Superantigen-Driven, CD8+ T Cell-Mediated Down-Regulation: CD95 (Fas)-Dependent Down-Regulation of Human Ig Responses Despite CD95-Independent Killing of Activated B Cells

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Superantigen-Driven, CD8+ T Cell-Mediated Down-Regulation: CD95 (Fas)-Dependent Down-Regulation of Human Ig Responses Despite CD95-Independent Killing of Activated B Cells

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Staphylococcal superantigens, including staphylococcal enterotoxin B (SEB), promote vigorous T cell-dependent Ig responses at low dose (0.01 ng/ml). In contrast, more mitogenic high dose SEB (100 ng/ml) profoundly inhibits the Ig responses. To assess the contribution of CD8+ T cells to this inhibition, high dose SEB-dependent killing of activated B cells and down-regulation of Ig responses were determined. Rapid killing (4 h) of activated B cells was effected by high dose SEB-activated CD8+ T cells (CD8+), but not by high-dose SEB-activated CD4+ T cells (CD4+), and required the presence of high dose SEB during the cytotoxicity assay. This killing was abrogated by chelation of extracellular calcium or by treatment with concanamycin A but was only modestly affected by treatment with brefeldin A, suggesting a perforin-based pathway of killing. Despite their widely disparate abilities to rapidly kill activated B cells, CD8+ and CD4+ demonstrated similar quantitative abilities to effect high dose SEB-dependent down-regulation of Ig responses. Antagonist anti-CD95 mAb substantially reversed high dose SEB-dependent down-regulation effected by CD8+ but had no appreciable effects on high dose SEB-dependent killing of activated B cells. These observations strongly suggest that the small fraction of activated B cells that secrete Ig are selectively sensitive to CD95-based killing but resistant to CD95-independent killing. This finding may help explain why clinical autoimmunity associated with increased titers of autoantibodies is a predominant feature of defects in CD95 or CD95 ligand.

perforin- and TNF-based killing as well (25, 33–38), we reasoned that CD8⁺ T cells would be especially effective high dose SEB-dependent down-regulators of Ig responses.

In this report, we demonstrate that the potency of CD8⁺ T cells to effect high dose SEB-dependent down-regulation of Ig responses is not much different from that of CD4⁺ T cells. In contrast to CD4⁺ T cells, which cannot effect rapid SEB-dependent killing of activated B cells, CD8⁺ T cells are capable of doing so via a CD95-independent, perforin-based pathway. Nevertheless, a CD95-dependent pathway predominates in CD8⁺ T cell-mediated high dose SAg-dependent down-regulation of Ig responses. The prominence of CD95-based down-regulation by both CD4⁺ T cells and CD8⁺ T cells in modulating SEB-driven Ig responses implies a vital role for CD95/CD95 ligand (CD95L) interactions in safeguarding against uncontrolled in vivo polyclonal Ig production following infection with SAg-producing microbial organisms.

**Materials and Methods**

**Cell populations**

PBMC were isolated from venous blood of healthy donors by Ficoll-Hypaque density gradient centrifugation (39). CD8⁺ T cells (≥90% CD4⁻), CD4⁺ T cells (≥80% CD8⁻), and B cells (50–90% CD20⁺ with undetectable CD3⁺ cells) were isolated by immunomagnetic bead negative selection (6, 17). In some experiments, CD4⁺ T cells (>99% CD4⁺) and CD8⁺ T cells (>99% CD8⁺) were further purified by positive-selection cell sorting with FITC-conjugated anti-CD4 mAb and phycoerythrin-conjugated anti-CD8 mAb (Dako, Carpinteria, CA).

**SAg**

SEB was purchased from Sigma (St. Louis, MO).

**Down-regulation of Ig responses**

To assess T cell-mediated down-regulation of Ig responses, two conditions had to be met. First, B cells had to be activated in a fashion that resulted in sufficient Ig production whose inhibition would be easily detectable. Second, only the experimental down-regulatory T cells could contact the target-activated B cells in order to eliminate potential confounding effects of other cells. To accomplish this, two separate protocols were utilized. In the first, two-chamber cultures (18) were established in RPMI 1640 medium supplemented with 10% FCS and glutamine and antibiotics in 24-well plates. Transwell inserts (Costar, Cambridge, MA) were used to separate inner chambers from outer chambers. The outer chambers contained 5 × 10⁶ CD4⁺ T cells that had been activated overnight in the presence of 2 × 10⁵ irradiated (3000 rad) B cells (serving as SPC) with high dose (100 ng/ml) SEB and the inner chambers contained 1 × 10⁶ B cells that had been activated overnight with high dose SEB + formalin-fixed heat-killed *Staphylococcus aureus* (SAC) (Life Technologies, Gaithersburg, MD; 1:10³ final dilution) + rIL-2 (100 U/ml). This two-chamber design permitted the activated B cells in the inner chambers to avoid T cell surface contact while being bathed by helper factors secreted by the activated CD4⁺ T cells in the outer chambers. After 4 days, CD4⁺ or CD8⁺ T cells (4 × 10⁶ unless otherwise indicated) that had been separately activated in parallel (in the presence of irradiated SPC as above) with high dose SEB (respectively denoted as CD4⁺ and CD8⁺) were washed and added to the inner chambers (containing activated B cells) with or without additional high dose or low dose (0.01 ng/ml) SEB. In all experiments, the day of reconstitution of target-activated B cells with effector T cells is considered as day 0. Plaque-forming cells (PFC) in the inner chambers were determined on day 2 by the reverse hemolytic plaque assay (39, 40). Each PFC was taken as an IgSC. Alternatively, IgG and IgM levels in the culture supernatants were determined at the indicated times by ELISA (41).

In the second protocol, B cells were continuously stimulated with SAC/ rIL-2 for 4 days. At this time, the activated B cells were washed and transferred to 96-well flat-bottomed plates (1 × 10⁶ cells/0.1 ml/well). CD4⁺ or CD8⁺ (4 × 10⁵ cells/0.1 ml/well) were added to the activated B cells with or without high dose or low dose SEB in the presence of rIL-2 (25 U/ml final concentration). Culture supernatants were collected 3 days later, and IgG and IgM concentrations were determined by ELISA.

**CD95-based protection from SEB-dependent down-regulation of Ig responses**

Antagonist anti-CD95 mAb M3 (10 μg/ml) or nonagonist/nonantagonist anti-CD95 mAb M33 (10 μg/ml) (42) were added to the experimental cultures at the time that CD4⁺ or CD8⁺ were added. Protection from down-regulation was calculated by the formula: [Ig in the presence of added CD8⁺/SEB with test Ab] − [Ig in the presence of added CD8⁺/SEB without test Ab] / [Ig in the absence of added CD8⁺/SEB] − [Ig in the presence of added CD8⁺/SEB without test Ab].

**B cell recovery**

Total viable cell numbers in the inner chambers of two-chamber cultures were determined by direct cell counting in the presence of trypan blue. B cell numbers were calculated by staining the harvested cells with FITC-conjugated anti-CD20 mAb, analyzing by flow cytometry, and multiplying the total viable cell count by the percentage of CD20⁺ cells (18).

**T cell-mediated B cell cytosis**

CD4⁺ and CD8⁺ were assayed on day 4 for cytolytic activity in a 4-h release assay against ⁵¹Cr-labeled activated B cells (17, 43). The target B cells either had been SAC/rIL-2-activated for 4 days or had been isolated by negative selection (6, 17) from CD4⁺ T cell + B cell cultures that had been stimulated with low dose SEB for 4 days. Cytotoxicity assays were performed in the presence of graded doses of SEB. Specific ⁵¹Cr release was calculated from the formula: (experimental cpm − spontaneous cpm) / (maximum cpm − spontaneous cpm), where spontaneous cpm was determined from wells containing only target cells without effector cells, and maximum cpm was determined from wells containing target cells lysed by 1% Triton X-100 detergent.

Calcium dependence of cytotoxicity assay was assessed by adding 4 mM EGTA + 3 mM MgCl₂ to the cytotoxicity assays. Repletion of extracellular calcium was accomplished by addition of 4 mM CaCl₂. Sensitivity of cytotoxicity to concanamycin A (CMA, 100 nM; Sigma) or to brefeldin A (BFA, 10 μM; Sigma) was assessed by adding them to the effector cells 2 h before addition of the labeled target cells. CMA and BFA were present throughout the cytotoxicity assay. Prior studies have demonstrated that their inhibitory effects are directed only against the effector cells and not against the targets (44).

**T cell-mediated B cell apoptosis**

B cells were activated with SAC/rIL-2 for 4 days and labeled with the fluorescent cationic membrane tracer, 1,1‘-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR) (18). The labeled B cells were cultured for two additional days with unlabelled CD8⁺ (ratio 2:5) + high dose SEB ± anti-CD95 mAb, stained with FITC-conjugated recombinant human annexin-V in calcium-containing binding buffer (Caltag Laboratories, Burlingame, CA), and analyzed by two-color flow cytometry. B cells undergoing apoptosis were taken as DiI-labeled (red) cells that stained positively for annexin-V (green).

**Release of soluble CD95L (sCD95L)**

Cultures (1 ml) of CD8⁺ T cells (2.5 × 10⁶) + irradiated B cells (1 × 10⁶) were stimulated with low dose SEB for 5 days, washed, and restimulated with graded doses of SEB. Culture supernatants were harvested at the indicated times following restimulation for sCD95L determination (18, 45).

**Statistical analysis**

All analyses were performed using SigmaStat software (Jandel Scientific, San Rafael, CA). The raw data were log transformed, and the paired t test and one-way repeated measures ANOVA test were used when comparing two groups and three or more groups, respectively. When the log-transformed data did not follow a normal distribution, the nonparametric Wilcoxon signed rank test and Friedman repeated measures ANOVA on ranks test were used, respectively. A p value of <0.05 was considered to be significant.

**Results**

**CD8⁺ effect down-regulation of Ig responses to a degree similar to that effected by CD4⁺**

To determine the ability of CD8⁺ to effect high dose SEB-dependent down-regulation of IgSC responses, two-chamber cultures were established, with the CD4⁺ T cells in the outer chambers...
supplying helper factors to the activated B cells in the inner chambers. (Preliminary studies showed that Ig responses in two-chamber cultures containing activated CD4+ T cells in the outer chambers were ~10-fold greater than those in single-chamber B cell cultures activated in the absence of T cells (data not shown)). In the presence of high dose SEB, CD8* added to the inner chambers inhibited PFC responses in a dose-dependent fashion (Fig. 1A), similar to our previous findings with CD4* (18). Also, as previously shown for CD4* (18), PFC responses were inhibited only when the effector CD8* and the target B cells were in the same chamber (p = 0.003, Fig. 1B). When CD8* were added to the outer chambers rather than to the inner chambers, cell-cell interactions between the CD8* and B cells were precluded, and no significant effects on PFC responses were observed.

No high dose SEB-dependent down-regulation was appreciated when the CD8+ T cells added to the inner chambers had not been previously activated (Fig. 2A). In contrast, high dose SEB-dependent down-regulation was substantial in the presence of activated CD8+ T cells, even if the CD8+ T cells had been activated by low dose SEB rather than by high dose SEB. CD8* and CD4* each effected significant high dose SEB-dependent down-regulation of IgSC responses (p < 0.001), and, strikingly, CD8* were no more potent than were CD4* in doing so (p = 0.939, Fig. 2B). Similar down-regulation was observed even when the effector CD8* and CD4* were generated from sort-purified CD8+ T cells and CD4* T cells (Fig. 2C), indicating that the down-regulation was not due to contaminating non-CD8* or non-CD4+ T cells, respectively.

Analysis of Ig levels in culture supernatants confirmed the PFC results. By the time CD4* or CD8* were added to the activated B cells in the inner chambers, IgG levels were already substantial. In unperturbed cultures, IgG levels steadily increased for at least a further 10-fold over the next 10 days (Fig. 3A). Addition of CD4* alone to the B cells had little effect on IgG production, and addition of CD8* alone had, at most, only modest inhibitory effects on IgG production. In contrast, addition of either CD4* or CD8* and high dose SEB completely blocked further increases in IgG levels. IgM production in these cultures was lower, but the pattern of response to CD4* or CD8*, with or without high dose SEB, was identical (data not shown).

To document that the reduction of Ig levels in the two-chamber cultures was not due to CD4* or CD8* blocking B cell responsiveness to the helper factors secreted by the CD4+ T cells in the inner chambers, SAC/IL-2-activated B cells were washed and transferred into single-chamber cultures (Fig. 3B). The modest IgG production was significantly retarded when CD4* or CD8* were added with high dose SEB (p < 0.001). In the absence of added CD4* or CD8*, high dose SEB had no inhibitory effects (p = 0.930), and neither CD4* nor CD8* were inhibitory even in the...
presence of low dose SEB ($p = 0.339$). Of note, CD8* were statistically again no more potent down-regulators than were CD4* in the presence of high dose SEB ($p = 0.071$).

**CD8* effect high dose SEB-dependent killing of activated B cells more potently than do CD4* via a calcium-dependent, CMA-sensitive, CD95-independent pathway**

Despite the similar down-regulatory potencies of CD4* and CD8*, CD8*, but not CD4*, effected considerable high dose SEB-dependent killing of activated B cells in a 4-h $^{51}$Cr release assay (Fig. 4A). Chelation of extracellular calcium with EGTA/MgCl$_2$ profoundly inhibited activated B cell killing by CD8*, and such cytotoxicity was restored by replenishment of extracellular calcium with CaCl$_2$ (Fig. 4B), suggesting a prominent role for perforin-based cytotoxicity (46–49). Unfortunately, perforin-based cytotoxicity cannot be inhibited with available anti-perforin mAb 8G9 (50) (data not shown), either because the mAb does not block the biologic activity of perforin or because the anti-perforin mAb cannot sterically reach the intimate cell-cell contact points between the CTL effectors and the target cells. Nevertheless, involvement of a perforin-based pathway can be inferred by pretreatment of effector CTL with the vacuolar type H$^+$-ATPase inhibitor, CMA, a process that inhibits perforin-based cytotoxicity but does not affect CD95-based cytotoxicity (44, 51). CMA completely blocked high dose SEB-dependent killing of activated B cell targets (Fig. 4C). In contrast, BFA, an inhibitor of intracellular glycoprotein transport that profoundly inhibits CD95-based cytotoxicity but has only modest effect on perforin-based cytotoxicity (44), had only a modest inhibitory effect on high dose SEB-dependent activated B cell killing (Fig. 4C). In addition, a CD95L fusion protein (52), which induces CD95-based apoptosis of activated B cells after overnight incubation, did not promote any specific $^{51}$Cr release in the 4-h assay (data not shown). Taken together, these experiments suggest that high dose SEB triggers a cytolytic perforin-based, CD95-independent pathway in CD8* that is either absent or markedly diminished in CD4*.

**CD8* effect high-dose SEB-dependent down-regulation of IgSC responses via a CD95-based pathway**

We have previously demonstrated that CD4$^+$ T cell-mediated high-dose SEB-dependent down-regulation of IgSC responses is CD95-based. This is associated with increased release of sCD95L by CD4$^+$ T cells following stimulation with high dose, but not low dose, SEB (18). Given the considerable CD95-independent killing of activated B cells by CD8* in the presence of high dose SEB, we anticipated that high-dose SEB-dependent down-regulation of IgSC responses mediated by CD8* would also be largely CD95 independent. Surprisingly, antagonist anti-CD95 mAb M3, but not nonagonist/nonantagonist anti-CD95 mAb M33, had a considerable protective effect (Fig. 5). In the four experiments performed, addition of CD8* to cultures containing high dose SEB resulted in a 94% drop in geometric mean PFC response. Addition of mAb M3 to such cultures restored the geometric mean PFC response to 58% of the level in cultures not containing CD8* ($p = 0.033$), resulting in a 65% geometric mean protective effect by mAb M3. In contrast, addition of CMA had no protective effect. However, since addition of CMA in the absence of CD8* or high dose SEB inhibited PFC responses by ~80% (data not shown), the lack of protection by CMA should be interpreted with great caution. Consistent with CD95-based down-regulation is that high dose, but not low dose, SEB markedly increased soluble CD95L (sCD95L) levels in CD8$^+$ T cell + irradiated B cell cultures (Fig. 6), similar to previous observations with CD4$^+$ (18).

**Antagonist anti-CD95 mAb M3 does not protect against high dose SEB-dependent activated B cell killing mediated by CD8***

CD8$^*$ are capable of effecting CD95-based, high dose SEB-dependent killing of activated B cells (18). Although such killing is not
Results are presented as geometric mean (circles) and day 2 (diamonds) and assayed for sCD95L levels by ELISA.

To confirm the lack of discernible protection by antagonist anti-CD95 mAb against B cell death, B (CD20<sup>+</sup>) cell numbers in the inner chambers of two-chamber cultures were assessed on day 2 (the time when PFC responses were assessed). When CD8<sup>+</sup> were added to the inner chambers without high dose SEB, geometric mean B cell recovery (× 10<sup>3</sup>) in the four experiments performed was 86.9. When high dose SEB was also added, geometric mean B cell recovery fell to 58.5. Addition of neither nonagonist/non-antagonist anti-CD95 mAb M33 nor antagonist anti-CD95 mAb M3 had any restorative effects on B cell recovery (respective geometric mean B cell recoveries 47.2 and 57.9). That is, with CD8<sup>+</sup> as effector cells, there was a striking dichotomy between the considerable ability of antagonist anti-CD95 mAb M3 to protect against high dose SEB-dependent down-regulation of Ig responses and the lack of mAb M3 to protect against high dose SEB-dependent killing of activated B cells. Thus, although CD8<sup>+</sup>, in the presence of high dose SEB, are potent killers of activated B cells via a perforin-based pathway, it is via a CD95-based pathway that CD8<sup>+</sup> effect the majority of their down-regulation of Ig responses.

**Discussion**

Microbial SAg elaborated by ubiquitous disease-promoting infectious organisms can lead to T cell-dependent polyclonal B cell differentiation in vitro (6–8) and in vivo (53–55) with increased titers of autoantibodies. Indeed, a compelling argument promoting a role for microbial SAg in the generation of pathogenic autoantibodies in vivo has been made (56). By inference, the normal ability to down-regulate SAg-driven polyclonal Ig responses may play an important role in maintaining an autoimmune disease-free state.

Regulation of polyclonal Ig responses to SAg, at least in vitro, is critically dependent upon SAg concentration. As long as surface contact between CD4<sup>+</sup> T cells and B cells can occur, low dose SAg promotes vigorous Ig responses in CD4<sup>+</sup> T cell + B cell cultures, whereas high dose SAg profoundly inhibits Ig responses (17, 18). This CD4<sup>+</sup> T cell-mediated high dose SEB-dependent down-regulation is effected via a cytolytic CD95-dependent pathway (18).

Since the bulk of cytolytic activity under physiologic conditions is likely effected by CD8<sup>+</sup> T cells, we focused our attention on CD8<sup>+</sup> as effectors of SEB-dependent down-regulation of Ig responses. As expected, CD8<sup>+</sup> were much more proficient than were CD4<sup>+</sup> in effecting high dose SEB-dependent killing of activated B cells (Fig. 4A). Importantly, the mechanism underlying the ability of CD8<sup>+</sup> to rapidly (4 h) kill activated B cell targets appears to be perforin-based and not CD95-based. First, cytotoxicity was highly calcium-dependent (Fig. 4B), and perforin-based pore formation and cytotoxicity is exquisitely dependent upon extracellular calcium (46–49). Second, cytotoxicity was highly sensitive to CMA but only modestly sensitive to BFA (Fig. 4C), consistent with perforin-based cytotoxicity but not with CD95-based cytotoxicity (44). Third, a biologically potent CD95L fusion protein (52) was incapable of inducing detectable 51Cr release in a 4-h time period (data not shown).

Despite this greater perforin-based killing of activated B cells by CD8<sup>+</sup> than by CD4<sup>+</sup>, high dose SEB-dependent down-regulation effected by CD8<sup>+</sup> was no greater than that effected by CD4<sup>+</sup> (Figs. 2 and 3). Restimulation of CD8<sup>+</sup> with high dose, but not low dose, SEB resulted in a dramatic increase in release of sCD95L (Fig. 6), similar to previous observations with CD4<sup>+</sup> (18). This suggested that a CD95-based pathway may substantially contribute to high dose SEB-dependent down-regulation effected by CD8<sup>+</sup>. Indeed, even though antagonist anti-CD95 mAb M3 had no appreciable effect on high-dose SEB-dependent killing of activated B cells mediated by CD8<sup>+</sup> (Fig. 7), antagonist anti-CD95 mAb M3 was highly protective of IgSC responses (Fig. 5). Thus, not only is a CD95-based pathway critical to Ig down-regulation effected by CD4<sup>+</sup> (18), but it is vital to Ig down-regulation effected by CD8<sup>+</sup> as well. This high dose SEB-dependent down-regulatory effect of CD8<sup>+</sup> on B cell function is a direct one requiring no other cells as intermediaries and requiring surface contact between effector
CD8* and target activated B cells (Fig. 1). This effect on B cells is distinct from a recently described CD95-based down-regulatory effect of murine SEA-activated CD8* T cells on SEA-activated CD4+ T cells (57). It may be that CD95-based, SAg-dependent, CD8* T cell-mediated down-regulation is realized in vivo via effects on both B cells and on CD4+ T cells. In any case, the redundancy of CD95-based down-regulation effected by both CD4+ T cells and CD8* T cells may be an important physiologic protective mechanism against development of autoimmunity even under conditions of selective T cell subset depletion (e.g., AIDS).

The preeminence of CD95-dependent down-regulation by CD8* despite their ability to rapidly kill activated B cells via a CD95-independent pathway suggests that the activated B cell targets of CD95-independent killing are preferentially not IgSc or their precursors. Only a small fraction of activated B cells differentiate into IgSc, and the degree of activated B cell death under our experimental conditions was always considerably less than 100% (Figs. 4 and 7 and data not shown). Thus, IgSc or their precursors may be selectively sensitive to CD95-based killing but relatively resistant to perforin-based killing. CD95-independent (perforin-based) killing of activated B cells could be quantitatively abundant while having a disproportionately low effect on ultimate IgSc responses. Nevertheless, our results suggest some role for CD95-independent down-regulation by CD8*. Although we previously showed that antagonist anti-CD95 mAb M3 completely (geometric mean 106%) protected against high dose SEB-dependent down-regulation mediated by CD4+ (18), mAb M3 incompletely (geometric mean 65%) protected against identical down-regulation mediated by CD8* (Fig. 5).

The modest CD95-independent contribution to high dose SEB-dependent down-regulation of Ig responses notwithstanding, our results underscore the vital role for CD95/CD95L interactions in Ig homeostasis. Systemic lupus erythematosus-like illness (including increased autoantibody production) has been described in association with genetic defects in CD95 or CD95L in man (58–61) and increased autoantibody production (including systemic lupus erythematosus-like illness (including increased autoantibody production)) has been described in association with genetic defects in CD95 or CD95L in man (58–61). Nevertheless, more recent studies using mice transgenic for CD86 have suggested that in vivo B cell elimination is also significantly affected by non-CD95-based mechanisms (70), and perforin deficiency exacerbated autoimmune features in mice whose background genes were 50% derived from autoimmune-prone MRL mice (71). Additional studies will be necessary to assess the in vivo effects of SAg on B cell function and B cell elimination (effected via CD95- and non-CD95-based pathways) and their ramifications for clinical autoimmunity.

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