Peripheral T Cells Undergoing Superantigen-induced Apoptosis In Vivo Express B220 and Upregulate Fas and Fas Ligand
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Summary
Staphylococcal enterotoxin B (SEB) is a bacterial superantigen (SAg) that predominantly interacts with Vβ8+ T cells. In vivo treatment of mice with SEB leads to an initial increase in the percentage of Vβ8+ T cells, followed by a decrease in the numbers of these cells, eventually reaching lower levels than those found before treatment with the SAg. This decrease is due to apoptosis of the SEB-responding cells. In the present study, we use the distinct light scattering characteristics of apoptotic cells to characterize T cells that are being deleted in response to SEB in vivo. We show that dying, SEB-reactive T cells express high levels of Fas and Fas ligand (Fas-L), which are implicated in apoptotic cell death. In addition, the B cell marker B220 is upregulated on apoptotic cells. Moreover, we show that the generation of cells with an apoptotic phenotype is severely impaired in response to SEB in functional Fas-L-deficient mutant gld mice, confirming the role of the Fas pathway in SAg-mediated peripheral deletion in vivo.

Materials and Methods
Animals and Treatments. 8–10-wk old BALB/c mice were obtained from Harlan Olac (Bicester, UK). C57BL/6 and congenic B6SmnC3H-gld mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The TCR Vβ8.2 transgenic mouse line (C57BL6.Tg93/lbm spf) (16) was kindly provided by Dr. H. Bluethmann (Hoffman-La Roche AG, Basel, Switzerland). These were backcrossed onto the BALB/c background. Staphylococcal enterotoxin B (SEB) (10 µg) from Toxin Technology (Sarasota, FL) was injected into each of the hind footpads.

Antibodies. The following directly labeled primary Abs were used: anti-CD4-PE (Becton Dickinson and Co., Palo Alto, CA), anti-CD8-PE (Boehringer Mannheim, Mannheim, Germany); anti-Vβ (F23.1) (17), anti-Vβ 6 (44.22.1) (18), anti-CD3 (17A2) and (Fab'2) anti-mouse Ig, were directly coupled to FITC in our laboratory. Anti-FasL mAb (24G2) was purchased from Pharmingen (San Diego, CA). Affinity-purified polyclonal rabbit anti–Fas-L (PE62) (19) and rabbit anti-Fas (AL59, made against the NH2-terminal 12 residues of mouse Fas, Muller, C., and J. Tschopp, unpublished data) antibodies were revealed by FITC-coupled donkey anti-rabbit Ig (Dianova, Hamburg, Germany). Anti-FcR mAb (24G2) was purchased from Pharmingen (San Diego, CA).

Flow Cytometric Analysis and Cell Sorting. Draining LN cells were stained with the relevant antibodies and either sorted using a FACStar® Plus or analyzed on a FACScan® (Becton Dickinson and Co., Mountain View, CA). Sorted cells were incubated at

Abbreviations used in this paper: Fas-L, ligand for Fas; FSC, forward scattering; SAg, superantigen; SEB, staphylococcal enterotoxin B; wt, wild type.

After in vivo challenge with conventional antigen or with superantigen (SAg), peripheral T cells undergo clonal expansion followed by apoptosis (1–6). The expression of cell surface markers has been extensively studied in resting and activated T cells, and a substantial body of knowledge exists concerning the conditions under which T cells can undergo apoptosis. By contrast, little is known about the surface phenotype of apoptotic cells and their expression of molecules that may be implicated in their death. One such molecule is Fas/APO-1 (CD95), an integral membrane protein of the TNFR family (7). Fas is expressed on a variety of cell lines and on in vitro–activated T cells (8). Cross-linking Fas with specific antibodies results in apoptosis (9). The ligand for Fas (Fas-L) is a member of the TNF family, and is expressed on certain cell lines, in vitro–activated T cells, and freshly isolated NK cells (7, 10). Fas-L can deliver a death signal by triggering Fas (11, 12). In this study, we take advantage of the distinct light scattering characteristics of dying cells on a flow cytometer (reviewed in reference 13) to characterize SAg-specific T cells undergoing apoptosis. We show that apoptotic CD4+ or CD8+ cells retain expression of their TCR (albeit at lower levels) and simultaneously coexpress the B cell–specific epitope of CD45 (B220) together with high levels of Fas and Fas-L. Moreover, in gld mice that lack a functional Fas-L (14, 15), SAg-specific T cells with an apoptotic phenotype are dramatically reduced despite a normal proliferative response to SAg in vivo.
37°C for 1 h (to allow apoptosis to occur [2, 3]) before further manipulation.

Simultaneous staining for BrdU and DNA content was performed as described (20). Briefly, cells were fixed in 70% ethanol for at least 30 min and the DNA was partially denatured in 3 N HCl, then neutralized with 0.1 M NaB₄O₇. The cells were subsequently resuspended in 100 μl PBS containing 2.5% FCS, and added to 200 μl PBS/FCS containing 3 μg/ml propidium iodide, 50 μg/ml RNase A (Sigma Chemical Co., St. Louis, MO), 50 mM Tris base, 50 mM NaCl, and 5 mM EDTA, pH 7.5. Cells were then incubated 5 min at 37°C and analyzed on a FACScan® equipped with a doublet discrimination module.

Results

SAg-specific T Cells Undergoing Apoptosis In Vivo Are Forward Scattering (FSC) low. Dying cells diminish in size before disintegrating and being cleared by phagocytes (21). Indeed, when undergoing apoptosis, thymocytes, transformed cells, and cultured splenocytes exhibit low FSC on a flow cytometer (13, 22-25), indicating decreased cell size. We have recently shown that in mice that were injected with SEB, massive apoptosis of responding Vβ8+ T cells (as assessed by subdiploid DNA content) is evident 7 d after administration of the SAg, coinciding with and accounting for the characteristic decrease in the percentage of these T cells (26). Fig. 1 A shows that there is an increase in the proportion of FSC low cells in the LN of BALB/c mice that were injected with SEB 7 d earlier. To confirm that the FSC low gate actually contains apoptotic cells, we analyzed the DNA content of Vβ8+ T cells from SEB-treated mice. Fig. 1 B shows that the FSC low, but not the FSC high, Vβ8+ T cell population contains a substantial proportion of subdiploid, apoptotic cells. To investigate whether the conversion to an FSC low phenotype is specific to SEB-reactive T

![Figure 1](image-url)

Figure 1. FSC low population is enriched in SEB-reactive, apoptotic T cells. Mice were injected or not with 10 μg SEB in each of the hind footpads. LN cells were isolated 7 d later, stained, and analyzed by flow cytometry as described in Materials and Methods. (A) Contour plot showing two distinct populations (FSC high or R1, and FSC low or R2) based on light scattering parameters. The percentages shown indicate the number of cells in R2 as a percentage of the number of cells in R1 + R2. (B) DNA content of sorted, FSC high or FSC low CD4+ Vβ8+ cells from SEB-injected mice. (C) Vβ8 or Vβ6 expression in FSC-gated, CD4+ T cells. These and all subsequent data are representative of at least two independent experiments.

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cells, analysis of TCR Vβ gene expression in LN T cells from naive mice or from mice that received SEB 7 d earlier was performed. Whereas Vβ8+ T cells represent roughly 30% of FSC<sup>low</sup> and FSC<sup>high</sup> CD4<sup>+</sup> LN cells of naive mice, the percentage of T cells bearing this TCR Vβ segment is dramatically increased in the FSC<sup>low</sup> CD4<sup>+</sup> subset from the SEB-treated mice (Fig. 1 C). Similar observations were made in the CD8<sup>+</sup> subset (data not shown). This increase was specific to SEB-reactive Vβ8<sup>+</sup> T cells since the proportion of SEB-unresponsive Vβ6<sup>+</sup> T cells was not increased in that same FSC<sup>low</sup> gate (Fig. 1 C). Interestingly, FSC<sup>low</sup> T cells express lower levels of TcR<sup>z</sup> than their FSC<sup>high</sup> counterparts (Fig. 1 C). This decrease in fluorescence intensity is unlikely to be simply a reflection of decreased cell size since other cell surface markers such as B220, CD4, or CD8 are expressed at similar levels in FSC<sup>low</sup> and FSC<sup>high</sup> populations (see below).

**SEB-reactive T Cells Undergo Normal Proliferation but Impaired Apoptosis in Fas-L-deficient gld Mice.** To investigate whether SAg-induced FSC<sup>low</sup> cells are generated through the Fas pathway, we quantitated the accumulation of Vβ8<sup>+</sup> T cells in the FSC<sup>low</sup> gate from SEB-treated wild type (wt) mice or congenic Fas-L-deficient mice carrying the gld mutation. As seen in Fig. 2, there is a substantial increase in FSC<sup>low</sup> CD4<sup>+</sup> Vβ8<sup>+</sup> T cells in the wt mice 7 d after injection with SEB, compared to no selective increase in FSC<sup>low</sup> Vβ8<sup>+</sup> T cells from gld mice.

Next, we investigated whether the decreased accumulation of FSC<sup>low</sup> cells in gld mice correlates with a lack of SEB-mediated deletion in these mice. Fig. 3 A shows a severely impaired deletion of CD4<sup>+</sup> Vβ8<sup>+</sup> cells in gld mice as late as 14 d after administration of the SAg. We have recently shown that proliferation is a prerequisite for SEB-induced T cell apoptosis (26). Thus, it was formally possible that the lack of SEB-mediated deletion in gld mice (Figs. 2 and 3 A) is the result of an intrinsic inability of their T cells to proliferate in response to SEB. We addressed this issue by quantitating the extent of BrdU incorporation (as a measure of proliferation) in BrdU-fed, SEB-injected wt and gld mice. We found that LN cells from gld mice incorporated BrdU to the same extent in response to SEB as those derived from the wt mice (Fig. 3 B), and that this proliferation correlated with a substantial increase in Vβ8<sup>+</sup> T cells (Fig. 3 A). However, unlike in wt mice where a drastic drop in the percentage of Vβ8<sup>+</sup> T cells and of BrdU<sup>+</sup> cells occurred starting at day 7 after SEB, only a slight decrease in Vβ8<sup>+</sup> cells could be observed in gld mice late in the response to SEB (day 14), correlating with a minor and late drop in the percentage of BrdU<sup>+</sup> cells (Fig. 3, A and B). The impaired deletion of SEB-reactive cells in gld mice is due to reduced apoptosis of these cells, which is in contrast to wt mice where a peak of subdiploid (apoptotic) BrdU<sup>+</sup> cells can be detected at day 7 (Fig. 3 C). Col-
FSC high

Rabbit Ig anti-FAS-L anti-FAS anti-FAS-L anti-FAS

Figure 4. Fas and Fas-L expression on FSC\textsuperscript{high} or FSC\textsuperscript{low} T and B cells. BALB/c mice were injected with SEB, and cells from the draining LN were stained 7 d later with the following Abs: anti-CD4 and anti-CD8 (a, b, c, f, g, and h) or anti-B220 (d, e, i, and j) in combination with control Ab (a and f), anti-Fas-L (b, g, d, and i), or anti-Fas (c, h, e, and j). (a-e) Cells that were gated on FSC\textsuperscript{high}, while cells in f-j were gated on FSC\textsuperscript{low}. CD4/8 indicates cells that were stained simultaneously with anti-CD4-PE and anti-CD8-PE at concentrations that allow discrimination between the two populations based on their different fluorescence intensities. The populations with a higher fluorescence intensity are CD8\textsuperscript{+} cells, and the populations with a lower intensity CD4\textsuperscript{+} cells.

lectively, these data indicate that a functional Fas-L is required for SAg-induced apoptosis to occur in vivo and that the generation of most FSC\textsuperscript{low} cells is dependent on this Fas-mediated apoptosis.

Fas and Fas-L Are Upregulated on the Surface of FSC\textsuperscript{low} T and B Cells. Having established that the FSC\textsuperscript{low} gate predominantly contains SAg-specific T cells undergoing Fas-dependent apoptosis in vivo, we studied the expression of Fas and Fas-L in FSC\textsuperscript{high} and FSC\textsuperscript{low} LN cells isolated from SEB-treated mice. Using novel antibodies (19), we found that in FSC\textsuperscript{high} cells, Fas is expressed at low but detectable levels on CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and on B cells (Fig. 4, c and e) whereas Fas-L is expressed constitutively at low levels on B cells, but not on T cells (Fig. 4, h and d). By contrast, Fas and Fas-L were substantially upregulated on both B and T cells in the FSC\textsuperscript{low} gate (Fig. 4, g–j). Fas and Fas-L staining was not due to FcR, binding since identical staining was obtained after preincubation with mAb to FcR or with an excess of normal mouse Ig (not shown). Furthermore, staining with each of these Abs was specifically blocked with an excess of the relevant peptide (not shown).

FSC\textsuperscript{low} T Cells That Express High Levels of Fas and Its Ligand Also Express the B Cell Marker B220. Apoptotic T cells accumulating in the liver express the B cell marker B220 (6). Interestingly, we also find B220 expression on a proportion of T cells in the FSC\textsuperscript{low} gate of LN from SEB-injected mice (Fig. 5 A). To further characterize these cells, the expression of CD4, CD8, B220, and either Fas or Fas-L was simultaneously examined on LN cells from SEB-treated mice. Cells were electronically gated on low for-
ward scatter. Fig. 5A shows that four distinct populations can be distinguished in the FSC<sub>low</sub> gate based on the expression of CD4/8 and B220: (a) a CD4<sup>+</sup>-B220<sup>-</sup> population (quadrant I), presumably containing cells that have lost their membrane integrity since they cannot be stained with any of a panel of antibodies (Fig. 5, and not shown); (b) CD4/8<sup>+</sup> (<sup>+</sup> or <sup>+</sup>) B220<sup>-</sup> T cells (quadrant II); (c) CD4<sup>+</sup>-B220<sup>+</sup> B cells (quadrant III); and (d) CD4/8<sup>+</sup>, B220<sup>+</sup> cells (quadrant IV). These latter cells were T cells by virtue of their expression of CD3, but not surface Ig, and were enriched in SEB-responsive Vβ<sup>+</sup> T cells (not shown).

The expression of Fas and Fas-L was then examined in each of these four populations. As expected, the CD4<sup>+</sup>-B220<sup>-</sup> T cells were negative for both molecules (Fig. 5 B, I). By contrast, B220<sup>-</sup> T cells showed a low staining with anti-Fas-L and an intermediate staining with anti-Fas (Fig. 5 B, II). B cells exhibited a bimodal (low and high) staining for Fas and Fas-L (Fig. 5 B, III). Interestingly, all B220<sup>+</sup> T cells expressed high levels of both Fas and its ligand (Fig. 5 B, IV).

It is noteworthy that FSC<sub>low</sub> cells with the phenotypes described above are also found in LN from untreated mice. However, the percentage of B220<sup>-</sup> LN T cells increases significantly in the FSC<sub>low</sub> gate upon treatment of mice with SEB (26% in untreated mice compared to 44% in day 7 SEB-treated mice), and this increase is due to a selective accumulation of SEB-reactive Vβ<sup>+</sup> T cells (data not shown).

**Discussion**

In this study, we have phenotypically characterized a population of apoptotic T cells generated during the course of the immune response to the bacterial SAg SEB in vivo. These cells, readily identifiable by their low forward scattering on a flow cytometer, a hallmark of apoptotic cells (13), express the CD4 or CD8 coreceptors, reduced levels of TCR, the B cell marker B220, and high levels of the apoptosis-associated molecules, Fas and Fas-L. This novel phenotype is presumably the product of a Fas-dependent, apoptotic process since the generation of these cells is impaired in Fas-L-deficient gld mice.

In a recent report, the surface phenotype of cultured thymocytes undergoing apoptosis was studied using a new method (TUNEL) that detects DNA strand breaks (25). It was shown that all apoptotic (TUNEL<sup>+</sup>) cells were also FSC<sub>low</sub> and vice versa, further validating the use of FSC<sub>low</sub> as a marker of apoptosis. An advantage of FSC as an apoptosis marker is that (unlike the fluorescent markers used to identify apoptotic cells) it does not occupy a fluorescence channel on the flow cytometer, thereby allowing the use of an additional marker in multiparameter cell phenotypic analysis of apoptotic cells.

Previous reports have shown impaired T cell deletion in response to SAgS in Fas-defective lpr mice (27, 28) or to antigenic peptide in TCR transgenic mice carrying the lpr mutation (29). We show here that this finding also applies to Fas-L-deficient gld mice immunized with SEB. We have recently shown that SEB-mediated T cell apoptosis has to be preceded by proliferation (26). The fact that Vβ8<sup>+</sup> T cells from gld mice respond to SEB by undergoing proliferation in vivo rules out the possibility that the impaired apoptosis in these mice may be due to some intrinsic proliferative defect. Rather, we show direct evidence for reduced apoptosis in the proliferating cell population. This finding is of relevance in light of the conflicting results regarding the susceptibility of lpr T cells to apoptotic cell death (30-33).

Since activation of T cells is a prerequisite for apoptosis in several experimental systems (26, 34-38), it is not unreasonable to expect that some markers found on apoptotic cells (such as B220, Fas, or Fas-L) would be expressed on their putative precursors, i.e., viable activated T cells. B220 has been reported to be widely expressed on activated T cells (6). We consistently find a small proportion (10%) of B220<sup>+</sup> T cells among FSC<sub>high</sub> LN blast cells from SEB-primed mice. Furthermore, the majority of these FSC<sub>high</sub> B220<sup>+</sup> T cell blasts upregulate their expression of Fas and Fas-L (not shown), suggesting that they may represent a transitional population that will eventually become FSC<sub>low</sub> B220<sup>+</sup> Fas<sup>+</sup> Fas-L<sup>+</sup> (apoptotic) cells. The accumulation of FSC<sub>high</sub> B220<sup>+</sup> T cells in lpr or gld mice defective in Fas-dependent apoptosis (reviewed in reference 8) is likewise consistent with the hypothesis that B220 upregulation on T cells represents an intermediate stage in the process leading to Fas-mediated T cell death.

If upregulation of Fas-L is required for induction of apoptosis (7, 39, 40), and since B220<sup>+</sup> T cells are the predominant LN cell population that expresses high levels of this molecule, it follows that B220<sup>+</sup> T cells are likely candidates to be involved in SAg-induced apoptosis. Since these same cells (in addition to some B cells) express high levels of Fas (also shown to be required for apoptosis [41]), three non-mutually exclusive mechanisms of Fas-dependent killing can be envisaged (42, 43): (a) B220<sup>+</sup> T cells kill each other (fractricidal killing) (44); (b) B220<sup>+</sup> T cells kill themselves in an autocrine fashion (suicidal killing) (45, 46); or (c) B220<sup>+</sup> T cells are killed by B cells. This latter possibility is consistent with the fact that a proportion of FSC<sub>low</sub> B cells express high levels of Fas-L and that activated B cells from normal, but not gld mice, can kill Fas-bearing target cells under certain conditions (Hahne, M., T. Renno, M. Schroeter, M. Immler, L. French, T. Bornard, H.R. MacDonald, and J. Tschopp, manuscript submitted for publication). The mechanism of B cell activation by SEB in vivo is not known but may involve either cognate T-B interaction or direct activation via MHC class II cross-linking (reviewed in reference 47).

In summary, we have analyzed the surface phenotype of cells undergoing apoptosis in response to SEB in vivo. We show that apoptotic, SEB-reactive T cells express the B220 marker and high levels of Fas and Fas-L. These data strongly implicate the Fas pathway in SAg-induced apoptosis in vivo; however, the actual mechanism of Fas-dependent cell death remains to be elucidated.
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