Antigen-specific memory in B-1a and its relationship to natural immunity

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In the companion article by Yang and colleagues [Yang Y, et al. (2012) *Proc Natl Acad Sci USA*, 109, 10.1073/pnas.1121631109], we have shown that priming with glycolipid (FtL) from *Francisella tularensis* live-vaccine strain (*i*) induces FtL-specific B-1a to produce robust primary responses (IgM >>IgG); (*ii*) establishes persistent long-term production of serum IgM and IgG anti-FtL at natural antibody levels; and (*iii*) elicits FtL-specific B-1a memory cells that arise in spleen but rapidly migrate to the peritoneal cavity, where they persist indefinitely but divide only rarely. Here, we show that FtL rechallenge alone induces these PerC B-1a memory cells to divide extensively and to express a unique activation signature. However, FtL rechallenge in the context of a Toll-like receptor 4 agonist-stimulated inflammatory response readily induces these memory cells to migrate to spleen and initiate production of dominant IgM anti-FtL secondary responses. Thus, studies here reveal unique mechanisms that govern B-1a memory development and expression, and introduce B-1a memory as an active participant in immune defenses. In addition, at a practical level, these studies suggest previously unexplored vaccination strategies for pathogen-associated antigens that target the B-1a repertoire.

B-1 | isotype-switching | memory B cells | inflammation

In a companion paper (1), we have shown that priming with a *Francisella tularensis* glycolipid (FtL), which induces long-term protection against lethal infection with *F. tularensis* live-vaccine strain (LVS) (2), also induces B-1a lymphocytes to produce primary IgM and IgG anti-FtL responses that fade rapidly but nonetheless persist indefinitely at very low "natural" antibody levels. FtL priming, we have also shown, induces long-lived IgM and IgG anti-FtL B-1a memory cells (IgM>>IgG) that migrate from spleen to the peritoneal cavity (PerC), where they persist indefinitely as largely quiescent cells available to replenish the very rare splenic anti-FtL plasma cells that maintain the persistent low-level anti-FtL titers in serum.

Studies here track anti-FtL B-1a memory cells from their T- and germinal center (GC)-independent inception in the spleen through their long-term quiescent persistence in PerC, their restricted response to FtL rechallenge alone (robust division in PerC, dynamic expression of activation genes, no migration to spleen, no plasma cell differentiation), and their migration and differentiation to anti-FtL–producing cells in spleen when the primed animals are rechallenged with FtL in an inflammatory context [Toll-like receptor (TLR) 4 agonist stimulation]. Importantly, we show that the mechanisms involved in the induction and the secondary responsiveness of these antigen-specific B-1a memory cells are clearly distinct from the T- and GC-dependent mechanisms responsible for the induction and the secondary responsiveness of the well-known B-2 memory cells.

Collectively, the findings we present open a view on previously unsuspected B-1a immune memory mechanisms that (*i*) enable long-term production of antigen-specific serum IgM and IgG antibodies at natural antibody levels; (*ii*) provide a resilient mechanism for rapidly increasing protective antibody production when the priming antigen is re-encountered under acute conditions; and (*iii*) introduce previously unexplored vaccination strategies for pathogen-associated antigens like FtL and other glycolipids and polysaccharides that target B-1 repertoire.

Results

Long-Term FtL-Specific B-1a Memory Cells in PerC. We have shown that anti-FtL B-1a arise in spleen during the FtL-evoked primary response and then migrate to PerC, where they divide initially but then persist indefinitely. Studies presented next demonstrate that these B-1a anti-FtL in PerC are appropriately designated as memory cells because they: (*i*) proliferate vigorously in PerC and express an activation-associated transcriptional signature in response to FtL rechallenge; (*ii*) give rise to strong adoptive anti-FtL secondary responses when appropriately transferred and boosted with FtL in the recipients; and (*iii*) migrate to spleen and produce anti-FtL responses when appropriately rechallenged with FtL. However, the conditions under which these B-1a memory cells are induced to mount in situ secondary responses differ profoundly from the well-known conditions that induce the T-dependent antigen-specific B-2 memory responses commonly taken as characteristic of B-cell memory.

Quiescent FtL-Specific B-1a Memory Cells in PerC Divide Rapidly and Express a Unique Activation Signature When Rechallenged with FtL; However, They Do Not Initiate Antibody Production. The quiescence of the anti-FtL B-1a that persist in PerC in FtL-primed animals does not indicate that these cells are anergic. In contrast, by 5 d after intraperitoneal rechallenge with FtL, roughly 80% have incorporated BrdU (Fig. S1*A*). Furthermore, a high proportion express K_i-67, an intracellular marker for cycling cells (Fig. S1*B*). Thus, FtL rechallenge induces anti-FtL B-1a memory cells to undergo robust proliferation in PerC.

FtL rechallenge also induces PerC anti-FtL memory B-1a to re-express a novel activation gene program that they expressed during the active phase of the primary response (Fig. 1). Thus, at day 5 after FtL priming, they have up-regulated activation-induced cytidine deaminase (AID) and down-regulated B-cell lymphoma 6 (BCL6) and paired box protein 5 (Pax5). With time, the expression levels of these markers tends to return to normal such that, by >70 d after FtL priming, the AID, BCL6, and Pax5 levels in PerC anti-FtL memory cells are roughly comparable to levels expressed by PerC B cells from nonimmunized mice (Fig. 1). Strikingly, at day 5 after FtL rechallenge, AID expression is once again elevated and BCL6 and Pax5 levels are down-regulated (Fig. 1), just as they were after priming.

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The authors declare no conflict of interest.

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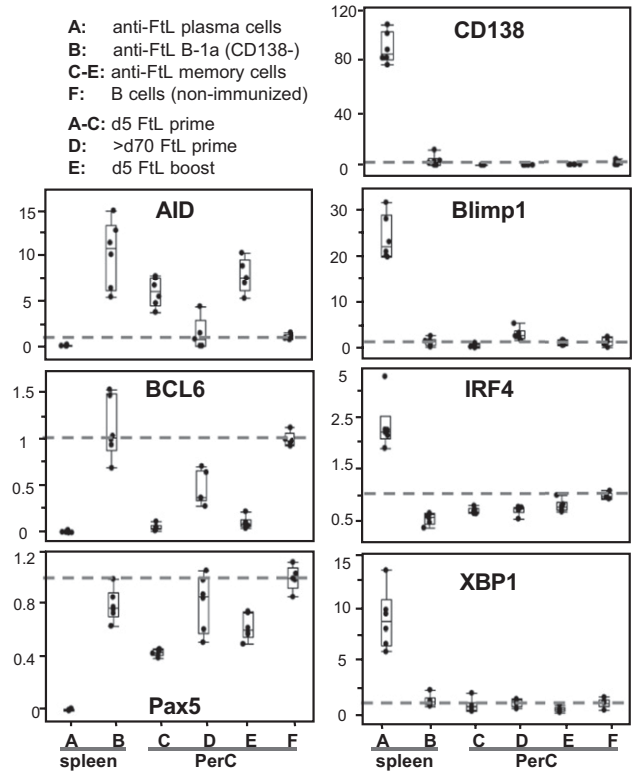

Fig. 1. FtL rechallenge induces anti-FtL memory B-1a in PerC to express a unique activation signature. Gene expression in sorted splenic or PerC anti-FtL B-1a subsets (groups A–E) from FtL primed or boosted C57BL6/J mice. The text *Top Left* defines the phenotype of the sorted subsets and the immune status of the donors. Each dot represents a single datapoint obtained for 100 sorted cells of the indicated subset (n = 6 data points per subset). Data are represented as fold-change relative to expression level (dashed line) of PerC B cells from nonimmunized mice (group F). Data for one of three experiments with similar results are shown.

FtL rechallenge does not, however, induce PerC anti-FtL memory B-1a to differentiate to plasma cells. Thus, in PerC, there are no detectable cells expressing either the typical plasma cell phenotype (CD138$^+$ intracellular Ig$^+$; figure S7 of ref. 1) or transcriptional signature for plasma cell differentiation [i.e., up-regulate CD138, B lymphocyte-induced maturation protein 1 (Blimp1), X-box binding protein 1 (XBP1), and interferon regulatory factor 4 (IRF4)] (3, 4) (Fig. 1). Furthermore, FtL rechallenge does not induce the anti-FtL memory B-1a to migrate from PerC to spleen and differentiate there to plasma cells. In sharp contrast to the primary response, anti-FtL B-1a cells are minimally detectable in spleen of primed mice following FtL rechallenge (Fig. 2*A* and Fig. S2) and serum anti-FtL antibody levels increase only minimally (Fig. 2*B*). Thus, despite undergoing extensive proliferation and dynamically expressing an activation-associated gene program, the anti-FtL memory cells in PerC do not mount a secondary anti-FtL antibody response to FtL rechallenge.

This failure of the secondary anti-FtL antibody response is not because of T-cell regulation, because primed TCR$\beta^{-/-}\delta^{-/-}$ mice also fail to produce anti-FtL (Fig. 2*B*). Instead, as adoptive cell-transfer studies in the next section show, this in situ response failure traces to complex mechanisms that operate in primed animals to prevent anti-FtL B-1a memory cells in PerC from generating secondary antibody responses except under acute inflammatory conditions.

Anatomic Localization and Host Immune Status Constrain Anti-FtL B-1a Memory Expression. Anti-FtL B-1a memory cells in PerC are fully capable of producing strong adoptive secondary anti-FtL responses when transferred intravenously either to naïve RAG-1$^{-/-}$ mice (Fig. S3) or to allotype-congenic recipients (Fig. 3). The PerC donor anti-FtL memory responses in the adoptive recipients parallel the primary responses mounted by endogenous anti-FtL B-1a to the FtL (re)challenge antigen: expanded populations of both donor and recipient anti-FtL B-1a appear both in recipient spleen and PerC (Fig. 3*A*); approximately half of the anti-FtL plasma cells in recipient spleen express donor allotype (Fig. 3*A*); and, both donor and recipient anti-FtL antibodies rise sharply in recipient serum (Fig. 3*B*). Interestingly, the serum anti-FtL antibody titers generated by naïve recipients are significantly lower than titers generated by untransferred naïve controls or naïve recipients that have received naïve PerC (Fig. 3*B*), suggesting that the transferred anti-FtL B-1a memory cell responses compete with the primary anti-FtL response mounted by the endogenous anti-FtL B-1a cells in naïve recipients.

Importantly, the route via which the PerC anti-FtL memory cells are transferred to naïve recipients is critical. PerC cells from FtL-primed donors that produce strong secondary anti-FtL antibody responses when transferred intravenously do not generate these responses when transferred intraperitoneally. Thus, although the naïve recipients themselves always mount a full

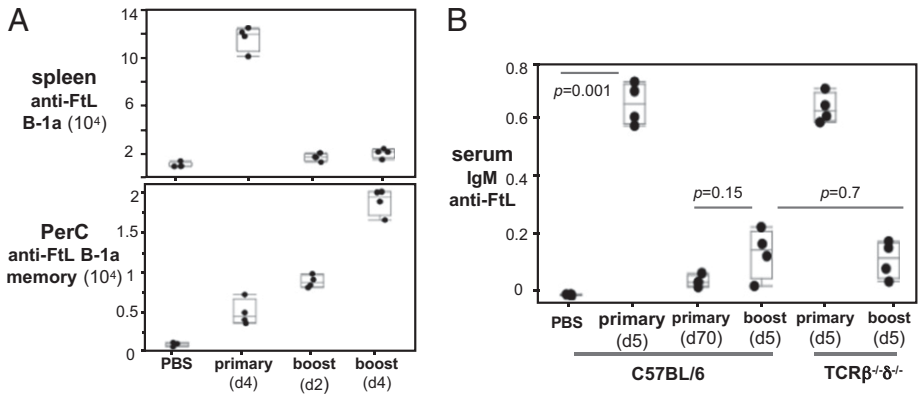

Fig. 2. FtL rechallenge does not induce activated anti-FtL memory B-1a in PerC to generate the secondary anti-FtL antibody responses. (*A*) Number of anti-FtL B-1a cells in spleen or PerC from BALB/c mice at indicated days after FtL priming or boost. (*B*) Serum IgM anti-FtL levels in C57BL6/J or TCR$\beta^{-/-}\delta^{-/-}$ mice with indicated immune histories. Each dot shows data for an individual mouse (n = 4 per group). Values are expressed as microliter equivalents of a standard serum pool from 5-d FtL primed C57BL6/J mice.

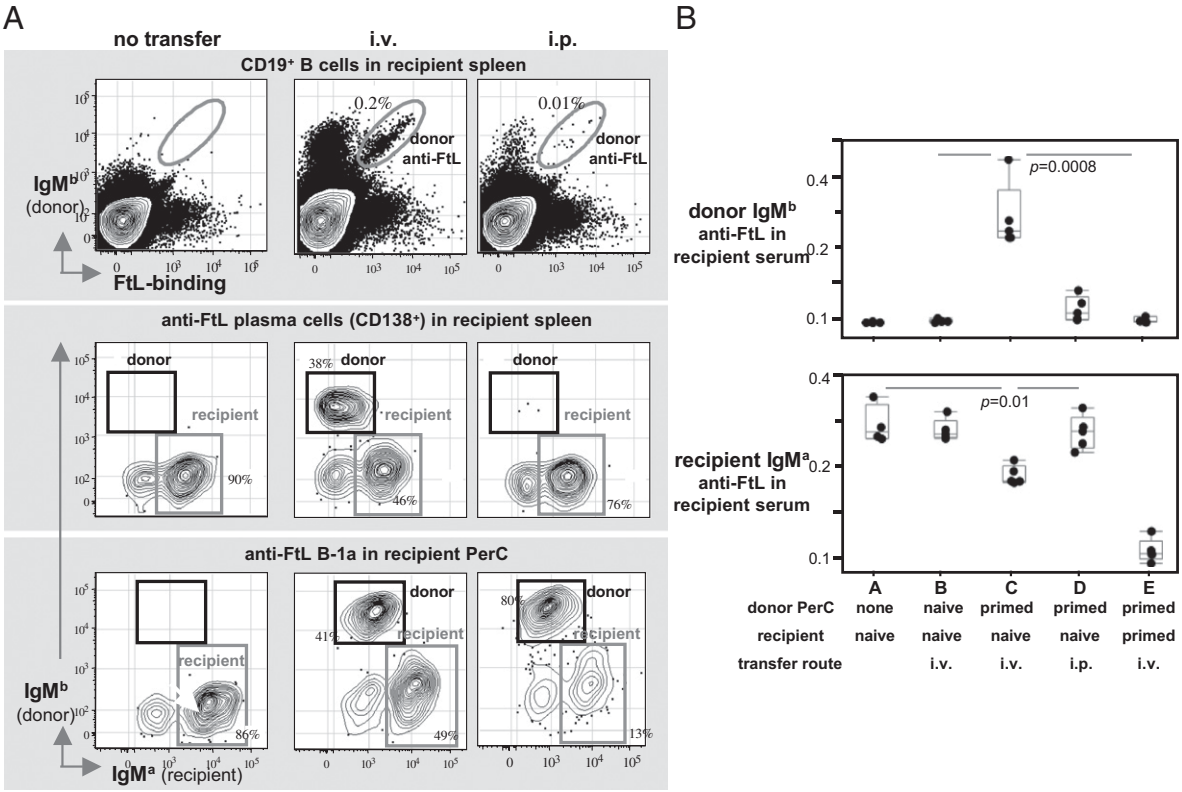

Fig. 3. PerC anti-FtL memory B-1a cells produce strong secondary anti-FtL antibody responses to FtL rechallenge when transferred intravenously to naïve allotype-congenic recipients, but not when transferred intraperitoneally either to naive recipients or intravenously to primed recipients. (*A*) PerC from FtL-primed *Igh*^{b} mice were transferred either intravenously or intraperitoneally to naïve allotype congenic *Igh*^{a} recipients that were immunized with FtL the next day. Anti-FtL responses in recipients or nontransfer mice were measured 5 d later after FtL immunization. (*Top*) Live splenic B (CD19$^+$) cells were gated to reveal donor anti-FtL cells (circled population). (*Middle*) Live splenic anti-FtL plasma cells (CD138$^+$) were gated to distinguish donor or recipient-derived anti-FtL plasma cells (boxed populations). (*Bottom*) Live PerC anti-FtL B-1a cells were gated to distinguish donor and recipient anti-FtL cells (boxed populations). (*B*) Donor IgM^b (*Upper*) or host-derived IgM^a (*Lower*) anti-FtL levels in recipient sera. n = 5–6 per group; each dot shows data for an individual mouse. Values are expressed as microliter equivalents of a standard serum pool from 5-d FtL primed nontransfer *Igh*^{a} or *Igh*^{b} mice.

primary response, PerC from FtL-primed donors selectively fail to generate anti-FtL antibody responses to the FtL rechallenge (Fig. 3*B*). In essence, when transferred intraperitoneally to naïve recipients, PerC anti-FtL memory cells respond just as they would have responded in situ: they proliferate extensively in the recipient PerC but generate minimal anti-FtL antibody responses (Fig. 3). Therefore, when PerC memory cells inhabit their natural long-term reservoir (PerC), their ability to produce secondary anti-FtL antibody responses is sharply constrained.

Adding to this complexity, the immune status of the recipient into which the cells are transferred also plays a key role in determining whether the transferred PerC anti-FtL memory cells generate antibody responses to FtL rechallenge. In essence, the PerC anti-FtL memory cells that produce strong secondary anti-FtL antibody responses when transferred intravenously to naïve recipients fail completely when they are similarly transferred (intravenously) and rechallenged in FtL-primed recipients (Fig. 3*B* and Fig. S4). Thus, in primed recipients, FtL rechallenge fails to generate secondary anti-FtL antibody responses either by the recipient or the transferred anti-FtL memory cells (Fig. 3*B*).

The response failure of the donor anti-FtL memory cells is not a result of the general inability of the PerC donor cells to enter recipient spleen, because donor B cells, the receptors of which not bind FtL, are readily detectable in the spleen of primed recipients (Fig. S4). Thus, FtL-priming induces an additional barrier that specifically prevents even intravenously transferred anti-FtL B-1a memory cells from generating splenic anti-FtL responses in the primed animals.

FtL Rechallenge in an Inflammatory Context (TLR4 Stimulation) Mobilizes PerC anti-FtL B-1a Memory Cells to Migrate to Spleen and Initiate Antibody Production. We have shown that FtL rechallenge induces vigorous proliferative responses and activation-associated transcriptional changes in PerC anti-FtL memory cells but does not induce these cells to produce secondary anti-FtL antibody responses in situ. However, we show here that rechallenging with FtL before or during an inflammatory response abrogates this response failure.

In essence, when FtL-primed animals are rechallenged with FtL and monophosphoryl lipid A (MPL) (a well-known TLR4 agonist) is administered intraperitoneally 2 d later, the number of anti-FtL memory cells in PerC 5 d after the FtL rechallenge is sharply decreased although the number of anti-FtL plasma cells in spleen (Fig. 4*A* and Table S1) and the levels of anti-FtL in serum increases sharply (Fig. 4*B*). Furthermore, unlike primary anti-FtL responses, there is only minimal de novo expansion of the anti-FtL B-1a population in spleen (Fig. 4*A* and Table S1). Thus, the anti-FtL plasma cells that appear in spleen during the MPL-facilitated secondary response to FtL rechallenge are derived from anti-FtL memory cells that were stimulated to migrate from PerC to spleen and to differentiate there to plasma cells.

Transfers of PerC from FtL-primed donors directly into the PerC (intraperitoneally) of naïve recipients confirm and extend this conclusion. In essence, immunizing the recipients with FtL and stimulating them 2 d later with MPL results in a sharp decrease in donor anti-FtL B-1a in recipient PerC and a concurrent increase in donor-derived anti-FtL plasma cells in recipient

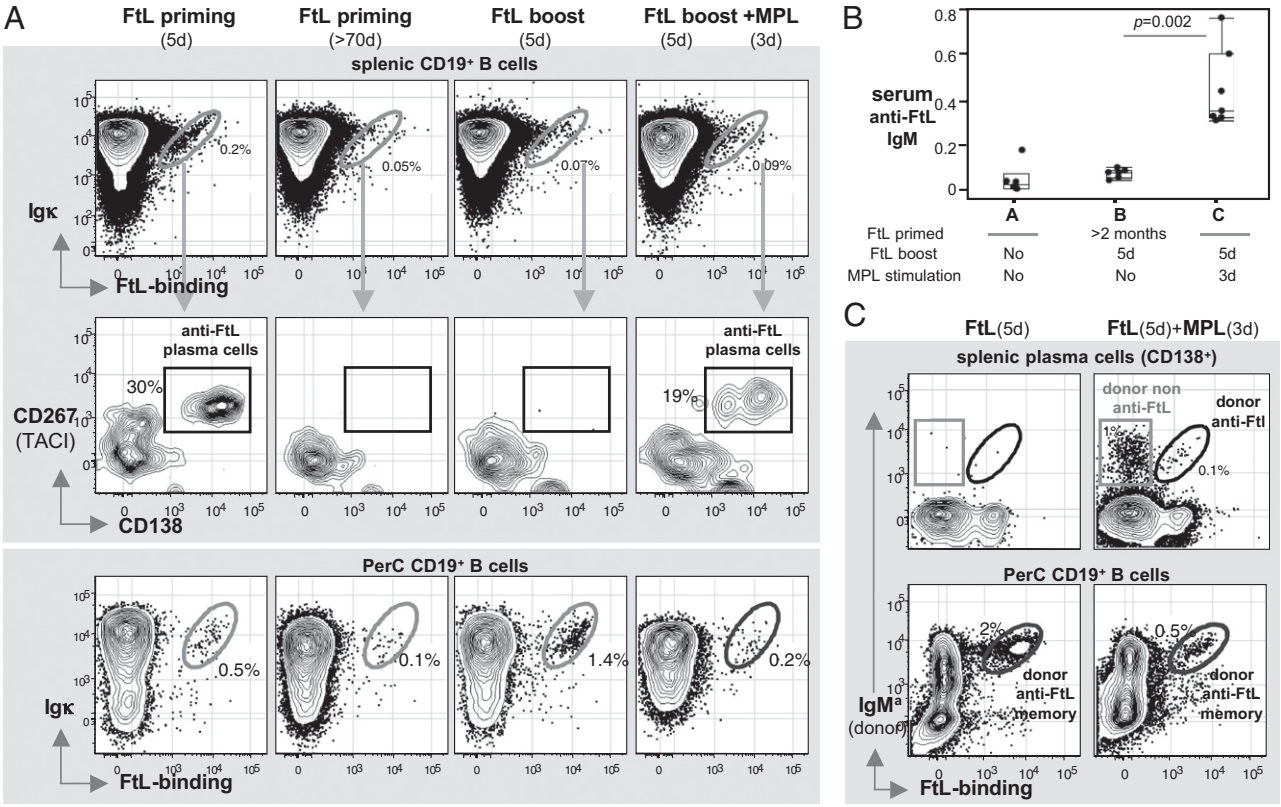

Fig. 4. FtL rechallenge in the context of MPL stimulation mobilizes antigen-activated anti-FtL memory cells to migrate from PerC to spleen, where they differentiate to plasma cell producing anti-FtL antibodies. (*A*) FACS analysis of spleen and PerC from C57BL6/J mice with indicated immunization history. Live splenic B cells (CD19$^+$) were gated to reveal anti-FtL B-1a (circled population, *Top*) and further gated to reveal anti-FtL plasma cells (boxed population, *Middle*). Anti-FtL B-1a (circled population) in gated PerC B cells are shown (*Bottom*). (*B*) Serum IgM anti-FtL levels in C57BL6/J mice. n = 6–7, per group. Each dot shows data for an individual mouse. (*C*) FACS analysis of naïve recipients to which PerC from FtL immunized donors was transferred intraperitoneally. (*Upper*) Live splenic plasma cells (CD19$^+$CD138$^+$) from recipients gated to reveal donor-derived anti-FtL (FtL$^+$) and other plasma cells (FtL$^-$) (circled and boxed populations). (*Lower*) Donor anti-FtL memory cells in recipients PerC (circled populations).

spleen (Fig. 4*C*). The timing of the MPL stimulation relative to the FtL rechallenge is not strict: results are similar when MPL is administrated with the FtL rechallenge or shortly (2 d) thereafter (Fig. S5). However, MPL stimulation is clearly required because, as we have shown (Figs. 3 and 4*C*), anti-FtL memory cells transferred intraperitoneally to naïve recipients and rechallenged with FtL alone only undergo proliferation in recipient PerC. The donor memory cells do not migrate to recipient spleen and generate anti-FtL antibody responses (Figs. 3 and 4*C*). Because MPL stimulation alone does not activate the donor PerC anti-FtL memory cells and does not induce the appearance of donor-derived anti-FtL antibody-secreting cells in recipient spleen (Fig. S5), we conclude that PerC anti-FtL B-1a memory cells must be activated by antigen (FtL) for MPL to mobilize them to migrate to spleen, where they differentiate to plasma cells producing secondary anti-FtL antibody responses.

Not surprisingly, the MPL-induced PerC B-1 migration is not restricted to anti-FtL memory cells. Like lipid A, which is well known to induce widespread antigen-independent migration of B-1 from PerC to spleen (5), MPL nonspecifically drives a large number of PerC B-1 cells to the spleen, where they differentiate to plasma cells that strikingly outnumber the anti-FtL plasma cells (Fig. 4*C*). Thus, the plasma cells derived from donor anti-FtL memory cells constitute less than 10% of total number of donor-derived plasma cells in recipient spleen (Fig. 4*C*).

Discussion

The FtL-induced robust primary and secondary B-1a antibody responses demonstrated here unveil previously unsuspected antigen-specific memory responses and introduce their potential relationship to natural (i.e., innate) antibody production. We have shown (1) that priming with FtL under noninflammatory conditions rapidly triggers splenic B-1a with FtL-binding BCR to proliferate, undergo AID-dependent isotype switching, and to differentiate to B-1a memory cells that migrate to PerC and persist there indefinitely, ready to generate recall responses following re-encounter with FtL under inflammatory conditions. Concomitant with inducing this long-term PerC-based B-1a memory, FtL priming induces initially robust primary IgM and IgG anti-FtL B-1a responses (IgM>>IgG) detectable in spleen and serum (1). These responses fade rapidly but persist indefinitely at characteristic natural antibody levels, apparently fed by the occasional migration and differentiation of anti-FtL B-1a memory cells from PerC to spleen. Because, except for B-cell receptor differences, the FtL$^+$ B-1a memory cells in PerC are similar to most other PerC B-1a, the B-1a that have accumulated in PerC collectively emerge as a potential reservoir that preserves the immunologic memory necessary to maintain organized production of what is commonly referred to as natural antibody in serum.

In addition, the B-1a memory pool in PerC emerges as a source of antigen-specific memory that can be triggered to rapidly generate protective antibody responses when the priming antigen is re-encountered during an invasive infection or in other inflammatory context. We have shown that FtL priming rapidly induces B-1a primary IgM anti-FtL responses and establishes long-term protection against *F. tularensis* LVS (2). Here, we demonstrate that this priming protocol induces anti-FtL memory

B-1a (IgM >>IgG) that persist in PerC and are triggered to migrate to spleen and differentiate to anti-FtL–secreting plasma cells when FtL is re-encountered in an inflammatory context. These findings suggest that many, perhaps the majority, of the B-1a in PerC are differentiated memory B cells that have already encountered their cognate antigens (exogenous or endogenous) and can give rise to antibody responses when their cognate antigens are re-encountered under inflammatory (or other acute) conditions.

Perhaps not surprisingly, the mechanisms that empower the induction, maintenance, and secondary responsiveness of the FtL-specific (and other) B-1a memory cells differ profoundly from the well-known (canonical) mechanisms that underlie B-2 memory. B-2 primary responses and memory induction typically involves T-cell help and GC formation, which supports AID-dependent isotype switching, antigen-dependent expansion and selection of high-affinity memory cells. In contrast, B-1a primary and memory response do not require T-cell and GC support. This process likely results in B-1a memory having lower average affinity that B-2 memory and, as we have observed, in substantially lower representation of isotype-switched cells in B-1a primary and memory responses. However, on the positive side, it enables speedier B-1a primary and secondary responses, and introduces a greater role for IgM antibodies, both of which may be beneficial in the disease modalities that B-1a addresses.

Consistent with GC and T-cell help being unnecessary for induction of anti-FtL responses, priming with FtL in the absence of adjuvant support or other inflammatory stimuli is sufficient to fully induce anti-FtL responses. FtL itself does not supply a substitute inflammatory stimulus because it has minimal TLR2 and TLR4 agonist activity and is shown not to induce inflammation in mice (6–8). Furthermore, primary anti-FtL responses in TLR4$^{-/-}$ (2) and TLR2$^{-/-}$ mice have been shown to be indistinguishable from WT (9).

Inflammation, however, is not a neutral factor in anti-FtL primary responses. As we have shown (1), priming with FtL during ongoing inflammation (induced by MPL, a potent TLR4 agonist) dampens rather than augments anti-FtL primary responses and decreases the development of anti-FtL memory cells in PerC. Thus, B-1a primary responses and the induction of B-1a memory is best accomplished in the absence of stimulation with adjuvants or other inflammatory stimuli.

The conditions required to induce B-1a memory responses similarly differ substantially from those that induce B-2 memory responses. B-2 memory responses are typically triggered by boosting with the lower dose of priming antigen in the absence of adjuvant. Boosting with FtL in the absence of adjuvant readily stimulates PerC anti-FtL B-1a memory cells to proliferate vigorously and to express an activation-associated gene program; however, it does not detectably raise anti-FtL antibody production above natural antibody levels. Thus, re-encounter with the priming antigen (FtL) in the absence of inflammation fortifies the existing B-1a memory population but dramatically fails to increase anti-FtL production in the immunized animal.

In contrast, boosting with the priming antigen during acute inflammation (e.g., TLR4 stimulation) readily mobilizes the activated memory cells to migrate to spleen, where they differentiate to plasma cells producing anti-FtL antibodies. Both the antigen and the inflammation are required to up-regulate B-1a antibody production. The antigen, as we have shown, is needed to activate the B-1a in PerC. In contrast, the inflammation is needed to mobilize the activated memory cells to the spleen and thus enable their differentiation to antibody-secreting plasma cells when they reach the spleen. This process can also be accomplished, we have shown, by intravenous transfers of PerC-containing anti-FtL memory cells to naïve recipients and (re)challenging with FtL alone. Collectively, these findings demonstrate that anti-FtL B-1a memory cells in PerC are fully capable of mounting a secondary antibody response. However, their ability to do so in situ is tightly constrained by the anatomical location at which they normally reside. Antigenic stimulation accompanied by inflammation releases this constraint.

Circulating antibodies have recently been implicated in suppressing T-independent antigen-specific memory B cells in secondary responses to 4-hydroxy-3-nitrophenylacetyl (NP)-Ficoll (10). However, several lines of evidence argue against antigen sequestration mechanism: (*i*) anti-FtL B-1a memory cells clearly respond to the FtL rechallenge; (*ii*) rechallenge with up to 10-times the initial immunizing dose of FtL fails to elicit anti-FtL antibody production (Fig. S4); and, most importantly, (*iii*) despite the presence of circulating anti-FtL antibodies, FtL rechallenge associated with TLR4 stimulation is sufficient to enable migration of FtL memory B-1a to spleen and their differentiation there to plasma cells. Thus, the failure respond to FtL rechallenge in the absence of inflammation traces to the collective action of mechanisms that rapidly empty the spleen of FtL-responsive cells capable of responding to priming antigen and restrict anti-FtL B-1a memory cells to reside in PerC, where they can proliferate upon re-encountering with priming antigen but cannot differentiate to plasma cells in PerC. Inflammatory (or perhaps other) conditions are required to break this impasse.

These findings lead to a startling conclusion: B-1a memory has evolved to maintain low-level antibody production to previously encountered antigens and to resist elevating antibody production to these antigens unless they are re-encountered under inflammatory conditions (e.g., TLR4 stimulation). These restrictive response properties are clearly sufficient to provide long-term protection against infection by certain pathogens, because FtL priming protocols that induce B-1a memory are necessary and sufficient to protect mice for months against lethal *F. tularensis* LVS infection (2) and, by extension, other pathogens (11–13).

Overall, the differences that we have demonstrated here between B-1 and B-2 memory responses thus offer key insights to how the immune system has evolved to endow the B-1 and B-2 lineages (14, 15) with complementary response capabilities to protect against invasion by pathogens. B-2 memory cell induction and its subsequent T-cell–supported maturation in GCs results in slower but higher-affinity primary responses that improve both the quality and the magnitude of recall responses. B-1 memory responses, in contrast, are induced more rapidly to provide more immediate protection against infection. However, these responses may lack typical affinity maturation and may be completely restricted to recall responses to antigens presented in an acute (e.g., inflammatory) context.

These differences have strong implications for vaccination strategies that target B-1 responses. Current vaccination protocols typically involve priming with an antigen in an inflammatory context (e.g., with adjuvant) and boosting with the antigen in the absence of adjuvant to test for success of the primary immunization (16). This protocol, however, will obscure protective responses to carbohydrate or glycolipid antigens, such as FtL, that target B-1a repertoire. In fact, as we have shown, FtL priming in the context of adjuvant-induced inflammation actually dampens B-1a primary responses and decreases of B-1a memory induction. In addition, once B-1a memory is optimally induced, boosting with the antigen will be minimally effective unless it is presented in the context of inflammation. Thus, our findings should be very useful for designing immunization strategies that will be effective for inducing long-term B-1 protective immunity against at least some carbohydrate and glycolipid antigens expressed by infectious pathogens.

Finally, the recognition that certain types of antigens can induce B-1a antibody responses and persistent B-1a memory may have etiological and therapeutic implications for autoimmune diseases, for B-1a neoplasias, and perhaps for allergy as well. In keeping with earlier thinking by many investigators, we believe

that much of the putatively natural antibody in circulation may trace to persistent B-1a memory cells induced by early encounters with self or environmental antigens (which may still be present in adults). Antibody production by these memory cells is normally kept under strict control by requiring both that the memory B-1a must meet the cognate antigens and that this meeting must take place under inflammatory conditions. Development of methods or drugs that either enhance or decrease this strict control of B-1a response could prove useful in diverse disease settings.

Materials and Methods

Mice and Reagents. All mice were purchased from the Jackson Laboratory except: AID$^{-/-}$ (C57BL6/J or BALB/c), from T. Honjo (Kyoto University, Kyoto, Japan); IL-7$^{-/-}$(C57BL6/J), from P. Vieira (Institute Pasteur, Paris, France); and, C.B-17 (BALB/c-Igh^{b}), from our colony. Seven- to 12-wk-old mice were used; study protocols were approved by the Stanford Animal Care Review Board.

Mice were immunized with 100 ng i.p. FtL (List Biological Laboratories) (7). When indicated, mice were rechallenged >6 wk after priming. For MPL stimulation, mice were injected intraperitoneally with 100 μg MPL (Avanti Polar Lipids). FtL for FACS staining was biotin-conjugated (2). For in vivo BrdU incorporation, mice were fed with BrdU-containing (0.8 mg/mL) water as indicated.

Adoptive Responses. Recipients were not irradiated. Pairs of IgH allotype congenic mice used as donors and recipients: (BALB/c-Igh^{a}/CB.17-Igh^{b} or C57BL/6-Igh^{a}/ C57BL/6-Igh^{b}). FtL-primed donors were immunized intraperitoneally with FtL at least 6 wk before transfer.

High-Dimension FACS Analysis. FACS staining was previously described (2). Briefly, cell suspensions were incubated with LIVE/DEAD Aqua (Invitrogen), washed, and then incubated with unconjugated anti-CD16/CD32 (FcγRII/III) mAb to block Fc-receptors. Next, cells were incubated on ice for 20 min with biotin-coupled FtL, stained with a "cocktail" of fluorochrome-conjugated antibodies, washed and resuspended with fluorochrome-labeled avidin. Fluorochrome-conjugated antibodies were purchased (BD, eBioscience, Invitrogen) or conjugated in our laboratory. Data were collected for 0.5–1 × 10^6 cells with LSR-II (BD Bioscience) and analyzed with FlowJo (TreeStar).

ELISPOT. ELISPOT assays (17) were used to count isotype/allotype anti-FtL secreting cells. Briefly, 2 × 10^5 to 10^7 cells were cultured for 5–6 h in 96-well microtiter plates precoated with 50 μL FtL (10 μg/mL). After washing, anti-FtL secreting cells were revealed by alkaline phosphatase (AP)-conjugated anti-IgM (331) (Sigma), or biotin-conjugated reagents followed by Streptavidin-AP (Sigma) [i.e., anti-IgG1 (A85-1), anti-IgG3(R40-82), anti-IgMa(DS-1) or IgMb(AF6-78)].

Serum Anti-FtL Antibody Analysis. Anti-FtL antibodies in serum were revealed with a fluorescence bead assay commonly used for measuring cytokines in serum or culture fluids (18, 19). Briefly, aliquots of avidin-coated beads (Spherotech) are incubated with biotin-conjugated FtL at a saturating concentration for 30 min at room temperature, washed, incubated with diluted serum samples for 30 min at room temperature, washed, and stained for 30 min at room temperature with fluorescent-labeled monoclonal antibodies to detect IgH allotypes (IgM^{a} or IgM^{b}) or isotypes (IgM, IgG1 or IgG3), separately or together, as the fluorescent label permits. After washing, the FACS-measured mean fluorescence intensity (MFI) of each fluorochrome bound to the beads is corrected for the volume of serum sample to determine MFI per microliter value for the sample. Finally, MFI per microliter are re-expressed as microliter equivalents of a standard anti-FtL serum pool based on MFI measured in each assay for serial dilutions of a standard BALB/C or C57BL6/J anti-FtL serum pool obtained from mice primed with FtL for 5 d previously.

Real-Time Quantitative RT-PCR Analysis. Anti-FtL B-1a or control B cells from C57BL6/J spleen or PerC we sorted with FACSAria (BD Bioscience) into a 96-well PCR plate. One-hundred cells per subset were directly sorted into 10 μL lysis buffer containing RNase inhibitor (Ambion SUPERase-In). Without RNA purification, a preamplified cDNA pool was generated for each sample in a themo-cycler in the presence of reverse-transcriptase reaction reagents (CellsDirect One-step qRT-PCR kit; Invitrogen) and a mixture of primers (Applied Biosystems). The cDNA products for each of the sorted wells, together with individual primer reagents, were loaded separately into the digital array chip (Fluidigm), and the real-time quantitative RT-PCR reactions were completed in the BioMark HD machine (Fluidigm).

Statistics. JMP genomics statistical software (SAS Institute) was used for all statistics procedures. Nonparametric Wilcoxon test was used as indicated.

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Supporting Information

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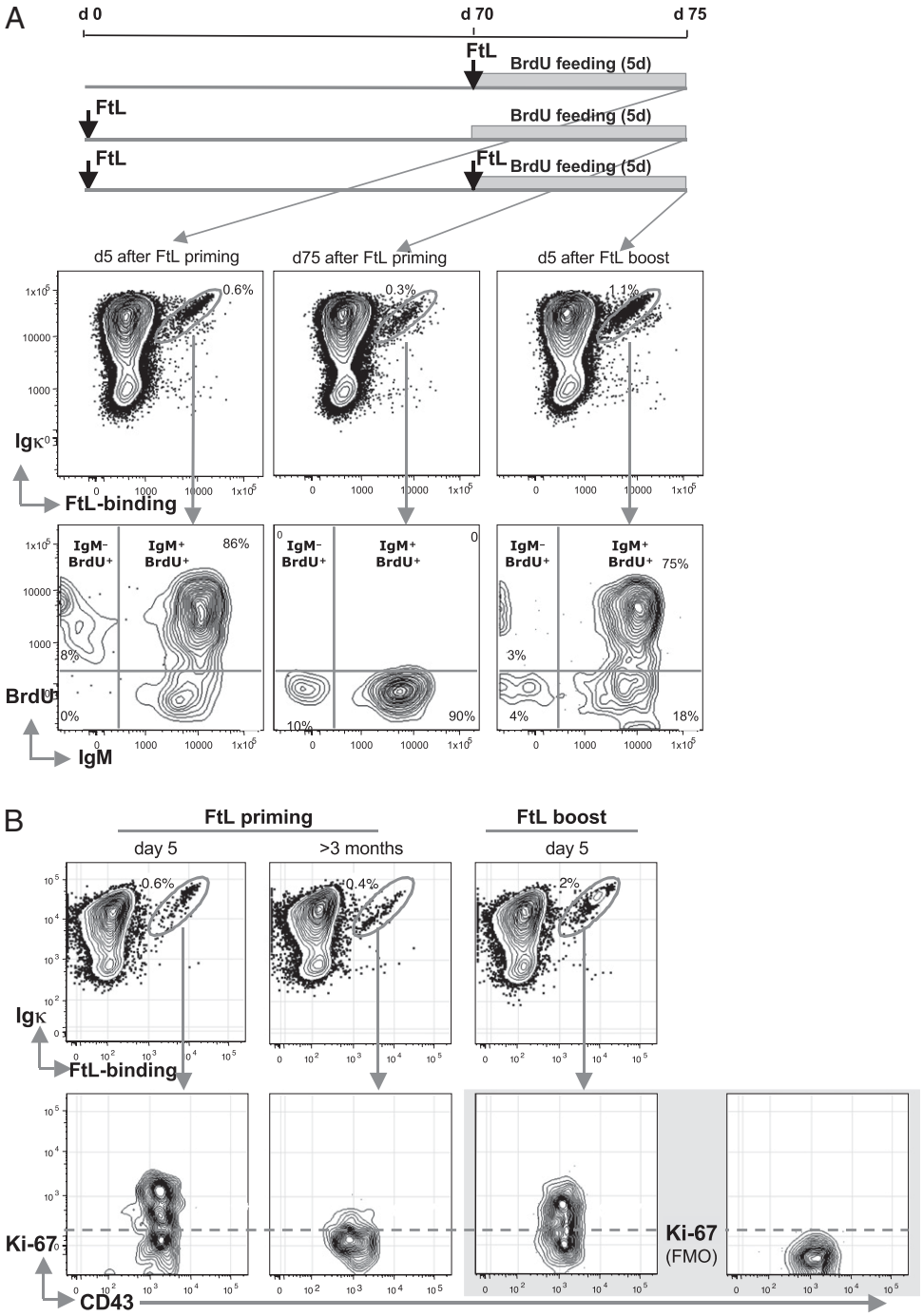

Fig. S1. *Francisella tularensis* glycolipid (FtL) rechallenge induces anti-FtL memory B-1a in peritoneal cavity (PerC) to undergo robust proliferate. (*A*) Cell division (BrdU incorporation) during anti FtL responses. BALB/c mice were primed, boosted and fed with BrdU-containing water for 5 d (gray bar) at indicated times. Live anti-FtL B-1a in PerC (circled population, *Upper*) were gated to reveal BrdU incorporation in IgM and isotype-switched (IgM$^-$) anti-FtL B-1a cells (*Lower*). (*B*) Live PerC B cells (CD19$^+$) of BALB/c mice primed or boosted with FtL at indicated days were gated to reveal anti-FtL B-1a cells (gray line circled populations), which were further gated to show intracellular expression of K_i-67. The FACS plot on the extreme right (*Lower*) show data for a fluorescence minus one (FMO) staining control for PerC cells from day 5 FtL-boosted mice. In FMO staining, fluorochrome-labeled anti–K_i-67 antibodies was omitted to enable setting the boundary (dashed line) for K_i-67 expression.

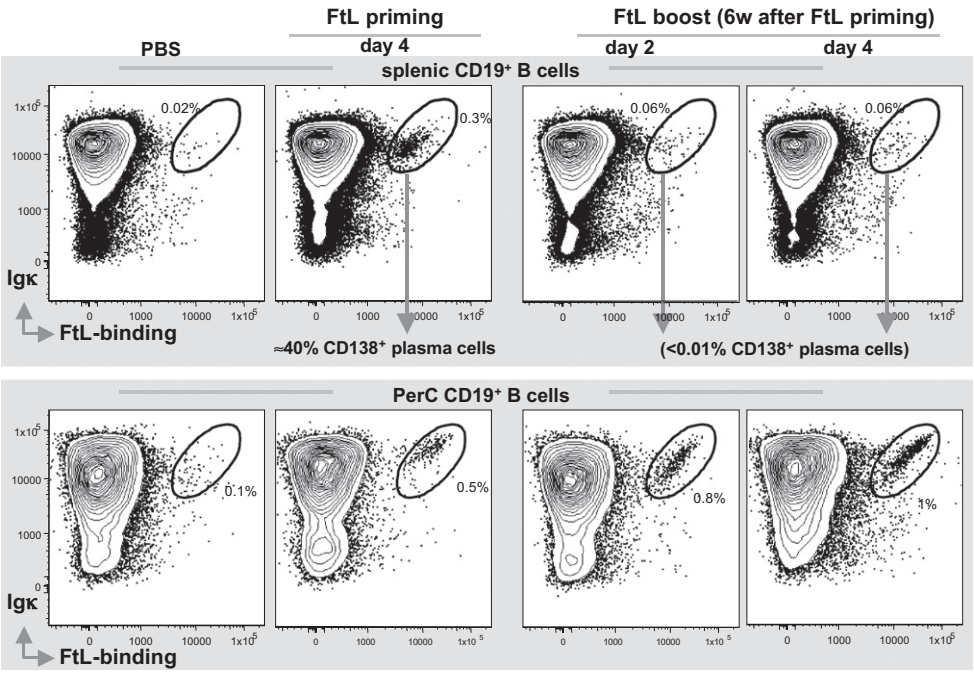

Fig. S2. FtL rechallenge does not induce activated anti-FtL memory B-1a in PerC to migrate to spleen generating the secondary anti-FtL antibody responses. Anti-FtL B-1a (circled population) were identified from gated live B cells from spleen (*Upper*) or PerC (*Lower*) of BALB/c mouse with indicated immune history shown above the FACS plots. The frequency of anti-FtL plasma cells (CD138$^+$) within the gated anti-FtL B-1a from spleen was also shown (*Upper*).

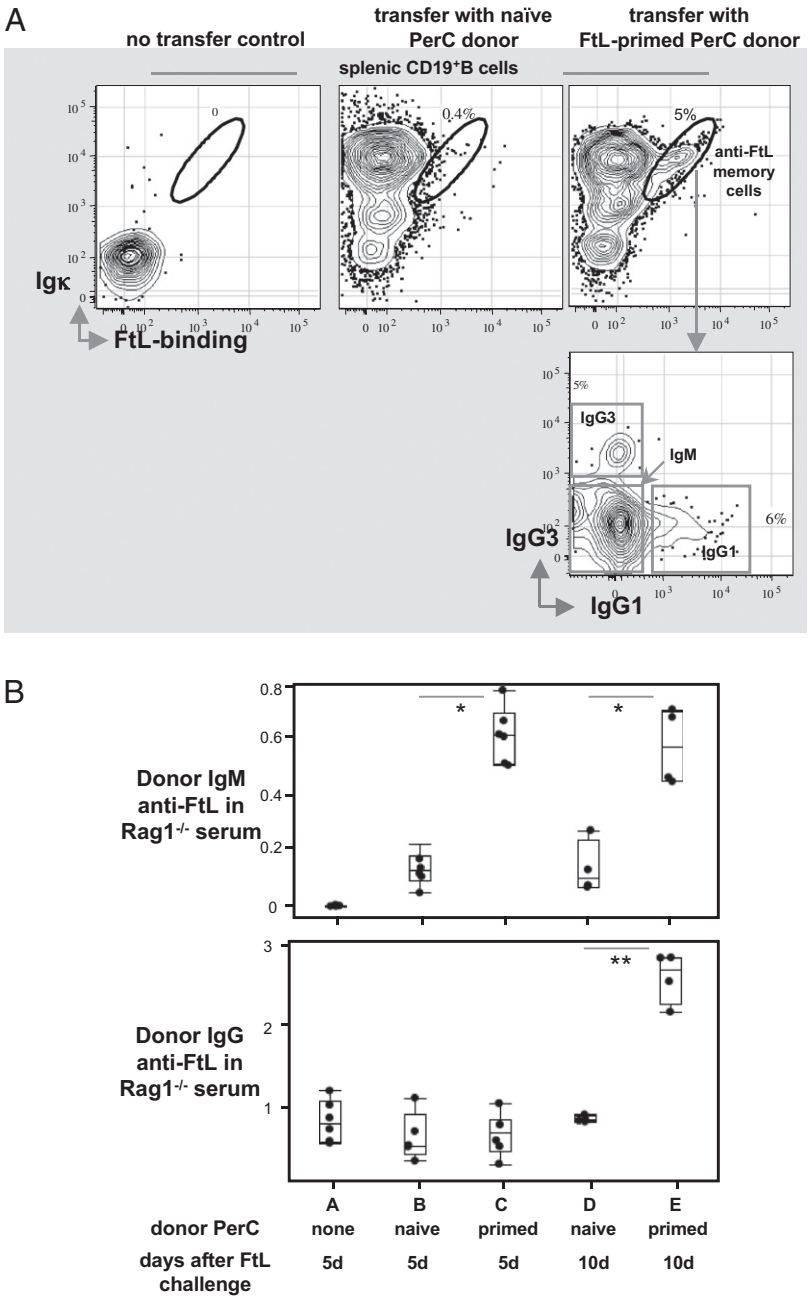

Fig. S3. When intravenously transferred to naïve Rag1$^{-/-}$ mice, PerC anti-FtL memory B-1a cells produce secondary IgM and IgG anti-FtL response to FtL rechallenge in recipients. (*A*) PerC from naïve or FtL-immunized BALB/c mice were transferred intravenously to naïve Rag1$^{-/-}$ recipients. The next day, recipients were immunized with FtL and anti-FtL responses were measured for each recipient or nontransfer Rag1$^{-/-}$ mice 5 d later. (*Upper*) Live splenic CD19^{+} cells from Rag1$^{-/-}$ recipients were gated to identify donor anti-FtL cells. Donor anti-FtL memory cells (circled population, right plot, *Upper*) were further gated to distinguish IgG1, IgG3, or IgM-expressing subset (boxed populations, *Lower*). (*B*) IgG and IgM anti-FtL level in serum from Rag1$^{-/-}$ recipients. Transfer status; days after FtL immunization are indicated for each group (A–E). Each dot represents data from an individual mouse (n = 4–6, per group). *P = 0.003; **P = 0.01 (nonparametric Wilcoxon test).

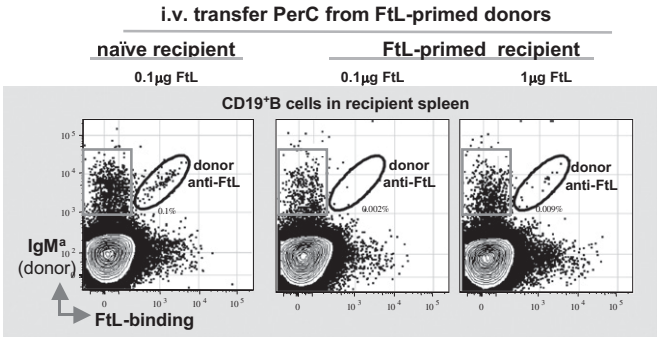

Fig. S4. PerC anti-FtL memory B-1a cells produce strong secondary anti-FtL responses to FtL rechallenge when transferred intravenously to naïve allotype-congenic recipients, but not when similarly transferred to FtL primed recipients. PerC from FtL-immunized *Igh*^{a} mice were transferred i.v. to naïve (*Left*) or primed (*Center* and *Right*) *Igh*^{b} congenic recipients. FtL challenge dose is indicated above each plot. Live splenic B cells were gated to reveal donor-derived (IgM^{a+}) anti-FtL (FtL^{+}) and FtL^{-} populations (circled and boxed population, respectively).

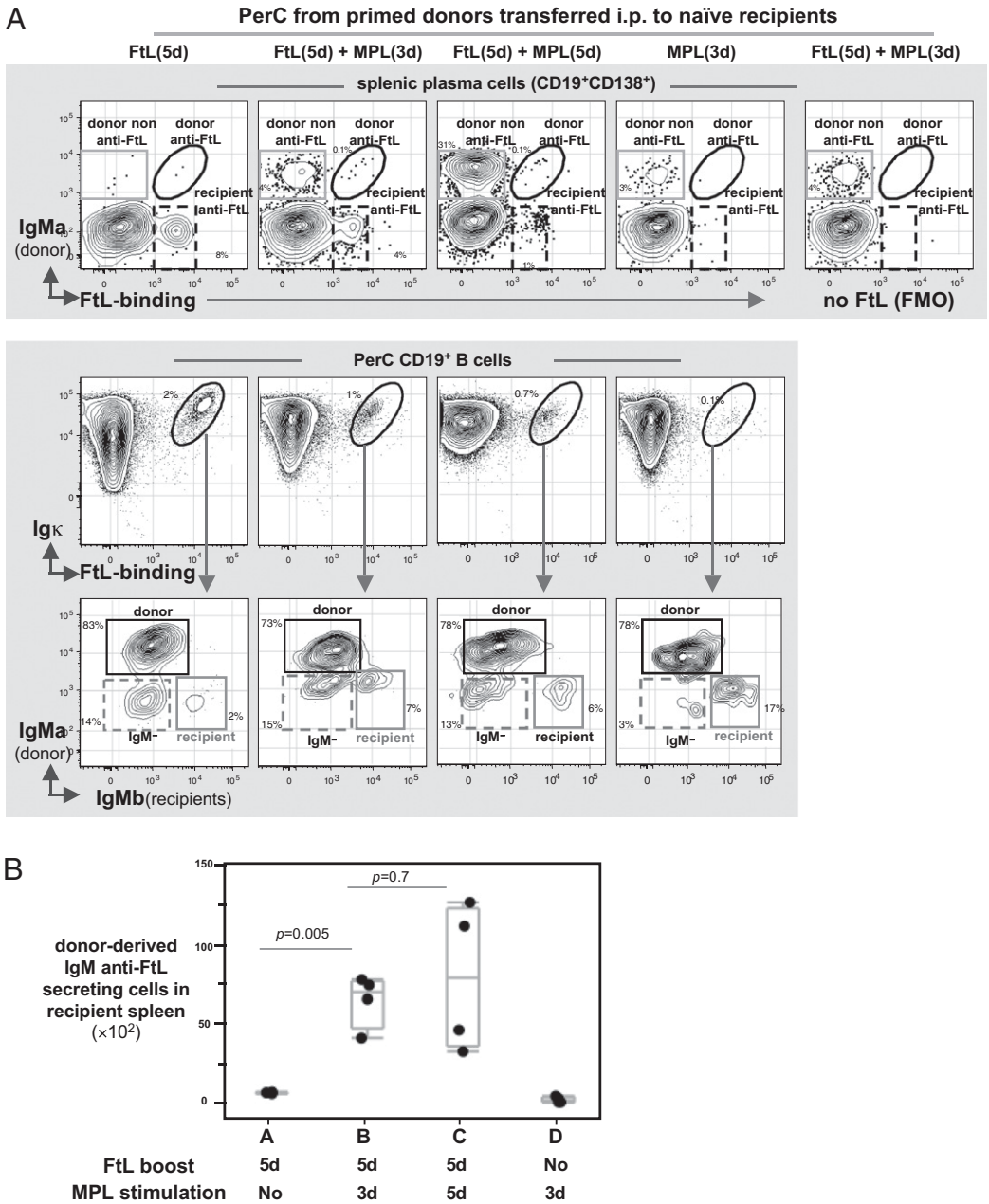

Fig. S5. FtL rechallenge together with MPL stimulation, but not MPL only, mobilize activated PerC anti-FtL memory cells to migrate to spleen, where they differentiate to plasma cells. (*A*) FACS analyze of spleen or PerC cells from recipients (Igh^b), which were transferred intraperitoneally with the same number of PerC cells from FtL immunized Igh^a congenic mice. Days after FtL challenge and MPL stimulation for each recipient is indicated above each plot. (*Upper*) Live splenic plasma cells ($CD19^+CD138^+$) from each recipient were gated to distinguish donor-derived ($IgMa^+$) anti-FtL (FtL^+) or other plasma cells (FtL^-) (circled and boxed populations, respectively). Cells in dashed box: >95% are recipient-derived ($IgMb^+$) anti-FtL plasma cells; the rest (about 5%) are class-switched (IgM^-) anti-FtL plasma cells from either donor or recipient. (*Middle* and *Lower*) PerC B cells ($CD19^+$) from indicated recipients were gated to reveal anti-FtL B-1a (circled population), which were further gated to distinguish donor or recipient-derived anti-FtL memory cells (boxed populations). Cells in dashed boxed represent class-switched anti-FtL B-1a from either donor or recipients. (*B*) Absolute number of anti-FtL antibody secreting cells that are derived from transferred donor PerC anti-FtL memory cells in recipient spleen. Immune status and immunization days for each group (A–D, n = 4, per group) are indicated.

Table S1. Number of anti-FtL B-1a and plasma cell in spleen or PerC of C57BL6/J mice

	FtL primed (5 d)		FtL primed (>2 mo)		FtL boost (5 d)		FtL boost (5d) + MPL stim (3 d)	
Tissue	Total	Plasma cells	Total	Plasma cells	Total	Plasma cells	Total	Plasma cells
Spleen (n = 5)	33.8 ± 8	9.9 ± 0.6	5.5 ± 1.5	0.04 ± 0.05	6.1 ± 1.6	0.04 ± 0.05	9.7 ± 3.5	1.1 ± 0.1
PerC (n = 5)	0.4 ± 0.1	<0.01	0.2 ± 0.1	<0.01	1.7 ± 0.3	<0.01	0.5 ± 0.2	<0.01

Values represent mean ± SE × 10^4.