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■ PIVOTAL ADVANCE

Pivotal Advance: Peritoneal cavity B-1 B cells have phagocytic and microbicidal capacities and present phagocytosed antigen to CD4$^+$ T cells

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ABSTRACT

Breaking the long-held paradigm that primary B cells are not phagocytic, several studies have demonstrated recently that B cells from fish, amphibians, and reptilians have a significant phagocytic capacity. Whether such capacity has remained conserved in certain mammalian B cell subsets is presently an enigma. Here, we report a previously unrecognized ability of PerC B-1a and B-1b lymphocytes to phagocytose latex beads and bacteria. In contrast, B-2 lymphocytes had an almost negligible ability to internalize these particles. Upon phagocytosis, B-1a and B-1b cells were able to mature their phagosomes into phagolysosomes and displayed the ability to kill internalized bacteria. Importantly, B-1a and B-1b cells effectively present antigen recovered from phagocytosed particles to CD4$^+$ T cells. However, these cells showed a much lower competence to present soluble antigen or antigen from large, noninternalized particles. B-1 B cells presented particulate and soluble antigen to CD4$^+$ T cells more efficiently than macrophages, whereas DCs were the most potent APCs. The novel phagocytic and microbicidal abilities identified in B-1 B lymphocytes strengthen the innate nature that has long been attributed to these cells. In the context of adaptive immunity, we show that these innate immune processes are relevant, as they enable B-1 B cells to present phagocytosable particulate antigen. These capacities position these cells at the crossroads that link innate with adaptive immune processes. In a broader context, these newly identified capacities of B-1 B cells further support the previously recognized functional, developmental, and evolutionary relationships between these cells and macrophages. *J. Leukoc. Biol.* 91: 525–536; 2012.

Introduction

In most animal species, phagocytosis plays an important role in the processes of nutrition, embryogenesis, tissue remodeling, and immunity [1]. In the context of immunity, phagocytosis has evolved from playing a role in innate immunity to having fundamental functions in adaptive immunity [1, 2]. Thus, phagocytosis plays an essential role in the uptake and destruction of microbes, as well as in the initiation and development of an adaptive immune response [2]. It is well-recognized that the process of phagocytosis involves the uptake and internalization of large particles (>0.5 μm), an active process that requires actin polymerization [2]. Upon ingestion, the phagocytosed particle is contained within phagosome vesicles, which fuse with lysosomes, thereby forming phagolysosomes. Lysosomes contain a variety of microbicidal enzymes that are pivotal for the killing and degradation of microbes. Subsequently, degraded microbial antigens can be proteolytically processed by intracellular endopeptidases, and the resulting peptides can then be loaded to MHC II molecules and presented to T cells [3], effectively initiating adaptive immune responses [2].

In mammals, phagocytosis is mainly carried out by cells of myeloid origin, namely the so-called professional phagocytes [4]. These include PMNs, monocytes, and macrophages. Certain cell types, including epithelial cells, fibroblasts, and plasmacytoid DCs, have also been shown to internalize large particles, although with a much lesser efficiency than professional phagocytes [4, 5]. Until recently, it was believed that primary B cells were not able to perform phagocytosis. Changing this

Abbreviations: anti-BCR bead=anti-BCR-coated bead, BM=bone marrow, BMDC=bone marrow-derived DC, IFC=imaging flow cytometry, LAMP1=lysosome-associated membrane protein 1, LB=Luria-Bertani, LEAF=low endotoxin azide-free, MFI=mean fluorescence intensity, MZ=marginal zone, OT-II=B6.Cg-Tg(TcraTcrb)425Cbn/J, OVA-beads=OVA-coated beads, PerC=peritoneal cavity, SN=supernatant, SPL=splenocyte(s)

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paradigm, we have demonstrated that B cells of teleost fish have a high capacity to phagocytose large particles, as well as the ability to kill ingested bacteria [6, 7]. This phagocytic capacity is also displayed by IgM^+ B cells from the amphibian *Xenopus laevis* [6]. The ability of B cells to internalize large particles has been confirmed in several other teleost fish species [8], as well as in reptilians [9]. Hence, it appears that the phagocytic capacity of B cells has remained evolutionarily conserved in several classes of vertebrates, including fish, amphibians, and reptiles. In mammals, however, it is generally accepted that primary B cells are not capable of performing phagocytosis. For example, we and others [5, 6, 10] have shown that murine B cells from blood and BM are not capable of internalizing large, inert particles or bacteria. On the other hand, a number of studies have shown that as opposed to primary B cells, mouse and human malignant B cells are able to phagocytose large particles [11]. In addition, lymphoblastoid cell lines with features of $CD5^+$ B-1 cells and macrophages have been reported to engulf inert particles and bacteria [11]. In the past, the biphenotypic characteristics of these cells pointed to a close developmental and evolutionary relationship between B-1 cells and macrophages [11–13]. A developmental relationship between these two cell types was demonstrated in mammals with the discovery of B/macrophage progenitors in fetal liver [14] and adult BM [15]. From an evolutionary perspective, it has been suggested that B cells could have evolved from macrophages or ancient phagocytic cells [12, 13].

The conservation of the phagocytic function in B cells from several classes of vertebrates, combined with the aforementioned functional, developmental, and evolutionary relationships between B-1 cells and macrophages, prompted us to evaluate the phagocytic capacity of primary murine B cell subsets. Here, we report a previously unforeseen phagocytic and intracellular killing ability of PerC B-1a and B-1b lymphocytes. Significantly for the initiation of adaptive immune responses, we also demonstrate an efficient capability of these cells to present antigen from phagocytosed particles to $CD4^+$ T cells, superior to that of PerC macrophages. These findings uncover novel immune roles of PerC B-1 B cells, which position these cells at the crossroads, linking innate with adaptive immune processes. Moreover, these findings further support the concept that B cells evolved from an ancestral phagocytic predecessor [12, 13].

MATERIALS AND METHODS

Mice

Eight-week-old, naïve C57BL/6 and OT-II mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in the Hill Pavilion Facility at the University of Pennsylvania (Philadlephia, PA, USA). For IFC experiments, 6-week-old, naïve C57BL/6 mice (Charles River, Wilmington, MA, USA) were maintained in a P-2-specific, pathogen-free facility in the Biosciences Animal Services Centre at the University of Alberta (Canada). All animals were maintained in accordance with the guidelines of NIH or the Canadian Council on Animal Care. Experiments were performed in accordance with protocols approved by University of Pennsylvania or University of Alberta Animal Care and Use Committee.

Cell isolation

Spleens were surgically removed, and SPL were isolated by mechanical disruption of the tissue, over a 70-μm cell strainer. Blood was collected by cardiac puncture with a 1-ml heparinized syringe. BM was expelled from femur and tibia using a 1-ml syringe with RPMI (Invitrogen, Carlsbad, CA, USA), and cells were brought to single-cell suspension. PerC cells were obtained by lavage of peritoneum with 10 ml RPMI. Erythrocytes from the different tissues were lysed in ACK buffer (0.15 M NH_4Cl, 10 mM $KHC0_3$, 0.1 mM Na_2EDTA) for 2 min on ice, and the remaining cell pellet was washed three times in RPMI and resuspended in RPMI supplemented with 5% FBS (HyClone, Logan, UT, USA) and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) for further use. Murine BMDCs were obtained by culturing BM cells in RPMI, supplemented with 10% FBS and 10 ng/ml of GM-CSF, replacing one-half of the culture medium with fresh GM-CSF-containing medium at Day 3 of culture. After 5 days culture, cells were analyzed by FACS with anti-CD11c (N418) and anti-$I\text{-}A^b$ (AF6-120.1) antibodies and obtained 75–90% of $CD11c^+$ $I\text{-}A^{b+}$ cells (DCs). To sort the different PerC leukocyte populations, cells were resuspended in PBS solution containing 10% FBS and incubated with LEAF-purified anti-mouse CD16/32 antibody (93; BioLegend, San Diego, CA, USA) on ice for 15 min before staining with a mix of antibodies labeled with FITC (dump): anti-CD3 (17A2), anti-CD11c, anti-F4/80 (CI:A3.1), and anti-CD49b (HMa2) and the Fab fragments of anti-CD5-PE (53-7.3), anti-CD11b-PE/Cy7 (M1/70), anti-CD19-allophycocyanin (6D5), and anti-B220-allophycocyanin/Cy7 (RA3-6B2) antibodies, all purchased from BioLegend. Pierce Fab Micro Preparation Kit was used to obtain the Fab fragments of the aforementioned antibodies following the manufacturer's instructions. Cell populations (B-2 cells: $dump^-/B220^{high}/CD11b^-/CD5/CD19^+$; B-1a cells: $dump^-/B220^{low}/CD11b^+/CD5^+/CD19^+$; B-1b cells: $dump^-/B220^{low}/CD11b^+/CD5^-/CD19^+$; macrophages: $dump^+/B220^-/CD11b^{high}/CD5^-/CD19^-$) were purified by FACS, using a FACSAria cytometer (BD Biosciences, San Jose, CA, USA), and were determined to be >94% pure by reanalysis of surface marker expression. Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

In vitro assessment of phagocytosis

To determine the capacity of phagocytosis of cells obtained from the different tissues, we followed the same strategy used previously by our group [6]. Briefly, SPL, blood, BM, or PerC cells were plated (2×10^5 cells/well) in 96-well plates (Thermo Fisher Scientific Inc., Waltham, MA, USA) in a final volume of 0.1 ml RPMI, supplemented with 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, and incubated for different times, depending of the experiment, in a humidified atmosphere of 5% CO_2 at 37°C with 1 μm fluorescent beads (Fluoresbrite Yellow Green Microspheres, Polysciences, Warrington, PA, USA) at a cell:bead ratio of 1:5, 1:10, or 1:20, depending of the experiment, or with 50 μg pHrodo *Escherichia coli* bioparticles. For inhibition of phagocytosis, cells were treated with different concentrations of cytochalasin D (Sigma-Aldrich, St. Louis, MO, USA) for 30 min before adding beads. After 6 h incubation, noningested beads or bacteria were removed by centrifugation (100 *g* for 10 min at 4°C) over a cushion of 3% BSA (Fisher Scientific) in PBS supplemented with 4.5% D-glucose (Sigma-Aldrich). To block FcRs, cells were incubated with LEAF-purified anti-mouse CD16/32 antibody on ice for 15 min. To analyze phagocytic populations, PerC cells were stained with anti-IgD-Pacific Blue (11-26c.2a), anti-CD5-PE or -FITC, anti-CD19-PE/Cy5, anti-CD11b-PE/Cy7, anti-IgM-allophycocyanin (RMM-1), and anti-B220-allophycocyanin/Cy7. Blood, SPL, and BM cells were stained with anti-IgD-Pacific Blue, anti-CD19-PE/Cy5, anti-CD23-PE/Cy7 (B3B4), anti-IgM-allophycocyanin, and anti-B220-allophycocyanin/Cy7. All antibodies used were purchased from BioLegend. Cells were analyzed on a FACSCanto with DiVa sofware (BD Biosciences). Data were analyzed with FlowJo software (Tree Star). B cell subsets analyzed were derived from: PerC, B-2 cells ($IgD^{high}/B220^{high}/CD11b^-/CD19^+/IgM^{low}$ or $IgD^{high}/B220^{high}/CD11b^-/CD5^-/CD19^+$), B-1 cells ($IgD^{low}/B220^{low}/CD11b^+/CD19^+/IgM^{high}$), B-1a cells ($IgD^{low}/B220^{low}/CD11b^+/CD5^+/C\ D19^+$), and B-1b cells ($IgD^{low}/B220^{low}/CD11b^+/CD5^-/CD19^+$); BM, Pro/Pre-B cells ($CD19^+/CD23^-/B220^+/IgM^-/IgD^-$), immature B cells ($CD19^+/CD23^-/B220^+/IgM^{high}/IgD^{-/low}$), and mature B cells ($CD19^+/CD23^+/B220^+/IgM^+/IgD^{high}$); blood, circulating B cells ($CD19^+/CD23^-/B220^+/IgM^{high}/IgD^{low}$);

and spleen, follicular B cells ($CD19^+/CD23^+/B220^+/IgM^{high}/Ig\ D^{high}$) and MZ B cells ($CD19^+/CD23^-/B220^+/IgM^{high}/IgD^{low}$).

To analyze the percent of internalized versus surface-bound particles on B cells, cells were labeled with anti-mouse CD5-PE (53-7.3) and anti-mouse IgM-allophycocyanin (II/41; BD Biosciences) for 30 min on ice. Cells were then washed twice with 1× PBS and fixed with 1% formaldehyde overnight at 4°C. Data were acquired on an ImageStream multispectral flow cytometer (Amnis, Seattle, WA, USA). B cell subsets analyzed were: B-1a ($CD5^+/IgM^{high}$), B-1b ($CD5^-/IgM^{high}$), and B-2 ($CD5^-/IgM^{low}$). At least 20,000 events were acquired/sample. Using a modified version of Ideas 4.0 software [16], a mask was created to identify the cellular membrane. This masking strategy allowed differentiation between internalized (degree of internalization >0) and bound (degree of internalization <0) beads or bacteria in the *x–y* axis.

Influence of BCR in phagocytosis of particles by B-1B cells and macrophages

To obtain anti-BCR beads, fluorescent (FITC) latex beads (1 μm, Polysciences) were incubated in PBS buffer with $F(ab)_2$ fragments of the mouse anti-IgM antibody (Novus Biologicals, Littleton, CO, USA) overnight at 4°C. To analyze the influence of the BCR in the uptake of beads by B-1B cells and macrophages, PerC cells were preincubated with anti-IgM $F(ab)_2$ for 30 min before adding plain beads or anti-BCR beads. After 6 h of incubation, PerC cells were processed, stained with anti-B220, anti-CD19, anti-CD11b, and anti-CD5, and analyzed by flow cytometry to assess phagocytosis, as described in the above section.

In vivo phagocytosis assay

Individual mice were injected i.p. with pHrodo *E. coli* bioparticles (500 μg) or fluorescent 1 μm beads (2×10^9) in 300 μl PBS. Control mice were injected with 300 μl PBS. After 3 h, PerC cells were isolated and labeled as described above. Cells were acquired on a FACSCanto with DiVa software, and posterior analysis was done with FlowJo software.

TEM

For TEM analysis, sorted phagocytic PerC cells were fixed with 2.5% (vol/vol) glutaraldehyde in PBS, pH 7.4, and processed and analyzed as described previously [6] at the Biological Imaging Core Laboratory of the University of Pennsylvania.

Phagolysosome formation assays

To study the formation of phagolysosomes after particle ingestion, PerC cells were loaded with 0.25 μg Dextran-FITC (Molecular Probes, Eugene, OR, USA) for 45 min. Cells were washed twice in incomplete media (no serum), followed by incubation in serum-containing media for 45 min to allow Dextran localization to the lysosomes. After this, "dextran-loaded" cells were incubated for 3 h with 1- or 2-μm nonfluorescent latex beads (Polysciences) at a 5:1 ratio (bead:cell). Following phagocytosis, cells were washed with $PBS^{-/-}$ and stained with CD5-PE and IgM-allophycocyanin, as described before. Cells were then washed, fixed for 10 min on ice with 1% formaldehyde, and analyzed with an ImageStream multispectral flow cytometer. Alternatively, sorted PerC cells were incubated for 6 h with nonfluorescent, 1 μm beads and fixed with 4% paraformaldehyde. After washing, cells were permeabilized with 1% saponin solution, and lysosomes were stained with anti-mouse LAMP1-Alexa Fluor 488 (1D4B, BioLegend) in 1% saponin buffer. Nuclei were stained with a solution of 100 ng/ml DAPI (Invitrogen) for 1 min at room temperature. Cells were examined by fluorescence microscopy using a Nikon Eclipse E600 microscope with iVision software.

Intracellular bacterial killing assays

To assess the intracellular bacterial killing capacity of phagocytic B cells, we used the gentamicin protection assay, as described previously [6]. Briefly, viable *E. coli* (5×10^6 CFU/well) were incubated with sorted PerC B cell subsets (5×10^5 cells/well) in 96-well plates for 2 h at 37°C in 5% CO_2. Nonadherent bacteria were removed via centrifugation (100 *g* for 10 min at 4°C) over a cushion of 3% BSA in PBS, supplemented with 4.5% D-glucose. Pelleted cells were resuspended in sterile RPMI medium, supplemented with 100 μg/ml gentamicin (Gibco) and incubated for 1 h at 37°C in 5% CO_2 to kill noninternalized bacteria. Cells were washed three times with PBS and resuspended in RPMI with 10 μg/ml gentamicin. Thereafter, aliquots of cells were pelleted after 0, 1.5, 3, and 6 h incubation and lysed with 0.9-ml sterilized water. Lysates were serially diluted, spread onto LB agar plates, and incubated at 37°C for 24 h. Single colonies (CFU) on the agar plates were counted.

Antigen presentation assays

Latex beads (1 μm and 6 μm) were used to prepare OVA-beads, as described elsewhere [17]. Briefly, 10^{10} latex beads were conjugated with OVA by incubation with 5 mg/ml OVA (Sigma-Aldrich) in citrate buffer (pH 4.2) at 4°C for 48 h. The amount of OVA adsorbed on the beads was determined by SDS-PAGE. To control and detect whether OVA bound to the bead could detach from them over time, the same amount of OVA-beads used for loading APCs was washed three times with RPMI and plated in a 96-well plate. After 3 days, SN was collected and was used as a source of antigen.

As APCs, we used SPL, BMDCs, sorted PerC B cells, or sorted PerC macrophages, which were subsequently loaded with varying amounts of OVA-beads, soluble OVA, or SN from OVA-beads. For loading the cells with the aforementioned forms of antigen, purified leukocytes were resuspended in RPMI, supplemented with 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, plated in round-bottom, 96-well plates (Nunc), and incubated overnight (5% CO_2 atmosphere at 37°C) with: 1 or 6 μm OVA-beads, 1 or 6 μm plain beads, SN from OVA-beads, or soluble OVA. For antigen-presentation inhibition assays, B cells or SPL were treated with chloroquine (Sigma-Aldrich) for 30 min before adding beads. Thereafter, the APCs prepared above were incubated with $CD4^+$ T cells purified from SPL of OT-II mice [18]. SPL were obtained as described above. Single-cell suspensions of $CD4^+$ T cells were purified using the Isolation Kit II (Miltenyi Biotec, Auburn, CA, USA), following the protocol provided by the manufacturer. Cell preparations contained >94% of $CD4^+$ T cells, as determined by flow cytometry, using anti-CD4-allophycocyanin/Cy7 (GK1.5, BioLegend). Purified $CD4^+$ T cells were then washed with PBS three times and labeled with 5 μM CFSE (Invitrogen) for 9 min at room temperature. Reaction was stopped immediately by adding PBS, supplemented with 10% FBS. Thereafter, CFSE-labeled $CD4^+$ T cells were cocultured for 3 days with purified PerC B cell subsets, PerC macrophages, and BMDCs, previously loaded with OVA-beads, plain beads, soluble OVA, or SN from OVA-beads, using a B cell:T cell ratio of 1:1 or 1:2, depending on the experiment. Viability of APCs was monitored with trypan blue at Days 2 and 3 after incubation with beads. For MHC II blocking assays, LEAF-purified anti-mouse I-A/I-E antibody (M5/114.15.2, BioLegend) was added to the well containing the APCs, 30 min before establishing the coculture. Expression of MHC II and costimulatory molecules (CD80 and CD86) was analyzed by FACS in B-1 B cell subsets after 3 days incubation with OVA-beads using anti-MHC II-PerCP-Cy5.5 (M5/114.15.2), anti-CD80-allophycocyanin (16-10A1), and anti-CD86-allophycocyanin (GL-1) antibodies (BioLegend).

To evaluate the capacities of the various loaded PerC B cell subsets, PerC macrophages, and BMDCs to present antigen to $CD4^+$ T cells, we measured proliferation of OVA-specific, CFSE-labeled OT-II $CD4^+$ T cells and IL-2 production by activated OT-II T cells after 3 days in coculture with the used APCs. Proliferation of $CD4^+$ T cells was assessed by flow cytometry. $CD4^+$ T cells were stained with anti-CD4-allophycocyanin/Cy7 and anti-CD3-PE-Cy7, and the decrease in CFSE fluorescence was analyzed for this population using FlowJo software. IL-2 levels in SN were determined by ELISA with the Legend Max ELISA kit for IL-2 with precoated plates (BioLegend), following the manufacturer's instructions.

RESULTS

Peritoneal B-1 B cells are phagocytic

We and others have shown that B cells from fish [6–8], amphibians [6], and reptilians [9] are capable of internalizing large, inert particles and bacteria. To determine whether this phagocytic ability has remained conserved in subsets of mammalian B cells, we analyzed this capacity in different B cell populations of lymphoid tissues and fluids from mice. To this end, blood, BM, spleen, and PerC cells were incubated with fluorescent particles (1 μm latex beads or pHrodo *E. coli* bioparticles), and phagocytosis was assessed as described previously [6]. We found that in blood, BM, and spleen, the percentage of phagocytic B cells was smaller than 1.6% of all B cells (**Fig. 1A** and **B**). In contrast, in the PerC, a significant percentage (11–14%) of B-1 B cells was phagocytic (Fig. 1A and B). Thus, our subsequent efforts to characterize further features of phagocytic B cells focused on the PerC B-1 B cells, which in the mouse, represent a significant portion (30–40%) of all PerC leukocytes.

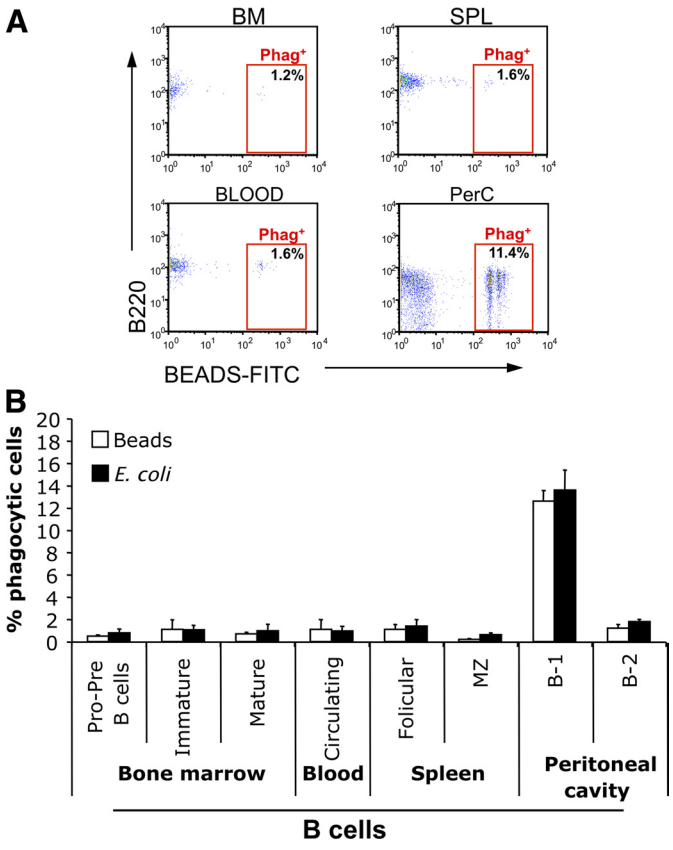

Figure 1. Phagocytic B cells in mouse tissues. (A) Flow cytometry of BM, spleen (SPL), blood, and PerC cells incubated with fluorescent (FITC), 1 μm beads and then stained with anti-B220 and anti-CD19. Dot plots show the gated population of CD19$^+$ cells. Phag$^+$, Phagocytic. Data are representative of four independent experiments (n=6). (B) Percentage of phagocytic B cells in BM, spleen, blood, and PerC. Cells were incubated with fluorescent, 1 μm beads or pHrodo *E. coli* bioparticles and then stained with anti-B220, anti-CD19, anti-IgD, anti-IgM, and anti-CD11b (PerC) or anti-CD23 (BM, spleen, and blood). Results are expressed as mean ± SEM of six mice from two independent experiments.

A more in-depth analysis of the in vitro phagocytic capacities of PerC leukocytes revealed that 9–17% of B-1a and B-1b lymphocytes were phagocytic (**Fig. 2A** and **B**). It is worth noting that the B-1a subset had a significantly higher percentage of phagocytic cells (14% and 17% of the cells internalized latex beads and *E. coli*, respectively) when compared with the B-1b cell subset (8.6% and 11.4%, respectively). In contrast, <1.5% of the B-2 cells had the capacity to internalize the same particles (Fig. 2A and B). Analysis of phagocytosis with several cell: bead ratios showed that higher ratios correlated with a larger number of phagocytic cells in all analyzed populations, except for the B-2 subset (Supplemental Fig. 1A). Moreover, there was a positive correlation between incubation time and the percentage of phagocytic cells, except in B-2 B cells (Supplemental Fig. 1B). In terms of the number of ingested beads/cell, it can be observed that with regards to the B-1 B cell subsets, higher cell:bead ratios resulted in an increased number of beads ingested/cell. More specifically, the increase was seen mostly in the number of cells ingesting three or four beads, especially in the B-1a B cell subset (Supplemental Fig. 1C). As expected, T cells were nonphagocytic (Fig. 2A), whereas a high percentage of CD11b^{high} non-B cells internalized both particles (Fig. 2A and Supplemental Fig. 2A). In mice, PerC CD11b^{high} non-B cells are known to be, for the most part, large macrophages, although a minor subset of small macrophages has been identified recently within the CD11b^{high} non-B cell population [19]. Experiments of phagocytosis in vivo with fluorescent beads and *E. coli* bioparticles corroborated the aforementioned in vitro data obtained within the B cell subsets. Thus, 3 h after bead or *E. coli* injection in the PerC, 10–15% of B-1a and B-1b cells had internalized these particles, whereas <2.5% of the B-2 B cells were phagocytic (Fig. 2C). Cytochalasin D inhibited the phagocytic capacity of murine B-1 B cells in a dose-dependent manner, indicating that the uptake and engulfment of particles involved an active process of actin polymerization and reorganization (Fig. 2D). Preincubation of B-1 B cells and macrophages with anti-BCR antibodies did not inhibit their phagocytic capacity, indicating that uptake of the tested particles was not mediated by the BCR (Supplemental Fig. 3).

The use of pHrodo bioparticles, which emit fluorescence upon acidification of only ingested particles (acidification occurs within maturing phagosomes), indicated that *E. coli* was being internalized rather than remaining attached to the surface of the cells. To confirm that the inert particles used here (FITC-latex beads) were also internalized by the phagocytic B cells, we used IFC, which allowed us to evaluate the percentage of internalized versus surface-bound beads in the FITC-associated leukocyte populations. Using this technology, we could image each acquired event and assess bead spatial distribution (Fig. 2E and Supplemental Fig. 2B). IFC yielded a similar percentage of FITC-associated B-1a, B-1b, and B-2 cells as that obtained by conventional flow cytometry (data not shown). From the analysis with Ideas software, it could be determined that >85% of beads in the FITC-associated PerC B-1 B cells were internalized, whereas >40% of the beads were surface-bound in the B-2 subset (Fig. 2F). In large, non-B cells, >90% of the beads were found to be internalized using IFC

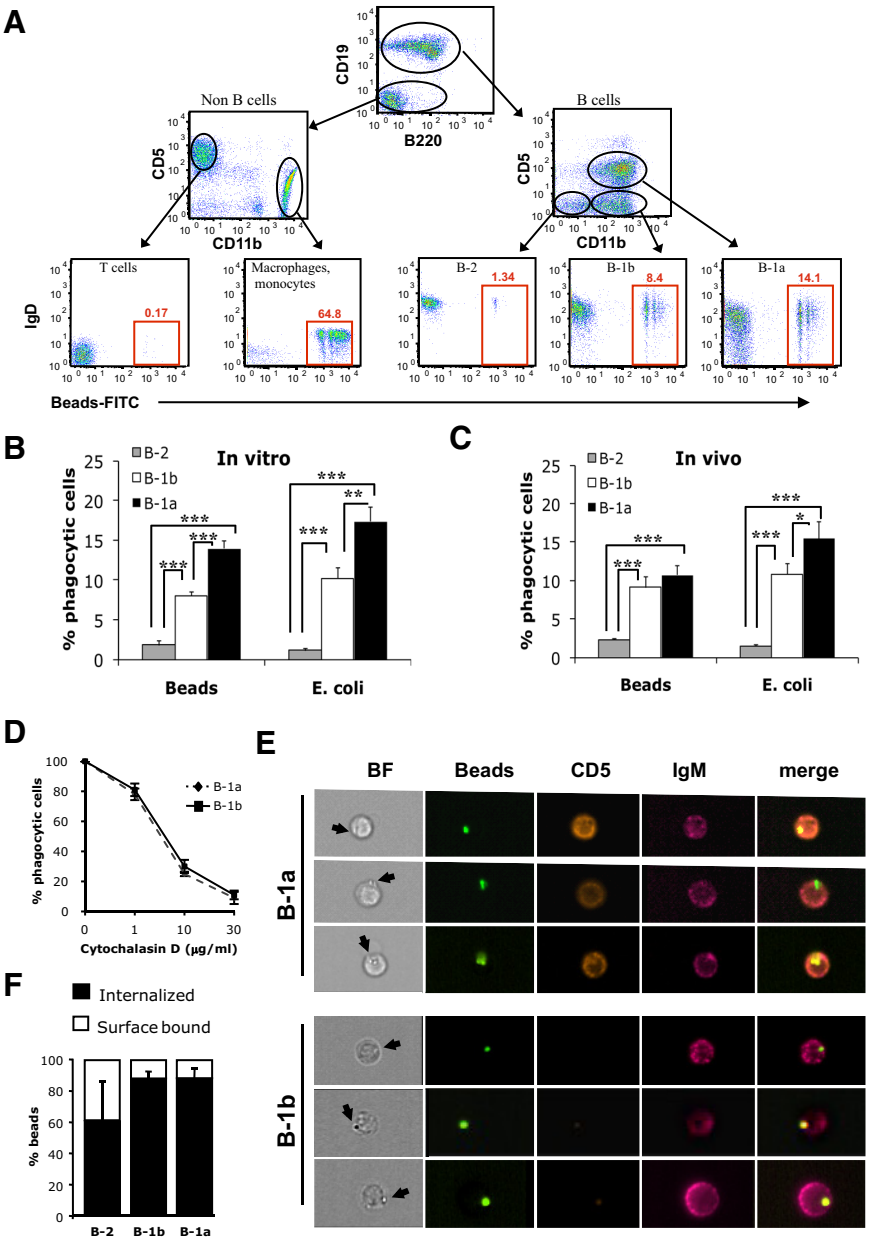

Figure 2. Phagocytosis by mouse PerC cells. (A) Flow cytometry of PerC cells incubated in vitro with fluorescent, 1 μm beads and then stained with anti-B220, anti-CD19, anti-CD11b, anti-CD5, and anti-IgD antibodies. Percentages of phagocytic cells are shown in red. (B) Percentage of phagocytic cells in PerC B cells incubated in vitro with fluorescent, 1 μm beads or pHrodo *E. coli* bioparticles, stained with the aforementioned antibodies (A), and analyzed by flow cytometry. Results are mean ± SEM of 10 mice from four independent experiments. (C) In vivo analysis of phagocytosis by PerC B cells. Mice were injected i.p. with 1 μm beads or pHrodo *E. coli* bioparticles. After 3 h, PerC cells were collected, stained with the aforementioned antibodies (A), and analyzed by flow cytometry. Results are presented as percentage of phagocytic cells and are mean ± SEM from three independent experiments (n=5). (B and C) Statistical differences were determined by unpaired Student's t test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (D) PerC cells were incubated with fluorescent, 1 μm beads in the presence of various concentrations of cytochalasin D and stained with the aforementioned antibodies (A). Results are presented as the percentage of phagocytic cells relative to that of PBS-treated controls. Results are expressed as mean ± SEM of four independent experiments (pool of cells from three mice/experiment). (E) Representative IFC images of PerC B-1 B cells incubated with fluorescent, 1 μm beads, stained with anti-IgM and anti-CD5, and acquired using an ImageStream multispectral flow cytometer. Black arrows point to internalized beads. BF, Bright field. (F) Percentage of internalized versus surface-bound beads in FITC-associated B cell subsets. Data were generated using Ideas software. Results are expressed as mean ± SEM of five independent experiments (pool of cells from five mice/experiment).

(Supplemental Fig. 2C). Internalization of beads by B-1a and B-1b was further confirmed by TEM (**Fig. 3**), which was also instrumental to examine the ultrastructural features of phagocytic PerC B-1 B cells. Similar to phagocytic B cells of fish and frogs [6], murine B-1a and B-1b cells with internalized beads were characterized by having a large nucleus, a thin agranular cytoplasm with a substantial number of mitochondria, and a considerable number of small dendrites surrounding the cell (Fig. 3). The large number of mitochondria and small dendrites observed is in agreement with a previous study reporting the ultrastructural features of murine B-1a and B-1b cells [20]. In the obtained TEM images, we could detect a large number of B-1a or B-1b cells with one to three beads internalized and less frequently, with four or more beads, corroborating data shown in Supplemental Fig. 1C. In contrast, CD11b^{high} non-B cells could often be found with >10–20 beads (Supplemental Fig. 2D–F). In PerC B-1 B cells, engulfment of beads was usually carried out by small, pseudopodia-like structures (Supplemental Fig. 4), as we have also reported for phagocytic B cells of fish and amphibians [6, 7].

Phagolysosome formation and bacterial killing by PerC B-1 B cells

Phagocytosis of particles by professional phagocytes results in the fusion of the phagosome containing the internalized particle with lysosomes, leading to the formation of phagolysosomes [2]. Enzymes contained within the lysosomes have microbicidal activities and are capable of killing ingested bacteria. Phagosomal acidification is known to occur during the process of phagosome maturation and phagolysosome forma-

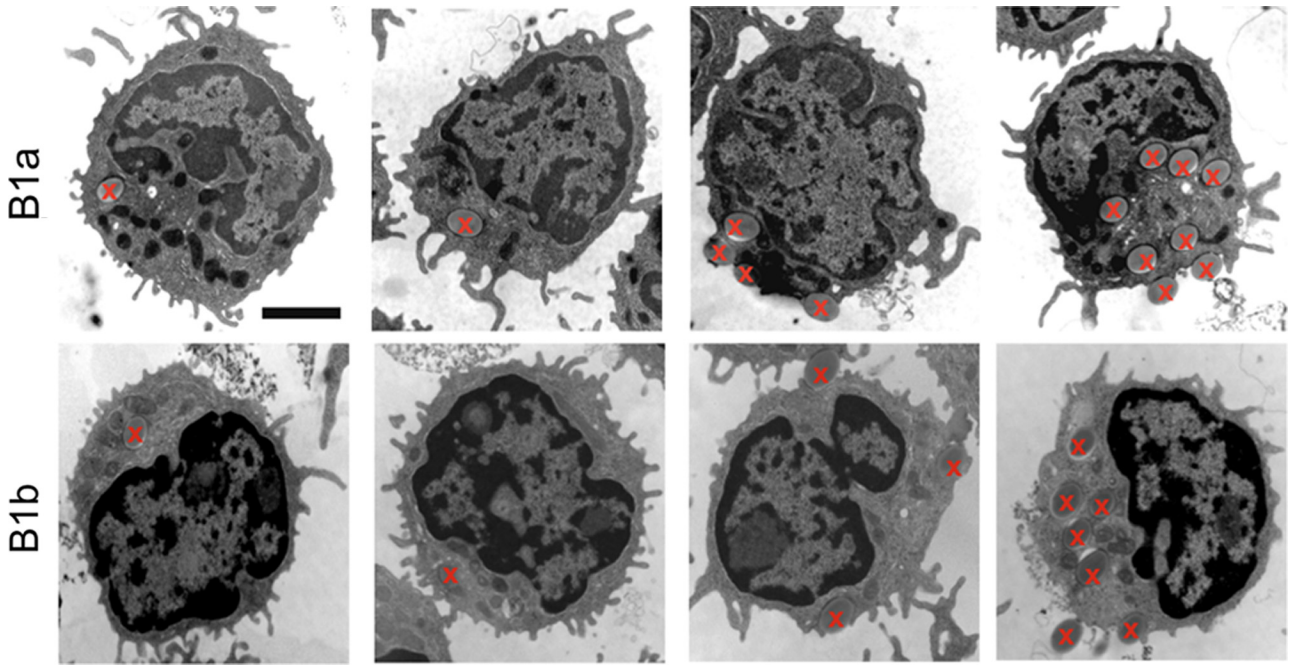

Figure 3. TEM analysis of phagocytic PerC B-1 B cells. TEM of PerC B-1 B cells incubated with fluorescent, 1 μm beads (red "X"). Phagocytic B-1a (upper) and B-1b (lower) cells were sorted, fixed, processed, and analyzed by TEM. Original scale bar, 2 μm. Original magnification, 12,000×. Data are representative of two independent experiments (pool of cells sorted from six mice/experiment).

tion [2]. Thus, internalization of pHrodo *E. coli* bioparticles by PerC B-1 B cells strongly suggested maturation of phagosomes and fusion with lysosomes within these cells (Fig. 2B and C). Here, we have obtained direct evidence of phagolysosome formation through the staining of phagocytic B-1 B cells with LAMP1 and fluorescent dextran-FITC. We observed that in the absence of beads, LAMP1 staining was localized within the cytoplasm of B-1a and B-1b lymphocytes (**Fig. 4A**, left panels, for B-1a and B-1b), thus pointing to the presence of lysosome vesicles in these cells. In phagocytic B-1a and B-1b cells, LAMP1 staining was seen partially or completely colocalizing with internalized beads, thus indicating fusion of lysosomes to the phagosome (Fig. 4A, center and right panels). Dextran staining patterns in phagocytic B-1a and B-1b cells (Fig. 4B) were very similar to those obtained with LAMP1, thus showing colocalization of dextran-FITC with internalized 1 μm (Fig. 4B, center rows) or 2 μm beads (Fig. 4B, bottom rows). Dextran-loaded cells without beads are shown in Fig. 4B, upper rows. As seen in Fig. 4, no differences were seen in phagolysosome formation between the two PerC B-1 B cell subsets. However, PerC B-1 B cells presented lower intensity in Dextran-FITC and LAMP1 staining compared with CD11b^{high} non-B cells (Supplemental Fig. 2E and F), probably as lysosomes in macrophages and monocytes are more numerous than in B-1 B cells.

Theses data demonstrate that B-1a and B-1b cells undergo phagolysosome fusion upon phagocytosis of particles. This suggested that these cells might have the capacity to kill ingested microbes, as lysosomes are rich in microbicidal enzymes. To test this hypothesis, we performed standard gentamicin protection assays, as described previously [6]. After internalization, bacteria could survive inside the PerC B-1 B cells for at least the first 8 h, after which, there was a marked decrease in the number of viable bacteria (Fig. 4C). After 18 h, ~80% of the internalized bacteria were killed by B-1a and B-1b lymphocytes. As B-2 cells internalized very few or no bacteria, they showed a basal level of CFUs in the same assay. CD11b^{high} non-B cells had significantly faster kinetics of bacterial killing when compared with B-1 B cells, and after 8 h, >80% of internalized bacteria had been eliminated (Supplemental Fig. 2G).

B-1a and B-1b lymphocytes present phagocytosed antigen to CD4$^+$ T cells

Phagocytosis is regarded as one of the main mechanisms for antigen uptake and presentation by macrophages and DCs [3]. We hypothesized that phagocytic B-1 B cells would have the capacity to present internalized particulate antigen through the MHC II pathway. To test this hypothesis, we used the OVA antigen model. OVA-responsive, transgenic CD4$^+$ T cells obtained from OT-II transgenic mice [18] were used to evaluate the antigen-presenting capacity PerC B cells loaded with OVA-beads or soluble OVA. First, we analyzed antigen-presenting capabilities of the PerC B-1 B cell using SPL as positive control. When B-1a and B-1b cells were loaded with 1 μm OVA-beads (equivalent to 1 μg OVA) and used as APCs, T cells proliferated significantly (**Fig. 5A** and **B**, left) and produced substantial amounts of IL-2 (Fig. 5C, left). The magnitude of the B-1a- and B-1b-induced T cell responses was similar to that elicited by the SPL. In contrast, when B-1a and B-1b were loaded with 1 μm plain beads (without OVA), T cell responses were nonexistent, thus indicating that such responses were in-

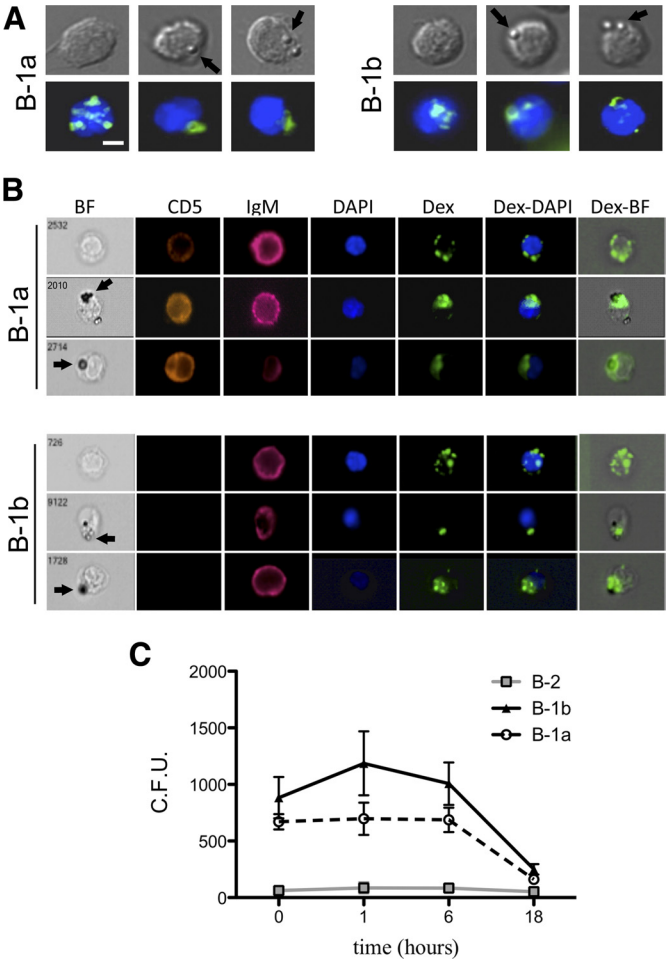

Figure 4. Phagolysosome formation and intracellular bacterial killing by phagocytic B-1 B cells. (A) DIC images (upper) and fluorescence microscopy (lower) of sorted B-1a and B-1b cells incubated with nonfluorescent, 1 μm beads, fixed and stained with anti-mouse LAMP1 (green). Nuclei were stained with DAPI (blue). Left rows are cells without ingested beads, whereas center and right rows show cells with ingested beads. LAMP1 is seen colocalizing with ingested beads (black arrows in DIC images point to internalized beads). Original scale bar, 2 μm. Original magnification, 1000×. (B) IFC images of PerC B-1a and B-1b cells loaded with fluorescent dextran (green) and incubated with nonfluorescent 1 μm or 2 μm beads. Cells were incubated with DAPI for visualization of nuclei (blue). Upper rows are cells without ingested beads, whereas middle and bottom rows show cells with ingested 1 μm and 2 μm beads, respectively. Dextran is seen colocalizing with ingested beads (black arrows in bright field images point to internalized beads). (A and B) Representative of three independent experiments (pool of cells from six mice/experiment). (C) Intracellular bacterial killing by sorted PerC B cell subsets incubated with live *E. coli*; attached bacteria were killed by incubation with gentamicin, after which, leukocytes were lysed at different time-points (time, horizontal axis). Live bacteria in lysates were counted after inoculation on LB agar plates. Results are expressed as mean ± SEM of four independent experiments (pool of cells from at least 10 mice/experiment).

duced by OVA coated onto the 1 μm beads (Fig. 5B and C, left). The possibility existed that T cell responses were induced by soluble OVA leaking from the beads throughout the 3-day coculture period of APCs with T cells. To test this, parallel experiments were carried out using SN from OVA-beads that had been incubated alone for 72 h in culture medium. This SN was then used as the source of antigen, and it was observed that it did not induce T cell responses (Fig. 5B and C, left). This indicated that the OVA-induced responses were exerted by OVA coated to beads. Supporting this data, we observed an increase in MHC II and CD86 surface expression in B-1a (MFI: 71.5±4.7 and 62.7±5.9, respectively) and B-1b (MFI: 90.2±0.3 and 81.7±3.5, respectively) after phagocytosis (Supplemental Fig. 5). No difference in expression of CD80 was observed.

We next asked whether OVA was acquired by B-1a and B-1b cells from internalized OVA-beads or from attached but noninternalized OVA-beads. In this regard, it has been shown that B cells have the capacity to extract antigen from large, noninternalized particles or surfaces in a BCR-mediated manner with subsequent presentation of these antigens to T cells [21]. Thus, to rule out the possibility that B-1 B cells might extract and present OVA from attached beads, we tested the ability of B-1a and B-1b cells to present OVA to $CD4^+$ T cells after their incubation with 6 μm OVA-beads. These beads are too large (almost as big as B-1 B cells) to be internalized by PerC B-1 B cells (data not shown). Loading of PerC B-1 B cells with these large OVA-beads induced very small T cell responses (Fig. 5B and C, right) when compared with 1 μm OVA-beads, thus indicating that internalization of the OVA-beads was a requirement to produce substantial T cell responses. In contrast to PerC B-1 B cells, B-2 cells that had been incubated with 1 μm OVA-beads induced very minor T cell responses (Fig. 5B and C, left), a result that was correlated with the very little capacity of these cells to internalize 1 μm latex beads (Fig. 2B, C, and F). As expected, B-2 cells incubated with 6 μm OVA-beads induced negligible T cell responses (Fig. 5B and C, right). During the performance of the aforementioned antigen-presentation experiments, no major differences in cell viability were found among the three PerC B cell populations.

All aforementioned T cell responses induced by phagocytic B cells could be inhibited when assays were carried out in the presence of anti-MHC II antibody, thus demonstrating that such responses were mediated through the MHC II pathway (Fig. 5D and E). In addition, T cell responses could be inhibited by incubation of the APCs with chloroquine, indicating that the presentation process required intracellular endosomal proteolysis of OVA (Fig. 5D and E).

Capacity of PerC B cells, PerC macrophages, and BMDCs to present soluble and particulate (phagocytosed) antigen to $CD4^+$ T cells

It is well-known that B cells can proficiently present soluble antigen to $CD4^+$ T cells [22, 23], and they can extract and present antigen from noninternalized large particles in a BCR-dependent manner [21]. To determine whether phagocytosed particulate antigen is more efficiently presented than soluble antigen and to test for possible differences in the antigen-presenting capacity among PerC B cells, PerC macrophages, and BMDCs, we tested the ability of these cells to present soluble and particulate antigen to $CD4^+$ T cells.

When OVA was coated to 1 μm latex, B-1 B cells and BMDCs required 100 times lower concentration of OVA (as

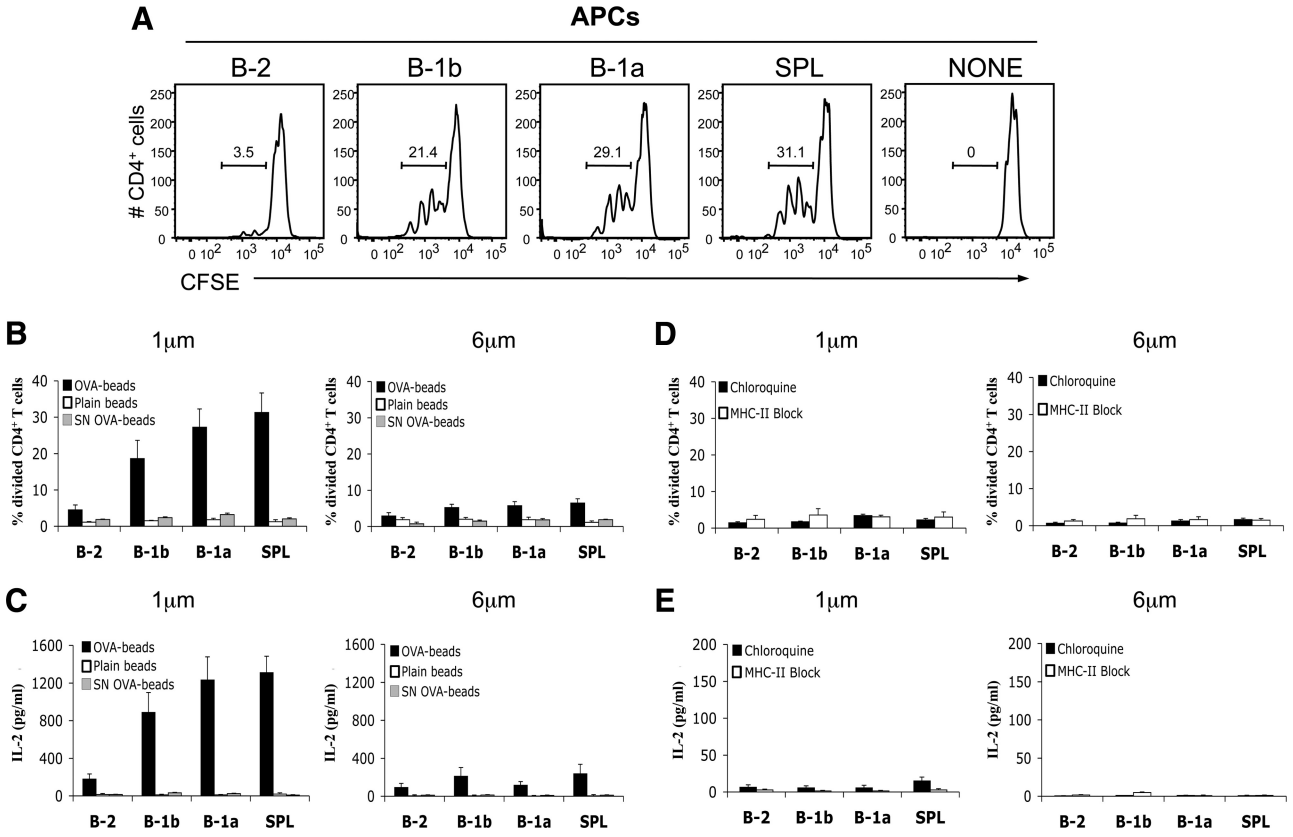

Figure 5. Antigen presentation by PerC B cells loaded with OVA-beads. (A) Representative histograms of $CD4^+$ T cell proliferation (percentage of CFSE dilution) after 3 days in coculture with sorted PerC B cell subsets or SPL preincubated for 12 h with 1 μm OVA-beads (1 μg OVA/well). Percentage of proliferative $CD4^+$ T cells is shown in each histogram. "NONE" panel represents T cells incubated with 1 μm OVA-beads without APCs. (B and C) Sorted PerC B cells and SPL were incubated for 12 h with 1 μm (left) or 6 μm (right) OVA-beads (1 μg OVA/well), plain beads, or OVA-beads SN and then cocultured with CFSE-labeled OT-II $CD4^+$ T cells for 3 days at a 1:2 ratio. Percentage of divided $CD4^+$ T cells (B) and IL-2 secreted by $CD4^+$ T cells (C) was measured by FACS and ELISA, respectively. (D and E) Sorted PerC B cells and SPL were incubated with 1 μm (left) or 6 μm (right) OVA-beads in the presence of chloroquine or anti-mouse I-A/I-E (MHC II block) prior to coculture with OT-II $CD4^+$ T cells for 3 days. Percentage of divided $CD4^+$ T cells (D) and IL-2 secreted by $CD4^+$ T cells (E) was measured (as described for B and C, respectively). Results are expressed as mean ± SEM of at least four independent experiments (pool of cells from 10 mice/experiment).

opposed to using soluble OVA) to induce proliferative $CD4^+$ T cells and IL-2 responses (**Fig. 6A** and **B**). Antigen presentation by PerC macrophages was also more efficient when antigen was in particulate form, although importantly, $CD4^+$ T cell responses induced by macrophages were approximately tenfold lower than those of B-1 B cells. On the other hand, BMDCs were the most potent APCs tested for soluble and particulate antigen (Fig. 6A and B). On the other end, B-2 B cells induced the lowest $CD4^+$ T cell responses, and no differences were seen when using soluble or particulate antigen. This is probably a result of the fact that B-2 B cells have an almost negligible capacity to internalize beads, as shown in Fig. 2. B-1a cells were more efficient presenting particulate antigen than B-1b cells (Fig. 6A and B), probably as a result of their greater phagocytic capacity when compared with B-1b cells (Fig. 2 B). However, the capacity of both B-1 B cell subsets to present soluble OVA was similar to that of B-2 B cells and macrophages (Fig. 6A and B).

DISCUSSION

In 2006, we showed [6] that B cells from fish and amphibians had the capacity to internalize inert particles and bacteria. This capacity was seen thereafter in other fish [6–8], as well as in reptiles [9]. In this study, we have demonstrated a previously unrecognized ability of primary PerC B-1a and B-1b lymphocytes to phagocytose large particles. In contrast to the PerC, the other lymphoid tissues analyzed contained negligible numbers of phagocytic B cells, probably because of the extremely low number of B-1 cells in these sites. B-1 B cells were able to ingest a substantially smaller number of particles when compared with macrophages, probably as a result of their smaller size. However, similar to macrophages, they were able to uptake particles through the formation of pseudopodia-like structures. Phagocytic PerC B-1a and B-1b lymphocytes had a morphology very similar to that reported previously for normal PerC B-1a and B-1b [20]. In the past, malignant mammalian B

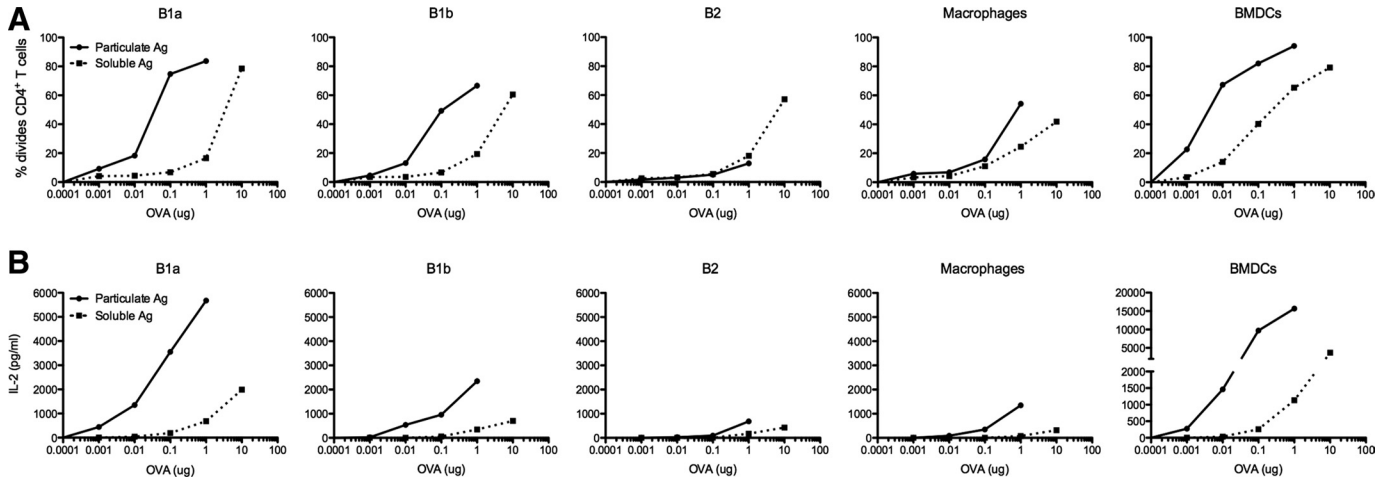

Figure 6. Capacity of PerC B cells, PerC macrophages, and BMDCs to present particulate and soluble antigen to CD4$^+$ T cells. Sorted PerC B cells, PerC macrophages, or BMDCs were incubated for 12 h with different amounts of 1 μm OVA-beads or soluble OVA and cocultured with OT-II CFSE-CD4$^+$ T cells for 3 days at a 1:1 ratio. Percentage of divided CD4$^+$ T cells (A) and IL-2 secreted by CD4$^+$ T cells (B) was measured by FACS and ELISA, respectively. Data are from one representative experiment of three performed.

cell have been shown to be phagocytic [11]. For example, cancerous B cells in hairy leukemia, as well as Ramos cells (a Burkitt's lymphoma-derived human B cell line), have been demonstrated to engulf large, inert particles and bacteria [24, 25]. In line with the aforementioned biphenotypic B/macrophage malignant cells, in vitro studies have illustrated subsets of mouse SPL that are capable of differentiation into phagocytic cells, displaying classical macrophage morphology and markers, while retaining some of the B-1 B cell markers, including CD5 and IgM [11]. Similarly, PerC murine B-1 cells have been shown to differentiate into a mononuclear phagocyte in vitro [26]. These cells were defined as B-1-derived mononuclear phagocytes; they adhered to plastic, had a macrophage-like morphology, and displayed the ability to internalize sheep erythrocytes and yeast [26]. On the other hand, mammalian primary B cells have been found unable to ingest large particles up until now [5, 6, 10]. However, previous studies had not analyzed primary B-1 cells from the PerC, the site that we have now identified to contain phagocytic B-1 B cells. Here, we show that this phagocytic ability of B-1 cells is confined to B-1a and B-1b subsets, whereas this capacity is almost negligible in B-2 cells. It is possible that the activation state of B-2 cells may influence their phagocytic capacity; thus, future work needs to explore to which degree the activation state of B cells modulates their phagocytic ability. Thus, this phagocytic competence strengthens the innate character that has long been ascribed to B-1 B lymphocytes [27].

It is interesting that the spleen MZ B cells were not found to be phagocytic, as these cells are often considered as part of the innate-like fraction of B cells, sharing with B-1 B cells an inherently high activation state compared with B-2 B cells [28]. Moreover, it has been reported recently that a B progenitor exists in a very early stage of development, which preferentially differentiates into B-1 and MZ B cells but not into B-2 cells [29]. However, despite the aforementioned similarities between MZ B cells and B-1 B cells, both B cell subsets clearly differ in their phagocytic ability. Perhaps the receptors involved in the uptake of the beads or bacteria are not present in the MZ B cells, or the location and state of activation of B-1 B and MZ B cells play a role in their phagocytic capacity. Those are points that need to be addressed in future work.

We propose that phagocytic PerC B-1 B cells are evolutionary, reminiscent of the phagocytic B cells described recently in fish, amphibians, and reptiles [6–9]. Therefore, in a broader context, the phagocytic capacity in B-1 B cells further supports the functional, developmental, and evolutionary relationships realized previously between B-1 B cells and macrophages [11–13].

Phagocytosis of particles leads to the fusion of the phagosomes containing these particles with lysosomes, thus enabling the formation phagolysosomes. Unless the phagocytosed pathogen uses mechanisms to escape the phagolysosome or inhibits its microbicidal potential, it will probably be degraded and die. We show that with regards to phagosome maturation and intracellular bacterial-killing abilities, B-1a and B-1b cells behave qualitatively similar to other professional phagocytes, although to a more-limited extent and with a slower kinetics than that observed here for CD11b^{high} non-B cells. This can probably be explained by the fact that we detected considerably more lysosome staining in CD11b^{high} non-B cells than in B-1a and B-1b cells. In support of our data, it has been described that macrophages have a higher level of lysosomal proteases than B cells [30]. The findings shown here are comparable with those found in fish phagocytic B cells, which also had a more limited capacity to form phagolysosomes when compared with macrophage-like cells and granulocytes [6]. Similarly, fish B cells also showed appreciably less staining for lysosomes than macrophages and granulocytes [6]. This indicates that although the processes of phagolysosome fusion and intracellular bacterial killing are conserved in subsets of B cells from fish to mammals, macrophages, monocytes, and granulo-

cytes from both animal groups have remained more proficient in these innate roles throughout evolutionary time.

It is well known that phagocytosis is a key process for the uptake of and presentation of particulate antigen, as reported for macrophages and DCs [3]. In that regard, proteolytic activity in the phagolysosome leads to the production of small antigenic peptides that can be presented on the APC surface through MHC I or MHC II molecules. In this study, we have shown that B-1a and B-1b lymphocytes are able to present OVA from phagocytosed, 1 μm beads to $CD4^+$ T cells, whereas when the beads are too large to be engulfed, presentation of the antigen to T cells is decreased dramatically. These data, together with the ability of phagocytic B-1B cells to up-regulate costimulatory molecules, uncover a previously unrecognized ability of PerC B-1 cells to efficiently present particulate antigen from phagocytosed material and prime naïve $CD4^+$ T cells. It has long been recognized that B cells are efficient APCs of soluble antigens [22, 23]. B cells can uptake soluble antigen in a specific manner through the BCR [31, 32] or nonspecifically, through pinocytosis [22]. It has been shown that soluble antigen uptake through the BCR is far more efficiently presented than pinocytosed antigen [31, 32]. In addition to presenting soluble antigen, B cells have been reported to extract antigen from noninternalized, large particles in a BCR-dependent manner [21]. The extracted antigens have been demonstrated to be efficiently presented to T cells in the context of MHC II molecules [21]. Although the antigen-presenting capacity of B-1 B cells has not been studied extensively, there are several studies that point to these cells as proficient presenters of soluble antigens. For example, B-1 B cells have been shown to be more efficient than B-2 cells in presenting soluble antigens, including OVA peptide [33] and DNP-keyhole limpet hemocyanin [23], to $CD4^+$ T cells. Our findings further strengthen the role of PerC B-1 B cells as potent APCs and extend this capacity to phagocytosed particulate antigen. Whether phagocytic B-1 B cells can cross-present particulate antigen to $CD8^+$ T cells is unknown at this point and is a subject that will be investigated in future studies. It is important to point out that the uptake of particulate antigen by B cells in a non-BCR-mediated manner might lead to the production of "antigen-nonspecific Ig" in a T cell-dependent or -independent manner. This may pose a dilemma, as phagocytic B-1 B cells might be producing a plethora of Igs whose specificity is unrelated to the antigen that they have internalized. It is possible that to avoid the aforementioned scenario, upon phagocytosis, B-1 B cells shut down their capacity to produce antibody, while their main role at that point would be to act as an APC. Alternatively, one could argue that the production of such antigen-nonspecific Ig upon phagocytosis could be beneficial in situations of acute infection, in which the B-1 compartment would produce evolutionarily conserved antibodies involved in a first line of defense. However, such scenario may also increase the chances of autoimmune reactivity. Perhaps, such "nonspecific" activation of B-1 B cells could explain part of the role that has been ascribed to B-1 B in autoimmune diseases [34]. Future work will have to explore these and other fascinating possibilities regarding the production of Ig by phagocytic B-1 B cells, the mechanisms involved, and the physiological consequences in the organism.

It is known that in macrophages and DCs, phagocytosed particulate antigen is more efficiently presented than soluble [35–37], In fact, several studies describe the use of encapsulated antigen as a strategy to increase the protective effect of vaccines [38–40]. Similar to the above mentioned APCs, PerC B-1 B cells required much less antigen to induce potent CD4-T cell responses when the antigen was in particulate form. In agreement with other reports, we show that BMDCs are more effective as APCs with regard to soluble antigens when compared with B cells or macrophages and more effective than macrophages when the antigen is particulate [35, 41]. One of the novel aspects of this study is that we show that PerC B-1 B cells are considerably more effective than PerC macrophages when presenting particulate antigen, whereas BMDCs remain the most potent APCs, even when antigen is particulate. To explain the higher capacity of BMDCs versus PerC B-1 B cells to present particulate antigen, one could argue that the phagocytic capacity of DCs is substantially higher than that of B-1 B cells, as they can ingest a larger number of beads/cell, and as a consequence, DCs would have more antigen at their disposal, thereby increasing their antigen-presenting abilities. This is not the case, however, for macrophages that can uptake a notably larger number of beads than B-1 B cells and BMDCs, yet their capacity to present particulate antigen is lower than that of the two aforementioned APCs. To understand this lower antigen-presenting capacity of macrophages, we should consider that in DCs and macrophages, regulation of phagosomal pH during phagocytosis has an important role in antigen presentation, as it has been shown that delayed acidification of the phagolysosome translates into increasing potency to present antigen, as is the case for DCs [42]. Thus, it is plausible that like DCs, the lower intensity in dextran staining that we have seen in B-1 B cells indicates lower acidification in their phagolysosome, as well as lower content of lysosomal vesicles. This, in turn, would lead to reduced degradation of antigen, thereby enhancing the antigen-presenting capacity of B-1 B cells when compared with that of macrophages. In addition, in line to the appreciably lower content of lysosomal staining that we have seen in B-1 B cells when compared with macrophages, B cells have been described to have lower levels of lysosomal proteases than macrophages, and because of that, they have been shown to retain antigen for a longer time period [30]. The latter may be another factor contributing to the greater capacity of B-1 B cells to present particulate antigen when compared with macrophages. Taking into account all of the above, as well as the much higher phagocytic capacity of macrophages when compared with B-1 B cells, one could predict that the main role of macrophages upon phagocytosis would be the killing of microbes and to a more limited extent, the presentation of phagocytosed antigens to T cells. Conversely, it is reasonable to infer that as a result of their more limited phagocytic and intracellular killing abilities, upon phagocytosis, B-1 B cells would have a more specialized and prominent role in presenting particulate antigen to T-cells. Moreover, phagocytic B-1 B cells may contribute to the killing of microbes, although to a much lower extent

than macrophages. Finally, we cannot rule out the possibility that macrophages and B-1 B cells present OVA with different efficiencies. In fact, it has been described that distinct epitopes in OVA are presented with different efficiencies by different APCs [43]. Future studies will have to corroborate further the aforementioned hypotheses.

In conclusion, in this study, we report a previously unrecognized phagocytic capacity of PerC B-1a and B-1b cells. The novel phagocytic and microbicidal capacities identified herein in B-1 B cells strengthen the innate nature that has long been attributed to these cells in systemic and mucosal compartments [27, 44, 45]. From an evolutionary perspective, we propose that phagocytic PerC B-1 B cells are reminiscent of the phagocytic B cells described recently in fish, amphibians, and reptiles [6–9]. Thus, our findings provide further support for the idea that B cells evolved from a phagocytic predecessor [12, 13, 46]. In that regard, the fact that the herein identified innate traits were mainly restricted to B-1 B cell subsets fits well with the concept of an evolutionarily layered immune system proposed by Herzenberg and Kantor [47]. Accordingly, and with regards to B cells, PerC B-1 B cells would constitute the evolutionary oldest layer, and as such, they embody more innate features than B-2 cells, the most evolved layer of all B cell subsets. Perhaps one should clarify that in this case, "most evolved layer" refers to the more specialized and greater role of B-2 cells in adaptive immunity. In the same vein of reasoning, there are clear morphological and functional parallels between mammalian B-1 cells and cold-blooded, IgM-bearing B cells. For example, they all secrete IgM, they can be activated directly by a number of PAMPs, and they both are capable of phagocytosis and intracellular killing. Therefore, it is tempting to speculate that the B-1 lineage evolved from IgM-bearing B cells of ectotherms, and later in evolution, B-2 cells emerged as a new B cell subset, probably poised to play a greater role in adaptive immunity. Although this is an attractive hypothesis, one cannot exclude at this point that other B cell lineages exist in cold-blooded vertebrates, perhaps some of which are not able to perform phagocytosis. Therefore, the evolutionary origins of B-1 and B-2 lineages cannot be delineated precisely at this point; to be able to do so, we will need to learn more about the putative presence of different B cell lineages in poikilothermic vertebrates, as well as their specific roles in innate and adaptive immunity.

Notably, the novel immune roles of PerC B-1 B cells uncovered here, including their capacity to phagocytose, degrade, process, and present particulate antigen, place these cells at the crossroads that link innate with adaptive immunity. Therefore, we propose that phagocytosis-mediated uptake and presentation of particulate antigen bridge innate and adaptive immune capacities of these cells, providing them with the potential to initiate T cell-dependent immune responses against pathogens and particulate self-antigens, a hypothesis that will be examined in future studies.

AUTHORSHIP

J.O.S., D.P., C.A.H., and D.R.B. designed research; D.P., A.M.R., J.L., Y.-A.Z., and L.M.R. performed research; D.P. and A.M.R. analyzed data; and D.P. and J.O.S. interpreted data and wrote the manuscript.

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