Division and differentiation of natural antibody-producing cells in mouse spleen

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B-1a cells reside in both the peritoneal cavity and the spleen. LPS stimulates splenic B-1a to differentiate to plasma cells producing natural IgM specific for microbial and self antigens. However, there are conflicting views as to whether the B-1a cells divide before this differentiation occurs, and hence how the resident B-1a population is maintained in the spleen. Studies here resolve this dispute in favor of both sides: we show that (some or all) B-1a cells resident in the spleen respond to LPS by differentiating to plasma cells immediately, without dividing; however, we also show that additional B-1a cells immigrate into the spleen after LPS stimulation and divide at least once before differentiating. Importantly, the studies we presently describe reveal the complex cell migration and differentiation events that collectively underlie the rapid production of natural antibodies in response to *in vivo* LPS stimulation. Thus, the studies present a different view of the roles that B-1a cells play in the early phases of the innate immune response.

B-1 | CD138 | CD5 | lipopolysaccharide | plasma cells

B-1a cells, which give rise to cells that produce most of the natural antibodies found in mouse serum, have unique localization and development patterns and display unique activation requirements (1–3). Although found mainly in peritoneal and pleural cavities in adults, small numbers of these cells are present in the spleen, where they express a phenotype similar to the peritoneal B-1a cells but lack the CD11b surface determinant that most of the peritoneal B-1a cells express. Much of the innate *in vivo* antibody response to systemic bacterial stimuli such as LPS has been shown to be produced by B-1a cells and/or their plasma cell progeny in the spleen (1).

LPS stimulation, both *in vivo* and *in vitro*, has been shown to rapidly trigger production of antibodies to phosphatidylcholine (PtC) (4). These responses are usually measured in terms of the number of plaque-forming cells detectable either in the spleen 2–3 days after i.v. LPS stimulation or, somewhat earlier, *in vitro*. Surprisingly, although cell division is well known to precede differentiation of antibody-forming cells (plasma cells) in antigen-stimulated responses to sheep erythrocytes and virtually all other T-dependent antigens, early *in vitro* studies demonstrated that cell division is not required for the LPS-stimulated anti-PtC response (5–8).

This finding, which was hotly discussed, was never (to our knowledge) shown to be incorrect. However, perhaps because the distinction between B-1a innate antibody responses and B-2 antigen-stimulated responses was not recognized until years later, the demonstrated absence of division in the B-1a anti-PtC response was ultimately "chalked up" to inadequate experimentation or as an *in vitro* artifact and largely forgotten.

We return to this issue in *in vivo* studies presented here, which focus on the differentiation of B-1a cells to plasma cells in the spleen in response to i.v. LPS stimulation. We confirm that LPS triggers B-1a cells to differentiate to IgM-producing plasma cells ($IgM^+CD138^+Blimp\text{-}1^{hi}$) without undergoing cell division. However, we also show that other splenic B-1a cells, principally those that have recently migrated from the peritoneal cavity, require cell division before initiating differentiation to plasma cells.

These findings, coupled with the demonstration that CD11b expression marks B-1a cells that have recently migrated to the spleen, suggest a model in which B-1a cells resident in the spleen provide a reservoir of rapidly responding precursors of plasma cells that produce innate antibodies. Our findings suggest that this reservoir is replenished/augmented by LPS-triggered migration of peritoneal B-1a cells into the spleen, where a small proportion of the immigrants go on to divide and contribute to the antibody response.

Results

We restrict our B-1 studies here to the B-1a subset, which has a characteristic phenotype that includes the expression of CD5 (i.e., $IgM^{high}IgD^{low/-}B220(RA3\text{-}6B2)^{low}CD23^-CD5^+$) and is the principal B-1 subset in the spleen and peritoneal cavity (PerC) (Fig. 1). Note that we use high-definition FACS methods that allow simultaneous detection of up to 10 colors to accurately determine the frequencies of cells expressing these and other markers indicated below.

B-1a and Their Plasma Cell ($CD138^+$) Progeny in Unstimulated Animals. Among the splenic B-1a in unstimulated animals, ≈4% are typical IgM plasma cells that express high levels of intracellular IgM and surface $CD138^+$ in addition to CD5 (Fig. 1). None (<0.01) of these plasma cells are detectable among PerC B-1a cells (FACS data not shown).

LPS Triggers Immediate Migration of PerC B-1a Cells to the Spleen. Intravenous LPS stimulation does not induce differentiation of B-1a to IgM plasma cells in the PerC (<0.01 $CD138^+$ PerC B-1a; data not shown). Instead, the absolute number of PerC B-1a cells decreases by half within 24 h of LPS stimulation (and recovers ≈2 days later), whereas the absolute number of splenic B-1a cells increases significantly 6 h after LPS injection (Fig. 2).

The LPS-stimulated increase in splenic B-1a cells is largely, if not wholly, due to migration of PerC B-1a cells to the spleen. To track this migration, we transferred 3×10^6 unmanipulated PerC B cells from IgM^b allotype (C.B/17) donors to unmanipulated congenic IgM^a allotype (BALB/c) recipients, which have roughly the same number of native PerC B cells. Two hours after transfer, we injected LPS i.v. into recipients and measured donor (IgM^b) B-1a cells in recipient spleen at appropriate intervals from 1 to 6 days thereafter.

Donor B cells are readily detectable in the recipient spleen 1 day after transfer in both LPS-stimulated and PBS-injected (control) mice. However, the number of donor cells in the spleen in LPS-stimulated recipients is always substantially higher than

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Abbreviations: PtC, phosphatidylcholine; PerC, peritoneal cavity; Blimp-1, B lymphocyte-induced maturation protein 1.

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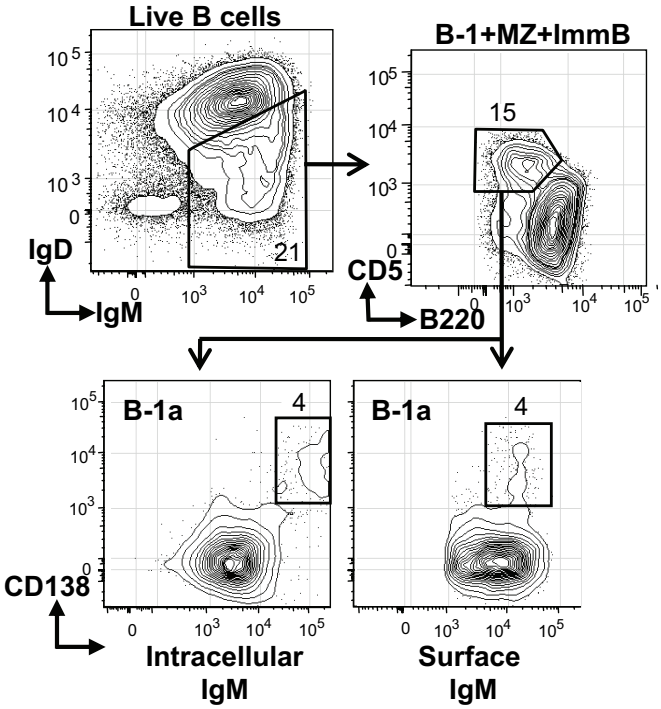

Fig. 1. Splenic CD5$^+$ (B-1a) plasma cells express surface and intracellular IgM. BALB/c spleen cells were stained with fluorochrome-conjugated antibodies in a 10-color-stain combination as described in *Materials and Methods.* Live B cells (B220+, IgM+) in spleens from unstimulated BALB/c mice (*Upper Left*) were sequentially gated as shown to reveal surface and intracellular IgM expression in CD138$^+$ plasma cells (*Lower*). (*Upper Left*) B-1, marginal zone B (MZ) and immature B cells are included in the IgM^{hi}IgD^{lo} gate shown for live B cells. (*Upper Right*) Gated population in *Upper Left* is next gated to reveal B-1a CD5$^+$B220^{lo} cells. (*Lower*) Gated population of B-1a cells from *Upper Right* is further gated to reveal intracellular IgM expression (*Lower Left*) and surface IgM expression (*Lower Right*) in plasma cells (CD138^{hi}).

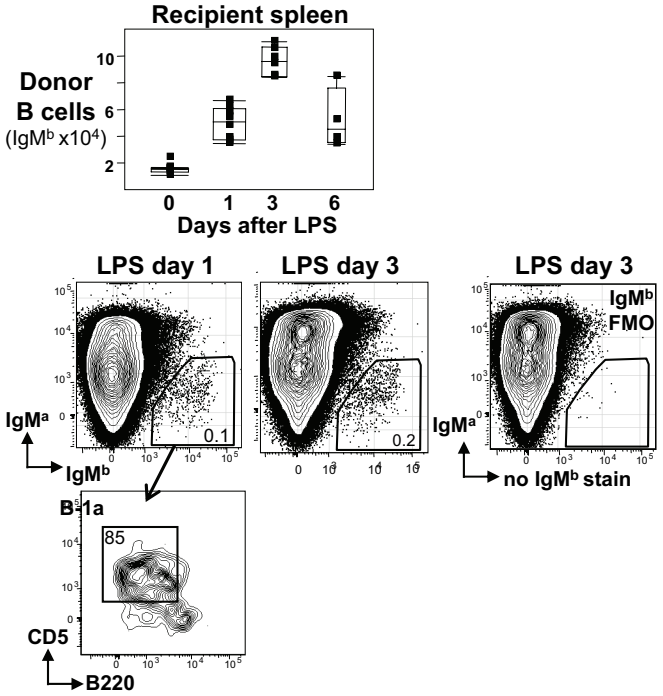

Fig. 3. LPS triggers migration of PerC B-1a cells to the spleen. (*Top*) C.B/17(IgM^b) peritoneal cavity cells (3×10^6) were injected into the peritoneal cavity of congenic BALB/c (IgM^a) mice and the mice were stimulated 2 h later with LPS (see *Methods*). At the indicated times after LPS injection, the absolute numbers of donor (IgM^b) B cells in the recipient spleen by using FACS to measure the frequency of donor B cells in each spleen cell suspension and by using a separate FACS/bead analysis to determine the total number of cells obtained from each spleen. Each dot in the quartile box (Fig. 2 legend) represents the absolute number of donor B cells in the spleen of an individual recipient mouse. (*Left Middle* and *Center Middle*) The gating used to determine the frequency of donor B cells in the recipient spleen (at days 1 and 3 after LPS, in the examples shown). (*Right Middle*) Data for the fluorescence-minus-one, or FMO, control, which reveals the background staining obtained when anti-IgM^b is omitted from the staining combination (see *Methods*). (*Bottom*) Roughly 85% of donor B cells in recipient spleen display the typical B-1a phenotype (CD5$^+$B220^{lo}), with the remaining cells being distributed between the B-1b (CD5-B220^{lo}) phenotype and a phenotype that approximates that expressed by follicular B-2 cells (full data not shown).

the number of donor B cells in PBS-injected recipients. The number of donor B cells peaks at day 3 in the LPS-stimulated recipients and, at all times, >85% of the donor B cells are B-1a (Fig. 3).

CD11b Initially Marks B-1a Cells That Migrate from PerC to the Spleen. Although the integrin CD11b/CD18 (Mac-1) is expressed on most PerC B-1a cells and is usually not found on splenic B-1a cells, IgM^b immigrant B-1a cells in recipient spleens clearly express CD11b 1 day after LPS stimulation. The CD11b is expressed at levels comparable to the typical CD11b levels expressed on B-1a in PerC and remains at this level for 2–3 days, after which it is gradually lost and is detectable only at very low levels 6 days after LPS stimulation (Fig. 4).

In the LPS-stimulated transfer recipients, a proportion of the native (IgM^a) B-1a cells in the spleen show the same CD11b expression kinetics as the donor (IgM^b) B-1a cells. These cells probably originate in PerC and comigrate to spleen with the donor B-1a cells (Fig. 4), because the same pattern of CD11b expression occurs in control LPS-stimulated animals that were not injected with donor cells (data not shown). In contrast, in unstimulated animals, the number of CD11b$^+$ cells is very low, suggesting that there is much less migration in the absence of LPS stimulation (Fig. 4).

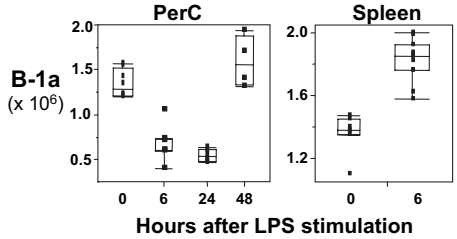

Fig. 2. The absolute number of PerC B-1a cells decreases for 6–24 h after LPS stimulation. The absolute number of cells in the PerC (BALB/c) was determined by injecting medium containing a fixed number of FACS-detectable microspheres per ml and computing the cell number based on the number of cells per microsphere corrected for the volume injected and volume recovered. Central horizontal lines indicate means; horizontal lines in the ''quartile box plots'' indicate the 25th, 50th (median), and 75th percentile values; and extreme horizontal lines indicate 5th and 95th percentile values.

IgM Plasma Cells Derived from B-1a Cells Increase Rapidly and Reach Their Maximum by Day 3 in the Spleen. As we have shown, ≈4% of splenic B-1a cells in unstimulated mice are IgM-producing plasma cells (CD138$^+$). In LPS-stimulated animals, the number of these cells in the spleen increases rapidly. The number of cells is substantially higher 1 day after LPS simulation, increases still further over the next 2–3 days, and starts to decline thereafter (Fig. 5*B*). This time course is somewhat delayed when donor B-1a (IgM^b)

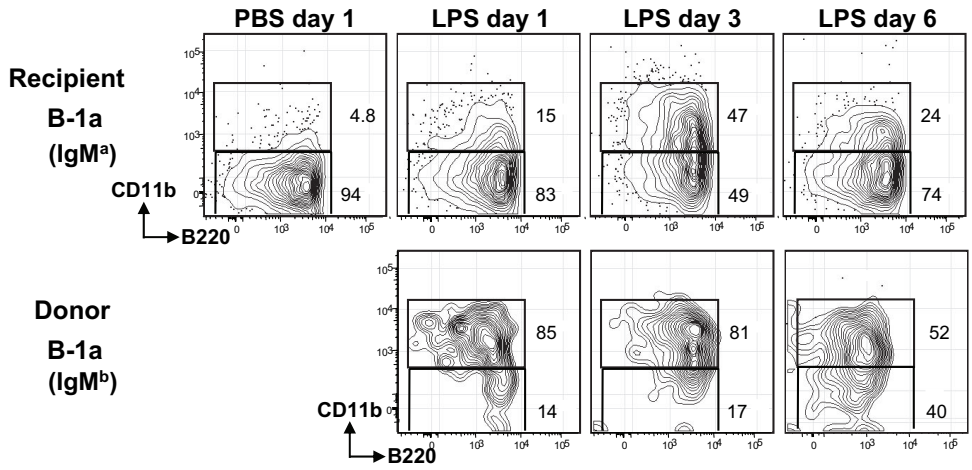

Fig. 4. PerC B-1a cells that migrate into the spleen express CD11b. PerC B-1a cells (IgM^b) injected into recipient PerC continue to express CD11b for several days after arriving in the recipient spleen (*Lower*). Recipient (IgM^a) B-1a cells probably represent comigrating B-1a cells from recipient PerC (*Upper*).

plasma cells in PerC transfer recipients are examined (Fig. 5*C*), suggesting that the initial LPS-stimulated increase in splenic B-1a plasma cells originates either in the spleen or elsewhere in the animal (rather than from PerC immigrants to the spleen).

In these studies, we define B-1a plasma cells as expressing both CD5 and CD138. Recent studies, however, have identified a transcription factor, B lymphocyte-induced maturation protein 1 (Blimp-1), as the principle regulator during plasma cell differentiation (9). Intracellular staining for Blimp-1 (Fig. 5*A*) shows that Blimp-1 is maximally up-regulated from day one onward in $CD138^+$ B-1a cells, confirming that LPS stimulation induces B-1a cells to differentiate to plasma cells.

Interestingly, the intracellular staining method that we use here for Blimp-1 also detects low levels of Blimp-1 in splenic (and PerC) B-1a cells that do not express CD138 (and hence are not plasma cells by either definition). The Blimp-1 levels in B-1a are higher than in B-2 cells (Fig. 5*A*), although they are still substantially below Blimp-1 expression levels in plasma cells. This low-level BLIMP-1 expression in B-1a cells, which we also detect in PerC B-1a cells (data not shown), may have escaped detection by other FACS methods but has been detected by bulk PCR analysis of PerC B-1a cells (10).

B-1a Cells That Migrate from PerC to the Spleen Divide Before/While Differentiating to Plasma Cells. To determine whether B-1a cells that migrate to the spleen divide in LPS-stimulated PerC transfer recipients, we initiated feeding of BrdU to the recipients at the time of transfer, and used high-definition FACS analysis to measure BrdU uptake by B-1a cells in the recipient spleen when the animals were killed. This method is widely used to recognize cells that have divided during the period of BrdU exposure (11–13).

Results from these studies (Fig. 6 *Right*) are quite striking: all B-1a plasma cells derived from the PerC donor incorporate BrdU and hence have divided at least once before, or during, plasma cell differentiation. In contrast, only some of the recipient B-1a plasma cells have incorporated BrdU (Fig. 6 *Left*). These recipient (native) B-1a plasma cells, which have undergone cell division, appear at roughly the same time as the donor B-1a plasma cells that have undergone division and probably represent native comigrants from the PerC.

A Proportion of Resident Splenic B-1a Cells ($CD11b^-$) Differentiate Rapidly to $CD5^+$ Plasma Cells Without Undergoing Cell Division. Importantly, more than half of the native (IgM^a) B-1a plasma cells in the recipient spleen 4 days after LPS stimulation have not incorporate BrdU (Fig. 6 *Left*). Because the number of native $CD5^+$ plasma cells in the spleen at this time is roughly 5- to 10-fold higher than the number of plasma cells in the unstimulated spleen, these findings demonstrate that a large proportion of the native plasma cells in the transfer recipients have differentiated in the absence of cell division.

Studies with "intact" LPS-stimulated animals (not transfer recipients) confirm and extend this conclusion. In essence, virtually none of the plasma cells that appear immediately after LPS stimulation (day 1) have incorporated BrdU (Fig. 7 *Upper*). Therefore, the first plasma cells to develop differentiate without dividing (days 1–1.5, Fig. 7 *Upper*). Because these cells do not express CD11b (Fig. 7 *Lower Right*), whereas the immigrant B-1a plasma cells do express this marker (Fig. 3), we conclude that the earliest appearing B-1a plasma cells derive from B-1a that are resident in the spleen (or possibly arrive from other locations at which CD11b is not expressed on resident B-1 cells).

The number of B-1a plasma cells that have divided in the spleen in the intact animals increases steadily from 1.5 to 3 days (Fig. 7 *Lower Left*). However, the plasma cells that have not divided are still detectable, just as they are in the native B-1a plasma cell population in the transfer recipients (Fig. 6 *Left*).

Discussion

B-1a cells are very important players in both innate and adaptive immunity (1, 14–19). In mice that have not been intentionally immunized or infected, they produce much of the "natural" serum IgM and, in mice stimulated with LPS or other microbial products, they produce much of the polyclonal antibody response (2, 3, 20).

Consistent with these earlier findings, we have shown here that B-1a cells differentiate rapidly to IgM plasma cells in LPS-stimulated animals. We have shown that these plasma cells continue to express CD5 after differentiating to fully mature plasma cells that express high levels of intracellular Blimp-1 and surface CD138. Preliminary studies indicate that CD5 expression begins to decay ≈5 days after LPS stimulation but persists at the normal levels for B-1a cells until this time (Y.Y., J.W.T., Leonard A. Herzenberg, and Leonore A. Herzenberg, unpublished work).

Studies here track the origin of the majority of the $CD5^+$ plasma cells in the spleen to B-1a cells that migrate rapidly from PerC to the spleen after i.v. LPS stimulation. We show that B-1a migrants from PerC express the peritoneal B-1a phenotype as do B-1a cells in PerC, including the expression of CD11b. However, CD11b declines after several days, and the migrants (including their plasma cell progeny) assume the typical $CD11b^-$ splenic

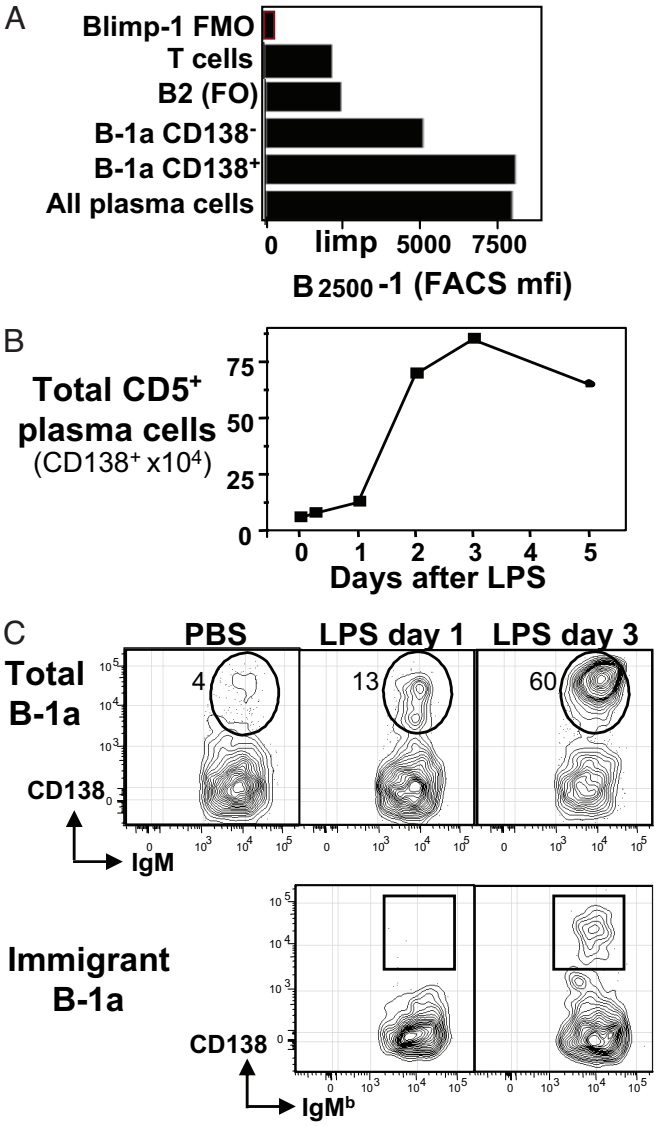

Fig. 5. LPS stimulates differentiation of $CD5^+$ B-1a plasma cells in the spleen. Intracellular Blimp-1, a key marker for identifying plasma cells, is present at the same level in $CD5^+CD138^+$ cells as in all plasma cells detectable in the spleen (*A*). FMO, fluorescence-minus-one; FO, follicular; mfi, median fluorescence intensity. The $CD5^+CD138^+$ plasma cells in the spleen increase with time after LPS stimulation (*B* and *C Upper*) in BALB/c mice. Donor (immigrant) B-1a plasma cells in PerC transfer recipients (see Fig. 3 legend) are also detectable in the recipient spleen, but their appearance is delayed (*C Lower*).

B-1a phenotype. BrdU incorporation studies show that all of the migrant-derived B-1a plasma cells have divided before or during their differentiation to plasma cells.

Interestingly, we have also identified a minority population of B-1a plasma cells that constitutes the first wave of B-1a plasma cells to differentiate and shown that these cells differentiate without undergoing cell division. These plasma cells, which appear 1–1.5 days after LPS stimulation (Fig. 7), are not derived from PerC B cells that migrate to the spleen because (*i*) they do not express CD11b expressed on the migrant-derived plasma cells (Fig. 7); (*ii*) they appear 1–2 days before the B-1a plasma cells derived from migrant B cells appear (Figs. 3); and (*iii*) they do not divide, whereas the plasma cells derived from PerC migrants all undergo cell division before or during differentiation (Fig. 6). Thus, we conclude that the initial wave of plasma cells generated in response to LPS stimulation differentiates

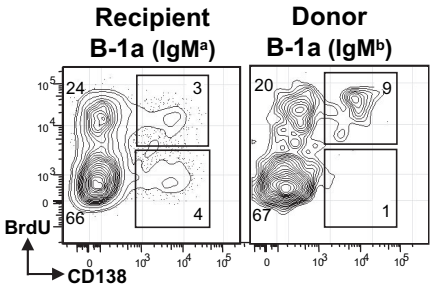

Fig. 6. All donor (immigrant) B-1a plasma cells divide at least once during differentiation. Donor (IgM^b) B-1a plasma cells have all incorporated BrdU (*Right*) in recipients fed with BrdU-containing water, 0.8 mg/ml for 4 days, starting at the time the PerC cells were transferred. Only a proportion of the recipient (IgM^a) B-1a plasma cells in the same animal (*Left*) have incorporated BrdU.

rapidly, without cell division, from B-1a cells that are resident in spleen and are ready to differentiate to antibody-producing cells in response to an appropriate stimulus.

These findings provide a detailed and surprising view of a special niche that evolution has created for B-1a cells. Some time ago, we suggested that the immune system evolved in layers and that, consistent with the idea that ontogeny recapitulates phylogeny, B-1a cells constitute the earliest layer(s) of the system and perform the most basic functions necessary for survival (3, 20–23).

B-1a cells are already well established as key producers of antibodies that provide innate immune protection against pathogen invasion. Our findings here endow them with a rapid response mechanism that "kick-starts" the production of these antibodies. In essence, it enables microbial invasion to trigger B-1a cells resident in the spleen to differentiate immediately to antibody-producing cells, without wasting time or energy on cell division.

This immediate response, as we have shown, overlaps with the recruitment of "reinforcements" that are triggered to migrate to the spleen from the PerC (and possibly elsewhere). These migrants divide and differentiate to create additional antibody-producing cells that quantitatively (and perhaps qualitatively) expand the innate immune response potential. In addition, it is likely that they replenish the ranks of the immediately responding resident B-1a population and ready the individual for the next onslaught.

Materials and Methods

Mice and Tissue Preparation. BALB/c and C.B/17 mice were bred and maintained in our colony at Stanford University. For cell transfer studies, PerC cells were harvested in deficient RPMI medium 1640 without serum as described in ref. 24. For FACS staining, PerC and spleen cells were harvested in serum-containing medium.

FACS Analyses (24). Cell suspensions from each mouse strain were preincubated with anti-CD16/CD32 mAb to block FcγRII/III receptors and stained on ice for 15 min with the following fluorochrome-conjugated antibodies in a 10-color staining combination: FITC-IgM^a (DS-1) PE-CD11b (M1/70); biotin-CD138 (281-2); PE-Cy5.5-CD19 (1D3); PE-Cy7-IgD (11–26); APC-B220 (RA3–6B2); APC-Cy5.5-CD23 (B3B4); APC-Cy7-IgM (331); biotin-IgM^b (AF6–78.25); and PE-Cy5-CD5 (53–7.3). Cells were then washed with staining medium and stained again on ice for 15 min with streptavidin-CyChrome (BD-PharMingen, San Diego, CA) to reveal biotin-coupled antibodies. Finally, the stained cells were resuspended in staining medium containing 10 μg/ml propidium iodide (surface stained cells).

For intracellular staining (Blimp-1, IgM), cells were stained initially with Fixable Live/Dead Dye (Invitrogen, Carlsbad, CA) to identify dead cells, then washed, stained with surface staining

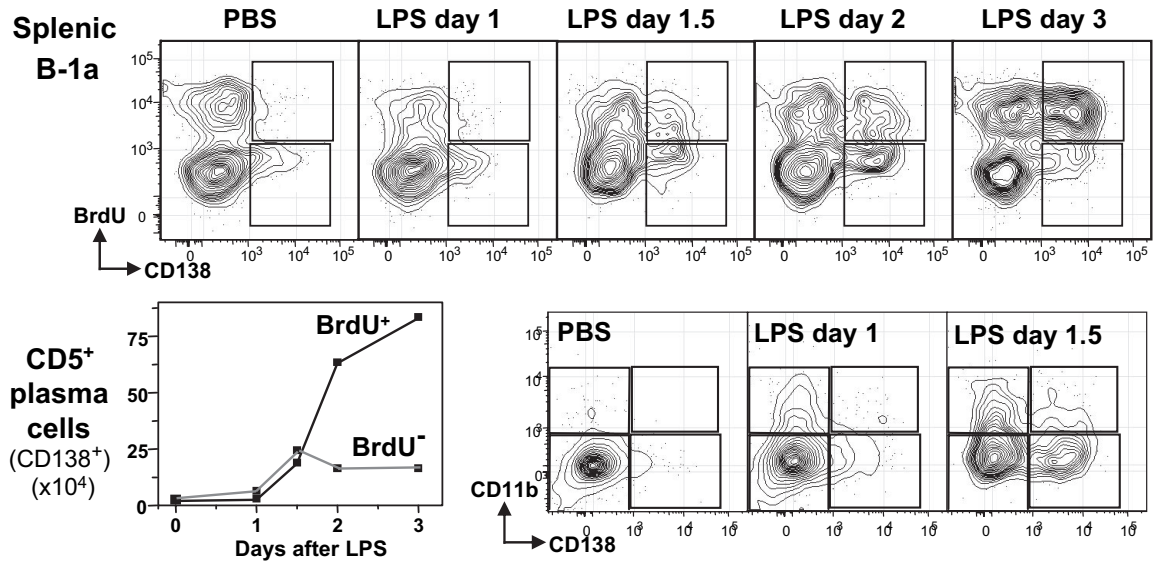

Fig. 7. Resident B-1a plasma cells (CD11b$^-$) differentiate without dividing. Most of the B-1a plasma that appear 1–1.5 days after LPS stimulation in BALB/c mice do not express CD11b (*Lower Right*) and do not incorporate BrdU (*Upper*). B-1a plasma cells that incorporate BrdU appear at the same time but continue to increase (*Lower Left*). These latter cells express CD11b$^+$ (data not shown).

reagents, fixed and permeablized with Becton Dickinson (San Jose, CA) Cytofix/Cytoperm, and finally stained with fluorochrome-coupled monoclonal anti-mouse antibodies: FITC-IgM or QuantumDot-605-Blimp-1 (25, 26). The fluorochrome-conjugated antibodies were either purchased from Invitrogen, Becton Dickinson Biosciences or custom-conjugated in our laboratory. The Becton Dickinson BrdU flow kit was used as directed to stain for BrdU uptake.

Data were collected for 5 hundred thousand to 1 million cells with the Stanford Shared FACS Facility FACSAria (Becton Dickinson). Staining protocols were designed with CytoGenie software (www.ScienceXperts.com) and data were analyzed with FlowJo software (www.TreeStar.com). To distinguish autofluorescent cells from cells expressing low levels of individual surface markers, we determined autofluorescence thresholds from fluorescence-minus-one (FMO) controls (27–29).

Peritoneal Cell Transfer. Peritoneal cells were harvested from C.B/17 donors by injecting 7 ml of serum-free, custom RPMI medium without biotin, L-glutamine, phenol red, riboflavin, and sodium bicarbonate (Invitrogen/GIBCO, Carlsbad, CA) into the PerC, withdrawing as much fluid as possible, and centrifuging to harvest the cells; the cells were then resuspended in 1 ml of serum-free deficient RPMI medium, and 3 million were injected into the PerC of BALB/c recipients.

LPS Injection. Fifteen micrograms of LPS (*Salmonella typhosa*, DIFCO Laboratories, Detroit, MI) was suspended in 200 μl of PBS and injected i.v. via the tail vein. Two hundred microliters of PBS was injected into control mice.

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