Antigen-specific antibody responses in B-1a and their relationship to natural immunity

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B-1a cells are primarily thought of as natural antibody-producing cells. However, we now show that appropriate antigenic stimulation induces IgM and IgG B-1a antibody responses and long-lived T-independent antigen-specific B-1a memory that differs markedly from canonical B-2 humoral immunity. Thus, we show here that in the absence of inflammation, priming with glycolipid (FtL) from *Francisella tularensis* live vaccine strain induces splenic FtL-specific B-1a to mount dominant IgM and activation-induced cytidine deaminase-dependent IgG anti-FtL responses that occur within 3–5 d of FtL priming and fade within 1 wk to natural antibody levels that persist indefinitely in the absence of secondary FtL immunization. Equally surprising, FtL priming elicits long-term FtL-specific B-1a memory cells (IgM>>IgG) that migrate rapidly to the peritoneal cavity and persist there indefinitely, ready to respond to appropriately administrated secondary antigenic stimulation. Unlike B-2 responses, primary FtL-specific B-1a responses and establishment of persistent FtL-specific B-1a memory occur readily in the absence of adjuvants, IL-7, T cells, or germinal center support. However, in another marked departure from the mechanisms controlling B-2 memory responses, rechallenge with FtL in an inflammatory context is required to induce B-1a secondary antibody responses. These findings introduce previously unexplored vaccination strategies for pathogens that target the B-1a repertoire.

B-1 | memory B cells | vaccine

B-1a lymphocytes mainly develop de novo during fetal/neonatal life and are maintained thereafter by self-replenishment (1–3). Although they and their plasma cell progeny are well known as natural antibody-producing cells, they are not commonly viewed as participating antigen-stimulated antibody responses. However, in this and a companion article (see ref. 4), we demonstrate that immunization with a glycolipid (FtL) isolated from *Francisella tularensis* live-vaccine strain (*Ft* LVS) readily induces splenic FtL-specific B-1a to produce T-independent antigen-specific IgM and IgG (IgM>>IgG) primary antibody responses, to develop long-term antigen-specific memory, and to produce secondary antibody responses when appropriately rechallenged with the antigen. Strikingly, although the B-1a memory responses that we identify share many of the properties of B-2 memory responses, they nonetheless differ in key ways that make the B-1a responses more suitable for the functional niche they occupy.

B-1 lymphocytes represent 1–5% of total B cells in adult mice. They are the principal B cells in peritoneal (PerC) and pleural cavities, are present at low but detectable frequencies in spleen and intestine, and are very rare in bone marrow (BM) and lymph nodes (1, 3). B-1a, which express low levels of CD5, predominate among PerC B-1, but B-1b, which do not express CD5, are present at much lower frequencies in the PerC (2, 3). Functionally, B-1a are well known to produce natural antibodies (2, 5–8) and to up-regulate the antibody production in response to Toll-like receptor (TLR) stimulation (9–11). Consistent with this function, our recent studies show that stimulation with *Salmonella typhimurium* LPS, a TLR4 agonist, nonspecifically induces PerC B-1a to migrate to spleen, where they join with resident splenic B-1a to augment polyclonal antibody production (10). In addition, intranasal influenza infection has been shown to induce B-1a migration, in this case to respiratory tract lymphoid organs where, without undergoing clonal expansion, the migrants produce IgM that includes virus-neutralizing natural IgM antibodies (12).

These findings fuel the prevailing view that B-1a do not mount antigen-induced antibody responses (13). However, B-1a are clearly known to produce specific antibody responses to certain antigens, including phosphorylcholine (14–16) and α1,3 dextran (17, 18). Most recently, foreshadowing studies presented here, we have shown that immunization with FtL, an atypical LPS isolated from *Ft* LVS, induces B-1a with FtL-binding IgM receptors to appear in spleen and to produce anti-FtL IgM primary antibody responses that protect against lethal *Ft* LVS challenge (19, 20). Consistent with B-1a mediating this protection, FtL priming does not similarly protect Bruton's tyrosine kinase (Btk)-mutant (*xid*) mice, which have B-1b but lack B-1a (21–23).

In contrast, B-1b generate protective antibody responses and provide long-lasting immunity against *Borrelia hermsii* infection such that transferring sorted PerC B-1b from *B. hermsii* infected mice intravenously to $Rag1^{-/-}$ mice confers long-term protection (24). Confirming that B-1b rather than B-1a mediate this protection, *B. hermsii* immunization also protects the Btk-mutant *xid* mice mentioned above, which lack B-1a (25). Thus, B-1a and B-1b have distinct repertoires and response properties.

Studies here and in a companion article (4) together show that the B-1a–mediated protection that FtL priming induces against lethal *Ft* LVS challenge (19) is accompanied by induction of anti-FtL B-1a primary responses and, importantly, by induction of anti-FtL B-1a memory cells that persist indefinitely in PerC (but not elsewhere) and remain ready to respond to FtL rechallenge under appropriate conditions. Activation-induced cytidine deaminase (AID)-dependent isotype switching occurs during development of a proportion of these FtL-specfic B-1a memory cells. However, unlike B-2 memory, B-1a memory cells develop in the absence of T-cell or germinal center (GC) influence. Furthermore, the induction of antigen-specific B-1a memory cells is inhibited when the antigen is initially encountered in an inflammatory context but their production of secondary antibody responses requires rechallenge with priming antigen presented in just such a context (TLR4 stimulation). Collectively, these findings open a view on previously unsuspected immune memory mechanisms and thereby introduce previously unexplored vaccination

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The authors declare no conflict of interest.

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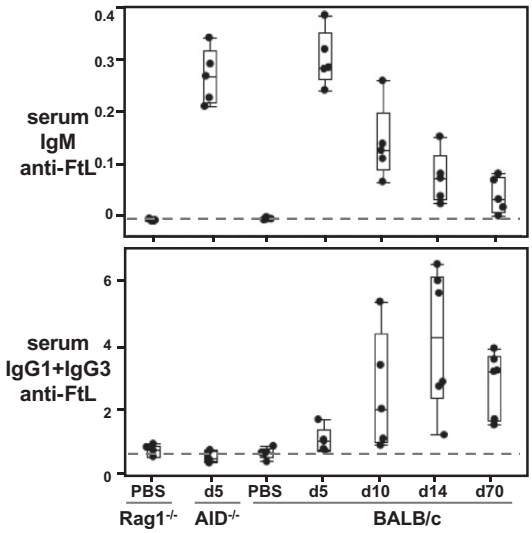

Fig. 1. FtL priming establishes persistent long-term production of serum IgM and IgG anti-FtL at natural antibody levels. IgM and IgG anti-FtL levels measured in sera from WT (BALB/c) and syngenic mutant (Rag1$^{-/-}$ and AID$^{-/-}$) mice primed with FtL or injected with PBS, as indicated. Each dot represents a single mouse; n = 5 per group. Horizontal lines in "quartile box plots" indicate the 25th, median, and 75th percentiles. The dashed line shows background levels (Rag$^{-/-}$ and AID$^{-/-}$ sera). Values are expressed as microliter equivalents of a standard serum pool from 5-d FtL primed BALB/c mice (Fig. S1 shows FACS data).

strategies likely to be suitable for immunization with pathogen-associated antigens that target B-1a repertoire.

Results

FtL Immunization Induces Antigen-Specific Isotype Switching and IgG Plasma Cell Development in Splenic B-1a. In addition to inducing IgM anti-FtL production (19), FtL priming induces anti-FtL (FtL-binding Igκ$^+$) B-1a in the spleen to undergo IgG isotype switching and to differentiate to plasma cells producing either IgG1 or IgG3 anti-FtL. Class-switched anti-FtL B-1a (including plasma cells) account for roughly 20% of total anti-FtL B-1a in spleen by day 5 after FtL priming (Fig. S1*A*); they express either IgG1 or IgG3 receptor (Fig. S1*A*) but not IgG2a, IgG2b, IgE, or IgA. Interestingly, IgG1 is expressed more in BALB/c mice than IgG3, but IgG3 predominates in C57BL/6 mice.

The class-switched plasma cells in spleens from FtL-primed animals are readily visualized as IgM$^-$IgD$^-$ anti-FtL cells that express a typical plasma cell phenotype (CD19$^+$CD138$^+$CD23$^-$CD5$^-$). These cells arise slightly later (day 4, rather than day 3) than IgM$^+$ anti-FtL plasma cells, and increase until day 5. Then, like their IgM counterparts, their numbers fall rapidly to below FACS-detectability by day 7 (Fig. S1*B*).

Consistent with the plasma-cell development kinetics in spleen, anti-FtL antibodies are not present in the natural antibody pool before FtL immunization. Instead, after being induced and reaching peak within 1–2 wk after FtL immunization, anti-FtL antibodies ultimately fall and merge into the natural antibody pool in FtL-primed mice. Thus, serum IgM and IgG anti-FtL antibodies are barely detectable before FtL immunization, rise quickly in FtL-immunized animals, and peak at days 5–7 for IgM and days 10–14 for IgG (Fig. 1). Notably, long after primary response resolves, both IgM and IgG anti-FtL persist indefinitely (>70 d) at levels slightly above background (Fig. 1).

As the persistent low-level serum anti-FtL production would predict, ELISPOT assays of spleen cells from long-term FtL-immunized animals reveal small numbers of plasma cells producing anti-FtL IgM (~2,000 per spleen) or IgG (IgG1+IgG3) (~300 per spleen) (Table S1). Of note, comparable analysis of BM from the same animals do not reveal either IgM or IgG anti-FtL plasma cells (Table S1), even though long-lived plasma cells originating from GC responses typically reside in BM (26, 27). Therefore, these anti-FtL antibody-secreting cells in spleen provide the sole source for the sustained low-level production of anti-FtL antibodies that we detect in serum from FtL-immunized animals.

Antigen-Induced Isotype Switching in B-1a Requires AID; T Cells and GCs Are Not Required. Primary IgM anti-FtL responses are comparable in WT, T-cell–deficient (TCRβ$^{-/-}$δ$^{-/-}$), and AID$^{-/-}$ mice (Fig. S2). As expected, IgG anti-FtL response fails in AID$^{-/-}$ mice (Fig. 2*A*). Consistently, AID expression is induced in anti-FtL B-1a cells (Fig. 2*B*). These findings demonstrate that FtL-induced isotype switching in B-1a requires AID. However, neither T-cell help

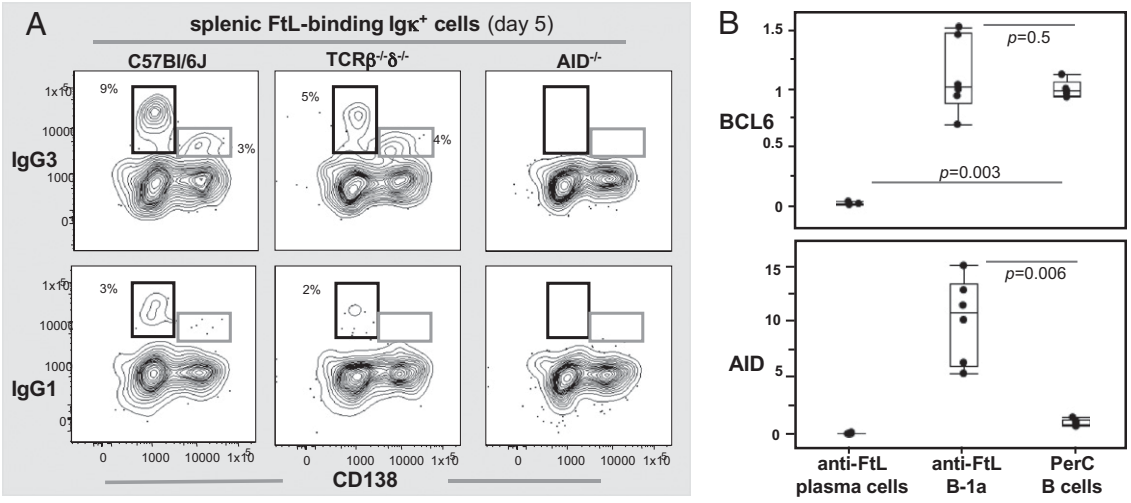

Fig. 2. FtL-induced isotype switching in anti-FtL B-1a requires AID but occurs without T-cell help or GC support. (*A*) Live FtL-binding Igκ$^+$ cells from spleen of C57BL6/J, TCRβ$^{-/-}$δ$^{-/-}$, and AID$^{-/-}$ syngenic mice 5 d after FtL immunization were gated to reveal CD138 and IgG expression. Boxes show IgG$^+$ CD138$^-$ and IgG$^+$CD138$^+$ plasma cells. One of four experiments is shown. (*B*) AID and BCL6 expression in sorted anti-FtL B-1a (CD138$^-$) and anti-FtL plasma cells (CD138$^+$) from spleen of day 5 FtL-primed C57BL6/J mice. Data for each gene is shown as fold-change relative to expression level (dashed line) in PerC B cells from nonimmunized mice. Each dot represents data for 100 sorted cells of each subset, n = 6. One of three experiments is shown.

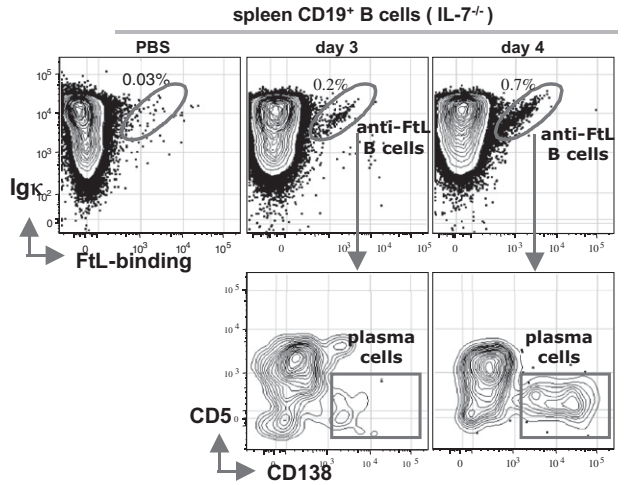

Fig. 3. FtL immunization induces splenic anti-FtL B-1a responses in IL-7$^{-/-}$ mice. Live splenic CD19$^+$ B cells from IL-7$^{-/-}$ mice injected with PBS or immunized with FtL for 3–4 d were gated to reveal FtL-binding Igκ$^+$ B cells ("diagonal" in circled population, *Upper*), and further gated to distinguish anti-FtL plasma cells (CD138$^+$CD5$^-$) (boxed population, *Lower*) from CD138$^-$ cells, >95% of which are CD5$^+$. (For serum responses, see Fig. S3*C*).

nor GC formation is required for these responses. Thus, anti-FtL IgG responses proceed normally in TCRβ$^{-/-}$δ$^{-/-}$ mice (Fig. 2*A*). GL7, a GC marker (27, 28), is not detected on the anti-FtL B-1a (CD138$^-$). Furthermore, anti-FtL B-1a disappear from the spleen within 1 wk of immunization (19), clearly before the time when GC usually starts developing (27–30). Finally, B-cell lymphoma 6 (BCL6) up-regulation, a hallmark for GC B cells (26, 31, 32), is not detected on anti-FtL B-1a (Fig. 2*B*). Consistent with its down-regulation on plasma cells (33), BCL6 expression is turned off in anti-FtL plasma cells (CD138$^+$) (Fig. 2*B*).

Anti-FtL Primary Responses Require Btk but Not IL-7. Although B-2 development fails entirely in IL-7$^{-/-}$ mice, substantial numbers of B-1a develop and are readily detectable in adult spleen and PerC (34, 35). Similar to WT mice, immunizing IL-7$^{-/-}$ mice with FtL induces the development of splenic anti-FtL B-1a (CD5$^+$B220^{low}) and anti-FtL plasma cells (CD5$^-$B220$^-$CD138$^+$) (Fig. 3 and Fig. S3*A*). Isotype switching also occurs normally (Fig. S3*B*) and serum anti-FtL IgM levels rise after FtL priming (Fig. S3*C*). However, the overall anti-FtL titers are about four-times lower than that in WT mice (Fig. S3*C*), which likely reflects the smaller spleens and the consequently lower numbers of anti-FtL B-1a in IL-7$^{-/-}$ mice (Table S2). These data suggest that IL-7 is not (or minimally) required either for the development of anti-FtL B-1a or for their responsiveness to FtL immunization.

As expected, B-1a anti-FtL responses fail entirely in *xid* mice, which lack functional Btk and show lack of B-1a (21, 22). Thus, FtL immunization of *xid* mice does not result in expanded anti-FtL B-1a (<0.01% of splenic and PerC B cells) and does not induce

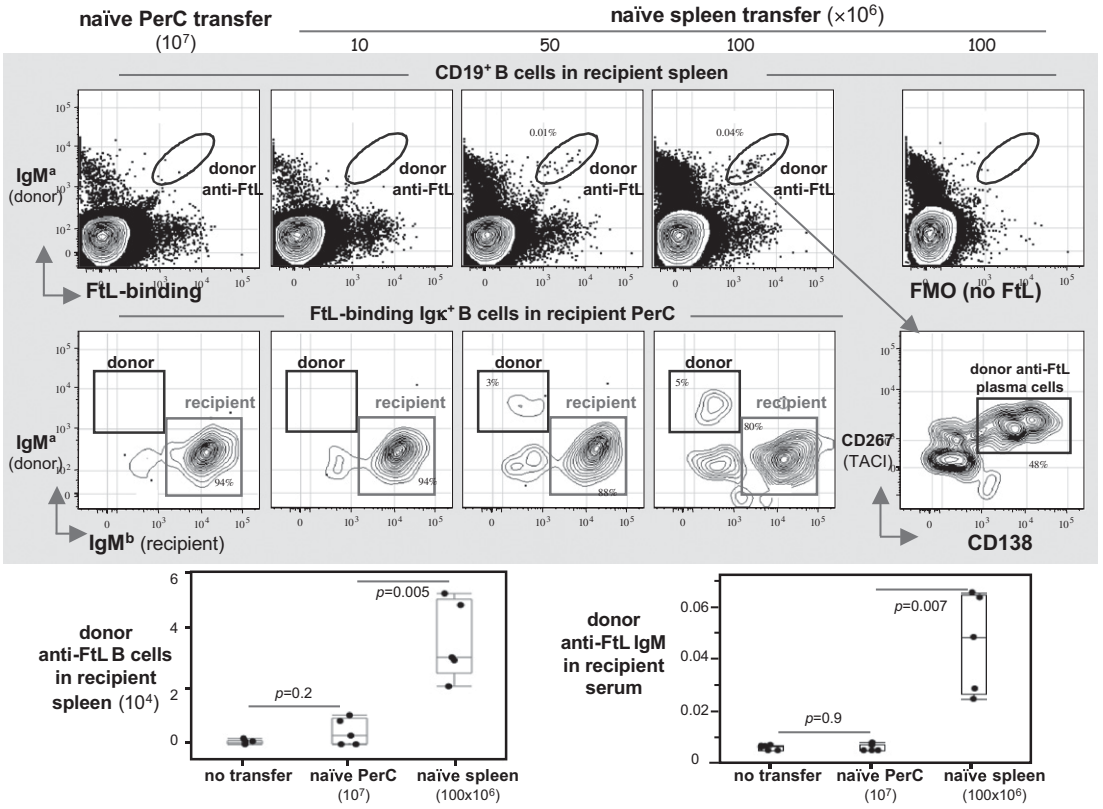

Fig. 4. Naïve spleen, but not naïve PerC, produce anti-FtL primary responses to FtL challenge when transferred intravenously to naïve recipients. (*Top*) 10^7 PerC (from one to two naïve mice) or indicated number of spleen cells from naïve *Igh*^{a} mice were transferred intravenously to naïve congenic *Igh*^{b} recipients immunized with FtL the next day. Donor and recipient anti-FtL responses were measured 5 d later. (*Middle*) Live B cells from indicated recipient spleen were gated to identify donor anti-FtL B-1a (IgM^{a+}) (circled population, *Top*), which are then further gated to show donor anti-FtL plasma cells (CD267^{hi}CD138$^+$, boxed population, *Rightmost Middle*). CD267, transmembrane activator, and calcium signal-modulating cyclophilin ligand interactor (TACI) is a marker induced during plasma cell differentiation. The boundary for FtL binding is determined from the fluorescence-minus-one (FMO) (40) staining control in which fluorochrome-labeled FtL was omitted from the stain-set (*Rightmost Top*). Live PerC anti-FtL B-1a were gated to distinguish donor or recipient-derived cells (boxed populations, *Middle*). (*Bottom*) Numbers of donor (IgM^a) anti-FtL B-1a in recipient spleen (*Left*); donor-derived (IgM^a) anti-FtL in sera of recipients (*Right*). Each point represents data from an individual mouse (n = 5, per group). Values are expressed as microliter equivalents of a standard serum pool from 5-d FtL-primed Igha mice.

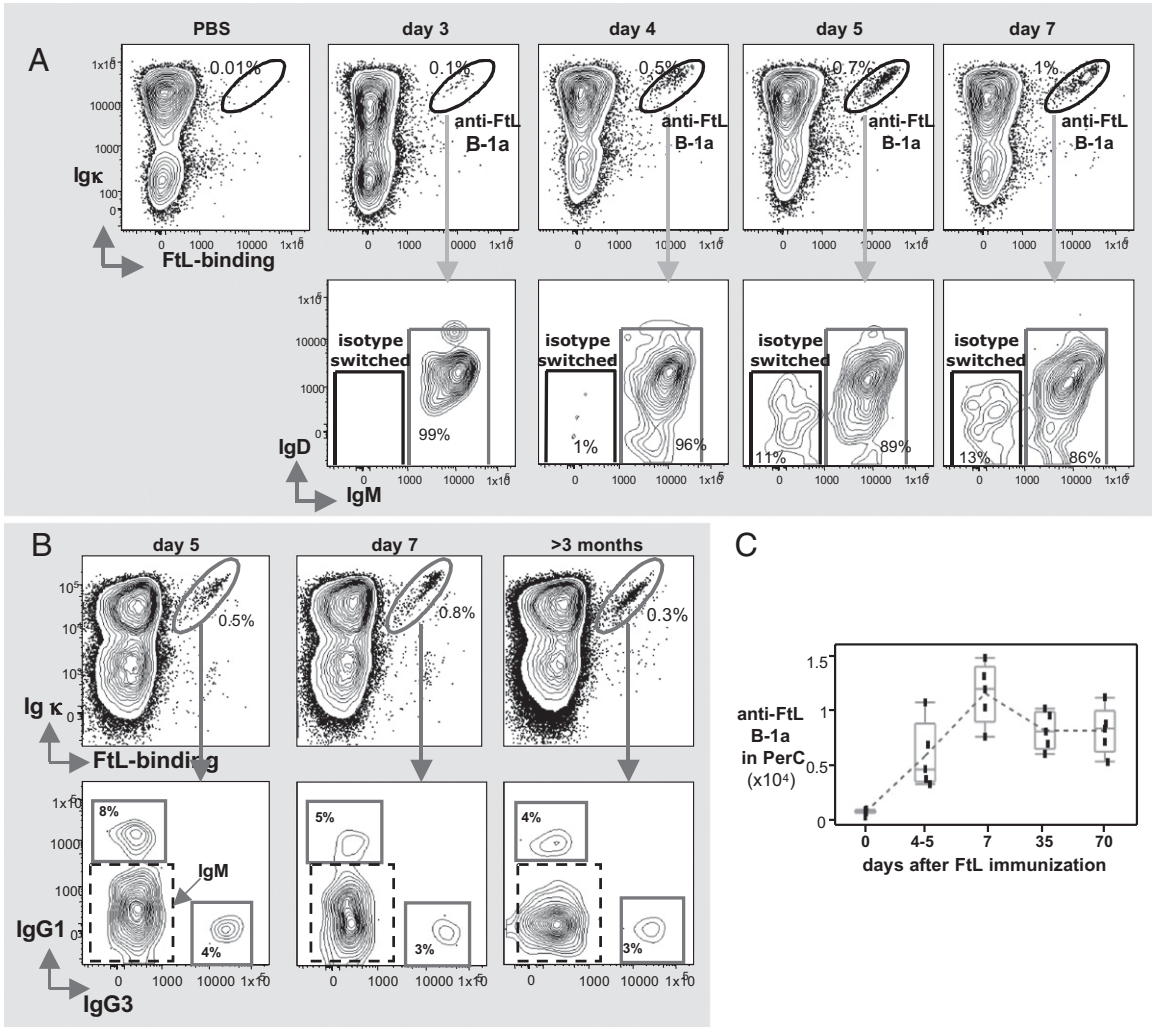

Fig. 5. FtL priming induces the development of anti-FtL B-1a (IgM>>IgG) populations that persist long-term in the PerC. (*A*) Anti-FtL B-1a (tight diagonal, *Upper*) in gated live BALB/c PerC B at indicated days after FtL immunization. IgM or isotype-switched (IgM^-) anti-FtL B-1a are shown in boxes (*Lower*). (*B*) Anti-FtL B-1a (circled population, *Upper*) gated from PerC B cells from FtL-immunized BALB/c for indicated days were further gated to reveal anti-FtL subsets expressing IgG1 or IgG3 (gray line boxed populations, *Lower*). The remaining cells ($IgG1^-IgG3^-$, dashed line boxed population) express IgM anti-FtL. (*C*) Numbers of PerC anti-FtL B-1a from BALB/c mice at indicated days after FtL immunization. Each point represents data from an individual mouse (n = 5, per group). Mean value for each group is connected by dashed line.

production of serum anti-FtL. This response failure is consistent with our previous demonstration that FtL immunization of *xid* mice does not protect against subsequent lethal *Ft* LVS challenge (19).

Spleen, Not PerC, from Naïve Animals Contains Cells That Give Rise to Adoptive Primary Anti-FtL B-1a Responses. Intravenous transfers of spleen cells from naïve donors to naïve allotype-congenic recipients results in the appearance of donor anti-FtL B-1a in recipient spleen (and PerC) shortly after the recipients are challenged with FtL (Fig. 4). However, similar transfers of 10^7 PerC cells harvested from one to two naïve donors give rise to minimal numbers of donor anti-FtL B-1a (Fig. 4). Consistent with these findings, donor anti-FtL antibody titers rise significantly in the serum of FtL-challenged recipients transferred with naïve donor spleen but not naïve donor PerC (Fig. 4). Thus, although we have shown that FtL priming triggers the appearance of anti-FtL B-1a in the PerC and spleen at approximately the same time in animals (day 3) (19), only the spleen from naïve animals contains cells that can give rise to primary anti-FtL responses in adoptive recipients.

PerC, Not Spleen, Is the Long-Term Reservoir for Anti-FtL B-1a in FtL-Primed Animals. Like anti-FtL B-1a in spleen, all of the anti-FtL B-1a in PerC express IgM at day 3 after FtL priming (Fig. 5*A*); by day 5, roughly 12% of anti-FtL B-1a in PerC have switched to IgG1 or IgG3 (Fig. 5 *A* and *B*). After 1 wk, however, the majority of anti-FtL B-1a disappear from the spleen (19), leaving the PerC as the long-term reservoir of these cells in primed animals (Fig. 5). Of note, although most of anti-FtL B-1a in the PerC have divided at least once (>90% $BrdU^+$) at day 5, their cell division rate drops precipitously (i.e., about 20% are $BrdU^+$ at day 7 but <5% are $BrdU^+$ at day 15) (Fig. S4). Thus, after the initial FtL-induced expansion, anti-FtL B-1a cells exit the cell cycle and persist in the PerC as quiescent cells in the absence of further antigenic stimulation.

Overall, the frequency of anti-FtL B-1a that appear in the PerC rises from undetectable (<0.01%) before FtL priming to a persistent 3–5% of total PerC B cells (Fig. 5 *B* and *C*). These cells express the typical PerC B-1a phenotype ($CD5^+CD43^+CD11b^+B220^{low}$) (Fig. S5) and dominantly express IgM (Fig. 5*B*). Similar findings with FtL-primed IL-$7^{-/-}$ and TCR$\beta^{-/-}\delta^{-/-}$ mice (Fig. S6) demonstrate that neither IL-7 nor T-cell support is

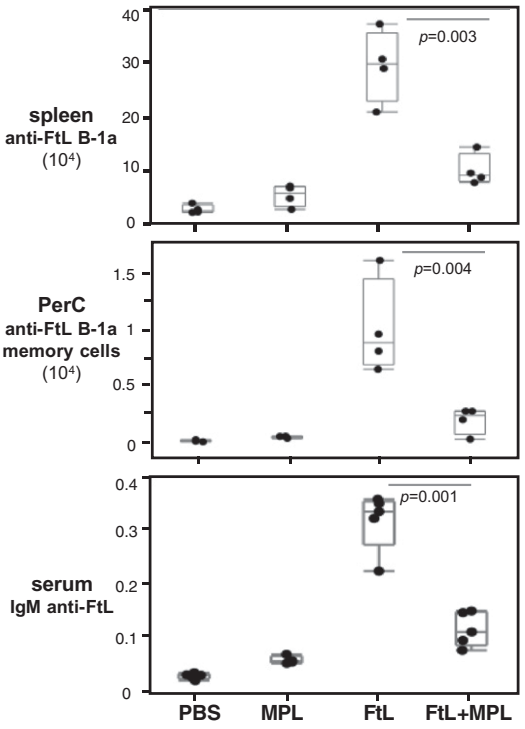

Fig. 6. MPL administered simultaneously with FtL priming dampens anti-FtL primary responses and inhibits development of FtL-specific B-1a memory in PerC. Absolute number of anti-FtL B-1a in spleen (*Top*) or in the PerC (*Middle*), and serum IgM anti-FtL antibodies levels (*Bottom*) in C57BL6/J mice at d 5 after PBS, MPL, FtL, or FtL plus MPL injection. Each dot represents data from an individual mouse (n = 4–5, per group). Values are expressed as microliter equivalents of a standard serum pool from 5-d FtL primed C57BL6/J mice.

required for the development or persistence of anti-FtL B-1a cells in PerC.

Importantly, despite their initial dividing and later quiescent persistence in PerC, anti-FtL B-1a cells do not differentiate to plasma cells at this location. Thus, ELISPOT does not reveal anti-FtL secreting cells in PerC from primed animals, even though such assays do reveal tiny numbers of anti-FtL plasma cells in spleen long after the primary response resolves (Table S1). Furthermore, PerC from FtL-immunized animals does not contain FACS-detectable anti-FtL plasma cells, defined as either $CD138^+$ cells or cells containing intracellular Ig (Fig. S7).

Priming with FtL in an Inflammatory Context Decreases, Rather than Augments, the Primary Anti-FtL Response. Monophosphoryl lipid A (MPL) is well-known as a TLR4 agonist that readily induces inflammation (36, 37). It is commonly delivered with priming antigens as an adjuvant to enable/increase primary antibody responses (37, 39). Surprisingly, however, priming naïve animals with FtL plus MPL substantially dampens the anti-FtL primary responses and dramatically decreases the development of anti-FtL B-1a cells in the PerC (an index of memory cell generation; see companion article, ref. 4) (Fig. 6 and Fig. S8). Thus, adding this inflammation-inducing stimulus during FtL priming is counterproductive for anti-FtL primary responses.

Discussion

The FtL-induced robust primary responses demonstrated here introduce key roles for B-1a in protective immunity and the production of "natural" (i.e., innate) antibodies in serum. Our findings demonstrate that B-cell receptor stimulation with a glycolipid (FtL) isolated from *Ft* LVS specifically triggers splenic FtL-binding B-1a to proliferate and, in a small proportion (about 15%), to undergo AID-dependent class-switching. These antigen-triggered events, which occur in the absence of accompanying adjuvant, T-cell support, or GC influence, result in the establishment of anti-FtL B-1a memory cells that migrate rapidly to the PerC and persist there indefinitely as a largely quiescent population in the absence of further antigenic stimulation.

We have also shown that FtL priming induces brief but robust development of anti-FtL B-1a plasma cells (IgM>>IgG) in spleen, but not in BM. These plasma cells largely disappear with a week of priming, although a few remain detectable as functional anti-FtL–secreting cells for months thereafter (essentially indefinitely). Consistent with these findings, the initial primary anti-FtL B-1a response is readily detectable in serum for 1–2 wk after FtL priming but fades rapidly thereafter to characteristic natural antibody levels that persist indefinitely as such in serum.

The anti-FtL B-1a memory cells (IgM>>IgG) that migrate to the PerC persist there as essentially quiescent cells similar in phenotype to the bulk of the B-1a in PerC. They do, however, divide occasionally, apparently as needed to replenish their numbers and feed the tiny persistent population of splenic anti-FtL–secreting cells that in turn feeds the serum anti-FtL pool. Thus, by likely analogy, B-1a memory emerges as the source of much of what is commonly referred to as "natural antibody" in serum.

In an accompanying article (4), we show that the typically quiescent anti-FtL B-1a memory cells in the PerC divide extensively and express a unique set of activation-associated signatures in response to FtL rechallenge. However, the FtL rechallenge must be administered in an inflammatory context (e.g., stimulation with a TLR4 agonist) to induce the migration of the PerC-based memory cells to the spleen and differentiate there to anti-FtL secreting cells. Thus, our findings suggest that initial encounter with a pathogen-associated antigen (e.g., FtL) under noninflammatory conditions establishes long-term low-level B-1a production of natural antibodies sufficient to provide a preexisting defense against a low level of pathogen re-encounter. To back up this minimal defense, the initial antigen encounter also induces long-term FtL-specific B-1a memory capable of rapidly ramping up anti-FtL production whenever the antigen is re-encountered in association with TLR4-stimulated or similar inflammation.

Materials and Methods

The methods for FACS staining, ELISPOT, real-time quantitative RT-PCR, serum anti-FtL antibody analysis, and the methods used for the cell transfer studies are all described in a companion article (see companion article, ref. 4), in which the same materials and methods are used. All of the animal experiment protocols are approved by Stanford Animal Care Review Board.

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Supporting Information

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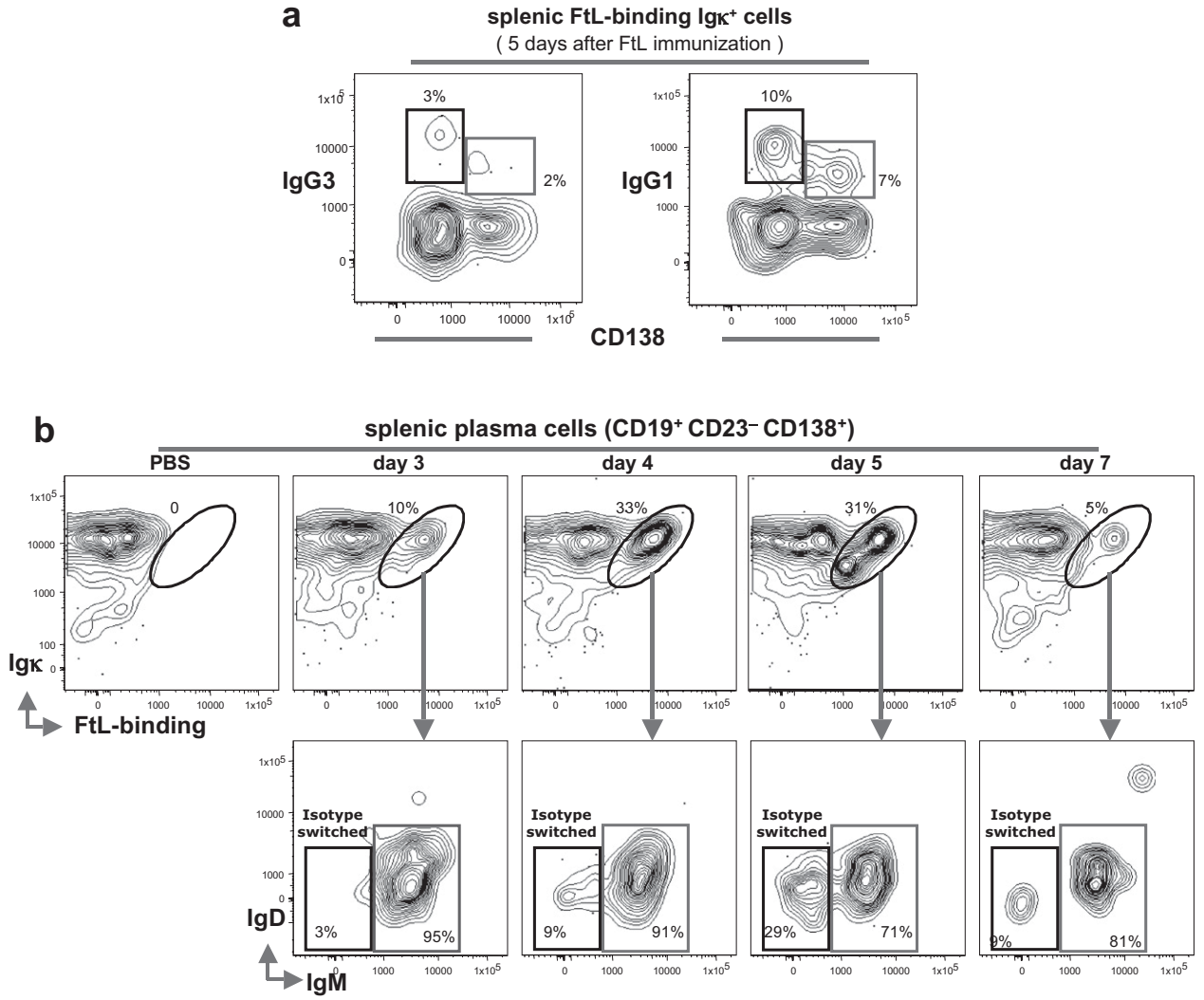

Fig. S1. Glycolipid (FtL) immunization induces splenic anti-FtL B-1a to undergo isotype switching and differentiation to IgG1 or IgG3 plasma cells. (*A*) Live FtL-binding Igκ$^+$ cells from spleen of BALB/c mice 5 d after FtL immunization gated to reveal IgG (IgG1 or IgG3) and CD138 expression. Gating strategy for splenic FtL-binding Igκ$^+$ population is shown in ref. 1. Black and gray boxes show IgG$^+$CD138$^-$ and IgG$^+$CD138$^+$ plasma cells, respectively. Data represent one of four experiments with similar results. (*B*) Live splenic plasma cells (CD19$^+$CD23$^-$CD138$^+$) from BALB/c mice injected with PBS or immunized with FtL for indicated days were gated to reveal anti-FtL plasma cells (circled population, *Upper*). The amount of FtL bound to the cells is proportional to the surface κ-light chain levels and hence is revealed as a tight "diagonal" population. Surface IgM and IgD levels on anti-FtL plasma cells distinguish class-switched plasma cells (IgM$^-$IgD$^-$) from nonswitched plasma cells (IgM$^+$) (*Lower*).

1. Cole LE, et al. (2009) Antigen-specific B-1a antibodies induced by *Francisella tularensis* LPS provide long-term protection against *F. tularensis* LVS challenge. *Proc Natl Acad Sci USA* 106: 4343–4348.

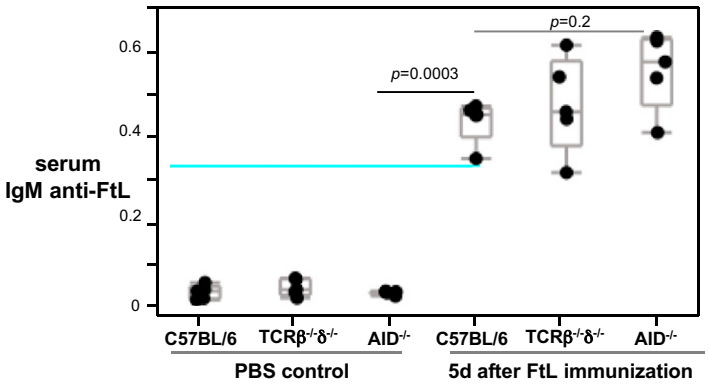

Fig. S2. FtL immunization of T-cell receptor (TCR)$\beta^{-/-}\delta^{-/-}$ or activation-induced cytidine deaminase (AID)$^{-/-}$ mice induces the primary IgM anti-FtL responses comparable to that of WT mice. Serum IgM anti-FtL level from C57BL6/J, TCR$\beta^{-/-}\delta^{-/-}$ and AID$^{-/-}$ syngenic mice injected with PBS or immunized with FtL for 5 d. Each dot represent data for individual mice (n = 5, per group). Values are expressed as microliter equivalents of a standard serum pool from 5-d FtL-primed C57BL6/J mice.

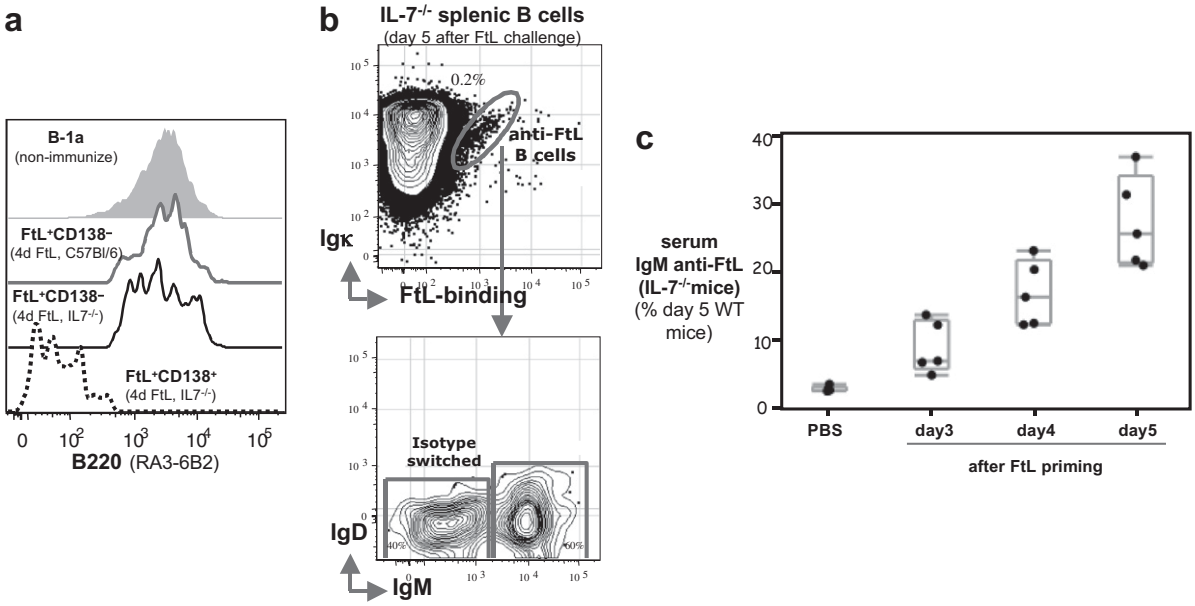

Fig. S3. FtL immunization induces splenic anti-FtL B-1a responses in IL-$7^{-/-}$ mice. (*A*) Histograms demonstrate that B220 (detected by clone RA3-6B2) expression by anti-FtL B-1a (FtL^{+}CD138^{-}) from C57BL6/J and syngenic IL$7^{-/-}$ mice is comparable to B220 expression by splenic B-1a from nonimmunized C57BL6/J. Anti-FtL plasma cells (FtL^{+}CD138^{+}) do not expressed B220. (*B*) Live splenic anti-FtL B-1a (circled population, *Upper*) in IL-$7^{-/-}$ mice at day 5 after FtL immunization were gated to distinguish cells expressing surface IgM (IgM^{+}IgD^{-}) and isotype-switched (IgM^{-}IgD^{-}) population (boxed populations, *Lower*). (*C*) Serum IgM anti-FtL level from IL-$7^{-/-}$ mice injected with PBS or at 3, 4, 5 d after FtL immunization. Each dot represents data from individual mouse (n = 5, per group). Data are the normalized values relative to the level from WT serum pool collected 5 d after FtL immunization.

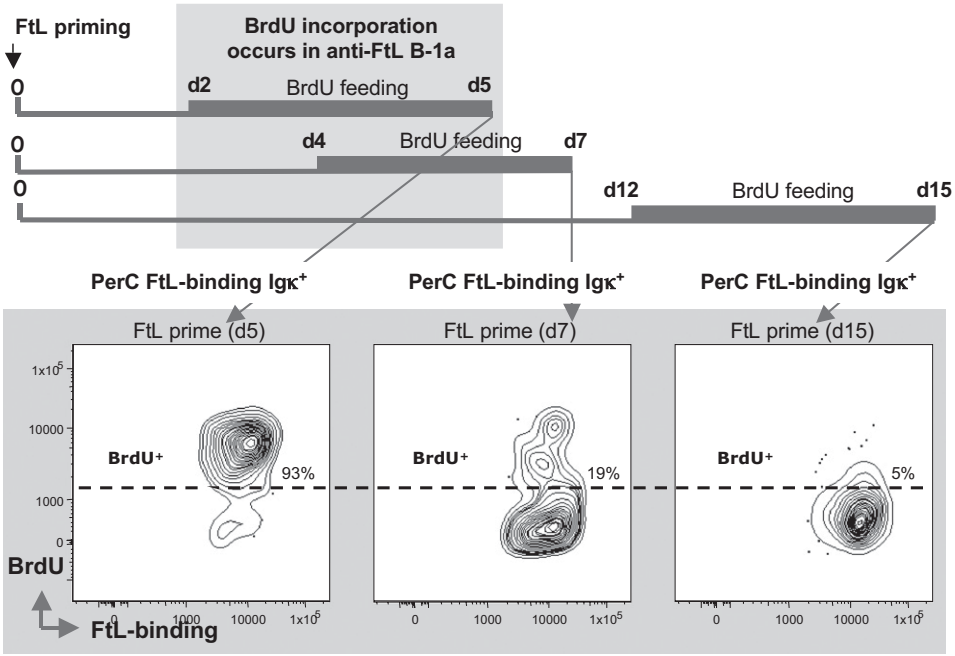

Fig. S4. Most anti-FtL B-1a cells divide in the peritoneal cavity (PerC) during the first week after FtL immunization but persist thereafter as largely quiescent cells. BALB/c mice were immunized with FtL on day 0 and then fed with BrdU-containing water for 3 d starting at the day indicated above the lateral gray bar. The gray box indicates the days after FtL immunization during which PerC anti-FtL B-1a proliferate (as indicated by BrdU incorporation). The FACS plots under the timing diagram show BrdU incorporation in gated PerC anti-FtL B-1a during the indicated feeding periods. Cells above the horizontal line incorporate BrdU (BrdU$^+$).

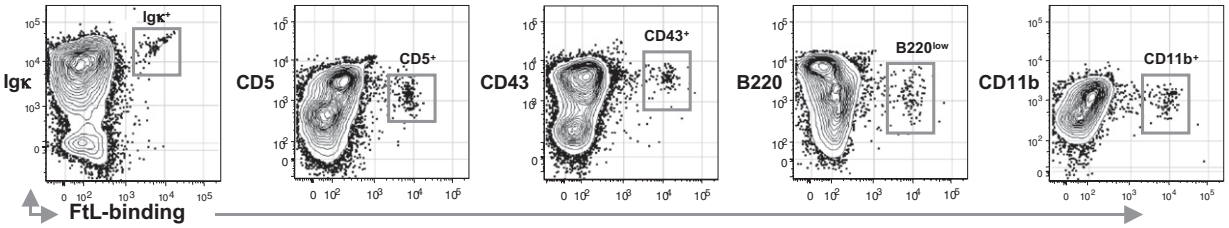

Fig. S5. PerC anti-FtL B cells express typical PerC B-1a phenotype. Live PerC B cells (CD19$^+$) from day 4 FtL-immunized C57BL6/J mice were gated to reveal FtL-binding B cells (population in square). The expressions of other surface markers (i.e., Igκ, CD5, CD43, B220, and CD11b are indicated in each plot). Data represent one of four experiments with similar results.

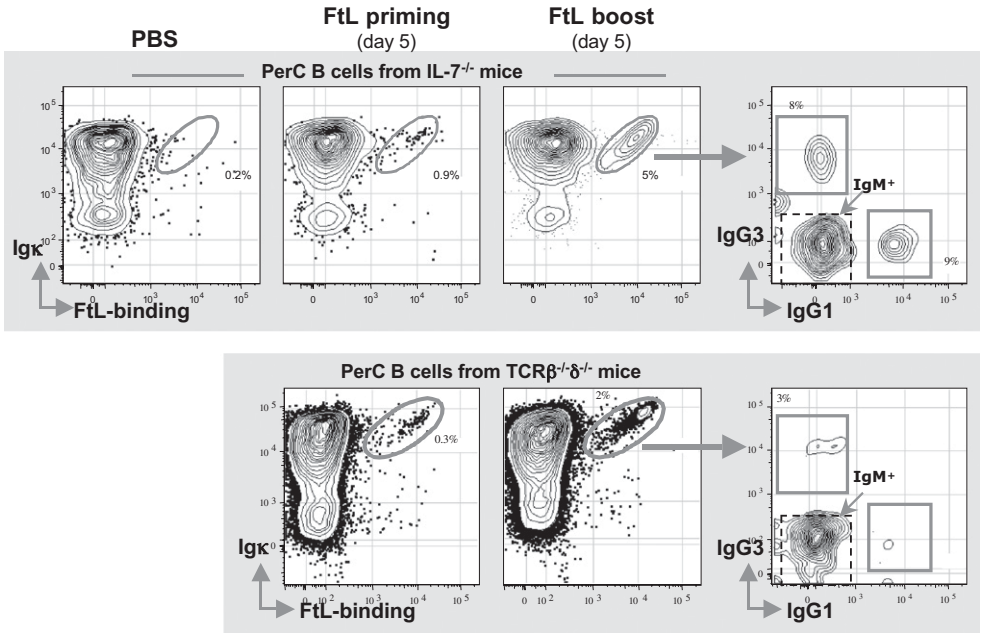

Fig. S6. FtL immunization of IL-7$^{-/-}$ and TCRβ$^{-/-}$δ$^{-/-}$ mice results, in both cases, in the development of PerC IgM and IgG anti-FtL B-1a memory cells, which increase in size in response to FtL rechallenge. Anti-FtL B-1a (circled populations) are identified from gated live PerC B cells from IL-7$^{-/-}$ (*Upper*) or T-cell–deficient (TCRβ$^{-/-}$δ$^{-/-}$) (*Lower*) mice. The FtL immunization history of each mouse is indicated. In both cases, anti-FtL B-1a memory cells are further gated to distinguish IgM, IgG1, or IgG3-expressing subsets (boxed populations, *Rightmost* FACS plots).

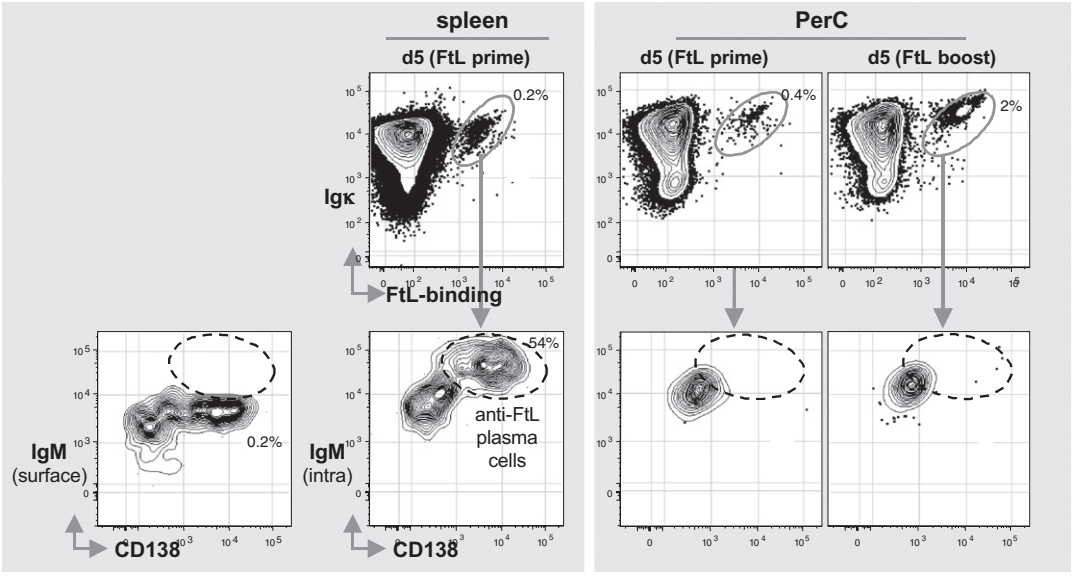

Fig. S7. Activated PerC anti-FtL B-1a either during the primary response or following FtL rechallenge do not express typical plasma cells phenotype. Live B cells (CD19^{+}) from spleen (*Left*) or PerC (*Right*) of BALB/c mice primed or boosted with FtL at indicated days were gated to reveal anti-FtL B-1a (gray line circled populations, *Upper*), which were further gated to show plasma cells expressing CD138 and high levels of intracellular IgM (dash-line circled populations, *Lower*). The FACS plot on the extreme left (*Lower*) show the surface IgM expression of anti-FtL B-1a from spleen of day 5 FtL-primed mouse.

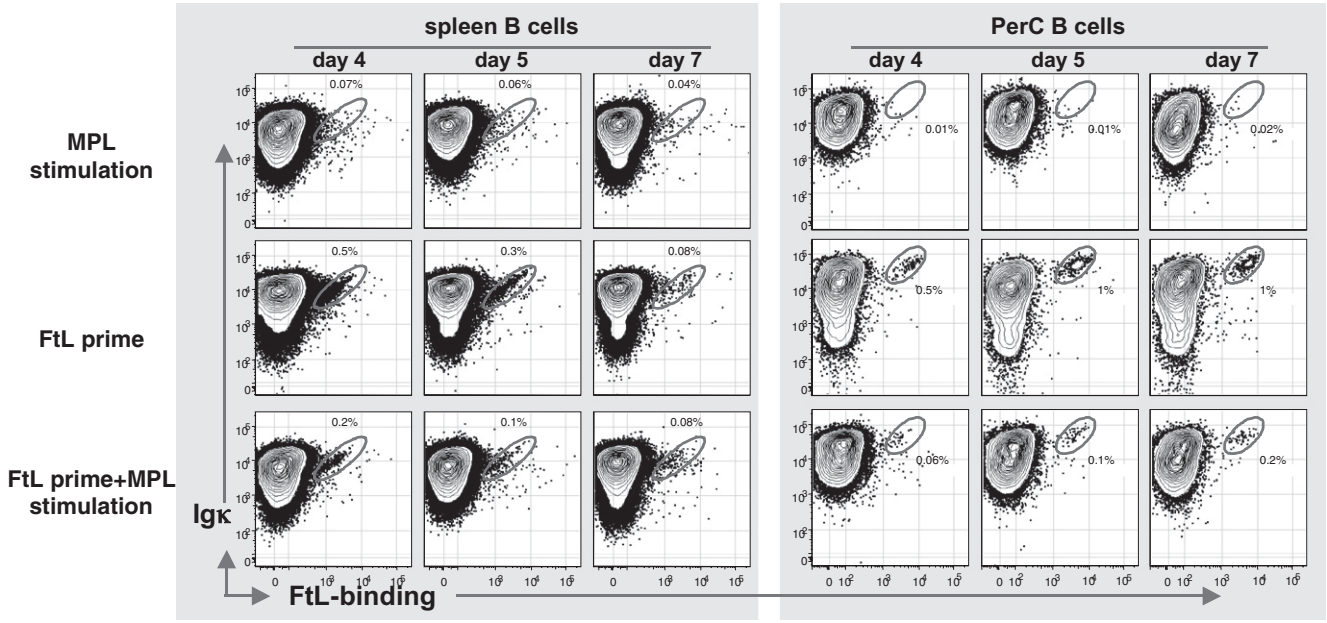

Fig. S8. Introducing monophosphoryl lipid A (MPL) stimulation simultaneously with FtL priming dampens anti-FtL primary response and the development of FtL-specific B-1a memory cells in PerC. Live splenic B cells ($CD19^+$) (*Left*) or PerC B cells (*Right*) from C57BL6/J mice by day 4, 5, or 7 d after MPL stimulation (*Top*), FtL immunization (*Middle*), or immunization with FtL plus MPL (*Bottom*) were gated to reveal anti-FtL B-1a ("diagonal" in circled population).

Table S1. Numbers of IgM and IgG anti-FtL secreting cells in tissues of BALB/c mice detected by ELISPOT

	PBS		FtL prime (day 5)		FtL prime (>2 mo)		FtL boost (day 5)	
BALB/c (n = 5) tissue	IgM	IgG1+IgG3	IgM	IgG1+IgG3	IgM	IgG1+IgG3	IgM	IgG1+IgG3
Spleen		$<1/10^7$	$9.1 \times 10^4 \pm 0.7$	$5 \times 10^3 \pm 2$	$1.9 \times 10^3 \pm 0.1$	$2.9 \times 10^2 \pm 0.9$	$1.7 \times 10^3 \pm 0.5$	$1.8 \times 10^2 \pm 0.5$
Bone marrow					$<1/10^7$			
PerC					$<1/10^7$			

Values represent means ± SE.

Table S2. Number of anti-FtL B-1a cells in spleen of C57BL6/J or IL-$7^{-/-}$ mice

		FtL immunization		
Mouse strain	PBS	Day 3	Day 4	Day 5
C57BL6 (n = 4)	1.9 ± 0.4	4.4 ± 1.1	8.7 ± 0.9	12.5 ± 1.3
IL-$7^{-/-}$ (B6) (n = 4)	0.2 ± 0.1	0.7 ± 0.1	1.4 ± 0.5	1.3 ± 0.6

Values represent means ± SE × 10^4.