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# Dysregulation of of phospholipid-specific phagocytosis by B1 B cells in diet-induced obese mice

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# DYSREGULATION OF PHOSPHOLIPID-SPECIFIC PHAGOCYTOSIS BY B1 B CELLS IN DIET-INDUCED OBESE MICE

by

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**DYSREGULATION OF PHOSPHOLIPID-SPECIFIC PHAGOCYTOSIS BY B1 B CELLS IN DIET-INDUCED OBESE MICE**

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## ABSTRACT

B1 B cells have received increasing attention recently due to their newly discovered phagocytic and microbicidal capabilities. Several studies have demonstrated that B1 cells can phagocytize polystyrene fluorescent particles, bacteria (Staphylococcus aureus, Escherichia coli), and even apoptotic cells. Nevertheless, little is known about the biological significance of this seemingly redundant function of B1 B cells as compared to that of conventional phagocytes. Here we investigate the unique phosphotidylcholine (PtC)-specific B1 B cell phagocytosis. PtC is a major phospholipid in the biological membrane and a classical antigen recognized by B1 B cell-derived natural antibodies. These antibodies play important roles in immune defense as well as tissue homeostasis. Here we report that B1 cells preferentially phagocytose PtC-coated beads, differing from that of conventional macrophages. We further attest that these beads were truly internalized and subsequently fused with hydrolytic lysosomes indicated by increasing fluorescent intensity of a pH-sensitive dye. Despite the differences in antigen specificity, phagocytosis of both B1 cells and macrophages can be inhibited by the microtubule-inhibitor, Colchicine, in a dose-dependent manner. Most intriguingly, upon chronic high-fat diet (HFD) consumption by the host, B1 cell phagocytosis starts to lose antigen-specificity for PtC. Morphologically, some of these B1 B cells in DIO mice show

enlarged cytosol and engulfed more beads, indicating a transition to macrophage-like cells. Our study suggests for the first time that B1 B cells have unique phospholipid-specific phagocytosis capacity, which is affected by diet-induced obesity.

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

CD ..... Control Diet

DIO ..... Diet-Induced Obesity

FACS..... Fluorescence-Activated Cell Sorting

FBS .....Fetal Bovine Serum

HBSS..... Hank's Balanced Salt Solution

HFD..... High-Fat Diet

LPS..... Lipopolysaccharide

nAbs ..... Natural Antibodies

OxLDL ..... Oxidized Low-Density Lipoprotein

PBL .....Peripheral Blood Lymphocytes

PBS ..... Phosphate Buffered Solution

PC.....Phosphorylcholine

PerC.....Peritoneal Cavity

PtC.....Phosphotidylcholine

RBC.....Red Blood Cell

RG ..... Rhodamine Green

TRITC .....Tetramethylrhodamine Isothiocyanate

# INTRODUCTION

As a first line of defense against many invading microbes and foreign pathogens, phagocytosis plays a critical role in innate immunity (1). Phagocytosis was first discovered in 1882 by Ilya Metchnikov and was later defined as an active process of engulfing particles that are larger than 0.5 μm in diameter (2). This complex process requires actin polymerization and reorganization of microtubules (20). Upon internalization, the phagocytosed particle is enveloped within a membrane-bound vesicle called 'phagosome' and subsequently fuses with lysosomes to form 'phagolysosome'. Lysosomes contain mainly hydrolytic enzymes with a low pH and are responsible for the degradation and killing of the ingested pathogens within the phagolysosome (36).

In most higher-vertebrate species, phagocytosis is considered a specialized function that has been attributed predominantly to these so-called 'professional' phagocytes, such as monocytes, macrophages, and polymorphonuclear granulocytes (PMN) (2). However, there are other 'non-professional' phagocytes that are also capable of performing phagocytosis such as epithelial cells (28), endothelial cells (16), mesothelial cells (42), and even adipocytes (41). In recent years, there have been many attempts to link phagocytosis to B lymphocytes, especially B1 B cells. Early reports showed that malignant CD5+ B cells have macrophage-like capacity to internalize large particles (9, 26, 39). Jun Li et al. also reported that fish B cells have phagocytosis capacity and those phagocytic B cells resemble B1 B cells in mammals (24). More recently, David Parra et al. reported that murine B1 B cells indeed have phagocytic and microbicidal capacities,

and most importantly, B1 B cells can present phagocytosed antigens to CD4 T cells (32). Furthermore, Ana Flavia Popi et al. reported that adoptive transfer of peritoneal B1 B cells into a macrophage-deficient op/op(-/-) mice restored peritoneal macrophage pool upon lipopolysaccharide (LPS) stimulation (40). This supports the notion proposed early in 1996 by Melinda Borrello and Richard Phipps that phagocytic CD5+ B cells are not a result of malignancy, but rather they are the evidence of the existence of B cell-to-macrophage trans-lineage differentiation (8).

One of the physiological functions of B1 B cells is to produce natural antibodies (nAbs). These nAbs are poly-reactive IgM that recognize common bacterial structures as well as self-antigens, such as cell membrane phospholipids and oxidized lipoproteins. Recent studies highlighted that B1 B cell-derived natural antibodies are essential in the maintenance of tissue homeostasis via clearance of apoptotic and altered cells, inhibition of inflammation, removal of mis-folded proteins and regulation of pathogenic autoantibody-producing conventional B2 B cells (22).

In mice, about 1% of B1 B cells produce anti-phosphorylcholine (PC) nAbs and up to 10% of B1 B cells produce anti-phosphatidylcholine (PtC) nAbs (13, 27). PC is the polar head group of PtC, which is a major phospholipid component of the cell membrane. Digestion of cell membranes by enzymes, such as phospholipase A2, renders the PC moiety exposed to the external aqueous phase and thus accessible for recognition by B1 B cells and their anti-PC IgM antibodies (23). Such exposure is critical for the non-inflammatory removal of senescent cells. Recent studies also revealed that numerous oxidized phospholipids within senescent or apoptotic cell membranes adopt

conformations in which the oxidized fatty acids protrude into the aqueous phase (18, 21). Interestingly, anti-PC IgM produced by B1 B cells also recognizes oxidized low-density lipoprotein (OxLDL) and prevents development of atherosclerosis (11). Reconstitution of anti-PtC IgM in IgM-deficient mice substantially reduced the bacterial load in a cecal ligation and puncture model (7, 31). In addition to bacteria, B1 B cell-derived nAbs also play an essential role in immune defense against influenza virus (5). In humans, 85% of the sera from clinically healthy subjects contain IgM anti-PtC, none of which contain IgG (10).

As mentioned above, B1 B cells play essential roles in maintaining tissue homeostasis, including clearing apoptotic cells, tissue debris or immune-complexes (IC). To test whether phospholipids are critical in B1 B cell phagocytosis, we investigated B1 B cells phagocytosis of PtC-coated beads. We compared the specificity of phagocytic B1 B cells and macrophages for this phospholipid component. In fact, we demonstrated that mouse B1 B cells in fact can internalize PtC-coated beads, and this process is carried out in a manner similar to phagocytosis with formation of phagolysosome and involvement of the cytoskeleton. However, B1 B cells specifically internalized PtC-coated beads, but not control beads, whereas macrophages internalized both types of beads. We also report for the first time that inflammatory conditions, such as obesity, could influence B1 B cell phagocytosis by diminishing their specificity for PtC.

## METHODS

### Mice

All C57BL/6J mice were purchased directly from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in the Laboratory Animal Science Center (LASC) facility at Boston University Medical Center. Male mice were used in this study to ensure consistency and exclude potential sex-related difference. The mice were housed in a group of four under a specific pathogen-free (SPF) environment and were sacrificed at indicated age in accordance with approved protocols and guidelines of the Institutional Animal Care and Use Committees (IACUC) of Boston University Medical Center.

### Diet-induced-obesity (DIO) model

Mice were fed with two different diets starting at the age of 6 weeks old: standard rodent chow diet (2918, Harlan Laboratories) as control diet (CD) with 18% kcal fat and high-fat diet (HFD) with 60 kcal % fat (TD.06414, Harlan Laboratories, South Easton, MA, USA). Most of the DIO mice were purchased from the Jackson Laboratory at 6 weeks of age and fed in house. Some DIO mice were purchased at 22 weeks of age. These DIO mice were put on a customized 60% fat diet (D12492, Research Diets, Inc.) starting the age of 6 weeks. A comparison of high fat diets from Harlan and Research Diets reveals that their products are interchangeable because they contain the same components (protein, carbohydrate, fat) and with 60% of total calories coming from fat (60% kcal fat). All diets were provided in metal hoops with easy access in sufficient amounts.

**Body Weight and Intra-peritoneal Glucose Tolerance Test (IPGTT)**

The body weight of each mouse was measured prior to the IPGTT procedure by using a standard 400 gram capacity Ohaus SC4010 Scout Scale (Pine Brook, NJ, USA). For IPGTT, mice were separated into individual clean cages prior to the test. After 6 hours of fasting, D-glucose (G7528, Sigma-Aldrich) solution (1 g/kg body weight) was delivered by i.p. injection. Blood glucose levels were measured via tail blood by using Contour blood glucose meter and test strips (Bayer, NJ, USA) before and after i.p. injection at 15 minute increments.

**Preparation of TRITC dye-doped silica beads**

Fluorescent silica beads were prepared by a modified Stöber process, in which a solution of Tetraethylorthosilicate (TEOS) in ethanol was hydrolyzed by adding water in the presence of ammonia (6). The beads with average diameter of 500-600 nm were synthesized as follows: First, dye precursor was prepared by reacting 0.2 μmol Tetramethylrhodamine isothiocyanate (TRITC) dye with 10 μmol (3-Aminopropyl)triethoxysilane (APTS) and stirring for 12 hours. Second, TRITC dye precursor was added to a solution containing 9 mL of ethanol with 90 μl of deionized water and 690 μl of ammonia (29.5%). Then, 390 μl of TEOS was added and mixed at a rate of 40 μl per hour and incubated for 6 hours. The system was stirred during the entire incubating process. The resulting solid was washed three times with deionized water by centrifugation. The washed beads were re-dispersed into 5 mL of deionized water. The beads were then coated with carboxyl acid and PEG 500. The coated beads were purified by centrifugation using the same procedures as described above.

**Preparation of L-α-Phosphatidylcholine (PtC) coated Rhodamine Green beads**

PtC (1.54 mg, 200 μM) in CHCl3 (10 mL) was added to a glass vial and the solvent was removed by rotary evaporation to provide a thin film (25). To ensure that all the $CHCl_3$ was removed, the film was dried for 12 hours in vacuum oven at 25˚C. Ultrapure H2O (10 mL) was added to the vial. The cloudy solution was stirred at 25˚C for 30 minutes. The solution was diluted 10 fold with Ultrapure H2O (15.4 μg, 2 μM). 20 μl of the diluted solution was added to 1 mL of 1 mg/mL Rhodamine Green bead in Ultrapure H2O and stirred for 12 hours at room temperature (0.3 μg PtC, 0.325 μM). Beads were centrifuged at 5,000 rpm for 10 minutes. The supernatant was discarded to remove unbound PtC. The yellow-green solid was collected and added another 1 mL of Ultrapure H2O. The centrifugation procedure was repeated. Finally, 1 mL of Ultrapure H2O was added to the beads and the solution was store at 4˚C.

**Preparation of Green beads with L-α-Phosphatidylcholine (PtC) and pHrodo-Red dye**

Reverse phase evaporation was used to coat green fluorescent silica beads (Discovery Scientific, USA) with a lipid bilayer coat (inner leaflet: DOTAP; outer leaflet: 85mol% PtC, 10% pHrodo-Red (P36600, Life Technologies, Grand Island, NY), and 5% DSPE-PEG2k). 0.288 mg DOTAP diluted in 877 μl chloroform was added to 2.08 mL methanol. At an approximate concentration of $6x10^{10}$ beads/mL, 6.67 μl of silica beads in distilled water was added to the DOTAP solution, and vortexed. After 30 minutes of incubation at room temperature, 1 mL of water and 1 mL of chloroform were added. The solution was briefly vortexed, then centrifuged at 830Xg for 7 minutes. The upper

aqueous phase was removed, and 0.231 mg PtC, 0.0543 mg pHrodo-PE, 0.0495 mg DSPE-PEG2k, and 1 mL water were added. The mixture was vortexed then bath sonicated for 4 minutes. The chloroform was subsequently removed via rotary evaporation (60°C, 476 mbar) and the resulting beads were washed three times via centrifugation. The bead size distribution, concentration, and zeta potential were measured with qNano (Izon, Oxfor, UK) and dynamic light scattering (90 Plus, Brookhaven, Holtsville, NY). The final beads had an average diameter of 530 nm.

**Cell isolation**

Peritoneal cavity (PerC) cells were obtained by injecting 10~15 mL of Hanks Balanced Salt Solution (HBSS) with 2% Fetal Bovine Serum (FBS) into the peritoneal cavity. Spleen (SPL) cells were isolated by mechanical disruption of spleen tissue and passed through a 70 μm nylon cell strainer. Shortly after the mice were euthanized, blood was collected by cardiac puncture with a 1 mL syringe that has been coated with heparin to prevent coagulation. To remove erythrocytes in the spleen and blood, cells were treated with 1X RBC lysis buffer (eBioscience, San Diego, CA) for 5 minutes at room temperature, centrifuged and the remaining cell pellet was suspended in supplemented RPMI 1640 media (10% FBS, 100 unit/mL Penicillin and Streptomycin, 2% 0.5M HEPES buffer, 1% Glutamine, and 1% Beta-ME).

**In vitro assessment of phagocytosis**

Cells isolated from different tissues were counted with an automated cell counter (Cellometer Vision, Nexcelom Bioscience, Lawrence, MA), centrifuged, and re-suspended in supplemented RPMI media. In each experiment, at least $1x10^6$ cells from

PerC, SPL, and blood were plated in 96-well tissue culture plates (Celltreat Scientific Products, Shirley, MA) in a final volume of 100 µl per sample. With a 10:1 cell-to-particle ratio, diluted fluorescent beads in supplemented RPMI media were added into each well to create an equal final volume per well. Unless otherwise indicated, 5 µg/mL of LPS was added in each well. Cells and fluorescent beads were co-cultured overnight in a 37˚C 5% CO2 incubator. For inhibition experiment, PerC cells were isolated and incubated over night with Rhodamine Green-fluorescent PtC-coated beads in the presence of different concentrations of Colchicine (SC-20300, Santa Cruz Biotechnology) without LPS stimulation. Colchicine powder was reconstituted in distilled water to a 100 mg/mL stock concentration. Then incubated cells were harvested the next day and subjected to flow cytometry and confocal microscopy analyses.

**Flow cytometry analysis**

After overnight incubation period, PerC, SPL, and blood cells were centrifuged and washed twice with PBS to eliminate non-ingested fluorescent beads. Before staining the cells, Fc receptors were blocked by incubating the cells with 50 µl of LEAF-purified anti-mouse CD16/32 antibody (Biolegend, San Diego, CA) for 15 minutes on ice. Each sample was then stained on ice for 45 minutes with 50 µl of staining antibody cocktail that contains the following antibodies: B220-PerCp-Cy5.5, CD5-eF450, Cd11b-APC-eF780, IgM-APC, Fixable Viability Dye eFluor® 506 (eBioscience, San Diego, CA). Cells were centrifuged and washed three times with FACS buffer (1X PBS with 2% FBS) and were ready for analysis with LSRII flow cytometer (BD Biosciences, San Jose, CA)

at Boston University Flow Cytometry Facility. Data was analyzed with FlowJo software (TreeStar, Ashland, OR).

**Immunofluorescent microscopy analysis**

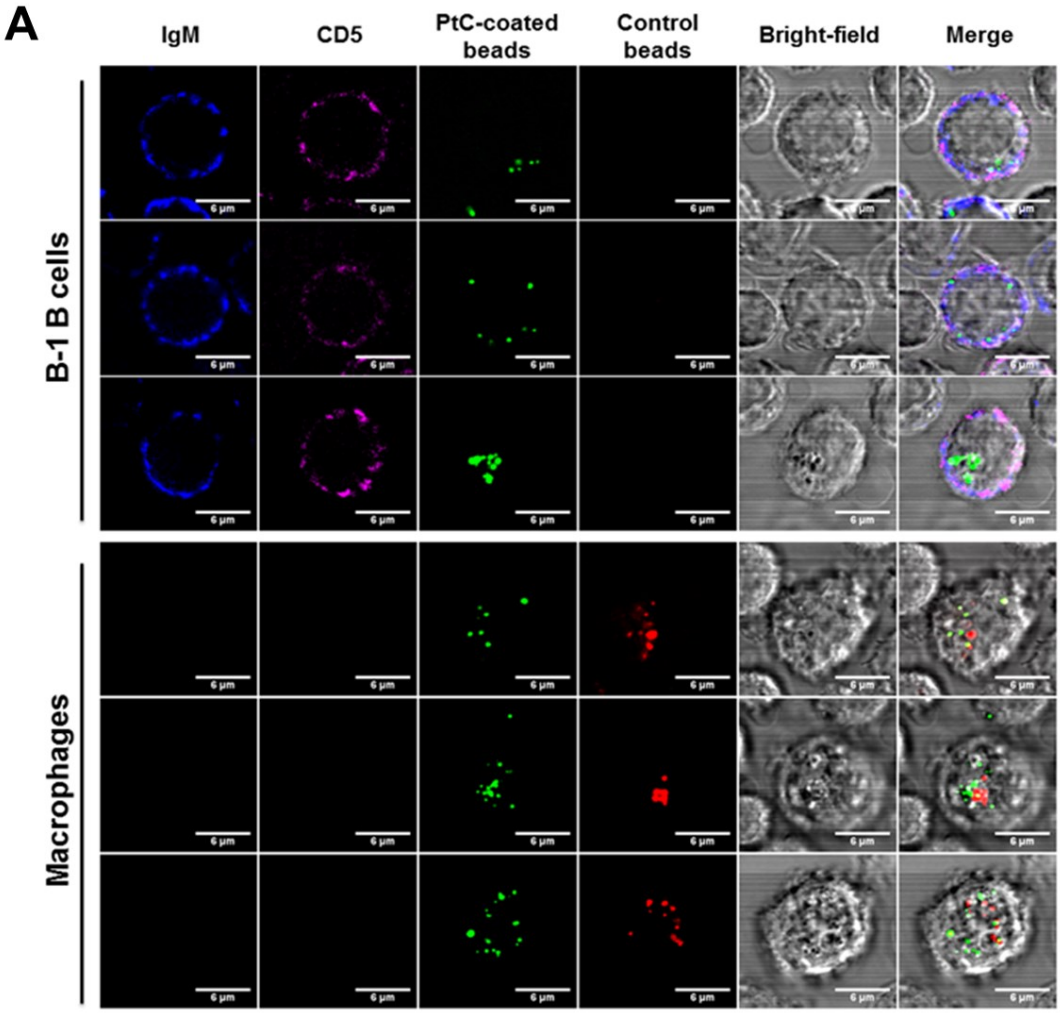
Cells were prepared similarly for microscopic analysis as for flow cytometry. After overnight incubation, cells were washed twice with 1X PBS by centrifugation to remove non-ingested beads. Afterward, washed cells were incubated with Fc blockers for 15 minutes on ice, and then stained with following antibodies: IgM-AF350 (Life Technologies, Grand Island, NY) and CD5-AF647 (Biolgend, San Diego, CA). For the experiment with pHrodo-Red dye, cells were stained with CD19-AF405 (AbD Serotec, North Carolina, USA) and CD5-AF647 (Biolegend, San Diego, CA). Stained cells were then washed with 1X PBS (GIBCO, Life Technologies, NY) three times by centrifugation, and re-suspended in a final volume of 200 µl. Each cell sample was then plated in a 35 mm glass bottom dish (MatTek Corporation, Ashland, MA). All images were taken at 63X oil-immersion objective with Leica TCS SP5 microscope (Leica, Buffalo Grove, IL) from Boston University Cellular Imaging Core.

**Statistical analysis**

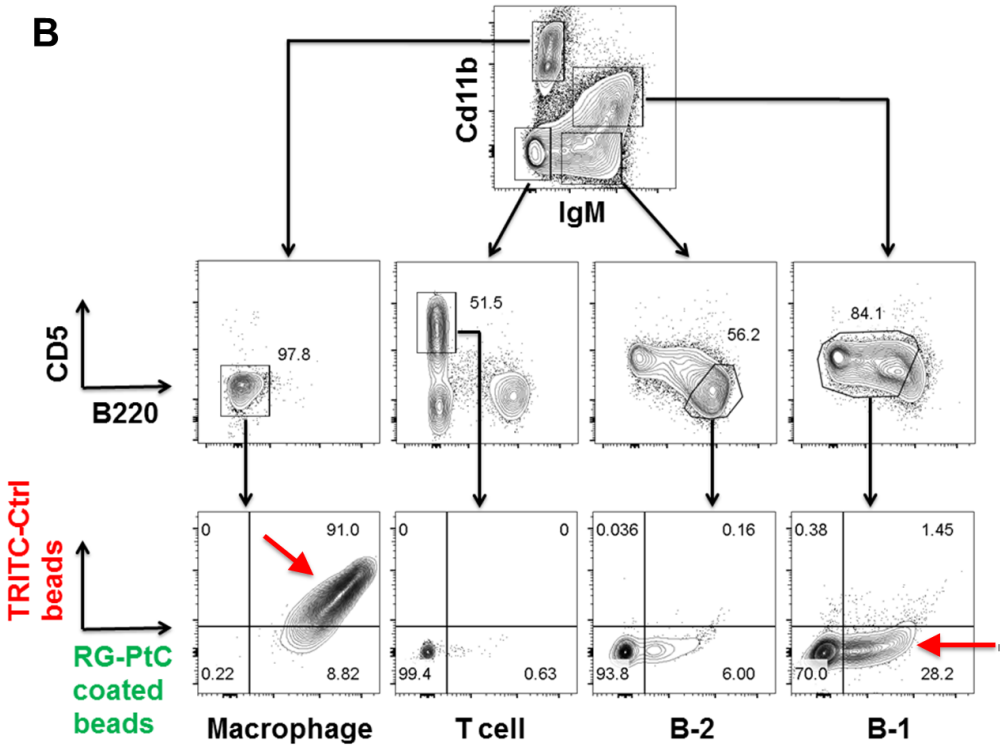
Statistical differences were calculated with Student's t-test method in Graphpad Prism version 6.01 software with $p<0.05$ accepted as the level of statistical significance (*P<0.05, **P<0.01, ***P<0.001).

## FIGURES AND FIGURE LEGENDS

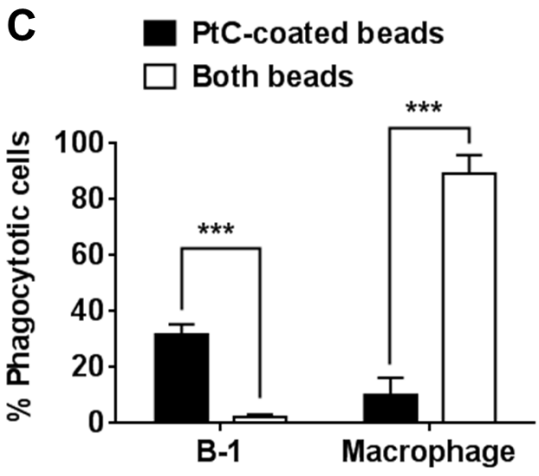
**Figure 1: PerC B1 B cells specifically phagocytose PtC-coated beads whereas macrophages indiscriminately phagocytose both PtC-coated and control beads**.




(***A***) Cells were isolated from peritoneal cavity (PerC) and incubated with Rhodamine Green (RG)-PtC-coated beads (green dots) and TRITC-control beads (red dots). Both types of beads were added at equal amount. Cells and beads were co-cultured overnight at 1:10 ratio in the presence of 5 μg/mL LPS. Cells were then washed and stained for IgM (blue) and CD5 (magenta) followed by microscopy observation. IgM-CD5 double positive B1 B cells were compared with IgM-CD5 double negative large macrophages for internalized red and green beads.

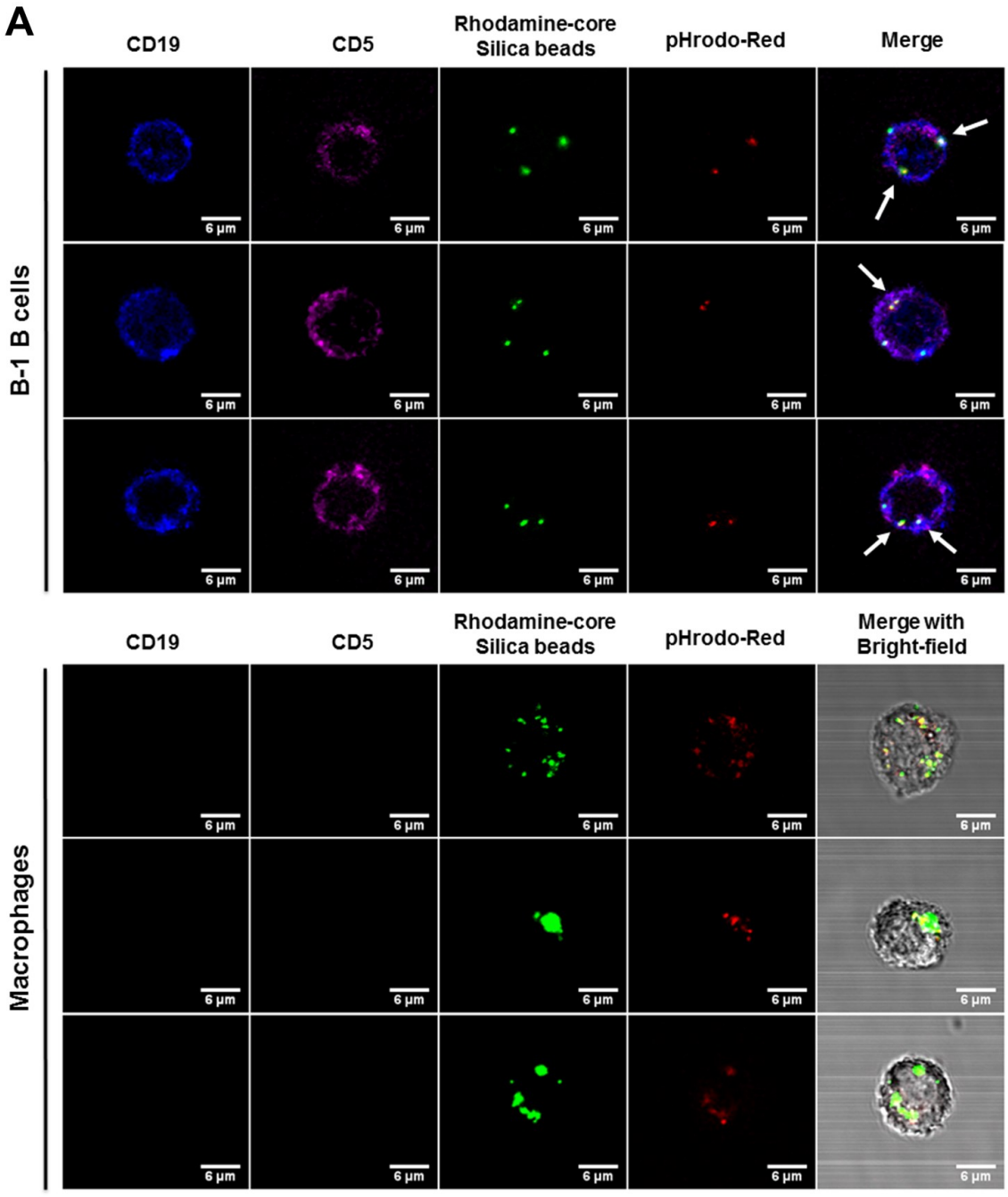



(***B***) Representative flow cytometry analysis of PerC cells incubated in vitro with fluorescent beads overnight, and then stained with anti-IgM, anti-CD11b, anti-B220, anti-CD5, and viability dye. Bottom panel shows the amount of green PtC beads and red control beads ingested by each type of cells. Cells taking in both beads are shown as double positive population.



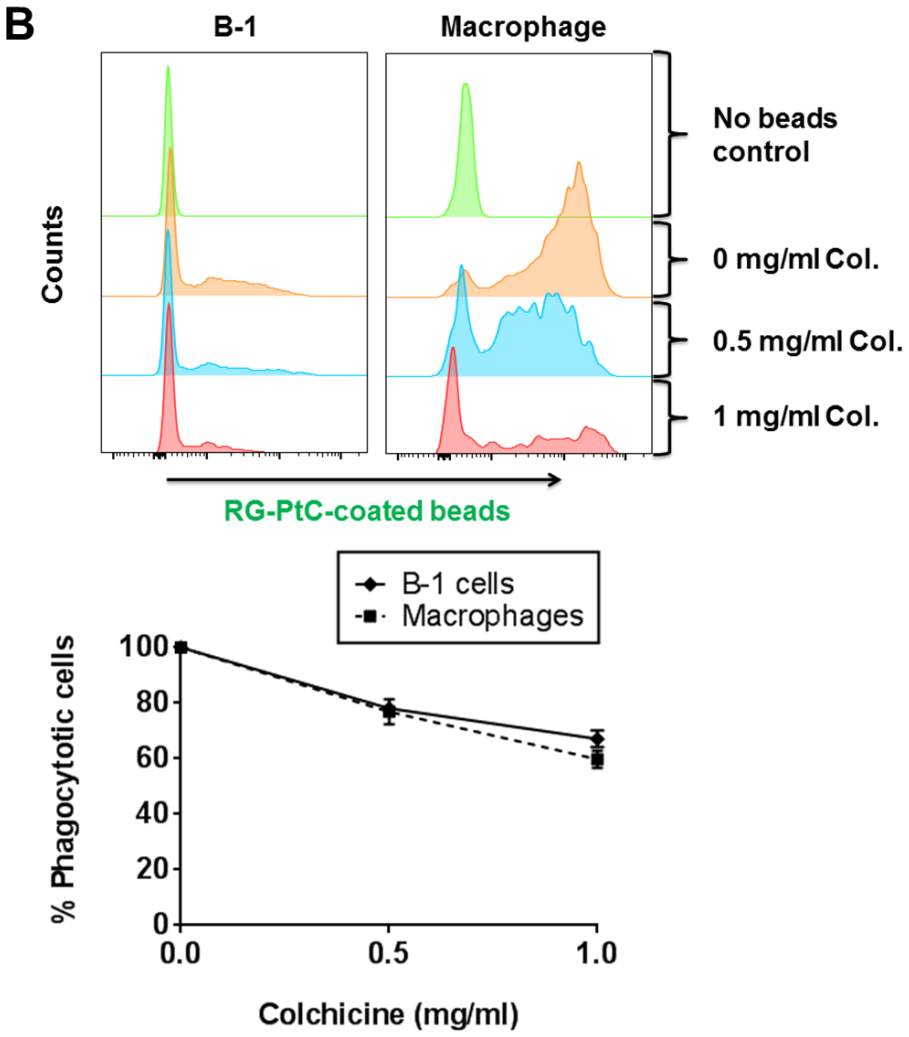

(***C***) Percentages of B1 B cells and macrophages that engulf PtC and/or control beads are compared. Results are mean ± SEM of 12 mice from 3 independent experiments.

**Figure 2: Tracking of phagolysosome formation and inhibition of B1 B cell phagocytosis with Colchicine.**



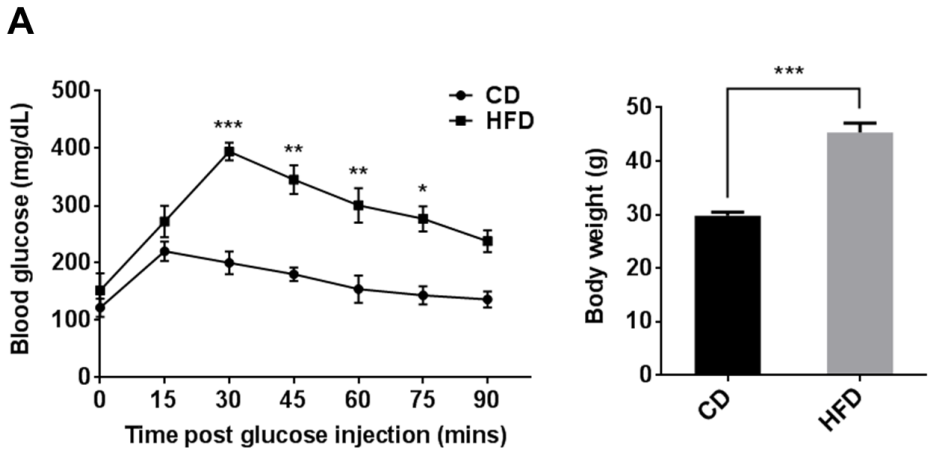

(*A*) Representative immunofluorescent microscopy images of phagocytic B1 B cells (upper-left panel) and macrophages (bottom-left panel). Images of B1 B cells (CD5+/CD19+) were merged without bright field due to difficulty to visualize internalized beads whereas macrophages (CD5-/CD19-) were shown with bright field

images because of the lack of fluorescent surface markers. Silica beads with Rhodamine green-core were coated with PtC and pH-sensitive pHrodo-Red dye. Surface-bound beads or ingested beads that were not fused with phagolysosome emitted only green fluorescence. Ingested beads within phagolysosome emitted both red and green (or yellow) fluorescence due to acidic pH that caused pHrodo-Red dye to produce strong red fluorescence (indicated by white arrows).

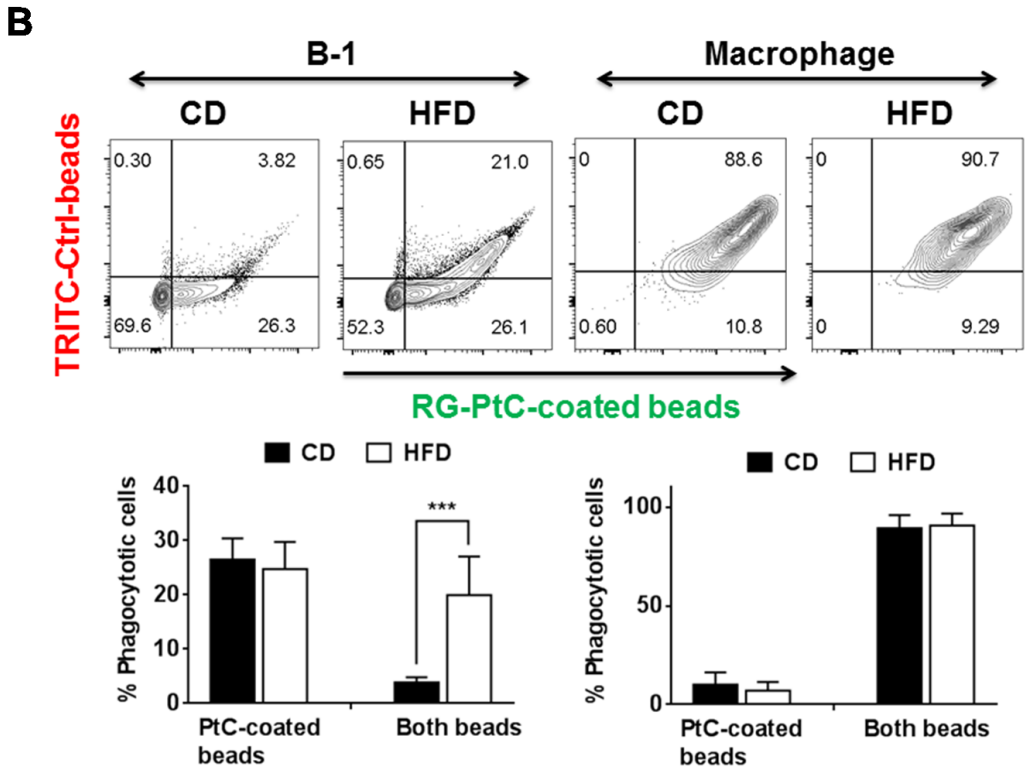



(***B***) PerC cells were incubated with RG-PtC-coated beads and Colchicine with indicated concentrations overnight at 37˚C and 5% CO2. Flow cytometry histograms (left panel) show decreasing green (FITC) signals with increasing doses of Colchicine. Right panel showed representative statistics of percentage of phagocytic cells relative to that of controls (calculated as mean ± SEM from 2 independent experiments)

**Figure 3: Diminished PtC-specific phagocytosis by B1 B cells in HFD-fed mice**

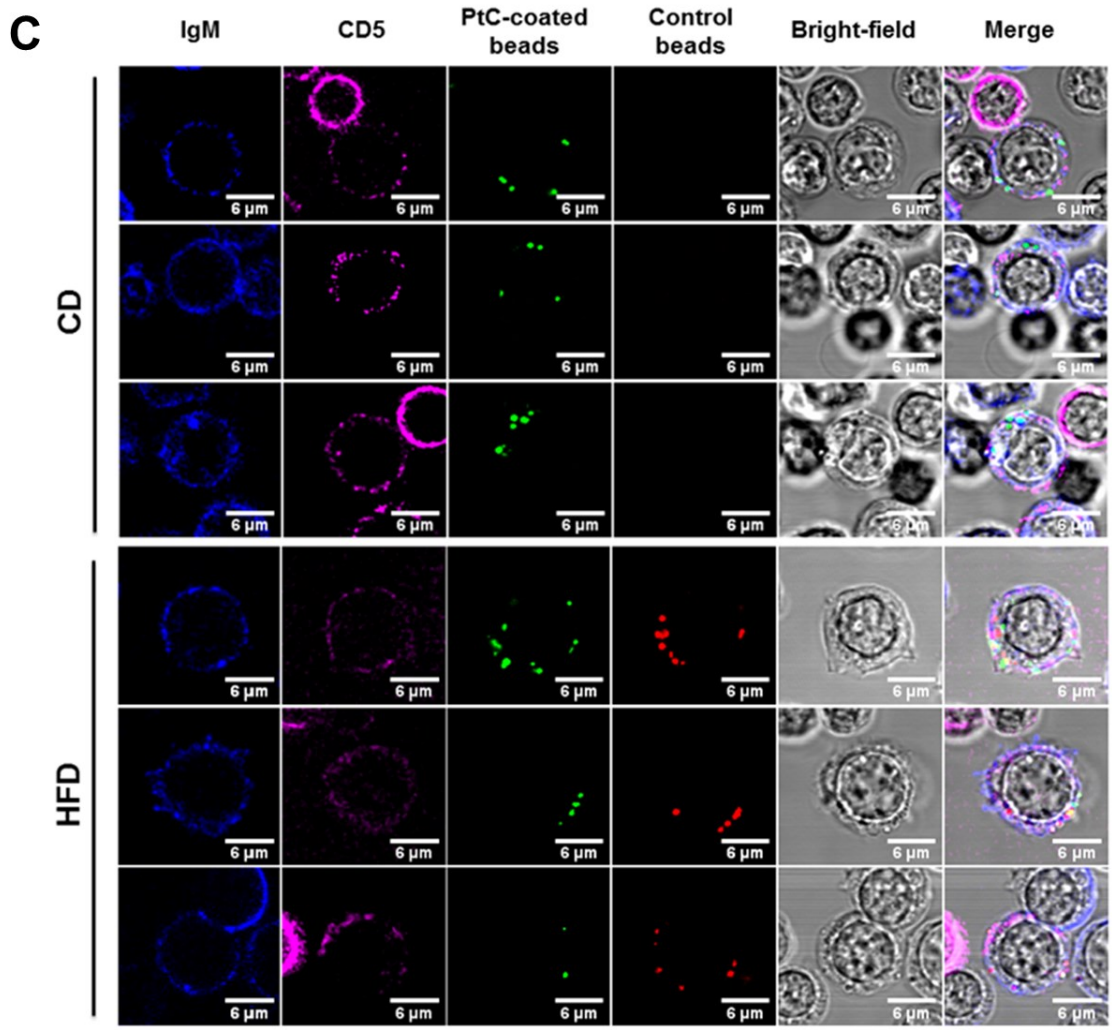



(**A**) Mice were kept on two different diets - control diet (CD) and high-fat diet (HFD) for 16 weeks. Measured body weight (right panel) and glucose tolerance test (left panel) demonstrated signs of diet-induced obesity in HFD group.

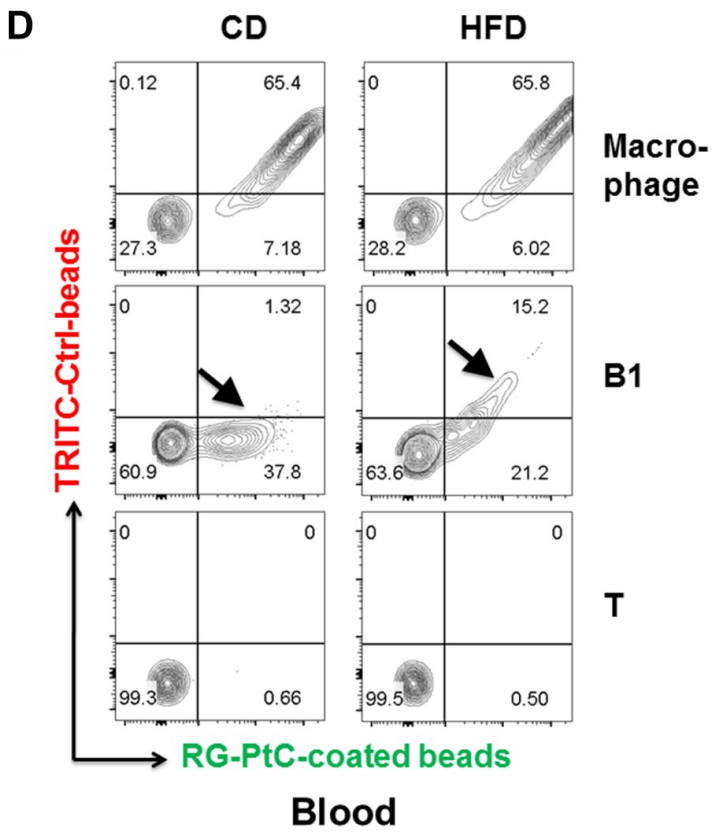



(**B**) Representative FACS analysis of PerC B1 B cells that were incubated with TRITC-control beads and/or RG-PtC-coated beads. Black arrows show example of increased

double positive population in HFD-fed mice as compared to CD-fed mice in B1 B cells. No difference was observed for macrophages in CD versus HFD groups. Bottom panel shows statistic comparison of percentage of phagocytic PerC B1 B cells (left) and macrophages (right) that engulfed PtC-beads alone or both PtC and control beads in CD (black bar) and HFD (white bar) groups (n=16 in each group).




(**C**) Representative immunofluorescence microscopy images of PerC B1 B cells in CD-fed and HFD-fed mice. B1 B cells were shown as double positive for IgM (blue) and CD5 (magenta). Images showed B1 B cells from CD-fed mice mostly phagocytosed PtC-beads (green) only, whereas B1 cells from HFD-fed mice phagocytosed both PtC-beads and control beads (yellow arrows). B1 cells from HFD group also have slightly enlarged cytosol and cell size.




(**D**) Pooled peripheral blood lymphocytes (PBL) from 8 HFD-fed mice and 8 CD-fed mice showed the same shift of specificity of phagocytosis in B1 cells (black arrows) in HFD-fed mice as compared to CD-fed mice.

## RESULTS AND DISCUSSION

### Peritoneal B1 B cells are distinct from macrophages in their specificity of phagocytosis

Several groups have recently reported that murine B1 B cells have phagocytic and microbicidal abilities (14, 17, 24, 32). To further investigate these novel functions of B1 B cells, our main focus is to examine the specificity of B1 B cell phagocytosis in comparison to that of macrophages, also known as "professional" phagocytes. As mentioned above, phosphotidylcholine (PtC) is a classical B1 B cell self-antigen that is recognized by 10% of total B1 B cells in the peritoneal cavity. Thus we generated PtC-coated fluorescent beads to test phagocytic capacity of B1 B cells and compared them against macrophages. Two types of silica-beads with different fluorescent cores were synthesized: green-fluorescent beads have PtC molecules attached to their surfaces and red-fluorescent beads with no surface PtC were used as control beads – both with an average diameter ranging from 500 nm ~ 600 nm. Cells isolated from the peritoneal cavity were incubated with both PtC-coated green beads and red control beads in the same well overnight in the presence of 5 μg/mL LPS at standard conditions of 37˚C and 5% $CO_2$.

Our FACS results revealed that 32 ± 3.7% of PerC B1 B cells ingested only green PtC-coated beads and less than 2% ingested both green and red beads (Fig. 1*B*). In contrast, over 90% of macrophages phagocytosed both types of beads (Fig. 1*B*). When observed under confocal microscope, most of the fluorescent beads ingested by macrophages were found in the central area of the cells whereas they were located in the

peripheral position in B1 cells (Fig. 1*A*). Compared to macrophages, B1 B cells have large central nuclei and very thin cytoplasm, which allow very little room for the cells to engulf foreign pathogens. Consequently, it is reasonable to find ingested beads in peripheral position within B1 cells. In addition, the number of engulfed fluorescent beads in B1 cells ranged from one to seven, whereas in macrophages, it can be anywhere between ten to thirty beads at a time (Fig. 1*A*). This demonstrated that macrophages have greater phagocytic capacity than phagocytic B1 B cells, possibly due to their larger cytoplasm and cell size.

While most of our microscope images showed that only green PtC-coated beads were found within B1 B cells, FACS analysis indicated that only 2% of B1 B cells were not discriminatory toward surface coating of fluorescent beads. Even so, B1 B cells clearly displayed a higher stringency in term of specificity of phagocytosis toward phospholipid (PtC) compared to macrophages. However it is not clear why nearly 30% of B1 B cells were able to specifically internalize PtC-coated beads whereas only 10% of B1 B cells express PtC-binding antibodies. It is most likely that another mechanism other than direct surface IgM-recognition or binding that might be involved in B1 B cell phagocytosis of this particular phospholipid.

**Phagolysosome tracking and inhibition of B1 B cell phagocytosis**

In order to exclude the possibilities that PtC-coated beads were simply absorbed into the cytosol or bound to surface IgM in our previous experiments, we coated our PtC-coated beads with a novel pH-sensitive dye called pHrodo-Red. This special dye remains colorless in a neutral pH environment and dramatically increases its fluorescent emission

as the pH changes from neutral to acidic. By coating the green-fluorescent-core phospholipid beads with the pHrodo-Red dye, these beads would emit yellow (combination of green and red) fluorescence when they are internalized and experience a drop in pH as they fuse with the lysosomes to form 'phagolysosome'. Any beads that emit only green fluorescence indicate that they remain either surface-bound outside of the cells or inside of the cell but are not yet fused with lysosomes yet. In fact, our hypothesis was confirmed through immunofluorescent microscopy analysis. Isolated PerC cells were incubated with PtC/pHrodo-coated beads and 5ug/mL LPS overnight, stained, and re-suspended in 1X PBS to maintain a neutral pH environment. Notably, our images revealed that a majority of the internalized beads emitted both green and red fluorescence in phagocytic B1 B cells as well as macrophages (Fig. 2*A*). This served as strong evidence that formation of phagolysosome did occur within phagocytic B1 B cells. In addition, our results also corresponded with a previous finding that phagocytic capacity of macrophages is greater than B1 B cells since there were more beads with dual fluorescence (yellow) within macrophages.

Another important characteristic of phagocytosis besides formation of phagolysosome is the rearrangement of the cytoskeleton which requires the involvement of actin filaments and microtubule system (2). Microtubules appear to play crucial roles in phagocytic activity since they are responsible for cell motility, changes in cell shape, and formation of pseudopods (20). Therefore, disrupting the fine balance of this microtubule cytoskeleton can critically reduce the engulfing ability of phagocytic cells. Colchicine is anti-mitotic agent that binds to the ends of microtubules and prevents their

polymerization, thus Colchicine has been used frequently in research and medicine to inhibit phagocytosis (12). Having the opposite effect to Colchicine, bacterial LPS can stimulate polymerization of microfilaments and microtubules in macrophages and monocytes (3, 34, and our data not shown). As a result, LPS was not used in our in vitro experiments to assess phagocytosis inhibition by Colchicine. The results obtained from FACS analysis showed that both phagocytic B1 B cells experienced a reduction in phagocytic capacity in a dose-dependent manner (Fig. 2*B*).

Considering the similarity with regards to phagolysosome formation and involvement of cytoskeleton between B1 B cells and macrophages, we conclude that internalization of PtC-coated beads by B1 B cells is through a genuine process of phagocytosis. However, phagocytosis is a dynamic and complex process, which requires more than just involvement of phagolysosome and microtubules. Generally held, phagocytosis has also been known to be an actin-dependent cellular process (1); therefore, evaluating phagocytic activity of B1 B cells toward phospholipid with agents that block actin polymerization is highly desired. In addition, the precise mechanism in which phagocytic B1 B cells employ to recognize and internalize PtC-coated beads requires further investigation. Besides the suspected surface-bound IgM which is highly expressed on B1 B cells, other mechanisms of phagocytosis should also be examined, such as Fc-receptor-mediated and complement-receptor-mediated phagocytosis. Finally, studies on morphological changes of B1 B cells during and after phagocytosis of phospholipid beads can provide us with further insight into this novel function of these lymphocytes.

**High-fat diet dysregulated the number and specificity of phagocytic PerC B1 B cells**

Previous studies have reported that high-fat diet can have adverse effects on the immune system and lead to a decrease in macrophage activity in mice (30, 35). To test whether high-fat diet affects PtC-specific B1 B cell phagocytosis, C57BL/6J male mice were kept on two different diets: control diet (CD) with 18% kcal from fat and high fat diet (HFD) with 60% kcal from fat. After diet duration of 16 weeks, there was a 52% body weight increase in HFD group compared to CD group ($P < 0.0001$). Glucose Tolerance Test (GTT) revealed signs of glucose intolerance in HFD mice (Fig. 3*A*). After overnight incubation of isolated PerC cells with fluorescent beads in the presence of 5 μg/mL LPS, cells were subjected to flow cytometry and immunofluorescent microscopy analysis. Unexpectedly, we found a significant 53% increase in phagocytic PerC B1 B cell population ($P < 0.0001$). Notably, PerC B1 B cells displayed a loss of specificity for PtC and started to phagocytize control beads along with PtC-coated beads (Fig. 3*B*). This shift in specificity of phagocytosis for PtC was also captured by immunofluorescent microscopy as shown in Fig. 3*C*. Not only the amount of internalized fluorescent beads by PerC B1 B cells in HFD group increased (more than 8 beads per cell), but also minor changes in morphology of these cells was observed such as slightly larger cytoplasm and cell size. These data suggested a B1-to-macrophage-like cell transition that may be induced by HFD. This might serve as a potential feedback mechanism to speed up the clearance of increased fatty acid and oxidized LDL in the peritoneal cavity released during adipose tissue inflammation in DIO mice at the costs of losing phagocytosis specificity.

It has been suggested that while B1 B cells primarily reside in pleural and peritoneal cavities in mice, they can also circulate in the body and are quickly mobilized during systemic events such as inflammation and infection (29). The human equivalent of murine B1 B cells were first reported to be present in peripheral and umbilical cord blood by Griffin et al (19). Although there are still controversial questions about the true identity of these putative human B1 cells, many believed that these cells can be detected promptly and reliably using whole blood (37). Therefore, we decided to examine whether HFD-induced changes in phagocytic PerC B1 B cells can also be detected in whole blood in mice. Blood of both CD and HFD groups was collected with cardiac puncture and isolated cells were incubated with the same condition as PerC cells (37˚C, overnight, with 5 μg/mL LPS stimulation). Flow cytometry analysis showed the same loss of specificity for PtC (indicated by black arrows), but no significant increase in phagocytic B1 B cell number was noticed in blood (Fig. 3*D*). The lack of increased phagocytic cell population in blood was probably due to the limited number of B1 cells present in this tissue. Similar loss of PtC-specificity due to effect of HFD between B1 B cells in peritoneal cavity and blood further supports the notion that B1 cells could be mobilized to migrate out of serosal cavities and circulate throughout the body in response to systemic events such as obesity or other systemic inflammatory diseases.

In summary, our study reported a new functional characteristic of B1 cells that could potentially be used to distinguish them from other B lymphocytes – the ability to specifically phagocytose PtC, one of the phospholipid classes. It is in our interest to further investigate phagocytic activity and specificity of B1 B cells with other

phospholipids such as phosphatidylserine which has been found as the “eat me” signal exposed on apoptotic and senescent cells (15, 38). Furthermore, if this phagocytic specificity of B1 cells can be established as one of the key features of murine B1 B cells, then it can also be employed as a useful parameter in the search for equivalent of murine B1 B cells in humans. In addition, acquiring macrophage-like phagocytosis by B1 cells in consequence of HFD strongly supported previous postulation that B1 cells could undergo differentiation to become phagocytes during inflammatory response (4, 33).

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