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Specific In Vivo Deletion of B-Cell Subpopulations Expressing Human Immunoglobulins by the B-Cell Superantigen Protein L

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Some pathogens have evolved to produce proteins, called B-cell superantigens, that can interact with human immunoglobulin variable regions, independently of the combining site, and activate B lymphocytes that express the target immunoglobulins. However, the in vivo consequences of these interactions on human B-cell numbers and function are largely unknown. Using transgenic mice expressing fully human immunoglobulins, we studied the consequences of in vivo exposure of protein L of *Peptostreptococcus magnus* with human immunoglobulins. In the mature pool of B cells, protein L exposure resulted in a specific reduction of splenic marginal-zone B cells and peritoneal B-1 cells. Splenic B cells exhibited a skewed light-chain repertoire consistent with the capacity of protein L to bind specific kappa gene products. Remarkably, these two B-cell subsets are implicated in innate B-cell immunity, allowing rapid clearance of pathogens. Thus, the present study reveals a novel mechanism that may be used by some infectious agents to subvert a first line of the host's immune defense.

Throughout evolution, the continuous interactions between the mammalian host and infectious agents have produced an array of innate and adaptive immune effectors able to combat insults by pathogens (30). Reciprocally, infectious agents have developed efficient countermeasures to persist in the infected host (63). These pathogens have developed mechanisms to mutate, exchange genetic materials, multiply rapidly, vary their phenotype, and occupy diverse ecological niches (61). One intriguing feature of some infectious agents is to produce proteins able to interact specifically with the immunoglobulin (Ig) heavy (H)- or light (L)-chain variable regions, independently of the conventional binding site. They are referred to as “B-cell superantigens” (SAGs) and include protein A of *Staphylococcus aureus* (SpA) (22, 29, 52, 57), gp120 of human immunodeficiency virus type 1 (HIV-1) (3, 20, 32, 33, 43, 50), staphylococcal enterotoxins A and D (7, 45), and protein L of *Peptostreptococcus magnus* (12, 13).

Although conventional antigens stimulate a small proportion of B cells, the B lymphocytes responsive to SAGs can be orders of magnitude higher. Because the B-cell SAG interacts primarily with the V_H or V_L portion of the Ig molecule, it can, in principle, trigger all B cells bearing the appropriate V_H or V_L, regardless of the other J_H, D, J_L, and pairing with V_H or V_L segments (58, 68). Since there are a limited number of V genes, this property results in stimulation of a large proportion of the repertoire. For example, the bacterial cell wall protein SpA has sites that interact with the Fab of many IgM, IgA, IgG, and IgE, and this interaction is restricted to the V_H3 gene family, leading to activation of ca. 40% of human polyclonal IgMs (58, 68).

The function of these proteins is unclear, but their ability to bind conserved portions of Igs suggests that they help the

bacteria to evade the host's immune system. Through direct interaction with host Igs, they have a potential to interfere with the humoral effector arm of the immune system and to modify the antibody response of the host. Since SAG interactions with the B-cell receptor (BcR) may, in principle, lead to activation, proliferation, differentiation, anergy, or induction of programmed cell death (68), this group of microbial molecules could interfere with mechanisms that shape the B-cell repertoire and could play a role in the pathogenesis of infectious diseases in humans. In HIV infection, for example, studies revealed that subjects infected with HIV have aberrant and unstable expression of Ig genes, a finding suggestive of humoral immune dysregulation and responses to HIV-associated antigens and SAGs (4, 6, 31).

Protein L is a cell wall protein produced by ca. 10% of strains of *P. magnus*, an anaerobic bacterial species (7). Depending on the bacterial strain from which it is isolated, protein L is a 76 to 106-kDa protein containing four or five highly homologous, consecutive extracellular Ig-binding domains (35). Protein L binds predominantly to κ-chains regardless of the H chain and consequently has affinity for all classes of Igs. Since ca. 60% of human Igs have κ-type L chains, protein L interacts with a significant proportion of Igs. Its binding does not block the antigen-binding site (45), and the affinity of the interaction ranges from $1.5 \times 10^9 \text{ M}^{-1}$ to $1 \times 10^{10} \text{ M}^{-1}$, depending on the L-chain and H-chain isotypes (1). The crystal structure of a human antibody Fab complexed through its V_L region to a protein L domain revealed that *P. magnus* protein L interacts with the framework part of the variable regions without contacting the hypervariable loops (23). In vitro, protein L appears to act as a SAG for human B cells (2) and induces BcR downmodulation (65). It also cross-links the V_L domains of IgE bound to Fcε receptors, resulting in the release of histamine by basophils and mast cells (49) and secretion of IL-4 and IL-13 by basophils (18).

Although the findings obtained in vitro suggest that B-cell

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SAGs play a pathogenic role, the impact of this group of microbial proteins on the human B-cell repertoire has been difficult to test, partly because of the lack of an experimental system. We used mice in which endogenous H- and L-chain genes were inactivated and instead were engineered to express human Igs (44). The Ig transgenes somatically recombine and mutate upon immunization to encode a functional human repertoire (44, 51). Here, we have determined the consequences of confronting B cells expressing human surface Igs with protein L *in vivo*. We were also able to follow the fate of various B-cell populations throughout development in the bone marrow (BM) and in secondary lymphoid organs after protein L treatment.

MATERIALS AND METHODS

Mice and immunogens. Generation of the "five-feature" transgenic mice used in these experiments has previously been described (44). Briefly, the endogenous loci coding for the H- and L-chains have been inactivated, and human H-, κ -, and λ -chain transloci were "knocked in." The H-chain translocus contains the core region of the human H-chain locus with the five most 3' V_H gene segments, the complete DH and JH loci, and the C _{μ} and C _{δ} exons in correct germ line configuration. The κ -chain translocus is a construction that contains 20 repeats of five V _{κ} gene segments linked to the core of the germ line locus, including the first three V _{κ} gene segments, the complete J _{κ} cluster, and the C _{κ} coding region. It contains 82 functional V _{κ} gene segments. Finally, the λ -chain translocus encompasses 28 V _{λ} gene segments (with 16 functional) attached to the J _{λ} and C _{λ} coding regions in the correct germ line configuration. All mice used in these experiments were bred in the animal facility of Broussais Hospital and used at 7 to 9 weeks of age. Animals were housed in a specific-pathogen-free barrier facility. Groups of five mice were treated with either hen egg lysozyme (HEL; Sigma-Aldrich, St. Louis, Mo.) or the B1-B4 recombinant domains of protein L (35) that was tested to exclude lipopolysaccharide contamination. Each mouse received five intraperitoneal injections of protein (1 mg in phosphate-buffered saline [PBS]) every other day. Mice were sacrificed 21 days after the first injection, and spleens, lymph nodes, BM, and peritoneal cells were collected.

Immunofluorescence analysis. Single-cell suspensions were isolated, and erythrocytes were lysed with Tris-buffered ammonium chloride, when necessary. Leukocytes (0.5×10^6 cells) were stained at 4°C by using predetermined optimal concentrations of different combinations of fluorochrome-labeled antibodies for 30 min. After washes, when necessary, biotin-labeled antibodies were revealed by using Streptavidin-CyChrome (BD Pharmingen, San Diego, Calif.).

Cell phenotype was determined by using the following reagents: anti-mouse CD5-phycoerythrin (PE; 53-7.3), anti-mouse CD45R/B220-fluorescein isothiocyanate (FITC), anti-mouse CD45R/B220-CyChrome (RA3-6B2), anti-mouse CD4-PE (H129.19), anti-mouse CD43-FITC (S7), anti-mouse CD21/CD35-FITC (7G6), anti-mouse CD23-PE (B3B4), anti-human IgM (HuIgM)-FITC, anti-HuIgM-PE (G20-127), anti-HuIgD-FITC (IA6-2), anti-HuIg κ -FITC (G20-193), biotinylated anti-HuIg λ (JDC-12), or Streptavidin-CyChrome. All monoclonal antibodies and their corresponding isotype controls were purchased from BD Pharmingen. Anti-mouse CD8-FITC (KT15) was obtained from Immunotech (Marseille, France).

After being washed and fixed in PBS-1% formaldehyde, the cells were analyzed by using a single laser FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). A minimum of 10,000 events was collected per sample, and data were analyzed with CellQUEST (version 3.1; Becton Dickinson).

Enzyme-linked assays. An enzyme-linked immunosorbent assay (ELISA) was used to quantify IgM(κ), IgM(λ), and IgM reactive with protein L in mouse plasma. Briefly, microtiter plates (MaxiSorp; Nunc-ImmunoPlate) were coated overnight with goat antibodies (1 μ g/ml in borate-buffered saline [pH 8.4]) to human κ chain (K3502; Sigma-Aldrich) or to μ chain (I0759; Sigma-Aldrich). After a blocking step with 1% PBS-bovine serum albumin, plasma samples diluted in 1% PBS-BSA were incubated 2 h at 37°C with the sensitized wells. Bound antibodies were detected either by incubation with biotinylated protein L (60 ng/ml), followed by the addition of horseradish peroxidase-labeled streptavidin, or directly with horseradish peroxidase-labeled goat anti-human κ and λ antibodies (A7164 and A5175, respectively; Sigma-Aldrich). The human monoclonal antibody (MAb) M3 (IgM, V_H3, and V _{κ} 1), provided by J. P. Bouvet (60), and a human serum pool were used as calibration standards.

Single-cell PCR analysis. B220⁺ fixed splenocytes from HEL- and protein L-treated mice were bulk sorted by using a Becton Dickinson FACS Vantage SE DiVa (Santa Cruz, Calif.), yielding 21,082 cells with 95% purity from the HEL-treated mice and 11,487 cells with 93% purity from the protein L-treated mice (8). B220⁺ cells were diluted in lysis buffer and aliquots containing 1 cell were dispensed into wells of a PCR plate (Robbins Scientific, Sunnyvale, Calif.).

Genomic DNA from cell lysates was amplified by using random 15-mers and 60 cycles of nested PCR as previously described (9, 14, 15). Sequences were analyzed on an automated capillary sequencer (ABI Prism 3100 Genetic Analyzer; Applied Biosystems, Foster City, Calif.). Germ line sequences were determined by using the JoinSolver sequence analysis program for the V_H amplifications and DNAPLOT (<http://www.mrc-cpe.cam.ac.uk/DNAPLOT>) for the V_L segments. A rearrangement was considered productive if the VDJ and V_LJ_L junction maintained the reading frame into the J_H or J_L segment. Rearrangements that were out of frame or introduced a stop codon during the rearrangement at a junction were considered nonproductive.

Statistical analysis. All experimental results were tested with analysis of variance (Fisher PLSD test) and Mann-Whitney nonparametric U test. The results were considered significant at a *P* of <0.05, to be highly significant at a *P* of <0.01, and to lack significance at a *P* of >0.05. The results of tests for significance are provided in the figure legends.

RESULTS

To investigate the *in vivo* consequences of confronting the human antibody repertoire with protein L, we made use of mice that were engineered to express human Igs. The transgenic mice produce B lymphocytes that secrete fully human antibodies (44). Their surface HuIgM⁺ (sHuIgM⁺) splenic B cells bind protein L in a way that is comparable to human peripheral blood B cells. Transgenic naive mice were injected intraperitoneally with 1 mg of recombinant protein L (or HEL as a control protein) on days 1, 3, 5, 7, and 9 to determine the effect on B-cell development and function and on other cells of the immune system.

Time course reduction of protein L-positive HuIgMs. The mice were bled periodically, and the concentration of HuIgM in the plasma of each mouse was determined. Throughout the observation period, there was a progressive increase of the mean HuIgM concentrations in plasma in both the HEL- and the protein L-treated groups (Fig. 1A). Since protein L targets κ -chain-positive Igs, we determined the concentrations of HuIgM reactive with protein L in the plasma of treated mice. Beginning at day 5, protein L⁺ HuIgM concentrations were depressed in the protein L-treated group and, after 3 weeks, there was a 68% decline compared to the concentrations at day 0 (*P* < 0.005) (Fig. 1B). This dramatic decrease was specific since in the HEL-treated mice protein L⁺ HuIgM concentrations exhibited minimal changes. Thus, repeated injections of soluble protein L to the transgenic mice led to a decrease in protein L⁺ IgM levels that was not observed in mice treated with a control protein.

Reduction of peripheral IgM(κ)⁺ B cells by protein L treatment. To determine whether protein L treatment affected B-lymphocyte development, we examined the numbers of B cells (defined as B220⁺) in the peripheral blood of the mice over time. Cells were stained with fluorescently labeled MAbs and analyzed by immunofluorescence-activated flow cytometry (FACS). There was a progressive increase of HuIgM-expressing lymphocytes (B220⁺ IgM⁺ cells) in both HEL- and protein L-treated mice, rising from 29.8% \pm 6% and 29.9% \pm 8% at day 0 to 67.9% \pm 5% (*P* < 0.0005) and 59.5 \times 10% (*P* < 0.05) at day 21, respectively. We then quantitated protein L-specific lymphocytes (B220⁺ protein L⁺ cells) in the peripheral blood

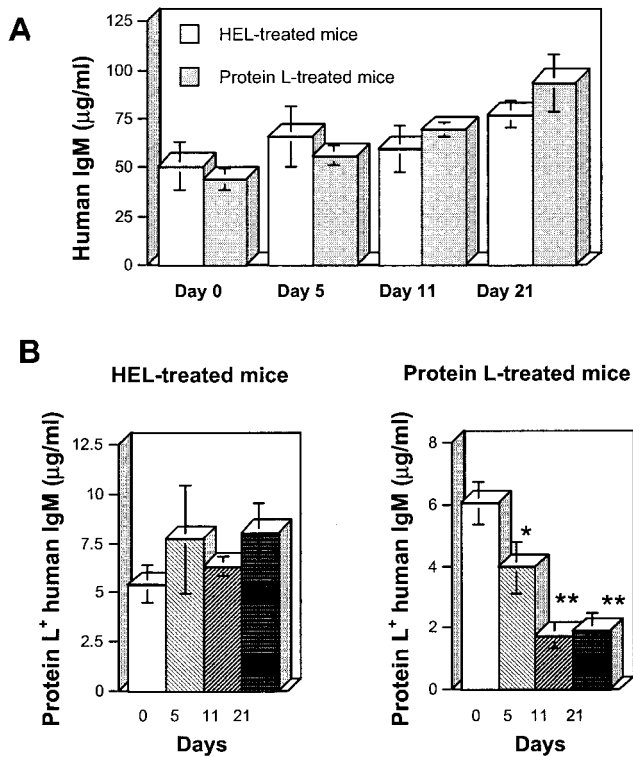


FIG. 1. Kinetics of HuIgM concentrations in the plasma of the HEL- and protein L-treated transgenic mice on days 1, 3, 5, 7, and 9. HuIgM concentrations were measured in the plasma of individual mice. (A) Total human IgM concentrations show no significant variations between the two groups. (B) Protein L⁺ HuIgM concentrations exhibit a dramatic specific decrease in the protein L-injected mice but not in the HEL-treated mice. *, *P* < 0.05; **, *P* < 0.005.

of treated mice. In mice receiving HEL, protein L⁺ B cells showed a slight increase, reaching 25.1% ± 2% at day 21 compared to day 0 (22.2% ± 2.3%). In contrast, protein L-treated mice exhibited a slight decrease of protein L⁺ B cells

at day 21 (19.2% ± 2%) compared to day 0 (21.9% ± 1.5%). Thus, injection of protein L had only a modest effect on peripheral B lymphocytes that express protein L⁺ IgM on their cell surfaces compared to HEL-treated mice at day 21.

Protein L targets immature B cells in the BM. To determine the developmental stage at which protein L acts in vivo, BM tissues, where early stages of B-lymphocyte development occur, were isolated, and cell populations were analyzed by FACS by triple labeling. Analysis of B220⁺ cells in the BM indicated no changes in the pro-B-cell (B220^{low} CD43⁺ IgM⁻) or pre-B-cell (B220^{low} CD43⁻ IgM⁻) compartments. Thus, the treatment does not affect the generation or differentiation of BM lymphoid progenitor cells that represent the majority of the B cells in the BM and that give rise to fully committed B lymphocytes. In contrast, immature B cells (B220⁺ IgM⁺ IgD⁻) were increased. The average percentages of immature B cells were expanded from 5.7% ± 1% to 16.7% ± 2% in the protein L-treated group (*P* < 0.005) but not in HEL-treated mice. In sharp contrast to this 66% elevation of the immature population, the percentages of cells that have progressed to the recirculating B-cell stage (B220⁺ IgM⁺ IgD⁺) in the BM were similar in the two groups.

Protein L treatment results in a specific loss of mature B-cell populations but has little effect on other cell lineages. To characterize the effect of protein L on other compartments of the immune system, the spleen and lymph nodes were isolated from the mice on day 21. The numbers of cells comprising splenic peripheral lymphoid compartments were determined in each mouse. Cell populations from each tissue were then stained with fluorescently labeled MAbs and analyzed by FACS in order to identify specific cell lineages in each of these organs. In the spleen, there was a decrease in the total numbers of viable cells in protein L-treated mice compared to HEL-treated and control mice. With protein L injections, the total splenocyte numbers were 1.7-fold lower than the splenocyte numbers in control untreated mice (Fig. 2A). More importantly, protein L had a profound effect on the representation of

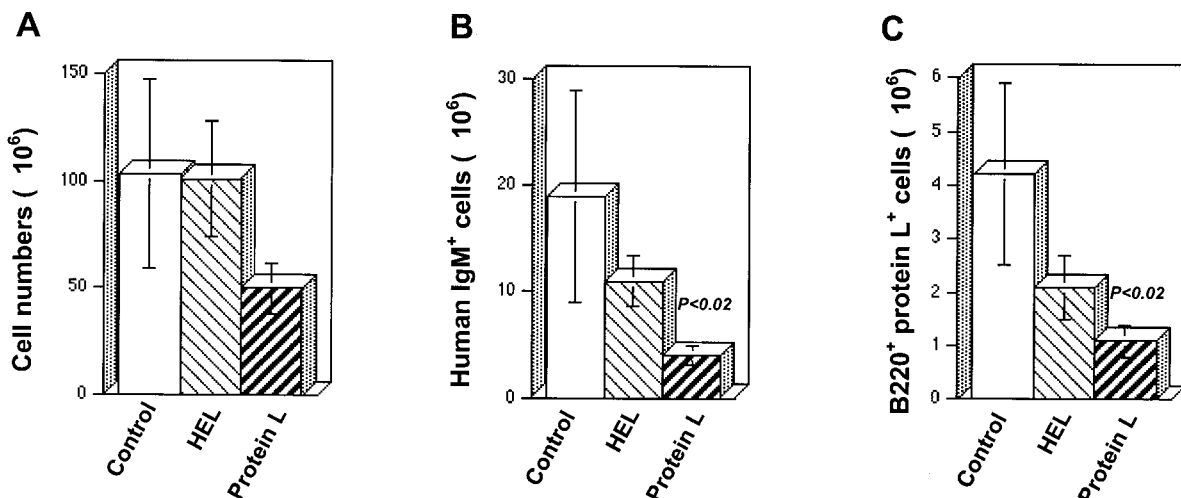


FIG. 2. Specific and marked reduction in the number of protein L-reactive cells in the spleens of transgenic humanized mice. Splenic cells of control (untreated) and HEL- and protein L-treated transgenic mice were depleted of erythrocytes by using Tris-buffered ammonium chloride and stained with cell surface markers. (A) Total number of splenic cells counted by using a Malassez cell; (B) HuIgM⁺ B220⁺ cells as detected by PE-labeled anti-HuIgM and CyChrome-labeled anti-mouse B220 MAbs; (C) B220⁺ protein L⁺ cells as detected by FITC-labeled anti-mouse B220 MAb and biotinylated protein L, followed by treatment with CyChrome-labeled streptavidin.

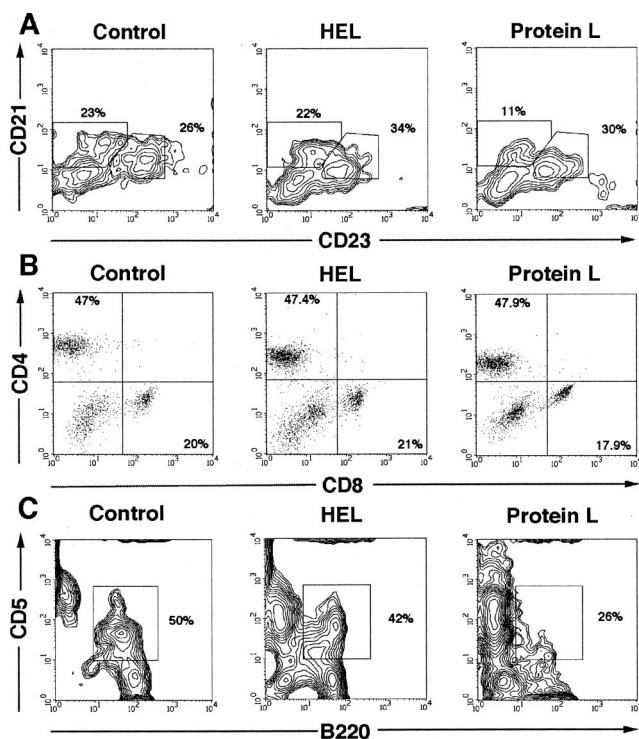


FIG. 3. Protein L targets B-cell subpopulations involved in innate immunity. Lymphoid cell subpopulations were identified by immunofluorescence staining in control (untreated) and in HEL- and protein L-treated transgenic mice. (A) Splenocytes were triple stained with CyChrome-labeled anti-mouse B220 MAb, FITC-labeled anti-mouse CD21 MAb, and PE-labeled anti-mouse CD23 MAb. Lymphoid cells were gated on the B220-expressing cells. MZ ($B220^+ CD23^{-low} CD21^+$) but not FO ($B220^+ CD23^+ CD21^+$) B cells are reduced in protein L-treated mice. (B) Lymph node cells were stained with PE-labeled anti-mouse CD4 and FITC-labeled anti-mouse CD8 MAb. The treatments do not affect T lymphocytes. (C) Peritoneal B1 cells are reduced in protein L-treated mice.

HuIgM⁺ B cells in this secondary organ, decreasing them from $18.9 \times 10^6 \pm 10 \times 10^6$ (control mice) to $4.3 \times 10^6 \pm 0.9 \times 10^6$ cells ($P < 0.02$, Fig. 2B). This marked reduction was specific for protein L-positive B cells. In the protein L-treated group, their mean numbers dropped from $4.2 \times 10^6 \pm 1.7 \times 10^6$ cells (control mice) to $1.1 \times 10^6 \pm 0.3 \times 10^6$ cells ($P < 0.02$, Fig. 2C). Remarkably, despite the substantial loss of mature B cells, protein L treatment had no effect on the percentages or total numbers of mature T cells defined by CD4/CD8 surface expression in the spleen and lymph nodes (Fig. 3B). Similarly, the CD5⁻ B220⁻ cell population was not affected significantly by the treatment.

Protein L treatment results in negative selection of B cells utilizing V_κ L chains. To determine whether protein L exposure affects the repertoire of expressed V genes, B220⁺ splenocytes from HEL- or protein L-treated mice were sorted and assessed for V_κ or V_λ genes from genomic DNA of individual B cells. This approach makes it possible to analyze both nonproductive and productive Ig rearrangements (8). Analysis of the nonproductive repertoire was informative to determine the frequency with which specific transgenic elements were rearranged because the nonproductive rearrangements do not en-

code an Ig molecule that can influence whether the B-cell is positively or negatively selected. Therefore, by comparing the nonproductive and productive repertoires, the impact of positive and negative selection of functionally rearranged B cells could be determined. A total of 82 L-chain PCR products from HEL-treated mice and 111 products from protein L-treated mice were analyzed. From either set of mice, there was a nearly equal proportion of nonproductive and productive rearrangements. From the HEL-treated mice, there were 47.6% nonproductive rearrangements and 52.4% productive rearrangements. Similarly, from the protein L-treated mice, 51.4% of the rearrangements were nonproductive and 48.7% were productive. The ratio of V_κ and V_λ gene utilization in the nonproductive repertoire was also similar in the two sets of mice. In the HEL-treated mice 48.7% of the nonproductive rearrangements utilized V_κ genes and 51.3% utilized V_λ genes, resulting in a nonproductive κ/λ ratio of nearly 1:1. In protein L-treated mice, 49.1% of the nonproductive repertoire utilized V_κ gene segments and 50.9% utilized V_λ gene segments resulting in a κ/λ ratio of nearly 1:1.

There were no significant differences between the κ/λ ratio in the nonproductive repertoires of the two sets of mice. Interestingly, the observed nonproductive κ/λ ratios were lower than expected considering the 5:1 ratio of 82 human V_κ genes and 16 human V_λ genes in the transgene loci of the mice (44).

In contrast to the nonproductive repertoire, there were marked differences in the V_κ and V_λ gene usage in the productive repertoires of the HEL- and protein L-treated mice. In the productive repertoire of HEL-treated mice, 44.2% of the L-chain sequences utilized V_κ gene rearrangements and 55.8% utilized V_λ genes, resulting in a productive κ/λ ratio of 5:6. Alternatively, in protein L-treated mice, only 33.3% of the productive rearrangements utilized V_κ gene segments, whereas 66.7% of the rearrangements utilized V_λ gene segments, resulting in a κ/λ ratio of 1:2.

Protein L binds the V_L region of Igs encoded by V_κ1, V_κ3, and V_κ4 subgroups but not V_κ5 or V_λ genes. To determine whether there was V_κ family specific deletion of B cells related to protein L administration, we analyzed in detail the utilization of V_κ genes in the nonproductive and productive repertoires of HEL- and protein L-treated mice (Table 1). Among the five V_κ genes in the translocus, one gene (V_κ1D-13) was not detected in the HEL- or protein L-treated mice. Another gene, V_κ3D-11, was detected only in the productive repertoire (10.5%) of HEL-treated mice and was not detected in the protein L-treated group. V_κ4-1 was the most frequently utilized gene in both the HEL- and protein L-treated mice. In the nonproductive repertoire of HEL-treated mice, it was utilized in 73.7% V_κ rearrangements and 64.3% of the V_κ rearrangements from the nonproductive repertoire of protein L-treated mice. In the productive repertoires, V_κ4-1 was utilized in 57.9% of the V_κ rearrangements of HEL-treated mice and 50% of the V_κ rearrangements from protein L-treated mice.

V_κ1D-12 was the second most utilized gene in HEL- and protein L-treated mice. V_κ1D-12 utilization in the HEL-treated mice was similar in the nonproductive and productive repertoires; 21.5% and 26.3%, respectively. By comparison, and consistent with the ability of protein L to bind the V_κ1 subgroup, in the protein L-treated mice, 28.6% of the nonproductive V_κ rearrangements utilized V_κ1D-12, whereas only

TABLE 1. Distribution of V_{κ} and V_{λ} genes in the nonproductive and productive repertoires from B220⁺ splenocytes obtained from HEL- or protein L-treated mice

Gene	% Distribution (<i>n</i>) ^a			
	Nonproductive rearrangements		Productive rearrangements	
	HEL	Protein L	HEL	Protein L
V_{κ}				
1D-12	21.5 (19)	28.6 (28)	26.3 (19)	16.7* (18)
3D-11	0 (19)	0 (28)	10.5 (19)	0* (18)
4-1	73.7 (19)	64.3 (28)	57.9 (19)	50* (18)
5-2	5.3 (19)	7.1 (28)	5.3 (19)	33.3* (18)
V_{λ}				
2	70 (20)	62 (29)	41.7 (24)	44.4 (36)
3	10 (20)	20.7 (29)	54.2 (24)	55.6 (36)
4	20 (20)	17.2 (29)	4.2 (24)	0 (36)

^a *, $P < 0.01$. P values were determined within each type of rearrangement by the χ^2 test.

16.7% of the productive V_{κ} rearrangements used V_{κ} 1D-12, suggesting that V_{κ} 1D-12 was deleted from the expressed repertoire.

Unlike V_{κ} 1, V_{κ} 3, and V_{κ} 4, the products of V_{κ} 5 gene do not bind protein L. Although the utilization of V_{κ} 5-2 is infrequent in the HEL-treated mice, V_{κ} 5-2 was utilized at the same frequency in the nonproductive and productive repertoires of HEL-treated mice (5.3%). In contrast, in protein L-treated mice 7.1% of the nonproductive and 33.3% of the productive V_{κ} rearrangements utilized V_{κ} 5-2. The increased utilization of V_{κ} 5-2 in the productive repertoire of protein L-treated mice could reflect positive selection. However, in view of its lack of reactivity with protein L, it more likely reflects a compensatory response to the deletion of V_{κ} 1D-12 and V_{κ} 4-1 genes, that could be exaggerated because of the limited number of human V_{κ} genes expressed in the transgenic mice. In marked contrast to V_{κ} gene expression, there was no positive or negative selection of V_{λ} gene rearrangements in relation to HEL or protein L treatment (Table 1). These results suggest that the cells that survive protein L treatment express a skewed L-chain repertoire consistent with the capacity of this SAg to bind specific V_{κ} -chain families.

Protein L specifically impairs marginal zone B-cell development in the spleen. We examined the numbers of splenic B-cell populations (defined as B220⁺) to determine the populations that were affected by the treatment with protein L versus the numbers of B cells affected by HEL. We used antibodies to CD21, CD23, HuIgD, and HuIgM to identify newly formed (NF), transitional 1 (T1), transitional 2 (T2), mature (M), marginal zone (MZ), and follicular (FO) B cells. Although the number of T1 (B220⁺ IgM⁺ CD21⁻), T2 (B220⁺ IgM⁺ CD21⁺), and M (B220⁺ IgM^{low} CD21^{low}) B cells did not vary significantly, there was a decrease in NF B-cell numbers (B220⁺ CD23⁻ CD21⁻) in the protein L-treated group ($P < 0.05$). There was also a clear block in development of MZ B cells (B220⁺ CD23^{-/low} CD21⁺) after exposure of mice to protein L, as manifested by a 70% decrease ($P < 0.01$). Despite these changes in MZ B cells, there was no apparent change in the mean numbers of FO B lymphocytes (B220⁺ CD23⁺ CD21⁺) in protein L-injected mice (Table 2 and Fig. 3A).

Thus, there is a clear loss of NF B cells in the spleen of mice exposed to protein L, whereas transitional cells remain unaffected. There is also a distinct block in the development of MZ B cells.

Protein L reduces the number of B-1 cells. The recirculating B-cell population is essentially composed of CD5⁻ B cells (called B-2 cells). In addition, a subpopulation of CD5⁺ B cells (termed B-1) has a unique tissue distribution and function. Most B-1 cells are located in the body cavities and make a dominant contribution to natural antibody production and T-cell-independent immune responses to exogenous antigens (27, 69). We therefore probed the B-1-cell subpopulation (B220⁺ CD5⁺) in the spleen and the peritoneal exudates. There were no apparent changes in B-1 cells in the spleen. However, there was a reduction of B-1 cells in the peritoneal cavity, dropping from 59.7% \pm 5% to 35.7% \pm 8% in mice that received protein L ($P < 0.05$). For comparison, B-1 cells averaged 56.8% \pm 7% in HEL-injected mice (Fig. 3C). Given the compelling evidence that B-1 and MZ subpopulations are involved in first-line immune defense against foreign invaders (38), these findings are in line with the reduction of MZ B cells in the spleen.

DISCUSSION

Work in recent years has revealed a novel mode of binding of some microbial proteins, termed SAGs, to the variable regions of Igs, independently of the paratope. Whereas the interactions have been substantially characterized at the immunochemical and structural levels, their consequences on the immune system in vivo have remained unclear. Using transgenic mice that express human Igs, we investigated the in vivo effects of protein L, a bacterial protein with well characterized in vitro SAg properties for soluble human Ig κ (23, 45) and for Ig κ -positive B lymphocytes (2). The experiments demonstrate that the introduction of soluble protein L to naive mice leads to a disruption in B-cell development without substantially affecting other cell populations, such as T-lineage cells or myeloid cell populations in the spleen, lymph node, and peritoneal cavity. Given the biological properties of protein L in vitro on human B cells, we can therefore conclude that protein L acts essentially on the B-cell lineage in vivo.

TABLE 2. Effect of protein L treatment on B-cell populations in the spleens of transgenic mice

B-cell sub-population	Mean cell number (10^6) \pm SE ^a		
	Control untreated mice	HEL-treated mice	Protein L-treated mice
NF	4.70 \pm 2.9	0.98 \pm 0.3	0.86 \pm 0.3*
T1	3.29 \pm 2.2	3.99 \pm 1.3	2.34 \pm 0.9
T2	5.08 \pm 2.2	3.36 \pm 1	1.98 \pm 0.4
M	1.80 \pm 0.7	2.23 \pm 0.4	2.12 \pm 0.5
MZ	1.87 \pm 0.4	1.17 \pm 0.2	0.54 \pm 0.1***
FO	1.74 \pm 0.3	2.62 \pm 0.4	1.66 \pm 0.4

^a Cell numbers are shown. B-cell subpopulations were identified by the expression of cell surface markers as follows: T1, B220⁺ IgM⁺ CD21⁻; T2, B220⁺ IgM⁺ CD21⁺; M, B220⁺ IgM^{low} CD21^{low}; MZ, B220⁺ CD23^{-/low} CD21⁺; FO, B220⁺ CD23⁺ CD21⁺; and NF, B220⁺ CD23⁻ CD21⁻. *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$. P values were determined by using the Mann-Whitney U test.

Impact of protein L on immature B cells. During B-lymphocyte development, ordered rearrangements of Ig gene segments are linked with cellular expansion and differentiation events (55). Initially, pro-B cells ($B220^+ CD43^+$) undergo rearrangement of Ig H-chain D to J_H gene segments, followed by V_H to DJH rearrangement. The expressed μ H-chain proteins then complex with the surrogate L-chain proteins (V_{preB} and $\lambda 5$) to form the receptor expressed on pre-B cells ($B220^+ CD43^-$). At this stage most Ig L-chain assembly occurs and the L chains produced will pair with the preexisting H chains, leading to BcR expression and differentiation of pre-B cells to immature B lymphocytes. After BcR expression, rearrangement, and negative selection of autoreactive B cells in the BM, a fraction of B cells migrates to secondary lymphoid organs. In protein L-treated mice, characterization of early B-cell development revealed a specific expansion at the immature B-cell stage in the BM. Alterations in B-cell development in this organ were restricted to the immature B-cell population and B-cell precursor populations (pro-B and pre-B cells) were unaffected by protein L administration. This stage-specific activity of protein L reflects the requirement of κ -chain expression on B cells, which marks the transition from the pre-B to the immature B-cell stage. The presence of normal B-cell precursor populations in the BM of protein L-treated mice suggests that B cells will repopulate the periphery after protein L is cleared from the animal.

NF B cells leaving the BM have a short half-life and remain largely confined to the blood circulation and defined areas of the spleen, since they lack adhesion molecules necessary for extravasation in peripheral lymphoid organs, such as lymph nodes. Only a fraction of these NF B cells eventually enter the pool of long-lived, recirculating B cells populating lymphoid organs, which are referred to as B-2 B cells. In the spleen, NF cells ($B220^+ IgM^{high}$) acquire more mature phenotypes with downregulation of IgM and upregulation of differentiation molecules, including CD21, CD23, and IgD, and transitional T1 cells ($CD21^{low} IgM^{high}$) give rise to transitional T2 cells ($CD21^{high} IgM^{high}$), which are the direct precursors of mature B cells. In marked contrast to the immature B-cell expansion in the BM, analysis of NF B cells in the spleen showed that protein L treatment resulted in a substantial loss of this immature B-cell population in the spleen. This observation has implications with regard to the mechanisms of B-cell selection. Although there is ample evidence that negative selection is an active process in the BM, it is unclear whether negative selection of immature B cells occurs in the peripheral compartment of conventional mice. The loss of NF B cells we noted supports the view that, in addition to receptor editing (42, 53), BcR-mediated affinity interactions result in apoptosis in immature peripheral B cells, probably representing a developmental checkpoint during B-cell maturation in the periphery.

The finding that protein L treatment results in a clear expansion of immature B cells in the BM and a loss of NF B cells in the periphery deserves consideration. First, it is possible that different antigen concentrations are reached in the BM and the periphery. Previous transgenic models demonstrated that deletion and anergy of autoreactive B cells are involved in B-cell tolerance, and different concentrations of antigen, or different forms, i.e., soluble versus membrane-bound, were invoked (42, 53). The concentration of protein L in our system can be

estimated not to be lower than that of membrane-bound antigens, suggesting that the concentration may be high enough to cause deletion in the periphery. However, it is difficult to assess the precise, effective protein L concentrations in the BM because its tissue distribution is unknown. Second, another aspect of antigen expression that may trigger different cell fates is the timing of contact between protein L and B cells. It may be that the duration of protein-L-B-cell encounters is longer in the periphery than in the BM. Third, the role of the microenvironment is likely to play a role (55). Since B-cell commitment, proliferation, differentiation, and survival are regulated by a finely tuned balance between cell-autonomous mechanisms (pre-BcR and BcR) and signals provided by the microenvironment (IL-7, stroma cells, competitor cells, and soluble signals), the B-cell response will be determined in large part by the developmental stage of the B-cell and its location within the immune system, as illustrated here by the *in vivo* effects of protein L.

Impact of protein L on V_{κ} -expressing B cells and circulating Igs. In addition to the expansion of the immature B-cell population in the BM, we noted that protein L induces a marked deficit in circulating protein L⁺ IgM. In the spleen, protein L-treatment deleted a portion of B cells utilizing $V_{\kappa}1D-12$ and $V_{\kappa}4$ genes, and the greatest impact of deletion was attributed to $V_{\kappa}1D-12$ rearrangements. The effect of protein L on V_{κ} gene expression was probably underestimated because single-cell V_{κ} gene analysis was performed on total splenic B-cell populations. We can postulate several mechanisms by which protein L perturbs humoral immunity. One possibility is that protein L acts on B-cell subpopulations that impart adaptive immunity. It may, for example, inhibit factors required for the maintenance of germinal centers or completely block germinal center formation. Protein L could also act directly by inhibiting the differentiation of B cells into Ig-producing plasma cells. Similarly, protein L may act by inhibiting the survival of mature B cells in the periphery, reducing the pool of cells that can develop into Ig-secreting cells. However, the characteristics of the deficit in circulating Igs suggest that protein L more likely targets B-cell subpopulations important in innate immunity. First, we observed a rapid (5 days after injection of soluble protein L) impact on Ig levels. Second, the deficit was specific for circulating κ -positive IgM, which are the targets of protein L. Third, as will be discussed below, we found no effect of protein L on FO B cells, which are involved in adaptive immunity.

Protein L affects innate B-cell immunity. The long-lived pool of B cells is composed of mature B-2 cells that are derived from NF B cells and that recirculate among the follicles, where they are termed FO B cells. Also derived from NF B cells that mature through the transitional stage in the spleen are MZ B cells that migrate to a more static cell compartment (reviewed in references 5 and 40). MZ B cells represent a distinct subset of lymphocytes. They are located in the periphery of the splenic periarteriolar lymphoid sheath at the border of white and red pulp and can be distinguished from FO recirculating B cells by the characteristic expression of cell surface markers. They have a preactivated phenotype, express high levels of B7-1 and B7-2 and, upon stimulation *in vitro*, rapidly differentiate into plasma cells (48). Remarkably, the route of antigen administration and the BcR specificity deter-

mine the relative contributions of FO versus MZ B-cell subpopulations. Because of their anatomical location, MZ B cells are the first cell population to encounter blood-borne antigens and are thought to play a critical role in host defense against pathogens (41). The properties of the MZ B-cell subset are reminiscent of those of B-1 cells, which are enriched in the peritoneal cavity and derived from fetal or adult precursor populations generated early in development (28, 67). B-1 cells have the capacity of self-renewal and are responsible for secreting most of the serum preimmune IgM, with multireactive binding properties and potentially protective properties (36). This B-cell subset has been shown to be deleted by injection of the SAg *S. aureus* protein A that targets V_H3^+ Igs (59).

Whether innate B-cell immunity is also affected in humans infected with protein L-expressing strains of *P. magnus* remains to be demonstrated. There are, however, observations suggesting that this could be the case. *P. magnus* is a member of the indigenous flora of the skin, the oral cavity, and the gastrointestinal and genitourinary tracts. Only a minority (~10%) of the *P. magnus* strains express protein L, and these isolates are more frequently connected with clinical infections, indicating that protein L is a virulence determinant (34). It is also noteworthy that significant amounts of protein L are found in the growth medium of protein L-expressing strains (34), suggesting that protein L also in vivo is released from the bacterial surface. Such a mechanism would allow the targeting of B-1 and MZ B cells during infection.

Analyzing various cell populations in the organs isolated from protein L-treated mice, we found no effect on mature recirculating FO B cells that have evolved to generate a huge repertoire able to mount T-cell-dependent B-cell responses with high-affinity and long-term memory (54). In contrast, we found that protein L alters the development of both MZ B cells in the spleen and B-1 B cells in the peritoneal cavity, two B-cell subsets that have evolved to provide a first line of defense against antigens acquired through the gut/peritoneum and the bloodstream. Both B-1 and MZ B cells exhibit a high antigen-presenting capacity (48, 62), preferentially secrete complement-fixing, potentially protective natural IgM (26, 46, 69). These cells proliferate rapidly and vigorously for more prolonged time periods to anti- μ and LPS stimulation than FO B cells (47). We therefore conclude that protein L acts predominantly on B-cell subpopulations with innate immune functions.

Targeting innate B cells, a novel escape mechanism used by some pathogens. The mechanisms underlying the specific suppression of the B-cell subpopulations by protein L remain to be elucidated. A reduction in the B-1 and MZ B-cell compartments could be caused by altered development, accelerated death, or defective retention at the appropriate microenvironmental site. It is known that different cellular responses are triggered at different signaling thresholds (64) and that balanced and focused BcR signaling seems to determine the fate of both B-1- and MZ B-cell populations. Mice lacking molecules important in the BcR signalosome have altered expression of these B-cell subsets. First, CD5, expressed on B-1 cells, is physically associated with sIgM in the BcR complex and may be a negative regulator of BcR signaling by acting via a pseudo-immunoreceptor tyrosine-based activation motif (17). Second, mice lacking key signaling effectors, such as CD19 and CD45,

show an absence or severe reduction of B-1 cells (5). Third, MZ B cells are markedly reduced in the absence of CD19 (37, 39), and they also fail to develop in the absence of Pyk-2 tyrosine kinase (25) and in mice lacking the transcription factors NF κ Bp50 (10) and Ailolos (66). Fourth, the highly motile behavior of MZ B cells is altered in Pyk-2^{-/-}, Lsc^{-/-}, Doc2^{-/-}, and CD22^{-/-} mice, leading to abnormal MZ development (16, 19, 25, 56). Fifth, enhanced BcR signaling facilitates FO B-cell activation and survival but impairs MZ B-cell generation (11). It is therefore tempting to suggest that protein L somehow delivers signals through the BcR that impact preferentially B-1 and MZ B cells. However, although a functional BcR is required for B-cell development, a variety of other receptors and signals from the microenvironment also seem essential. In addition to BcR-mediated signaling, the selecting stimuli probably depend on environmental factors, including stroma cells, competitor cells and soluble signals. For example, the TACI longevity factor influences the survival of B-1 and MZ populations (24). Therefore, we cannot formally exclude the possibility that, through indirect effects, protein L affects B-1 and MZ B-cell development.

It is notable that, compared to protein L that appears to affect innate B-cell populations specifically, another B-cell superantigen, *S. aureus* protein A, targets V_H3^+ mature B lymphocytes in all compartments, including B-1, MZ, and FO cells (21). Whether these disparities reflect differences in signals delivered by B-cell SAgS that bind V_H gene products (protein A) or V_κ gene products (protein L) or differences in the capacity of human versus murine surface Ig molecules to provide signals to B cells in vivo or other features of the two B-cell SAgS remains to be determined.

Finally, as discussed above, B-cell subsets are distributed in a predetermined fashion, probably to cope selectively with different kinds of exogenous antigens. The ability to elicit adaptive and innate B-cell responses to different environmental stimuli probably requires both ligand-dependent and ligand-independent functions, providing a mechanism for enhanced flexibility in responding to exogenous insults. The ability of the microbial protein we have studied to target B cells responsible for innate immunity reveals a novel escape mechanism that may be used by some infectious agents to evade the host's immune system.

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