Temporal and Dose-Dependent Relationships between In Vivo B Cell Receptor-Targeted Proliferation and Deletion-Induced by a Microbial B Cell Toxin

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*J Immunol* 2006; 176:2262-2271; doi: 10.4049/jimmunol.176.4.2262

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Temporal and Dose-Dependent Relationships between In Vivo B Cell Receptor-Targeted Proliferation and Deletion-Induced by a Microbial B Cell Toxin

Carl S. Goodyear, Fujimi Sugiyama, and Gregg J. Silverman

The effective functioning of the adaptive immune system requires careful clonal regulation within the B cell compartment. Some microbial pathogens produce virulence factors, like staphylococcal protein A, which interact at high frequencies with B lymphocyte through unconventional binding sites in BCR variable region frameworks conserved during evolution. We have characterized the in vivo effect of staphylococcal protein A treatment on peripheral B cells bearing susceptible BCR, and found a dose-dependent direct relationship over the range of 2 mg to <0.2 μg in the magnitude of induced BCR-targeted supraclonal cell death. Significantly, some level of targeted B cell proliferation was always detectable, with greatest interim supraclonal expansion demonstrated at 2 days after 20-μg treatment. Subsequently, this transient expansion always collapsed. In direct comparisons, i.p. treatment was more efficacious than i.v. treatment, although at higher doses this finding was less marked. These studies elucidate a general paradigm in which in vivo encounters with a B cell superantigen are uniformly associated with proliferative expansion followed by deletion that is more rapid and complete with higher doses, whereas lower doses lead to greater transient in vivo expansion with delayed deletion to levels at later times that are still quantitatively proportional to the dose. Our results document the potent in vivo B cell-targeted properties of a microbial B cell superantigen, even at submicrogram doses associated with great molar excess of circulating Ig, and clearly illustrate the intertwined relationships between targeted proliferative cycling and apoptotic death that is induced by a microbial B cell superantigen. The Journal of Immunology, 2006, 176: 2262–2271.

The induction of Ag-specific responses and the subsequent maintenance of immune memory require the careful regulation of clonal selection and expansion, with the subsequent elimination of redundant cells (1). In general, the fate of a B lymphocyte clone is largely determined by signals received through its Ag receptor (BCR), which can result in either positive selection with proliferation and enhanced survival, negative selection due to deletion or functional inactivation, or escape due to receptor editing. A lymphocyte clone can also potentially be affected by encounters with more than one type of Ag, because a BCR can be cross-reactive with unrelated exogenous and self-antigens. The coevolution of microbial pathogens with the adaptive BCR can be cross-reactive with unrelated exogenous and self-antigens, which have been termed superantigens (SAgs), based on their capacity to interact with conserved binding sites in the variable (V) regions of Ag receptors. These SAgs interact with sets of lymphocytes that are orders of magnitude more frequent than for conventional Ags that typically are bound through interactions with the CDR3 loops of the V regions of the Ag receptor (for review see Ref. 2).

We and others have shown that protein A of Staphylococcus aureus (SpA) interacts with large supraclonal B cell sets via a conserved site in the H chain V regions of many BCR (3–5). SpA is composed of five homologous extramembrane domains in tandem (6), and each domain possesses both the well-known Fcγ-binding specificity and a separate site specific for BCR with VH regions from the structurally related clan III families (7). The structural basis for the SAg properties of SpA were elucidated in crystallographic studies that characterized the conformational surfaces that provide the interface in a complex of a domain of SpA and a human Fab (8). Contacts from the Fab were remote from the conventional ligand binding site, because this surface is formed by side chain contact residues from four β strands in the VH framework (FR)1 and FR3 subdomains. Although not previously appreciated to have functional roles aside from supporting the overall β barrel Ig fold, this B cell SAg binding site has been conserved during the evolution of the adaptive immune system and common expression in the immune systems of diverse mammalian species, amphibian, and avian species that have been studied (9). In the human system, this BCR-mediated binding site is expressed by VH3 family genes of clan III, which represent about half of all inherited VH genes (10). It is also prevalent in the mouse, because the homologous S107, J606, 7183, and DNA4 genes also commonly convey this binding activity (11, 12). In naive adult BALB/c and C57BL/6 mice, ~3–5% of mature B cells display this nonimmune Fab-mediated SpA-binding interaction in flow cytometric analysis, and it is also constitutively expressed by ~10% of IgM-secreting splenic cells and a comparable proportion of circulating natural IgM (13, 14). Significantly, in vivo exposure to SpA

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Received for publication July 15, 2005. Accepted for publication November 18, 2005.

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1 This work was supported by Grants CA104815 from the National Cancer Institute and from the National Blood Foundation (to C.S.G.), and Grants AI40305, AR47360, AR50659, and AI46637 from the National Institutes of Health, and from the Alliance for Lupus Research (to G.J.S.).

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4 Abbreviations used in this paper: SAg, superantigen; V, variable; SpA, protein A of Staphylococcus aureus; FR, framework; MZ, marginal zone; AICD, activation-induced cell death; mSpA, chemically modified SpA; slg, surface Ig; LN, lymph node; ISC, IgM-secreting cell.

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rapidly depletes V_{H}-targeted B cells by a T cell-independent process, and these induced changes in the composition of mature B cell pools persist long after exposure (14–16).

In recent studies, we have begun to characterize the distinct sequence of events by which SpA induces the programmed cell death of V_{H}-susceptible B cells (15). Within minutes of infusion, SpA passes through the circulation to target lymphocytes throughout the body and rapidly induce a down-regulation of surface Ig (sIg) and up-regulation of phenotypic markers of activation (e.g., CD80, CD86, CD40, CD95, and MHC class II) on B cells fated for apoptotic deletion. Within several hours, targeted B cells then display a progressive dissipation of mitochondrial membrane potential that leads to death, although this fate can be aborted by in vitro exposure to second signal stimuli (e.g., CD40L or IL-4). The mitochondrial death induced by in vivo SpA exposure is further accelerated with the subsequent induction of DNases and catabolic hydrolases that include activated caspases. Overall, induced B lymphocyte death progresses to completion over ~4 days, with more rapid and efficient deletion of immature and marginal zone (MZ) B cells than other affected B-lineage cells (16).

Because the B cell death induced by SpA shares many conceptual and mechanistic features with the activation-induced cell death (AICD) of T cells that is induced by microbial SAgs (17–19), we have therefore wondered whether there are also other central features that are common to the pathways by which B cells and T cells undergo Ag receptor-induced death. There are, however, many inherent differences between B cells and T cells, including their capacities for autocrine/paracrine stimulation, the availability and dependence upon distinct second signals, and inherent differences in their Ag-receptor functional/structural properties. In specific, our hypothesis poses an inherent seeming paradox, because B cells, unlike T cells, express both membrane-associated (i.e., BCR) and soluble Ag receptors (i.e., circulating Ig). Moreover, even naive hosts have high titers of Ig capable of such nonimmune Fab-mediated binding interactions with SpA. In fact, because we initially anticipated that circulating Ig may act as inhibitors, we previously evaluated the in vivo responses to only high doses of SpA. By contrast, it has been reported that even very small doses of some T cell SAgs can result in massive supraclonal responses, demonstrating that microbial SAgs can be among the most potent of T cell mitogens. We have therefore now performed a series of time-course analyses of in vivo dose responses to SpA exposures, and assessed the balance of induced proliferation vs deletion that follows exposure to even relatively low SpA doses. These investigations have demonstrated unexpected shared features of immunomodulatory properties common to the SAgs for T cells and B cells.

Materials and Methods

Mice and immunogens

C57BL/6 and congenic CD45.1 mice were obtained from The Jackson Laboratory, and T15L "knock-in" mice were bred under specific pathogen-free conditions under the supervision of the University California San Diego Animal Subjects Program. Adult mice, at least 6 wk of age, received either recombinant SpA (Repligen), or a control protein, OVA (Sigma-Aldrich), as indicated, with removal of contaminating endotoxin (<0.5 endotoxin units/ml). Mice received protein in 250 μl of pyrogen-free saline instilled into the peritoneal cavity (i.e., i.p.) or injected into the tail vein (i.e., i.v.). Each experiment involved a control group that received saline alone and a group that received control protein. After mice were killed, tissues were harvested for immediate ex vivo analysis. Mice were age- and sex-matched in all experiments.

Flow cytometry analysis

Adapting previously reported methods (14–16), mononuclear cells were stained in the presence of Fc block (BD Biosciences) as appropriate. As described previously (10), endotoxin-free recombinant SpA (Repligen) was chemically modified to create a form of SpA, termed modified SpA (mSpA), that retains V_{H}III Fab-binding specificity but is devoid of Fcγ-binding activity. In studies of C57BL/6 mice, we used PE- or biotin-labeled mSpA, or isotype controls used with streptavidin-labeled APC (BD Biosciences), and applied previously validated methods, to detect B cells expressing V_{H}III clan III genes for the quantitation of B cells capable of binding this B cell SAg (10, 13–15). BrdU incorporation was determined using the BrdU–APC kit (BD Biosciences). Data were acquired using a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star). Forward and side scatter gates included only nucleated viable cells, dead cells were excluded based on light scatter, and 7-aminoactinomycin D uptake, as possible.

Studies of induced proliferation

For studies of adoptively transferred mononuclear cells, isolated splenocytes were suspended at 5 × 10⁷/ml in RPMI 1640, labeled with 1 μM CFSE (Molecular Probes), and incubated at 37°C for 10 min. The cells were washed three times in ice-cold RPMI 1640 with 5% FCS and resuspended in PBS. A total of 3 × 10⁶ splenocytes were transferred i.v. through the tail vein. After 24 h, mice received either SpA or control protein as described above. Control studies confirmed that CFSE treatment did not compromise the ability of B cells to respond to SpA or mitogens (C. S. Goodyear and G. J. Silverman, unpublished observations).

To assess levels of in vivo proliferation, at the time of SpA or control treatment, mice received a 1-μg i.p. injection of BrdU suspended in PBS, and thereafter all consumed water from foil-wrapped bottles contained BrdU at 0.8 ng/ml, replenished each 48 h.

Enzyme-linked assays of Ab response

A standard ELISA was used to quantify the Ab responses to mSpA and BSA as a control Ag. Briefly, microtiter plates (Corning Costar) were coated overnight with protein (5 μg/ml) in PBS. After blocking with 2% BSA-PBS, serum samples diluted in block were incubated for 4 h at room temperature. The amount of bound Ab was determined by incubation with HRP-labeled affinity-purified goat F(ab')₂ anti-mouse IgM or IgG (Jackson ImmunoResearch Laboratories), with values obtained after incubation of substrate for 15 min. To compare Ab responses, IgM anti-mSpA values from different groups of mice were compared with a standard curve of a monoclonal clan V_{H}III IgM, and IgG were compared with a polyclonal standard, as described previously (12).

ELISPOT assays

To quantitate the frequency of spontaneous IgM- and specific Ab-secreting cells, assays were performed on splenocytes. Multiscreen HA plates (Millipore) were precoated overnight at 4°C with 4 μg/ml affinity-purified goat anti-mouse IgM (Jackson Immunochemicals), mSpA, or as controls, OVA or BSA, in PBS. Plates were washed once with 0.05% Tween 20/PBS and blocked for 1 h at room temperature with PBS containing 3% BSA and 1% casein. Freshly isolated splenocytes in 100 μl of supplemented RPMI 1640 medium were added to triplicate wells with 2-fold dilutions starting at 400,000 cells/well and incubated in 5% CO₂ at 37°C for 4 h. To identify the isotype-specific Ig/Ab secretors, plates were vigorously washed to remove cells, and then HRP-conjugated anti-mouse IgM or IgG (Jackson ImmunoResearch Laboratories), anti-IgG1, or anti-IgG2a (Southern Biotechnology Associates) was incubated overnight at 4°C. Plates were washed six times before adding 80 μl of 3-amino-9-ethylcarbazole substrate (Sigma-Aldrich) to the well and were developed at room temperature until red spots were visible. Plates were scanned and analyzed on an Immunospot ELISPOT reader (Cellular Technology). When possible, values were based on the mean of replicate wells containing >10 but <100 spots/well.

Statistical analysis

Using Instat (GraphPad), significant differences between treatment groups were determined using a factorial ANOVA and reported as F (x, y) = z. Wherein x is the ANOVA value, and x and y are the degrees of freedom. Degrees of freedom are the number of independent pieces that are free to vary (N-1) in a statistical test. Further analysis was performed using a Student’s t test, p < 0.05 was considered statistically significant.
Results
Low doses of SpA induce greater in vivo proliferation of T15i B cells

To begin to assess whether the specific induced supraclonal fate of susceptible B cells is affected by the relative size of the SpA dose, we extended our earlier investigations in T15i+/+ knock-in mice (20), in which almost all B cells express an S107 transgene that conveys the high-affinity V_{H} FR-associated SpA-binding motif (12) paired with diverse V_{L} regions. To evaluate for induced proliferation of splenic B cells, cellular DNA was in vivo labeled by BrdU, and we found that at 48 h after a 20-μg (0.4 mg/kg) dose there was a trend toward an increase in B cell proliferation (Fig. 1 and Table I). We also observed a slight decrease in total B cell numbers, 20.8 × 10^6 ± 4.7 (mean ± SEM) in the control compared with 15 × 10^6 ± 2.4 in the 20-μg SpA-treated group (Table I). By contrast, under these conditions after a 2000-μg (~40 mg/kg) SpA dose, there was already ~47% deletion of B cells (p = 0.03) (15) at 48 h due primarily to the loss of CD9^+ B cells (Ref. 16 and data not shown), even with this dramatic deletion there was also a selective increase (>3-fold; p = 0.04) in the splenic B cell proliferation, compared with control-treated mice (Fig. 1 and Table I).

Studies were also performed to evaluate the outcome at 6 days after treatment, when the induced B cell death has progressed to completion (15). At this later time point, the 20-μg SpA treatment resulted in a trend toward a modest increase (~10%) in the representation of B cells in the spleen, with ~24% increase of newly generated B cells (Fig. 1A and Table I). Significantly, at this later time point a 2000-μg dose induced an overall mean ~84% deletion of splenic B cells (p = 0.002), compared with control treatment, representing the same level of deletion previously documented after two sequential 1000-μg SpA doses (15). At this time point, although ~40% of detectable splenic B cells were recently generated (i.e., BrdU^+), this represented less than half the number of newly generated B cells in the control-treated mice (p = 0.04).

In this system, it was unclear whether the increased representation of SpA-susceptible newly generated B cells in the spleen derived from the proliferative expansion of peripheral mature B cells, or alternatively that low-dose SpA treatment enhanced the entry into the mature peripheral pool of B cells newly generated from precursors in the bone marrow.

To remove the possible contribution of the central compartment, we evaluated the response of fluorochrome-labeled T15i+/+ splenocytes after transfer into congenic CD45.1 mice (21), using a previously validated system (15). Confirming our earlier studies, at 48 h after treatment, even though some T15i B cells had undergone up to four rounds of proliferation, we found that higher SpA doses (i.e., 1 mg) induced an overall net ~43% B cell deletion in the spleen (p = 0.002) (Fig. 1, B and C, and Table I). Significantly, after treatments with much lower SpA doses, there was a net expansion. For instance, 20-μg treatments yielded a net 64% increase of T15i B cells (p = 0.05), whereas the 4-μg treatment resulted in a net ~69% increase of T15i B cells at this time point (p = 0.02) (Fig. 1, B and C, and Table I). We also found that the lowest SpA doses was associated with the greatest number of detectable residual B cells that had undergone a single round of proliferation. With the exception of the 4-μg dose, these lower doses were also associated with more residual B cells undergoing two or three rounds of proliferation. Each of the tested doses of SpA significantly altered the representation of B cells that underwent 1, 2, 3, or 4 rounds of proliferation (Fig. 1C and data not shown).

By correcting for proliferative expansions, we also evaluated the outcome of SpA exposures on the proportion of the originally transferred B cell progenitor clones that persisted after treatment (15, 22). These progenitor analyses also showed that higher SpA doses resulted in the maximal deletion of the transferred B cells, whereas decreasing SpA dose resulted in increased clonal survival, because following the lowest doses one or more daughter cells from each transferred T15i^+ B cells survived to this time point, resulting in the documented overall supraclonal B cell expansion (Fig. 1D).

In these studies, we have also found that higher SpA doses cause marked and prolonged decreases in detectable levels of surface IgM (15), due to the formation of SpA-sIg cocomplexes (C. S. Gooyear and G. J. Silverman, unpublished observations), and this effect persists on B cells fated to die. We were therefore intrigued to find that detectable levels of CD79α, a membrane-associated component of the BCR cocomplex, were also greatly depressed by SpA treatment, and these levels also increased with later sequential rounds of proliferation (Fig. 1E). These findings were independently verified by data obtained for the analysis of sIgM and sIgG levels (data not shown). These findings are consistent with the notion that the seemingly depressed sIg levels on affected B cells represents a signature for BCR occupancy by SpA, which has special binding capacities linked to its inherent oligovalency (14). These observations are also consistent with the reported persistence of SpA-Ig complexes in the circulation and tissue (23).

Because we have previously found that additional deletion can occur after 48 h, we also assessed the outcome at later time points using a system that can detect transferred cells even after greater numbers of proliferative cycles might impair detection of fluorochrome-labeled transferred cells. Therefore, we repeated these studies of transferred CFSE-labeled T15 B cells that bear the CD45.2 marker, which can readily be distinguished from congenic host CD45.1 cells. With this unambiguous cellular tracking system, we found that even though the 20-μg SpA dose caused overall expansion at 48 h, by 96 h there was now only a net 6% expansion of detectable T15i B cells (Fig. 1F and Table I), which represented a net 68% deletion (0.56×10^6 ± 0.13 (mean ± SEM) for control compared with 0.18 ± 0.01 for 20 μg of SpA) based on B cell progenitor frequencies.

Polyclonal C57BL/6 B cells display dose-dependent quantitative supraclonal deletion

To assess the relevance of these findings to interactions in mice with polyclonal repertoires, comparable studies were performed in adult C57BL/6 mice that have B cells with a broad range of SpA-binding affinities. Confirming previous observations, in naive and control-treated adult C57BL/6 mice 2.8% ± 0.01 (mean ± SEM) (or 1.6 × 10^6 ± 0.15; mean ± SEM) of total splenic B cells displayed Fab-mediated SpA-binding capacity, based on flow cytometric analyses (Fig. 2A and Table I). At 72 h after 2000-μg SpA treatments, we found only 0.2% ± 0.06 (0.07 × 10^6 ± 0.02) of splenic B cells displaying Fab-mediated SpA-binding capacity, which represents a ~93% loss (p < 0.0001). By contrast, after treatment with a much smaller SpA dose (i.e., 20 μg), we found only a mean ~66% reduction of detectable splenic B cells 0.94 ± 0.1 (0.3 × 10^6 ± 0.05) with SpA-binding capacity at this time point (p < 0.0001).

We also evaluated the outcome at a later time point and found that after 28 days, the 2- or 20-μg treatments induced comparable levels of deletion (~50%) (i.e., 0.87 × 10^6 ± 0.26 and 0.88 × 10^6 ± 0.14, respectively). Significantly, treatments with 200- and 2000-μg SpA doses induced somewhat greater (~60%) overall deletion by this later time point (i.e., 0.72 × 10^6 ± 0.1 and 0.67 × 10^6 ± 0.04, respectively) (Table I). Although these studies did not consider the potential for regeneration of this cellular splenic pool,
FIGURE 1. High SpA doses induce predominant targeted B cell deletion, whereas low doses induce net suprACLonal expansion of T15i splenic B cells.

A, Adult T15i+/+ mice were treated i.p. with a high (2000 μg) or low (20 μg) dose of SpA on day 0, and harvested on either day 2 (top panels) or day 6 (bottom panels). Cells depicted were pre gated for B220+. The absolute number of BrdU+ cells ± SEM are shown. B, T15i+/+ splenocytes were labeled with CFSE and adoptively transferred into congenic C57BL/6 recipients. In this study, CFSE identifies mononuclear cells derived from the T15i+/+ donor, and dilution of staining intensity identifies cells that have undergone cell division. The following day, mice were i.p. treated with control protein or declining doses of SpA. Splenic mononuclear cells were harvested and evaluated 48 h after treatment. Top panel, Viable (7-aminoactinomycin D+) B cells are identified as B220+. Mean values for percentage B cell deletion (−) or proliferation (+) in treatment groups compared with control mice are indicated. Lower panels, The percentage representation of viable CFSE+ B and T cells are shown.

C, The mean absolute number of viable CFSE+ B cells in each proliferative round is depicted.

D, To estimate the number of original transferred cells (i.e., progenitor cells) that survived treatment, calculations were based on the evaluation of sequential rounds of proliferation, as previously described, using the equation as follows:

\[
\text{Progenitor cells} = \text{Round 0} + \left( \frac{\text{Round 1}}{2} \right) + \left( \frac{\text{Round 2}}{4} \right) + \left( \frac{\text{Round 3}}{8} \right).
\]

Mean values ± SEM are shown. E, To illustrate the dose- and proliferation-dependent down-regulation of BCR on affected B cells, the mean fluorescent intensity (MFI) of CD79a for each round of proliferating B220+CFSE+ is depicted. F, Congenic CD45.1+ hosts that received T15i+/+ CFSE-labeled CD45.2+ splenocytes were i.p. treated with 20 μg of SpA or control treatments. Splenic mononuclear cells were harvested and evaluated 96 h after treatment. Top panel, Previously gated on CD45.2 (T15i+/+ splenocytes) B cells are identified as B220+. The mean value for percentage B cell proliferation (+) is indicated. Lower panels. The percentage representation of viable CFSE+ B and T cells are shown. In addition, analysis of B cell progenitor frequencies also revealed a net 68% deletion because SpA-treated mice had 0.18 ± 0.01 (mean ± SEM) T15i B cells in the spleen, compared with mean 0.56 × 10^6 ± 0.13 in the control-treated mice (data not shown). Representative of three experiments. Statistical significance by Student’s t test is indicated: *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
we have recently shown that affected mature splenic B cell populations recover very slowly from SpA-induced deletion (>t1/2 life of ~13 wk) (16). Therefore, we believe that the dose-dependent persistent loss of SpA-reactive B cells documented in this study at 28 day predominantly reflects the overall level of targeted peripheral B cell deletion induced by i.p. treatment with this microbial toxin.

**Lower doses of SpA induces greater proliferation of polyclonal C57BL/6 B cells**

In pilot studies, we first evaluated control-treated C57BL/6 mice after 72 h of BrdU incorporation, and found great variations in the overall proportions of lymphocytes that had undergone interim proliferation, but within each of these mice the splenic B and T lymphocytes displayed very similar rates of incorporation, which presumably reflects their homeostatic proliferation (data not shown). Moreover, when evaluating only the BrdU-labeled splenic B cells, a comparable proportion (4.1% ± 0.7, mean ± SEM) of newly generated splenic B cells displayed Fab-mediated SpA-binding activity in different control-treated mice (Fig. 2B). Hence, control studies confirmed that there was no overall bias in the representation of SpA-reactive splenic B cells within the BrdU-labeled lymphocyte populations that arise from homeostatic proliferation.

In this system, after high-dose SpA treatment (i.e., 2000 μg) we found that in the newly generated B cells only 0.2% ± 0.1 of the population had SpA-binding capacity compared with a mean 4.1% population in the control groups (Fig. 2B and Table 1), representing a mean 88% reduction at 72 h (p < 0.0001). In contrast, after low-dose SpA treatment (i.e., 20 μg), the representation of newly generated splenic B cells (i.e., BrdU+) with SpA-binding capacity was greatly increased (7.2% ± 1.2), representing a doubling of these B cells with SpA-binding capacity (p = 0.01). Although these newly generated SpA-binding B cells were in fact few in number (<10⁵ splenic mononuclear cells) in the control group, after SpA treatment an increased proportion of these BrdU-labeled B cells displayed SpA-binding activity (i.e., 9% after 2-μg treatment, and ~33% after 20-μg treatment). However, because affected B cells may still have down-regulated BCR at 72 h, these flow cytometry-based estimates, based on surface phenotype, may underestimate the real frequency of SpA-reactive B cells.

When equivalent groups were evaluated 28 days after treatment, single doses between 2 and 2000 μg resulted in 52–63% depletion of SpA-reactive B cells. In addition, whereas we found that low-dose SpA treatment yielded preferentially incorporation of BrdU into V1H-targeted B cell subsets at 72 h, this interim suprACLonal expansion did not result in the persistence of these expanded cells at later time points.

Taken together, these findings directly document that at early stages of exposure, the in vivo SpA exposure level determines the balance between proliferative expansion and deletion of the targeted polyclonal splenic B cells in C57BL/6 mice. Overall, at 72 h we found that higher dose treatments induce greater targeted deletion with only limited detectable expansion, whereas low dose results in substantially greater proliferation with little or no deletion. Notably, in comparable studies performed 6 days after SpA treatment, there was no longer evidence of preferential expansion of newly formed SpA-reactive cells (data not shown). Therefore, the B cells that are expanded at early time points after low-dose exposure do not survive for long because deletion of susceptible B cells still occurs, albeit their death was somewhat delayed.

**Intravenous treatment induces dose-dependent but less efficient quantitative clonal deletion**

To determine whether the mode of parenteral administration can affect the kinetics or magnitude of the response, comparative surveys were performed after i.p. compared with i.v. SpA treatment, without the use of adjuvant or other vehicle (Fig. 3). As expected,

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**Table I. Outcome of intraperitoneal SpA dosing on splenic B cell representation**

<table>
<thead>
<tr>
<th>Model System</th>
<th>Type of SpA-Reactive B Cell</th>
<th>Dose Regime (μg)</th>
<th>Interval to Evaluation (days)</th>
<th>Proliferation</th>
<th>Net B cell change†</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo in T15i+/+ with BrdU labeling</td>
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<td>20</td>
<td>2</td>
<td>+/−</td>
<td>28% ↓ *</td>
</tr>
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<td>10% ↑ *</td>
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<td>47% ↓</td>
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<td></td>
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<td>2000</td>
<td>6</td>
<td>+</td>
<td>84% ↓</td>
</tr>
<tr>
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<td>+</td>
<td>69% ↓</td>
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<td></td>
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<td></td>
<td>2</td>
<td>+</td>
<td>43% ↓</td>
</tr>
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<td>+</td>
<td>6% ↓</td>
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<td></td>
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<tr>
<td>In vivo in C57BL/6 with BrdU labeling</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2000</td>
<td>3</td>
<td>+</td>
<td>83% ↓</td>
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<td>28</td>
<td>−*</td>
<td>62% ↓</td>
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*Essentially all peripheral T15i B cells are capable of binding SpA. For studies of C57BL/6 B cells, direct Fab-mediated SpA binding activity was assessed. Whereas SpA exposure can cause transient modulation of BCR on targeted B cells, this effect resolves by 96 h of exposure.

†In this system, a 3-day time point in C57BL/6 may be associated with an understated expansion of SpA-susceptible B cells. The detection of these B cells is dependent on a surface IgM flow cytometric assay, and most affected B cells may still have down-regulated BCR.

Although proliferation of B cells was detected, there was no preferential expansion of SpA-reactive B cells. All results represent means values with groups of three or greater from multiple experiments.

↑ = net increase, ↓ = net decrease. Differences in all groups were significant (see text), with the exception of those indicated (*).
in these studies of adult C57BL/6 mice, the maximal induced decreases in detectable circulating SpA-binding B cells were found at 2 and 7 days after treatment. Quantitative depletion was documented after i.p. treatment with only 0.2 μg of SpA (i.e., ~0.01 mg/kg) (p < 0.05). While comparing the routes of administration, it was observed that a 20-μg SpA i.v. treatment only induced about the same level of depletion as a single 2-μg i.p. dose. Greater levels of depletion were also documented after 28 days following i.p. treatment with 2 μg (>30% depletion; p = <0.001) than after i.v. treatment with 20 μg of SpA (p = <0.001) (Fig. 3). Consistent with the above-described results, whereas response/recovery kinetics were parallel in each of the treatment groups, larger doses induced greater decreases. Hence, in general, we found that low-dose i.p. treatment induces greater decreases than i.v. treatment, although the differences using these two modes of administration were much less marked at higher doses.

Compared with mice in the control groups, reiterating observations found at 28 days in blood, there was significant depletion of the absolute number of SpA-reactive B cells in the spleen after i.p. treatment with 2 μg and higher doses of SpA (Fig. 4A) (F(4,14) = 9.9; p = 0.0005). The evaluation of the percentage of SpA-reactive B cells in the spleen after i.p. treatment with 2 μg and higher doses of SpA (Fig. 4C) showed similar trends (F(4,14) = 109; p < 0.0001). In comparison, analysis of the absolute number of SpA-reactive B cells in the spleen after i.v. treatment showed no statistical significance (Fig. 4B), whereas analysis of the percentage of SpA-reactive B cells in the spleen after i.v. treatment with 20 μg and higher doses of SpA (Fig. 4D) resulted in significant change (F(4,15) = 30; p < 0.0001).

To determine whether the depletion of B cells is consistent in other peripheral lymphoid tissue, we also evaluated the depletion in lymph node (LN). The percentage of SpA-reactive B cells after i.p. treatment with 2 μg and higher doses of SpA (Fig. 4E) showed the same trends documented in the spleen (F(4,14) = 87; p < 0.0001). The outcome of i.v. doses on LN depletion was also comparable to that observed in the spleen because a dose of 20 μg and higher resulted in significant depletion of SpA-reactive B cells (F(4,15) = 23; p < 0.0001) (Fig. 4F).

Dose-dependent decreases of SAg-reactive IgM-secreting cells (ISC) and circulating IgM

Because earlier studies have shown that SpA also has the capacity for inducing long-term reductions in the representation of ISCs, as an independent functional measure we also performed ELISPOT analyses (Fig. 5A). In naive adult C57BL/6 mice, ~10–12% of all splenic ISCs can bind SpA as detected by this more sensitive method (14). When mice were assessed 28 days after i.p. exposure, we found that doses of at least 2 μg caused significant reductions in the representation and proportion of SpA-reactive ISCs (F(4,13) = 9; p = 0.0011) (Fig. 5A). Moreover, the magnitude of the induced deficit increased with higher doses, such that there was a mean 34% reduction after 2-μg i.p. treatment, and 2-mg i.p. doses were associated with a mean 76% reduction. When we evaluated the impact of i.v. treatments, only doses of 20 μg and higher resulted in significant levels of depletion detected (49%) (p < 0.01), with progressive greater levels of depletion documented (F(4,13) = 10; p = 0.0005) (Fig. 5A).

Confirming the above-described induced changes in the cellular frequencies of IgM-secreting splenocytes, we found that SpA treatment induced comparable persistent quantitative depletion in the representation of circulating SpA-reactive IgM (Fig. 5B). Illustrating that i.p. treatment resulted in a significant change in the levels of IgM at days 2 (F(4,14) = 25; p < 0.0001), 7 (F(4,14) = 18; p < 0.0001), and 28 (F(4,14) = 7; p = 0.0031). The administration of SpA via the i.v. route also resulted in significant changes in
the levels of IgM at days 2 \((F(4,14) = 18; \ p < 0.0001)\), 7 \((F(4,14) = 14; \ p < 0.0001)\), and 28 \((F(4,14) = 11; \ p = 0.0003)\).

These findings clearly demonstrate that SpA treatment at doses as low as 2 \(\mu\)g result in both a cellular and functional impairment of this SpA-reactive component of the B cell compartment that persists to day 28. Taken together, these assays of the frequency of ISCs, which represent a functional readout unaffected by transient BCR-SpA complexes, confirmed the dose-dependent loss of targeted peripheral B cells (and their products) even with SpA doses at molar ratios much lower than the levels of circulating Ig.

**Discussion**

In the current studies we have investigated the nature of in vivo responses to a microbial toxin with the properties of a B cell SAg, and defined conditions that affect the balance between induced suprACLonal deletion and proliferation. After a single exposure, we found that SpA doses ranging from 2000 to 2 \(\mu\)g (or less) induced persistent changes in peripheral lymphoid compartments, and in general the greater the dose the greater the magnitude of the induced suprACLonal deletion. Moreover, direct dose-dependent stoichiometric deletion of \(V_{H}\)-targeted B cells was consistently demonstrated in flow cytometric determinations of residual B cells, and confirmed in ELISPOT assays of Ig-secreting cells, and in altered levels of circulating Ig. Taken together, these studies extend our earlier demonstration that equivalent doses with different forms/variants of SpA induced quantitative levels of BCR-targeted suprACLonal deletion that directly correlated with the intrinsic level of \(V_{H}\)-restricted Fab-binding activity (14). The current studies have provided the first characterization of a dose-response relationship to this model B cell SAg.
Our studies confirmed that SpA-induced targeted B cell deletion is always associated with concordant induced proliferation. Significantly, while higher doses induced only limited detectable BCR-targeted proliferation, lower in vivo SpA doses induced much greater levels of proliferation and delayed the subsequent kinetics of deletion. Although we have previously shown that large (i.e., 1000 μg, 20 mg/kg) doses of fluorochrome-labeled SpA rapidly travel through the circulation to affect B cells at remote anatomic sites, the current results can only be interpreted as evidence that in vivo lymphocyte-specific targeting and quantitative deletion at remote sites is also induced with doses of 2 μg (0.04 mg/kg) or lower. Our findings therefore lead to the incontrovertible conclusion that the immunomodulatory activities of this B cell targeting agent, even at low doses, is not effectively inhibited by high molar excess of circulating SpA-binding Ig, and we presume that the pentavalent structure of SpA may make functional inhibition by soluble Ig analogues inefficient (or impossible).

Our findings were consistently demonstrated in a range of in vivo model systems, with proliferation documented by both BrdU incorporation studies and adoptive transfer of fluorochrome-labeled cells. In addition, SpA treatment induced similar changes in the B cell compartment of T15i knockin mice, in which nearly all B cells are targeted by SpA, and in the polyclonal B cell populations of a “normal” C57BL/6 mice, which express the full diversity of Ab gene usage and different murine VHIII genes that display nonconservative variations, resulting in a range of SpA-binding affinities (12). However, while higher levels of suprachromal expansion were documented in the adoptive transfer model, this may in part reflect that much smaller number of detectable T15i B cells in the spleen of the C57BL/6 recipients (i.e., ~1.5 × 10^6), while naïve T15i/+ adult mice have >15 × 10^6 splenic B cells, representing an estimated 10-fold greater representation of SpA-susceptible B cells. Hence, in the adoptive transfer model the greater apparent induced suprachromal expansion may be due to higher dose-dependent stoichiometric ratios per affected B cell. However, in both systems, our results demonstrated a massive net deletion with higher doses of SpA, and greater proliferative expansion and/or increased survival of residual mature B cells after low-dose (i.e., < 20 μg) SpA treatment.

In our earlier studies, we showed that lower affinity/avidity forms of SpA induce lower levels of suprachromal deletion (14), which is consistent with current evidence that lower doses of SpA induce greater levels of induced interim proliferation and less overall deletion of T15i B cells. Similarly, the greater induced proliferation and preferential expansion found in some B cell clones in C57BL/6 mice likely represents their lower affinity SpA interactions of SpA, which is more easily detectable at lower doses.

It remains possible that the relatively lower representation of SpA-reactive B cells at 3 days after treatment reflects the level of induced deletion and an inability to detect some B cells with downregulated BCR. However, we believe that by 28 days after treatment these detection issues are no longer relevant, and the values depicted in the current report are also highly consistent with data previously obtained at 1 wk and 2 wk after high-dose treatment in C57BL/6 mice (16).

Although suprachromal expansion/deletion can be induced by different parenteral approaches, we found that i.p. administration induced more potent B cell effects than i.v. treatment, although effects were induced with relatively small doses by either site of administration potent. In studies using the same recombinant SpA preparation, it has been shown earlier that i.v. administration results in the rapid development of circulating SpA-IgG complexes, and the composition of complexes shifted over time, but were detectable in the circulation and tissue for prolonged periods (23). Although we did not evaluate the nature of the resulting SpA-immune complexes in our studies, from our findings we speculate that the greater efficacy of i.p. dosing likely reflects a more protracted rate of systemic entry of SpA, and/or delayed subsequent clearance, or perhaps reflect differences in the composition of the SpA-Ig and/or SpA-BCR complexes that subsequently form in vivo.

Our findings are reminiscent of a report that the staphylococcal T cell SAgs, staphylococcal enterotoxin B, induces targeted T cells to undergo a fixed number of rounds of proliferation until massive suprachromal collapse and death ensues from presumed cytokine exhaustion. With the same labeling transfer study design, Staphylococcal enterotoxin B was reported to induce Vβ-restricted lymphocyte deletion that was always preceded by a specific number of rounds of proliferation (24), although in fact the potential impact of different levels of dosing was not considered in these studies. The findings in our studies evoke the question of whether the clonal fate outcome of SpA-induced in vivo BCR triggering is influenced by B cell autocrine capacity, or the availability of B cell extrinsic in vivo survival/growth signals, akin to enhanced survival/proliferative effects we have shown for subsequent in vitro cytokine or cognate “second signal” exposure (15). The current studies provide further support for the notion that higher SpA doses provide a strong “signal 1” that is imbalanced with the availability of growth/survival stimuli, the signal induced by lower doses are better balanced by sufficient endogenous growth/survival signals to enable greater proliferation (25, 26). As such, survival would also be determined by the relative fitness of a B cell, and the number of B cells in vivo that compete for limited survival niches, which may differ in T15i and C57BL/6 mice.

We have recently shown that SpA induces a death pathway that is associated with early induced changes in mitochondrial membrane potential, and Bcl-2 over expression interferes with this clonal fate, whereas there is no requirement for the Fas death pathway (15) or the TNF R1 death receptor (C. S. Goodyear and G. J. Silverman, unpublished observations). The death induced by SpA is therefore consistent with the sequence of changes described for AICD that follows BCR triggering. Although controversy persists, recent studies have also challenged the role of death receptors for in vivo TCR-mediated death, and have instead implicated the mitochondria changes (i.e., intrinsic pathway) as playing a central role, resulting from altered bioenergetic demands that follow the exhaustion of prosurvival cognate and/or cytokine (second) signals that leads to clonal collapse and death (15, 19, 26, 27). The current studies therefore provide further insight into the outcome of BCR-mediated AICD, and the many surprising and unexpected functional parallels between microbial SAgs that target B and T lymphocytes.

One of the major relevant observations is the somewhat unexpected observation that even very small doses of SpA can greatly alter the composition of the in vivo B cell repertoires. These findings were perhaps previously unexpected, in part because it was previously anticipated that circulating Ig would block interactions with small doses of SpA. Although the levels of SpA produced during clinical infection are currently unknown, evidence that single doses as low as 0.2 μg under the right conditions can have lasting effects on B cell compartment. These observations are therefore very relevant to understanding the possible roles of SpA and other B-microbial B cells SAgs, on the pathogenesis of infection. In support, in a recent report, the immunologic influence of the SAgs properties of SpA was...
evaluated during murine infection, based on comparisons made between the SpA-producing S. aureus 8325-4 strain with an isogenic SpA-deficient mutant created by allelic replacement (28). In fact, primary infection with modest inoculum of SpA-expressing staphylococci gave rise to significantly higher serum levels of V_H3-encoded Abs compared with the isogenic spa deletion mutant strain. Moreover, larger staphylococcal inoculum correlated with a significantly impaired capacity to make V_H3 Ab responses. These findings suggested that SpA expression during staphylococcal infection can modulate SpA-reactive B cell clonal responses, with V_H3-dependent mechanisms inducing either supraclonal expansion or deletion.

How could the expression of a B cell SAg benefit a microbial pathogen? Survival from an infection may be directly dependent on the capacity of the host to localize and limit an infection before the pathogen proliferates and spreads, and Ab responses are often key factors in such defenses. Hence, we have speculated that this type of bacterial toxin may have developed for a pre-emptive first strike mechanism for the microbe to specifically target those B cells most essential for host defense. Although it remains uncertain which staphylococcal conventional Ags are most important for host immune recognition, clones from the B-1 and/or MZ B cell pools are likely important for these responses. In support, whereas immunization with a non-protein staphylococcal adhesin (poly-N-acetyl glucosamine) can induce protective immune responses in mice, immunization with whole staphylococcal bacteria does not (29). Speculatively, the SpA in staphylococcal extracts could contribute to the finding that such immunogens do not induce protective immune responses to later infection. This virulence mechanism may also partly explain why a clinically effective staphylococcal vaccine is not yet available.

These findings may also have therapeutic implications. As we discuss at length in a recent commentary (30), patients with autoimmune diseases may already be benefiting from the immunomodulatory activity of SpA, even though the therapy they receive was not intentionally designed for this purpose. During treatment with a Federal Drug Administration-approved apheresis column that consists of SpA on an inert silica base, ~200–µg doses of SpA leach off of the column. A therapeutic regimen may include a series of 12 or more treatments, which could result in the cumulative infusion of several milligrams of SpA (31, 32). Data presented in this study illustrate that doses of <2 µg (0.1 mg/kg) in the mouse are sufficient for the induction of targeted B cell deletion. This murine dose corresponds to a human equivalent dose (33) that is well within the range of SpA exposures that may be infused during an apheresis column treatment.

Recent studies have suggested that MZ B cells, which we have shown are especially sensitive to SpA-induced deletion (16), can be recruited into pathologic autoimmune diseases to become sources of pathogenic autoantibodies (34). In fact, SpA-apheresis has been approved for the treatment of idiopathic thrombocytopenic purpura and rheumatoid arthritis, and the diagnostic autoantibody responses have been reported to have overrepresentation of V_H3 gene products (35, 36). Therefore, although not optimized for this purpose, for some patients the in vivo delivery of SpA may already provide clinical benefits akin to those now being explored with other B cell deletion agents, like the anti-CD20 Ab, Rituximab (37). The current studies may therefore provide insights into effective dose ranges, and preferred routes of administration, for the development of SAG-based therapeutic approaches that may provide special benefits for B cell-driven autoimmune and neoplastic diseases.

Acknowledgments We thank Kathleen Warmoth for help with statistical analyses.

Disclosures Through the University of California, San Diego, T. S. Goodyear has had a lab service agreement with Repligen.

References

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