments discussed above indicate that sIg-induced Fas-resistance requires NF-κB yet takes place in the absence of Btk. The reason for these seemingly conflicting results remains unclear. It may be that prior CD40L stimulation establishes a new intracellular signaling pathway through which sIg activates NF-κB in the absence of Btk. Or it may be that induction of Fas-resistance requires only a very minimal threshold level of NF-κB. Alternatively, inhibitors of NF-κB activation may at the same time inhibit another signaling mediator, and this latter mediator, not NF-κB, is the important molecular effector. These possibilities are currently being tested.

Two terminal effectors of Fas-resistance: Bcl-x_L *and FLIP*

The identity of gene products whose synthesis might be responsible for inducible Fas-resistance was sought by testing likely prospects according to two criteria: upregulation of expression under conditions that produce Fas-resistance, and, induction of Fas-resistance by upregulated expression in the absence of receptor engagement. Initial experiments designed to detect such molecules focused on members of the Bcl-2 family, some members of which interfere with mitochondrial cytochrome c release[67]. A number of Bcl-2- homologous genes were assayed for induction by anti-Ig treatment of CD40L-stimulated B cells through RT-PCR. Only bcl-x_L was upregulated. Bcl-x_L expression was strongly induced at the RNA level within an hour of anti-Ig addition, and at the protein level within 6-12 h[68]. B cells obtained from Bcl-x_L-overexpressing transgenic mice were then tested for susceptibility to Th1 cell mediated cytotoxicity after stimulation by CD40L alone, in order to determine the capacity of Bcl-x_L, overexpressed in isolation, to produce Fas-resistance. These B cells were (3-fold) less sensitive to Fas killing than wild-type littermate control B cells, despite similar levels of upregulated Fas expression, and similar levels of activation as judged by thymidine incorporation[68]. Thus, Bcl-x_L appears to account for at least a portion of sIg-induced Fas-resistance because, expression was upregulated coordinately with induction of Fas-resistance (in both murine and human B cells, reference[69]), and, because isolated overexpression diminished B cell susceptibility to Fas killing separate and apart from any other potential effects of sIg engagement (although in connection with this last point it should be mentioned that the capacity of Bcl-x_L to inhibit Fas-mediated apoptosis in B cell lines has been questioned, reference[70]). Further, the reduction in susceptibility to Th1-induced cytotoxicity produced by Bcl-x_L suggests that Fas death signaling in B cells involves mitochondrial cytochrome c release; this notion is supported by our observation that inducibly Fas-resistant B cells are protected against apoptosis produced by C2-ceramide,

the cytotoxicity of which has been associated with mitochondrial damage[71-75][71-75].

There are, however, several indications that Bcl-x_L does not fully account for Fas-resistance produced by anti-Ig: 1) the loss of Fas-sensitivity in (CD40L-stimulated) Bcl-x_L-overexpressing B cells was incomplete, and was less dramatic than the loss of Fas-sensitivity observed in wild-type B cells stimulated by anti-Ig plus CD40L; 2) anti-Ig treatment of (CD40L-stimulated) Bcl-x_L-overexpressing B cells produced a further (approximately 3-fold) reduction in the already muted Fas-sensitivity of these B cells; and, 3) Bcl-x_L}protein did not appear in normal CD40L-stimulated B cells treated with anti-Ig until after the initial onset of diminished sensitivity to Fas. These considerations strongly suggest that at least one other anti-Ig-responsive molecule plays a role in blocking Fas killing following sIg engagement.

Additional experiments designed to elucidate the identity of gene products potentially responsible for sIg-induced Fas-resistance focused on evaluation of FLIP. FLIP is a FLICE/caspase 8 homolog that lacks an active enzyme site and thus competitively inhibits downstream signaling that normally occurs following FLICE/caspase 8 binding to FADD[76-79]. Our early Northern blot experiments suggested that some FLIP expression was induced by anti-Ig treatment of CD40L-stimulated B cells, but the data were inconclusive owing to the very low level of FLIP expression detected. However, definitive results were subsequently obtained by RT-PCR, which showed that FLIP RNA expression was induced within an hour of adding anti-Ig to CD40L-stimulated B cells, and that FLIP protein expression was upregulated within 6 h. These results were recently extended by reports indicating that FLIP overexpression interferes with Fas-mediated apoptosis in primary B cells and in B cell lines[80],[81]. These studies together strongly suggest that FLIP is involved in bringing about sIg-induced resistance to Fas-mediated apoptosis because the two criteria discussed above were met, as described earlier for Bcl-x_L: FLIP expression was upregulated coordinately with induction of Fas-resistance by anti-Ig, and isolated FLIP overexpression diminished B cell susceptibility to Fas signaling for cell death. (In these studies a number of other known anti-apoptotic genes screened as outlined above failed to meet the criterion of expression coincident with induction of Fas-resistance, including A1, A20, BAG-1, Bcl-2, IAP-1, IAP-2, Ich-1S, IEX-1L and survivin, references[82-90].

Identification of a novel Fas apoptosis inhibitory molecule (FAIM)

To identify additional genes that might be responsible for resistance to Fas-mediated apoptosis without regard to conserved motifs or known properties, transcripts induced as a result of anti-Ig treatment that produces Fas-resistance were detected using a differential display strategy[91]. Through this approach, a completely novel 1.2 kb sequence was cloned that contains an open reading frame encoding a protein product of 179 amino acids predicted to reflect a stable, soluble, β-strand-rich molecule that is slightly acidic[51]. The function of this gene was evaluated with BAL-17 murine B lymphoma cells, the activation responses of which mimic those of primary B cells in a

number of ways[92], including upregulated Fas expression and increased sensitivity to Fas killing following CD40L stimulation. BAL-17 B cells were transfected with the gene cloned in pBKCMV, or with empty vector, selected in G418, and then stimulated with CD40L for 24 h and examined for susceptibility to Th1 cell-mediated cytotoxicity. Cytotoxicity was assessed in two ways, by chromium release after exposure of transfectants to FasL-bearing Th1 effector cells, and by propidium iodide-staining for subdiploid DNA after treatment of transfectants with Jo-2 anti-Fas antibody. Regardless of the means by which Fas was engaged or cytotoxicity was assessed, Fas-mediated apoptosis was markedly reduced in B cells expressing the gene of interest as compared to vector transfectants, using both G418-resistant pools and stably transfected lines cloned by limiting dilution[51]. Considering the dose-response curve with Th1 effector cells, Fas-resistance produced by the gene of interest amounted to a greater than 9-fold level of protection against Fas-mediated apoptosis.These results indicate that the differentially expressed gene encodes a Fas Apoptosis Inhibitory Molecule, which we termed FAIM. The anti-apoptotic function of FAIM was demonstrated further at a biochemical level by evaluating cleavage products of poly-ADP ribose polymerase (PARP) that result from caspase activation[93, 94]. PARP fragments were detected within 40 minutes of treating vector-transfected BAL-17 B cells with Jo-2 anti-Fas antibody, whereas such fragments were not observed in faim-transfected BAL-17 B cells up to 60 minutes after antibody treatment[51]. This correlated well with the transgenically enhanced level of FAIM expression, which was demonstrated by Western blotting to be greatly increased over the endogenous levels present in vector-transfected controls. These results indicate that FAIM blocks Fas killing by interfering with a step prior to terminal caspase activity.

As might be expected from the original selection conditions for differential display analysis, faim mRNA increased within an hour of adding anti-Ig to CD40L-stimulated B cells, and expression continued to rise over the next 5 h, as shown by Northern blotting. Along the same lines, FAIM protein increased within 6 h of treating CD40L-stimulated B cells with anti-Ig, and expression continued to rise, peaking at 12 h, as detected by Western blotting[51]. The finding of substantially increased FAIM expression in splenocytes obtained from sheep red blood cell-immune animals, as compared to splenocytes obtained from control animals injected with saline, suggests that these in vitro studies of inducible FAIM expression bear relevance for physiological immune responses. Thus, upregulation of FAIM expression coincides with B cell treatment by Fas-resistance-inducing regimens in vitro, and with induction of foreign antigen-specific immune responses in vivo.

The murine faim sequence contains no known effector domains; that is, it lacks kinase or phosphatase domains, caspase or CARD domains, PH or SH2 domains, and so forth. However, we readily identified highly homologous faim sequences in other species, indicating strong evolutionary conservation[51]. Human faim was obtained by constructing a consensus sequence from overlapping expressed sequence tag (EST)

fragments that share some homology with mouse faim, plus direct sequencing of a single EST clone that completely spans the putative human transcript[95]. The predicted amino acid sequence of human FAIM is 90% identical to the predicted amino acid sequence of mouse FAIM. These results were extended by Southern analysis showing hybridization by a mouse faim probe to genomic DNA from all mammalian species tested (human, monkey, rat, mouse, dog, cow, and rabbit) (unpublished observations). Worm faim was identified by directly sequencing cDNA amplified by PCR, using primers based on our own manual analysis of the putative exonic structure of a C. elegans genomic sequence of unknown function that contains discrete regions of homology with mouse faim. The predicted amino acid sequence of C. elegans FAIM is 50% identical to the predicted amino acid sequence of mouse FAIM. Fly faim was obtained by computer-aided searching of the recently posted D. melanogaster genome, using accepted algorithms to define putative exon/intron structure. The putative predicted amino acid sequence of D. melanogaster FAIM is 36% identical to the predicted amino acid sequence of mouse FAIM, with the caveat that the fly sequence has not been amplified directly from cDNA [96]. Examination of the predicted FAIM sequences from these 4 species indicates that specific regions of very high identity exist within the amino terminal region that likely represent novel anti-apoptotic effector domains or domains required for FAIM association with another mediator or modulator protein. Two distinct short stretches of 12 and 13 amino acids each are 69-75 % identical across all four species[96]. The evolutionary conservation of faim strongly suggests that the faim gene product is an important regulatory molecule that has been favored for retention throughout phylogeny, and thus likely plays a key role in cellular viability or other processes. (Since there is no known Fas homolog in C. elegans or D. melanogaster, it is presumed that FAIM carries out a similar, but possibly not Fas-specific, anti-apoptotic function in the worm and the fly.)

Two other characteristics of FAIM suggest that it plays an important role in regulating cellular processes. 1) The faim gene is broadly expressed. High level faim gene expression was documented in murine brain, thymus, kidney and spleen by Northern blotting, and in additional normal tissues by RT-PCR[51],[96], suggesting that FAIM performs an essential function in non-lymphoid, as well as in lymphoid, cells. 2) The faim gene localizes to a cytogenetic region associated with disease. Human faim was localized to chromosome 3q22-25 by fluorescence in situ hybridization (FISH), and to chromosome 3q25 by radiation hybrid analysis of a faim-containing EST[96]. This region contains loci associated with B cell lymphoma, acute non-lymphocytic leukemia, and squamous cell carcinoma of the lung[97-99]. The mechanism by which mutation of a gene that blocks Th1 cell-mediated cytotoxicity might inure to the benefit of malignant cells is speculative at best, but such changes might increase the level of FAIM expression or enhance the anti-apoptotic activity of FAIM. Recently, inhibition of Fas-mediated apoptosis by overexpression of FLIP was shown to increase lymphoma tumorigenicity[100],[101], supporting the concept that modulation of Fas signaling for cell

death plays a role in determining the effectiveness of anti-tumor immunity, and providing a rationale for a connection between tumor-associated loci and anti-apoptotic genes like faim.

During our initial cloning efforts the faim sequence was obtained from two sources- a mouse thymus library and, subsequently, a mouse brain library. However, the two sequences differ. The original thymus-derived FAIM sequence, now termed FAIM-Short (FAIM-S), is shorter by 22 5' amino acids in comparison to the brain-derived sequence, now termed FAIM-Long (FAIM- L)[96]. Recently we identified a human faim-L homolog among publicly available EST fragments, which similarly encodes 22 additional 5' amino acids, 21 of which are identical to the additional sequence in murine FAIM-L, indicating a high degree of homology in this region, although this sequence contains no presently recognized effector motifs. To clarify the origin of the two faim sequences, the mouse faim genomic locus was characterized[96]. Mouse faim was localized to a region (9f1) syntenic to the chromosomal location of human faim by FISH analysis. Three BAC clones containing the faim locus were obtained by screening a murine 129Sv genomic library, and one of these clones was used as a template for analysis. All intron/exon boundaries were confirmed by direct sequencing[102]. The murine faim locus was shown to consist of 6 exons. Exons IV and VI in M. musculus were each found to represent the fusion of 2 exons identified in C. elegans, so that the intron/exon organization is simplified in the higher organism, which although unusual is not unprecedented[103-107]. Two putative translation initiation sites were identified on separate exons (II and III), indicating that faim-L and faim-S result from alternative splicing. Through PCR amplification utilizing a 5' primer that corresponds to the 5' untranslated region, faim-L was shown to be expressed in primary tissue, although among multiple tissues examined, expression of faim-L was extremely tissue-specific, and was strictly limited to the brain[96]. The function of FAIM-L is presently unknown, and the possibility that FAIM-L is active only in the brain, or that FAIM-L fulfills a unique role in the brain, has not been ruled out.

These results indicate that faim is a novel gene without precedent in publicly available databases, whose protein product inhibits Fas-mediated apoptosis, and whose expression is upregulated coordinately with induction of Fas-resistance by sIg engagement in vitro and during antigen-specific immune responses in vivo. These features suggest that FAIM is involved in the physiological regulation of immune cell activity, inducibly suppressing Fas signaling for cell death along with Bcl-x_L and FLIP. The multigenic nature of sIg-induced Fas-resistance suggests, in a larger sense, that the relative susceptibility of B cells to Fas-mediated apoptosis is determined by the balance between multiple pro- and anti-apoptotic molecules, whose expression changes rapidly in response to shifting environmental conditions, such as the presence of antigen and/or T cell-derived lymphokine.

Speculative role for Fas-resistance

T cell activation results in upregulation of CD40L expression[108-110], as well as upregulation of FasL expression[8],[1111-114]. As much as engagement of (constitutively expressed) B cell CD40 is necessary for T-dependent stimulation, CD40 triggering also upregulates Fas expression, and engagement of B cell Fas by T cell FasL initiates apoptotic events. In view of this, activated T cells represent a potential threat to B cell viability, and this may well be the point at which inducible Fas-resistance plays a key role, by protecting B cells against FasL expressed by interacting Th1 cells and thereby promoting B cell immune responses. Two manifestations of this have been documented. 1) Fas-resistant B cells present antigen more effectively than Fas-sensitive B cells. This was demonstrated by experiments in which antigen-pulsed, mitomycin-treated Fas-resistant B cells produced greater proliferation of antigen-specific Th1 cells than did similarly treated, Fas-sensitive B cells[115]; improved antigen presentation presumably occurred because Fas-resistant B cells were protected against the threat of death posed by initially activated T cells, and thus remained viable to present antigen to another round of T cells. 2) Fas-resistant B cells block activation-induced cell death (AICD) of T cells, whereas Fas-sensitive B cells do not. This was demonstrated by experiments in which primed T cells were exposed a second time to antigen (SEB or HEL) presented by B cells; the resulting Fas-induced cytotoxicity (AICD) of T cells was markedly reduced when antigen was presented by Fas-resistant B cells, presumably because Fas-bearing, apoptosis-resistant B cells competitively inhibited T cell FasL, preventing interaction with T cell Fas[116]. These results suggest that Fas-resistance induced in B cells may enhance and perpetuate T, as well as B, cell responses. The idea that inducible Fas-resistance modulates B and T cell deletion, and promotes B and T cell responses, is further supported, somewhat indirectly, by the observed durability of the receptor-specific phenotype, which was unchanged when B cells were washed and re-cultured for 24 h in medium prior to assay of Th1 cell-mediated cytotoxicity.

Fas-resistance in tolerant B cells

The model described above, which suggests that inducible Fas-resistance acts to protect B cells from FasL-bearing, activated T cells, raises the issue of autoreactive B cells, the very B cells that are dysregulated in the absence of Fas expression. Inasmuch as these B cells appear to be eliminated in a Fas-dependent fashion[15],[117-121], the induction of Fas-resistance (as a result of interaction with ever present self antigen) could lead to deleterious consequences, because of the possibility that normal processes of autoreactive B cell deletion might be thwarted. It would seem necessary for the rules governing the regulation of Fas-mediated apoptosis to be different for autoreactive B cells in comparison to normal (foreign antigen-specific) B cells.

To address this issue, B cells were obtained from transgenic mice expressing anti-HEL antigen receptors, and from double transgenic mice that express both the anti-HEL receptor as well as a transgene encoding soluble HEL (anti-HEL/sHEL). B cells

from anti-HEL/sHEL mice are continually exposed to what is, for them, autoantigen, as a result of which these transgenically autoreactive B cells are tolerant, and thus do not signal normally through sIg[122-124]. B cells from both kinds of transgenic mice were stimulated with CD40L alone or with CD40L in combination with soluble HEL, and tested for susceptibility to Fas-mediated apoptosis. The addition of soluble HEL produced resistance to Fas killing only in B cells from anti-HEL mice; there was no effect of sHEL on the Fas-sensitivity of CD40L-stimulated B cells from anti-HEL/sHEL mice, despite the fact that upregulated levels of Fas expression and Fas-sensitivity produced by CD40L were similar for B cells obtained from single transgenic and double transgenic mice[36]. Thus, specific antigen differentially induced Fas-resistance in non-autoreactive and in autoreactive B cells, succeeding in the former but failing in the latter. However, it was noted that anti-Ig produced similar levels of Fas-resistance in CD40L-stimulated B cells from single and double transgenic mice, suggesting that autoreactive B cells are not totally intractable to induction of Fas-resistance through sIg signaling but, instead, may simply require a higher degree of sIg crosslinking for this outcome. To examine this issue, B cells from anti-HEL/sHEL mice were stimulated with CD40L alone or with CD40L in combination with biotinylated sHEL, with or without additional crosslinking imposed by streptavidin. In the presence of streptavidin, autoreactive B cells from anti-HEL/sHEL mice acquired resistance to Fas-mediated apoptosis, similar in magnitude to that produced by anti-Ig[36]. These results strongly suggest that in autoreactive B cells an elevated threshold of sIg signaling must be exceeded for induction of Fas-resistance, as compared to the degree of sIg signaling needed for induction of Fas-resistance in foreign-antigen-specific B cells. In this way the possibility that self antigen might induce Fas-resistance through sIg signaling in autoreactive B cells is minimized, and sIg-induced Fas-resistance is unlikely to contribute to serological autoimmunity under normal circumstances.

The situation with IL-4 is very different. Similar levels of Fas-resistance were produced by IL-4 in CD40L-stimulated B cells obtained from single and double transgenic mice[36]. Thus, the capacity of IL-4R engagement to produce Fas-resistance was not altered in autoreactive B cells. These results suggest the possibility that autoreactive B cells might escape Fas-dependent deletion because of IL-4R signaling (which, under normal circumstances, would be averted by mechanisms that maintain T cell tolerance). In support of this notion, serum samples (5 out of 5 tested) obtained from IL-4-overexpressing transgenic mice[125] were found to contain autoantibodies by indirect immunofluorescence on HEp-2 cells[37]. HEp-2-staining serum autoantibodies were similarly reported in sera obtained from a separate IL-4 transgenic line[126]. More recently, the role of IL-4 has been examined using the defined anti-HEL/sHEL system by breeding triple transgenic (anti-HEL/sHEL/IL-4) animals and then testing sera for anti-HEL antibody. Preliminary results indicate that these mice (10 out of 10 examined) produced increasing amounts of serum anti-HEL autoantibody with increasing age, and in so doing broke tolerance. Although none of these studies precludes the possibil-

ity that IL-4 affects a non-B cell target, all are consistent with the ability of IL-4 to induce Fas-resistance and thereby interfere with the known Fas-deletional pathway for elimination of autoreactive B cells. This conclusion is supported by the observation that constitutive expression of B7.2 led to a breakdown in tolerance in anti-HEL/sHEL transgenic mice[127], which our results infer was due to complete stimulation of T cells to produce IL-4 which in turn induced B cell Fas-resistance.

The results discussed above suggest that induction of Fas-resistance, dysregulation of autoreactive B cells and disruption of B cell tolerance, along with the appearance of autoantibodies, could result either from sIg stimulation that exceeds a certain threshold, as might be produced by immune complexes, or from IL-4R engagement not directly related to cognate interactions, as might occur during periods of heightened immunological stress that may be accompanied by lymphokine overproduction[128].

SUMMARY AND DISCUSSION

The most important conclusion to be drawn from these studies is that B cells are not monomorphic targets for Th1 cell-mediated cytotoxicity but rather modulate their own intrinsic susceptibility to Fas-mediated apoptosis in response to specific environmental cues in a dynamic fashion in which Fas- sensitivity changes rapidly as a result of specific receptor binding. Engagement of the B cell antigen receptor or the IL-4 receptor triggers at least partially non-overlapping, inducible pathways that result in a durable state of Fas-resistance that is mediated by Bcl-xL, FLIP, and FAIM, and possibly other molecules that are as yet unknown. Fas-resistance likely plays an important role during critical, early B cell:T cell interactions, protecting B cells from the inherent threat to survival posed by FasL expression on the part of activated T cells. In this way inducible Fas-resistance is hypothesized to enhance serological immune responses and, perhaps, to promote T cell responses by improving antigen presentation and by blocking AICD. Notably, Fas-resistance, as represented by the absence of functional Fas expression in mutant mice or by overexpression of FLIP in retrovirally transduced chimeras, produces serological autoreactivity, and thus may constitute an etiologic factor in clinically important autoimmunity[26], [28],[80]. In other words, the presence or absence of receptor-induced Fas-resistance is likely to be a key element in determining the usefulness or aberrance of real world immune responses.

Modulation of Fas-sensitivity is not limited to B cells. T cells may upregulate Fas expression but still fail to undergo apoptosis in response to Fas signaling. This occurs shortly after activation through the T cell receptor in vitro. With time and continued stimulation, the Fas-resistant phenotype reverses, and T cells become Fas-sensitive, without any change in the elevated level of Fas expression, perhaps as a result of IL-2R signaling and alterations in the levels of Bcl-xL and FLIP, although the precise mechanism remains uncertain[76],[129-133]. Additional reports suggest that Fas-sensitivity is modulated in anergic T cells, memory T cells, primed T cells and Th2 cells[134-139].

Thus, inducible changes in the sensitivity of T cells to Fas-mediated apoptosis likely play a role in T cell homeostasis and in T cell immune responses.

Malignant cells, both lymphoid and non-lymphoid, manifest resistance to Fas-mediated apoptosis[140-149]. Inasmuch as inhibition of Fas signaling for cell death is associated with tumor development and progression, it appears that Fas-mediated apoptosis constitutes at least a portion of anti-tumor immunity, and that some malignancies take advantage of mechanisms that block Fas killing to maintain viability and co-exist within the framework of a normal immune system[100,101], [150-153]. The mechanisms utilized by malignant cells may represent perversions of the physiological means by which B cells normally inhibit susceptibility to Fas-mediated apoptosis in a receptor-specific fashion. If true, molecules involved in establishing sIg-induced Fas-resistance might form targets for pharmacologic manipulation of the apoptotic balance between host and tumor, including NF-κB, Bcl-x_L, FLIP, and/or FAIM[51,56, 68,76,154, 155].

The sum of the work described above, in conjunction with evidence provided by other investigators, strongly suggests that the regulation and dysregulation of Fas-mediated apoptosis contributes to the level and importance of normal immune, autoimmune, and tumor-immune responses. New insights are likely to arise from further study of the inter-relationships between surface receptor engagement and susceptibility to Fas signaling, and of mechanisms and molecules responsible for inducible Fas-resistance, which may provide new targets for influencing normal and abnormal immune activity.

ACKNOWLEDGEMENTS

This work was supported by United States Public Health Service grants AI40181 and AI45112 awarded by the National Institutes of Health and by a grant from the Arthritis Foundation.

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