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In Vivo $V_L$-Targeted Activation-Induced Apoptotic Supraclonal Deletion by a Microbial B Cell Toxin

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To interfere with host immune responses, some microbial pathogens produce proteins with the properties of superantigens, which can interact via conserved V region framework subdomains of the Ag receptors of lymphocytes rather than the complementarity-determining region involved in the binding of conventional Ags. In recent studies, we have elucidated how a model B cell superantigen affects the host immune system by targeting a conserved $V_H$ site on the Ag receptors of B lymphocytes. To determine whether these findings represent a general paradigm, we investigated the in vivo immunobiologic properties of protein L of *Peptostreptococcus magnus* (PpL), a microbial Ig-binding protein specific for a V region site on Ig L chains. Our studies confirmed that PpL binding is restricted to a subset of murine $V_{\lambda}$-expressing B cells, and found that B cells with stronger PpL-binding activity are associated with certain B cell subsets: splenic marginal zone (CD21$^{\text{high}}$ CD23$^{\text{low}}$), splenic CD1$^+$, peritoneal B-1a (IgD$^{\text{low}}$ CD5$^+$), and CD21$^{\text{high}}$ CD24$^{\text{high}}$ B cells in peripheral lymph nodes, mesenteric lymph nodes, and Peyer’s patches. Infusion of PpL triggered a sequence of events in B cell receptor (BCR)-targeted B cells, with rapid down-regulation of BCR, the induction of an activation phenotype, and limited rounds of proliferation. Apoptosis followed through a process heralded by the dissipation of mitochondrial membrane potential, the induction of the caspase pathway, DNA fragmentation, and the deposition of B cell apoptotic bodies. These studies define a common pathway by which microbial toxins that target V region-associated BCR sites induce programmed cell death. *The Journal of Immunology*, 2004, 172: 2870–2877.

To facilitate their relationships with the host, certain bacteria and viruses have developed proteins that are capable of specific high avidity-binding interactions with the secreted and membrane-associated Ag receptors of B lymphocytes (BCR) of the adaptive immune system (1). Several of these Ig-binding proteins have practical utility as affinity purification and detection moieties in a wide range of applications. However, the true biologic roles of these proteins remain poorly understood. Whereas these factors do not appear to be essential to the microbial life cycle, they have been assumed to be involved in the pathogenesis of infections.

Following the discovery that protein A of *Staphylococcus aureus* (SpA) has the capacity for specific interactions restricted to the products of defined V region gene families (2), we initiated a series of investigations that have better defined the immunobiologic properties of this prototypic Fab-binding protein. In these studies, we have characterized the molecular requirements for Fab-binding specificity of SpA (3), and demonstrated the capacity for a high frequency of interactions with human (4) and murine B lymphocytes (5–8). In collaborative studies, we also reported the crystallographic structure of this Ig-binding protein in cocomplex with a human $V_{\lambda}^3$ Fab, which demonstrated that these interactions are mediated via BCR V region framework determinants (9) that have been highly conserved during the evolution of the immune system (7). In parallel, we have shown that SpA can induce the in vivo deletion of $V_{\lambda}$-targeted supraclonal sets of B lymphocytes (5), resulting in effective long-lasting immunologic tolerance to a common dominant microbial epitope important for immune defense from many infectious agents (6). Extending these investigations, we have recently reported on our dissection of pathways by which SpA induces $V_{\lambda}$-targeted in vivo activation-induced apoptotic death of B cells (8).

From our investigations, we have developed a structure-function model that we believe rationalizes how the capacity of SpA to induce apoptotic death of targeted B cells is directly linked to its structural organization (6, 8). To consider whether these properties represent a paradigm common to microbial Ig-binding proteins, we have now examined the host cellular response to protein L of *Peptostreptococcus magnus* (PpL), a secreted membrane protein of *P. magnus* (also termed *Finegoldia magna*) (10). This anaerobic bacterium is part of the indigenous flora of the gastrointestinal and genitourinary tracts, and the skin, although it has also been implicated in various infections (11, 12). Bacterial virulence has been correlated with expression of PpL, which is a 76- to 106-kDa protein (13–15) composed of four or five highly homologous Fab binding domains in tandem (16), reiterating a structural theme common to SpA. Furthermore, PpL binding has been shown to involve a framework-associated site shared by many Ig $V_{\lambda}$ gene products of the human immune system (17).

To test our hypothesis, we sought to determine whether PpL, by interactions with the L chain-associated Fab binding site (18), has the capacity to affect the B cell compartment of the adaptive immune system. In studies designed after those we have recently reported for SpA (8), we found that PpL is capable of inducing activation-associated apoptosis of B cells that are targeted via the...
Ve regions expressed in their membrane-associated BCR. Cumulatively, these findings demonstrate general principles that may also apply to the potential host interactions of microbial proteins of diverse fine Ig-binding specificities.

Materials and Methods

Mice and immunogens

T15i knockin (19) and AB29 transgenic (20) mice, which had been back-crossed to C57BL/6 background, were the kind gifts of K. Rajewsky (Harvard Medical School, Boston, MA) and H. Tighe (University of California, San Diego, CA). Other mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and bred under specific pathogen-free conditions under the supervision of the University of California Animal Subjects Program. Applying a regimen previously used in studies of SpA (5–8), adult mice, at least 6 wk of age, received rPpL (Tetralink, Amherst, NY) or OVA as a control protein (Sigma-Aldrich, St. Louis, MO), from which contaminating endotoxin had been removed to a low level (<0.5 EU/ml). Unless otherwise indicated, mice received doses of 1 mg in 25 µl of pyrogen-free saline, instilled into the peritoneal cavity. After sacrifice, tissues were harvested for immediate ex vivo analysis, and aliquots were also placed in culture. In studies intended to quantitate residual deletion after longer time intervals, mice received a dose of 0.4 mg of protein in saline, which was repeated after 2 and 4 days, with sacrifice 7 days after the first dose. In all experiments, mice were age and sex matched.

Flow cytometry analysis

Adapting previously reported methods (5–7), subsets of mononuclear cells were identified by staining with FITC-labeled anti-IgD (clone 11-26c.2a) or anti-CD21 (7G6), CD86 (GL-1), PE-labeled anti-CD1 (B11), CD23 (2G12, Southern Biotechnology Associates, Birmingham, AL), CD24 (M1/69), CD3 (145-2C11) or CD5 (53-7.3), PerCP-labeled B220, Tricolor-labeled anti-Igκ, anti-Igλ (Southern Biotechnology Associates), or isotype controls, used with streptavidin-labeled PerCP or allophycocyanin (BD PharMingen, San Diego, CA), in the presence of Fc block (Ethyl BioTech, San Diego, CA), as appropriate. Data were acquired using a FACSCalibur (BD Biosciences, San Diego, CA), and analyzed with FlowJo software (Treestar, Ashland, OR).

To measure the activity of a proapoptotic pathway, cells were permeabilized and stained with specific affinity-purified rabbit IgG anti-activated caspase 3 (Cell Signaling Technology, Beverly, MA) and detected with PE goat anti-rabbit IgG (Southern Biotechnology Associates). Changes in mitochondrial transmembrane potential (ΔΨm) were detected by staining with JC-1 (Intergen, Purchase, NY) (21).

Adoptive transfer studies

Isolated splenocytes were suspended at 5 × 10⁷/ml in RPMI 1640, labeled with 2 µM of CFSE (Molecular Probes, Eugene, OR), 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 × 10⁷ splenocytes was transferred i.v. through the tail vein. After 24 h, mice received either PpL or control protein, as described above, or 500 µg of the immunostimulatory CpG phosphorothioate oligonucleotide (5′-TGA CTG TGA ACG TTC GAG ATG A-3′), which was used as a positive control for proliferation (i.e., mitogen) (22). Control studies confirmed that CFSE treatment did not compromise the ability of B cells to respond to PpL or to mitogens (data not shown).

In vitro studies

In certain studies, freshly isolated splenocytes were placed in culture at 2 × 10⁶ cells/ml at 37°C, 5% CO₂ in RPMI 1640 with 5% FCS, supplemented with l-glutamine, 10 mM of HEPES, amino acid solution, 1 mM of sodium pyruvate, 60 µM of 2-ME, and penicillin/streptomycin.

Histology

Spleens were placed in OCT compound embedding medium (Fisher Scientific, Pittsburgh, PA) and frozen in a 2-methyl butane/dry ice bath. Sections (7 µM) were cut and mounted on Superfrost Plus Slides (Fisher Scientific). Slides were fixed in 3.7% formaldehyde in PBS (pH 7.4) for 20 min at room temperature, blocked in PBS, 5% FCS for 1 h at room temperature, then stained with PE-labeled anti-B220 (diluted in blocking buffer), washed, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice, washed, and then stained with the TUNEL assay kit (Roche). After mounting with Prolong (Molecular Probes), digitized images were captured at low power (i.e., ×10) magnification.

Statistical analysis

Comparisons between different groups used the two-tailed Student’s t test, with p values <0.05 taken as significant.

Results

PpL-binding interactions with a subset of peripheral B cells

To investigate the cellular implications for the Ig-binding activity of PpL, we first assessed for potential binding interactions with peripheral mononuclear cells from naive adult mice. As shown in Fig. 1, we found that binding of labeled PpL was restricted to B220⁺ mononuclear cells, and in both BALB/c and C57BL/6 mice PpL interacted with more than one-half of the B lymphocytes. Significantly, PpL binding was overwhelmingly associated with Igκ-expressing B cells, as little or no binding by Igλ-bearing B cells was detected. Moreover, although certain Igκ-expressing B cells displayed higher PpL-binding activity, there was no distinct separation of PpL binders and nonbinders, which may indicate that κ-bearing B cells can display a continuous range of PpL-binding affinities. We also assessed binding in T15i knockin and AB29 transgenic mice, in which we found PpL binding was also restricted to a subset of Igκ-expressing B cells, confirming that H chain usage does not directly influence PpL binding. These findings were therefore consistent with previously reported in vitro binding studies with soluble Ig (23), as we confirmed that interactions with PpL are associated predominantly (if not solely) with a subset of κ-bearing B cells. These results, however, were different from those in an earlier flow cytometry report that also found PpL binding by a fraction of CD19⁺/CD3⁺ mononuclear cells (24), as presumably the B cell restriction demonstrated in our studies reflected a methodologic approach that excluded cytoplasmic FcR-mediated interactions.

Differential PpL binding to distinct B cell subsets

In the mouse, there are distinct sets of mature B cells believed to have specialized BCR repertoires associated with different functional roles (reviewed in Ref. 25). To determine whether PpL...
binding is more commonly associated with particular B cell subpopulations, we assessed PpL binding in different anatomic compartments. In studies of adult C57BL6 splenocytes, we applied a phenotyping strategy shown to discriminate subpopulations of mature B cells from the B cell precursors that continually emerge from the bone marrow (26). In representative studies illustrated in Fig. 2A, we compared total surface Igκ (sIgκ) expression on transitional bone marrow emigrants; transitional type 1 (T1) (B220<sup>+</sup>CD24<sup>high</sup>CD21<sup>low</sup>) and type 2 (T2) (B220<sup>+</sup>CD23<sup>high</sup>CD21<sup>high</sup>), with marginal zone (MZ) (B220<sup>+</sup>CD21<sup>high</sup>CD23<sup>low</sup>) and mature follicular (i.e., B-2) (B220<sup>+</sup>CD21<sup>low</sup>CD23<sup>high</sup>) B cells. Notably, while these B cell sets displayed only relatively limited differences in their mean levels of sIgκ expression, there was a clear hierarchy in the representation of B cells with strong PpL-binding activity, as T2 > MZ > T1 > B-2, which was highly reproducible in independent studies. In parallel studies, we also found that splenic B220<sup>+</sup>CD1<sup>high</sup> B cells, a set overlapping with MZ and perhaps T2 cells (27), displayed a significantly greater representation of higher PpL binders than for B220<sup>+</sup>CD1<sup>low</sup> B cells, even after considering for differences in sIgκ levels (Fig. 2B). These findings are consistent with the hypothesis that there is biased PpL-binding activity in different splenic mature B cell sets, with an apparent selection for PpL binders into the MZ set, and a relative paucity of PpL binders in the conventional B-2 set.

In studies of peritoneal mononuclear cells (Fig. 2C), we also found evidence of biased expression of PpL-binding capacity in B-1a (B220<sup>+</sup>sIgD<sup>low</sup>CD5<sup>+</sup>) compared with both B-1b (B220<sup>+</sup>sIgD<sup>low</sup>CD5<sup>+</sup>) and conventional mature peritoneal B cells (i.e., B-2 cells) (B220<sup>+</sup>sIgD<sup>high</sup>CD5<sup>+</sup>). These correlative findings provide direct evidence that the V<sub>λ</sub> repertoire of peritoneal B-1a cells can be functionally distinguished from those of local conventional B cells, which is consistent with the notion that these B cells may be responsive to, and selected by, different BCR ligands.

We also examined the expression of PpL-binding activity in other peripheral lymphoid tissues. As shown in Fig. 2D, while sIgκ expression was relatively uniform among B cells at these different sites, we found a somewhat higher level of PpL binding in B220<sup>+</sup> cells in peripheral lymph nodes (LN) (i.e., pooled axillary and inguinal LN) compared with mesenteric LN and Peyer’s patches (PP). Because at these sites there appeared to be two peaks of PpL-binding intensity, especially in the peripheral LN samples, we performed additional phenotypic characterization. These studies demonstrated that in all samples PpL binding was higher in the CD21<sup>high</sup>CD24<sup>high</sup> B cells, compared with the CD21<sup>low</sup>CD24<sup>low</sup> B cells, and these differences could not be explained based on levels of sIgκ expression (Fig. 2E). Hence, these findings were also consistent with our studies of splenic B cells, in which the splenic CD21<sup>high</sup> subsets (i.e., MZ and T2 B cells) also displayed higher levels of PpL-binding activity. These findings may reflect a distinct BCR repertoire of the CD21<sup>high</sup>CD24<sup>high</sup> B cell subset in LN, which in recent reports has been implicated to have special regulatory roles in the gut (28, 29).

**FIGURE 2.** Preferential PpL binding by B cell subpopulations in the spleen, peritoneal cavity, LN, and gut-associated lymphoid tissue. A. Splenic B cells were pregated on B220 and then stained for CD21 and CD23 expression to determine the representation of MZ, mature follicular (B-2), and T2 B cells. To identify T1 cells, we used CD21<sup>high</sup>CD24<sup>high</sup>B220<sup>+</sup>, which discriminates these recent B cell emigrants from other B cell types that are also CD21<sup>high</sup>CD24<sup>high</sup> (26, 28). B. Splenic B cells were gated with CD1 and B220 to discriminate CD1<sup>low</sup> B cells. C. Peritoneal B cells were gated with B220, IgD, and CD5 to discriminate B-1a, B-1b, and B-2 cells. D and E. Cells harvested from mesenteric LN (MLN), peripheral LN, and PP were analyzed as total B cells, and subdivided into CD21<sup>high</sup>CD24<sup>high</sup> (R1) and CD21<sup>low</sup>CD24<sup>low</sup> (R2) cells. The associated PpL and Igκ binding of the different B cell subpopulations is illustrated in adjacent histograms with mean fluorescence intensity depicted in a corresponding color. Results depicted are from representative studies of adult C57BL6 mice.
As shown in Fig. 3A, when examined 16 h after in vivo PpL treatment, a subset of \(\kappa\)-bearing splenic (B220\(^+\)) (top, right) displayed significantly down-regulated sIg, while Ig\(\lambda\)-bearing B cells were not affected (bottom, right), compared with mice that were instead treated with a control protein (at left). We also found that PpL treatment resulted in a transient increase in the representation of Ig\(\kappa\)-bearing B cells in the spleen (data not shown), which most likely represents an induced early trafficking of affected B cells into the spleen akin to that documented after SpA treatment (8).

We found that PpL treatment also induced affected Ig\(\kappa\)-bearing B cells to up-regulate CD86, an early marker of cellular activation (Fig. 3B). By contrast, PpL treatment did not affect CD86 expression on Ig\(\lambda\)-bearing B cells. In other studies, we confirmed that this induced activation phenotype was restricted to the B cells with down-regulated V\(\kappa\)-associated sIg.

**In vivo clonal fate after PpL treatment**

We next evaluated whether PpL treatment altered the clonal fate of the targeted B cells. For these studies, C57BL/6 splenocytes were labeled with CFSE and then adoptively transferred into C57BL/6 recipients. At 48 h after treatment, the spleens of treated recipient mice were harvested and evaluated. In a representative study shown in Fig. 4, treatment with the B cell-specific mitogen, CpG DNA (a positive control), was shown to induce proliferation in \(~40\%\) of labeled B cells. However, in these mice, there were no significant differences in the responses of sIg\(\kappa\)-bearing B cells (or the subset of B cells detected based on PpL-binding capacity) or Ig\(\lambda\)-bearing B cells (data not shown), which confirmed that there was no BCR selectivity in the response to this mitogen. By contrast, following PpL treatment, we found that a smaller proportion (\(~5.5\%, p < 0.002\)) of Igk-bearing B cells had undergone one or two rounds of proliferation, but this was still greater than the low background levels detected in control protein-treated mice. Importantly, in analysis focused on PpL-binding B cells, a significantly larger proportion (\(~9\%, p < 0.0006\)) of these targeted B cells was found to have undergone proliferation. These levels of induced proliferation for Igk- and PpL-binding B cells were also much higher than those detected for Ig\(\lambda\)-bearing B cells (data not shown), although the small number of sIg\(\lambda\)-bearing B cell events detected in our flow cytometric studies made quantitation less meaningful.

**PpL treatment induces supraclonal deletion of a subset of V\(\kappa\)-bearing splenic B cells**

In our studies of transferred CFSE-labeled splenocytes, we also evaluated whether PpL treatment results in detectable changes in

![FIGURE 3](image)

**FIGURE 3.** Phenotypic changes induced by PpL in C57BL/6 splenic B cells. A, PpL induces down-regulation of membrane-associated BCR on B cells 16 h after treatment. B, In vivo PpL exposure induces an activation phenotype on V\(\kappa\)-expressing B cells. Results depicted are from representative studies of three independent experiments in adult C57BL/6 mice.

![FIGURE 4](image)

**FIGURE 4.** In vivo clonal fate after PpL treatment. C57BL/6 splenocytes were labeled with CFSE and adoptively transferred into C57BL/6 recipients. In this figure, CFSE identifies mononuclear cells derived from the C57BL/6 donor, and dilution of staining intensity identifies cells that have undergone cell division. B cells are identified as B220\(^-\). The in vivo outcome to treatments with OVA (control), PpL, and immunostimulatory DNA (mitogen) is depicted in cells recovered from the spleens. Mean values for percentage of proliferation of gated B cells from groups of four mice are indicated in the table. Data were pooled from two independent experiments.

<table>
<thead>
<tr>
<th>Round of Proliferation (% of cells)</th>
<th>3</th>
<th>2</th>
<th>1</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>untreated</strong></td>
<td>0.7</td>
<td>0.6</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>PpL-treated</strong></td>
<td>8.2</td>
<td>5.4</td>
<td>4.5</td>
<td>3.6</td>
</tr>
<tr>
<td><strong>mitogen-treated</strong></td>
<td>12.0</td>
<td>10.5</td>
<td>9.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

In the subsequent representation of mature B cells at 48 h after treatment. Applying previously described methods (8), we looked for altered representation based on B-T cell ratios. In the spleens of the mitogen-treated mice, we found a mean 296 ± 18% increase of labeled B cells, which was consistent with the above-described evidence of massive proliferation. Significantly, despite the limited proliferation that followed PpL treatment, there was still a 34 ± 7.5% (mean ± SD) overall decrease of labeled splenic B cells in the PpL-treated mice compared with the control-treated mice (\(p < 0.004\)) (from groups of four mice pooled from two independent experiments). Notably, we also evaluated the outcome at 72 h after treatment, and although we found that a limited number of B cells had now undergone three rounds of proliferation, the same net levels of B cell deletion were still documented (data not shown).

These studies document that even though PpL can induce proliferation detectable in a small subset of surviving Igk-bearing B cells (or control protein) and were then evaluated 7 days after the initial treatment. Consistent with the prediction that by this time point there should also have been normalization of any induced
caspase 3 represents an essentially irreversible
mitigation that reflects the early phase of the intrinsic apoptosis
pathway. As shown in Fig. 6, akin to our recently reported findings
in SpA-treated mice, compared with control-treated mice we found
limited changes in mitochondrial membrane changes (ΔΨm) in
a subset of B cells as early as 4 h after PpL treatment (data not
shown), and even greater changes in ΔΨm of B cells were readily
detected 48 h after PpL treatment, while T cells (i.e., CD3+ 
) in the same samples were unaffected.

We also looked for evidence of induction of the caspase pathway,
which are cysteine proteases that often play a central role in
apoptotic death pathways. In several studies, at 16 h after in vivo
exposure, splenocytes were harvested and placed in culture with-
out additional stimulants. After 24 h in culture, we found that B
cells from PpL-treated mice displayed progressive increases in the
expression of activation-associated surface phenotypic
markers. This was followed by progression of a death pathway
that rigorously documented by the
mitochondrial membrane protein of a common anaerobic isolate of clinical in-
fections, can induce Vλ-targeted in vivo apoptotic death. In murine strains with polyclonal B cell repertoires, PpL
elicited a sequence of events initiated by sIg down-regulation and
increased expression of activation-associated surface phenotypic
markers. This was followed by progression of a death pathway
involving alterations in mitochondrial membrane potential that led
to apoptosis. In fact, the induction of in vivo apoptotic death was
rigorously documented by the finding of in situ deposition of

Deletion reflects apoptotic death
Based on our experience with mice treated with SpA, we wondered
whether the PpL-induced loss of B cells represented supranclonal
deletion due to the induction of an apoptotic pathway, so we
looked for evidence of induced mitochondrial membrane perme-
ableization that reflects the early phase of the intrinsic apoptosis
pathway. As shown in Fig. 5, akin to our recently reported findings
in SpA-treated mice, compared with control-treated mice we found
limited changes in mitochondrial membrane changes (ΔΨm) in
a subset of B cells as early as 4 h after PpL treatment (data not
shown), and even greater changes in ΔΨm of B cells were readily
detected 48 h after PpL treatment, while T cells (i.e., CD3+ 
) in the same samples were unaffected.

To verify that these findings accurately reflect the apoptotic
pathway of in vivo death in Vλ-targeted B cells, we also performed
immunohistochemical analyses that looked for induction of cellular
DNA fragmentation in targeted B cells using the TUNEL assay,
which detects DNA 3′-hydroxyl ends resulting from caspase-acti-
vated DNA cleavage (30, 31). As shown in Fig. 7B, we compared the spleens of control protein-treated mice with those that had received either SpA or PpL treatment. At 48 h after treatment, we found that both SpA treatment and PpL treatment resulted in the increased shift in FL-1 and lower levels of staining with the specific fluores-
cent dye detected in FL-2. Results of splenic lymphocytes are depicted for BALB/c mice at 48 h after a single treatment.

Discussion
In the current studies, we have demonstrated that PpL, a secreted
membrane protein of a common anaerobic isolate of clinical in-
fections, can induce Vλ-targeted BCR-mediated in vivo apoptotic
death. In murine strains with polyclonal B cell repertoires, PpL
elicited a sequence of events initiated by sIg down-regulation and
increased expression of activation-associated surface phenotypic
markers. This was followed by progression of a death pathway
involving alterations in mitochondrial membrane potential that led
to apoptosis. In fact, the induction of in vivo apoptotic death was
rigorously documented by the finding of in situ deposition of
apoptotic bodies, demonstrated by the TUNEL assays, in primary follicles in the peripheral lymphoid organs (Fig. 7). These studies support our fundamental hypothesis that, despite completely distinct fine binding specificities within the Fab portion of the BCR, protein L can induce a supraclonal B cell fate, which is the same as we have recently shown following in vivo exposure to SpA (8). Before we performed these studies, the only available reports on the cellular interactions of PpL had been limited to binding studies on naive mononuclear cells (24), and in vitro assays demonstrating that PpL (like SpA) is capable of triggering mast cells and basophils via their membrane-bound Ig (32). In fact, we were uncertain whether PpL was capable of deleting B cells, as we were concerned that PpL might not induce a suitable BCR signal due to subtle differences in affinity or avidity, or perhaps because affected B cells might have the potential capacity to recruit additional second signals. We were gratified to determine that using similar in vivo doses, while SpA was shown to induce deletion of mean 68% of Ig transgene-expressing susceptible splenic B cells (8), PpL induced a mean 44% loss of polyclonal Igκ-expressing splenic B cells. Moreover, the frequencies of the deposition of B cell apoptotic bodies in the spleen (Fig. 7) were also proportional to the representation of polyclonal B cells capable of these binding interactions, as detected by flow cytometry. Admittedly, although in our studies the most apparent overall induced lymphocyte fate was apoptotic death, minor sets of B lymphocytes may well have been induced to undergo alternate fates, which may include functional inactivation (i.e., anergy), supraclonal expansion, or end-differentiation, possibly due to variations in their intrinsic binding affinities for protein L, or other factors. In particular, it remains potentially possible that certain B cells responding to PpL may escape through receptor editing (33, 34) or by receptor dilution (35), mechanisms that may be more commonly linked to L chain-targeted interactions.

Our results document similar potencies of two unrelated microbial proteins to induce BCR-mediated effects on B cells, despite the fact that these proteins target distinct conserved sites on host B
cell Ag receptors. These proteins also embody two very different structural approaches to convey similar functional capacities. An Ig-binding domain of SpA uses side chains α helices 2 and 3 to compose a V₃H₃ Fab binding site, which is separate and functionally independent (9) from the binding site for Ig Fcγ that involves helix 1 and 2 (as originally described by Deisenhofer (36)). By contrast, in a recent report, Graille et al. (18) also showed that one 61-residue polypeptide domain derived from PpL contains a Vκ binding site that is formed by two pairs of antiparallel β strands and a helix that lies on top of the sheet. Although much less is known about the interactions of PpL compared with SpA, these authors suggested that a single domain of PpL may actually have two separate Igκ binding sites of the same, or similar, binding specificities (37).

Although SpA is exquisitely specific for the products of structurally and genetically related homologous genes within the V₃H₃ clan, the genetic and structural relationships of Vκ genes within and between species are less well defined. Moreover, it is also currently unclear whether this alternate structural strategy of PpL provides a similar restriction, or greater promiscuity, for potential BCR-binding interactions. Nonetheless, the current studies dramatically demonstrate the functional equivalence of PpL-binding interactions that may affect one-half or more of Vκ-expressing B cells in both BALB/c and C57BL/6 mice. The Vκ binding site of PpL and the V₃H₃ binding site of SpA both convey one-point binding interactions with Fab in the μM range, and binding is greatly enhanced by the avidity inherent to the four or more domains present in each of these native proteins (18, 38, 39). We hypothesize that these structural features impart the capacity to target specific B cells for superantigen-mediated lymphocyte deletion. These insights also suggest that these two different Fab-binding proteins arose by convergent evolution to convey very similar immunobiologic activities. Although our findings document a V region-targeted B cell apoptotic deletion, interactions of PpL (or SpA) with non-B cells may still provide in vivo cofactors that contribute to the B cell deletion process.

Although actual sequence data are limited, reported studies have shown that PpL has the capacity for binding interactions with isolated Ig L chains from the human Vκ1, 3, and 4 gene families. However, it is unknown whether these interactions are common to all Ig products derived from these families, and binding to some λ L chains has also been reported (40). Significantly, the Ig products of these particular Vκ gene families share similar key residues, which include first framework subdomain residues implicated as contact sites in the reported PpL-Fab co-crystal (18). However, rigorous examinations of the requirements and limitations for these interactions have not been reported. In preliminary studies, we have identified murine monoclonal Ig of known V region sequences, which display PpL-binding capacity (our manuscript in preparation). These functional observations are consistent with genetic and sequence correlations reported by Zachau and coworkers (41), as our data suggest that murine analogues of human Vκ1, 3, and 4 families may also commonly convey PpL-binding activity. However, these comparative studies are complicated by the fact that, whereas humans have 7 human Vκ families (of which only 4 are commonly expressed) (42), the mouse expresses 18 distinct Vκ families that embody much greater genetic and structural diversification, and ~30% of potentially functional murine genes have uncertain or unknown human analogues (41). In any case, the current findings contribute to the notion that diverse mammalian species (i.e., human and mouse), separated by many million years of evolution, have adaptive immune systems that have conserved highly expressed V₃H₃ and Vκ binding sites for microbial products of common colonizing/pathogenic microbes.

We were also intrigued to discover that the representation of murine B cells that can bind, and presumably can be modulated by PpL, varies in distinct B cell subsets at different sites in the body. Among mature B cell populations, there was a much greater representation of strong PpL binders among B-1a cells, with somewhat lower level among MZ B cells and splenic CD1₃high B cells, while strong binders were much less common among conventional recirculating follicular B cells. Although the maturational relationship between these B cells and precursors from the central compartment is still controversial, we found circumstantial evidence suggesting great relative enrichment of PpL binders among T2 cells, compared with the T1 cells from which they derive. One interpretation is that from newly emergent B cells, there is also a selection bias for PpL binders into the MZ and B-1 pools, while there is a concurrent selection against PpL binders into the pool of B-2 cells, which may later be recruited into germininal center responses to protein Ags. In addition, we were intrigued to find B cell sets with differential PpL-binding activity in LN and PP. Our results indicate that CD21₃high B cells at these sites have a Vκ repertoire with the same increased representation of PpL binders as found in splenic CD21₃high B cells (i.e., T2 and MZ), representing a bias compared with other B cells. In earlier studies, splenic CD21₃high cells have been shown to be more susceptible to in vitro BCR-mediated death (43), and we have recently shown they also have increased susceptibility to SpA-induced BCR death (manuscript in preparation). We wonder whether the roles that CD21₃high B cells play in the defense from infection also make them attractive targets for the development of microbial virulence factors.

In a limited survey of clinical isolates, PpL expression was associated with only 10–15% of P. magnus isolates, but PpL production appeared to correlate with virulence (14). Although a role in pathogenesis is uncertain, Bjorck and colleagues (14) recently described an innovative murine model of infection in which the introduction into Streptococcus gordonii of PpL expression facilitated murine vaginal colonization and clinical features akin to those of P. magnus-associated vaginosis. These studies, however, did not experimentally address the mechanistic basis for this altered host-pathogen relationship. Although enhanced adhesion capacity was discussed, evidence that PpL can be a secreted microbial factor during colonization/infection is consistent with the notion that PpL may affect large sets of host B lymphocytes, potentially by acting either locally or systemically. It should be appreciated that in the current studies, while PpL doses were administered into the peritoneal cavity, dramatic effects were demonstrated in lymphocytes at remote anatomic sites. Moreover, as ~40% of circulating murine Ig are capable of PpL-binding interactions (44), our findings document that soluble PpL can travel through the bloodstream, unhindered by the great molar excesses of circulating Ig, to induce altered B cell clonal fates at distant sites in the immune system.

We wonder whether the B cell modulatory properties of PpL can play a role in infection. From our surveys, it is likely that B cells enriched in BCR-mediated PpL-binding activity may normally be important in the defense from bacterial invasion. To explain these findings, we wonder whether the specificity of PpL was shaped by the goal of pre-emptive inactivation of B cell defenders of mucosal surfaces. It may therefore not be a coincidence that B cells targeted by PpL are especially enriched in B cell populations that are specific for nonprotein bacterial Ags (i.e., MZ and B-1a cells). In future investigations, it may therefore be important to examine whether infections by microbes that produce Ig-binding proteins are especially effective at perturbing B cell function and clonal regulation at sites directly adjacent to (or remote from) sites of infections.
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