Differential regulation by leukotrienes and calcium of Fcγ receptor-induced phagocytosis and Syk activation in dendritic cells versus macrophages

Claudio Canetti,*,† David M. Aronoff,*,‡ Mun Choe,* Nicolas Flamand,* Scott Wettlaufer,* Galen B. Toews,* Gwo-Hsiao Chen,* and Marc Peters-Golden*,[1]

*Divisions of *Pulmonary and Critical Care Medicine and ‡Infectious Diseases, University of Michigan Health System, Ann Arbor; and †Departamento de Farmacologia, Universidade do Estado do Rio de Janeiro, Brazil*

Abstract: Macrophage (MØ) phagocytosis via the Fc receptor for immunoglobulin G (FcγR) requires the spleen tyrosine kinase (Syk) and serves an important antimicrobial function. We have reported previously that FcγR-mediated ingestion and Syk activation in MØ are amplified by and depend on the proinflammatory lipid mediator leukotriene B_4 (LTB_4). Although FcγR-mediated ingestion is also important for antigen uptake, there is no information about LTB_4 regulation of these processes in dendritic cells (DCs). In this study, we compared murine bone marrow (BM)-derived DCs to MØ from BM, peritoneum, and the pulmonary alveolar space. Neither phagocytosis nor Syk activation in DCs was influenced by exogenous LTB_4. Unlike the various MØ populations, Syk activation in DCs was likewise unaffected by pharmacologic or genetic strategies to inhibit endogenous LTB_4 synthesis or to block the high-affinity LTB_4 receptor BLT1. DCs were refractory to regulation by LTB_4 despite the fact that they expressed BLT1 and mobilized intracellular calcium in response to its ligation. This resistance to LTB_4 in DCs instead reflected the fact that in contrast to MØ, Syk activation in DCs was itself entirely independent of calcium. These results identify a fundamental difference in FcγR signaling between DCs and MØ, which may relate to the divergent, functional consequences of target ingestion in the two cell types. *J. Leukoc. Biol.* 79: 1234–1241; 2006.

Key Words: *innate immunity · lipid mediators · cell signaling*

INTRODUCTION

Phagocytosis is a key process for immune responses initiated by dendritic cells (DCs) and macrophages (MØ). Among several mechanisms of internalization, the ingestion of immunoglobulin G (IgG)-opsonized targets via the Fc receptor for IgG (FcγR) is essential for DCs to process and subsequently present antigens to T lymphocytes in acquired immunity and for MØ to clear microbes in innate immunity.

The protein spleen tyrosine kinase (Syk) plays an essential role in various signaling cascades necessary for immune cell responses, including the ingestion of IgG-coated targets by MØ [1] and DCs [2]. Following FcγR engagement, the two N-terminal Src homology 2 domains of Syk bind to the immunoreceptor tyrosine-based activation motifs of the γ-chain of FcγRI and FcγRIII and with the cytoplasmic domain of FcγRIIA. Following this interaction, Syk becomes phosphorylated on tyrosine and is itself activated, initiating a cascade of signaling events leading to actin polymerization and phagocytosis [3, 4].

Leukotrienes (LTs) are lipid mediators of inflammation derived from the 5-lipoxygenase (5-LO) pathway of arachidonic acid (AA) metabolism. The enzyme 5-LO, in conjunction with its helper protein 5-LO-activating protein (FLAP), oxygenates AA to form LTA_4. This intermediate can be hydrolyzed to form the potent leukocyte activator and chemoattractant LTB_4 or conjugated with glutathione to form cysteinyl-LTs (cysLT; LTC_4, LTD_4, and LTE_4), which elicit smooth muscle contraction and microvascular permeability [5]. Important in vivo roles for LTs have been established in acquired [6, 7] and innate [8] immune responses.

We have demonstrated previously that endogenous and exogenous LTB_4 enhances FcγR-induced phagocytosis in MØ [9] and neutrophils [10]. One mechanism by which LTB_4 does so in MØ is by amplifying FcγR-induced Syk activation, a process that was itself dependent on Ca^{2+} influx [11]. In the present study, we sought to extend this work to DCs by evaluating the role of LTB_4 in modulating FcγR-induced phagocytosis and Syk activation in these professional antigen-presenting cells (APC). In contrast to our findings with MØ, we now demonstrate that FcγR-induced phagocytosis in DCs is independent of LTB_4 and that Syk activation in DCs is itself independent of LTB_4 and Ca^{2+}.

---

[1] Correspondence: Division of Pulmonary and Critical Care Medicine, 6301 MSRB III, 1150 W. Medical Center Drive, University of Michigan Medical Center, Ann Arbor, MI 48109-0642. E-mail: petersm@umich.edu

Received July 9, 2005; revised August 18, 2005; accepted February 22, 2006; doi: 10.1189/jlb.0705374.

 0741-5400/06/0079-1234 © Society for Leukocyte Biology

MATERIALS AND METHODS

Animals

Seventeen 5-LO knockout (KO; 129-Alox5^{tm1Fun}) mice on a 129 background were bred in the University of Michigan Unit for Laboratory Animal Medicine (Ann Arbor) from breeders obtained from The Jackson Laboratories (Bar Harbor, ME). Strain-matched wild-type (WT) mice as well as CBA mice and Wistar rats were obtained from Charles River Laboratories (Portage, MI). The University Committee on Use and Care of Animals approved animal protocols.

Generation of bone marrow (BM)-derived DCs (BM-DCs) and BM-derived MØ (BM-MØ)

BM cells were harvested from flushed marrow cavities of femurs and tibiae of mice under aseptic conditions. BM-DCs were generated from mouse BM cells (1×10^6 cells/ml) cultured for 4 days in culture medium [RPMI with 10% fetal calf serum (FCS)], supplemented with 20 ng/ml granulocyte MØ-colony stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ) and 20 ng/ml interleukin (IL)-4 (PeproTech), as described by Asavaroengchai et al. [12]. The BM-DC population was enriched by collecting the low-density interface following 14.5% (by weight) metrizamide density gradient centrifugation (15 min, 4°C, 2000 revolutions per minute) and washing twice in Hanks' balanced saline solution (HBSS). Purified BM-DCs (~90% purity) were further cultured in 60 mm dishes at 1×10^6 cells/ml for 24 h before use.

Flow cytometry analysis of cell surface antigens

To prevent nonspecific FcγR-mediated staining, the cell suspension (except the one for FcγR staining) was preincubated with anti-CD16/CD32 (FcγRIII/II) monoclonal antibody (mAb), according to the manufacturer's instructions (PharMingen, San Diego, CA). The following mAb were used for staining of surface antigens: anti-F4/80 (murine MØ marker), anti-CD16/CD32 (FcγRIII/II), anti-CD11c (integrin α_x chain), anti-CD80 (B7-1), anti-CD86 (B7-2), anti-I-A^k [major histocompatibility complex (MHC) class II], and the appropriate isotype controls (all from PharMingen). For flow cytometry, the cells were adjusted to a concentration of 5×10^6 cells/ml in staining buffer [fluorescent assay (FA) buffer (Difco, Detroit, MI) with 0.1% sodium azide and 1% FCS]. Cells (5×10^5) were stained with saturating antibody concentrations for 30 min at 4°C. The samples were washed in FA buffer and fixed with 1% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) in buffered saline. Stained samples were stored in the dark at 4°C until analyzed on a flow cytometer (Coulter Elite ESP, Palo Alto, CA). Flow cytometry data were analyzed by using the FlowJo 5.4.5 software (Tree Star, San Carlos, CA).

Adherent BM-DCs used in this study were considered to be immature BM-DCs, a fact confirmed by flow cytometry, demonstrating a higher expression of CD11c [mean fluorescence intensity (MFI) of 110.1 vs. 87.6] and FcγR (MFI 73.1 vs. 47.1) and a lower expression of MHC class II (MFI 75.6 vs. 159.5), CD80 (MFI 88.1 vs. 200.4), and CD86 (MFI 77.7 vs. 138.7), as compared with more mature, nonadherent BM-DCs. BM-MØ (>95% pure) were obtained from mouse BM cells prepared as described above and cultured at 1×10^6 cells/ml for 6 days in medium (RPMI with 10% FCS) supplemented with 20 ng/ml GM-CSF following a modified protocol published previously [13].

Isolation and culture of alveolar and peritoneal MØ

Resident alveolar MØ were obtained by lung lavage from rats as described previously [14]. Resident peritoneal MØ were harvested by peritoneal lavage of mice with 5 ml Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY). The cell suspensions were enumerated using a hemocytometer, adhered in flat-bottom, six-well plates (Becton Dickinson, Franklin Lakes, NJ) for 1 h at 37°C in a 5% CO_2 atmosphere, and nonadherent cells were removed by washing. After adherence, the cultures were composed of more than 98% MØ, as assessed by a modified Wright-Giemsa stain (Diff-Quik, American Scientific Products, McGaw Park, IL). MØ monolayers were cultured overnight in DMEM with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). The cells were washed, and the medium changed to DMEM without serum 20 min before the challenge with phagocytic targets.

Microcolorimetric erythrocyte phagocytosis assay

The phagocytosis of sheep red blood cells (sRBCs) was assessed as described previously [15]. Briefly, BM-DCs, BM-MØ, and rat alveolar MØ were plated for 24 h in 96-well, culture-treated dishes (BD Biosciences, San Jose, CA) at a density of 2×10^5 cells/well. Cells were then washed twice with warm DMEM and preincubated with the phagocytosis inhibitor cytochalasin D (Cyto D; 5 μg/ml, 45 min), LTC_4 (10 nM, 2 min, Cayman Chemical, Ann Arbor, MI), or LTB_4 (10 nM, 2 min, Cayman Chemical). RBCs (ICN Pharmaceuticals, Costa Mesa, CA) were opsonized with a subagglutinating concentration of polyclonal rabbit anti-sRBC IgG (Cappel Organon Teknika, Durham, NC) as described previously [16]. Following preincubation, opsonized RBCs were added at a target:cell ratio of 50:1, and cultures were incubated for an additional 90 min at 37°C. Wells were then washed three times with phosphate-buffered saline (PBS) to remove noningested erythrocytes, and 100 μl 0.3% sodium dodecyl sulfate (SDS) in PBS was added to each well for 10 min. Serial dilutions of known amounts of RBCs were added to separate wells before the addition of the SDS solution to derive a standard curve. Lastly, 100 μl *o*-phenylenediamine dihydrochloride solution was added to each well as a chromogen. Following a 30-min incubation (at 22°C) in the dark, the absorbance at 450 nm was evaluated with an automated reader (VERSAmax, Molecular Devices, Sunnyvale, CA). The number of RBCs per well was derived from absorbance data at 450 nm using the standard curve made with known amounts of RBCs. Independent experiments were performed in septuplet.

Quantification of fluorescein isothiocyanate (FITC)-dextran endocytosis by flow cytometry analysis

To analyze endocytosis, cells (2×10^6) were incubated in RPMI-1640 medium containing 3% FCS and incubated with FITC-dextran (Sigma Chemical Co.) at a final concentration of 1 mg/ml at 37°C for 60 min, as described previously [17]. After incubation, cells were washed with ice-cold FA buffer, then fixed in 4% paraformaldehyde, and were analyzed by flow cytometry. The FITC-dextran uptake was determined as the mean fluorescence relative to the background staining of the respective sample incubated with FITC-dextran at 4°C.

Mixed leukocyte reaction (MLR)

To assess lymphocyte proliferation in response to APC, RBC-depleted, allogeneic spleen cells were cultured in triplicate at 5×10^5 cells/well in 96-well plates together with 10^4 BM-DCs or BM-MØ cells as stimulators in a total volume of 200 μl RPMI-1640 medium containing 10% heat-inactivated FCS. Prior to culture, stimulator cells were irradiated (3000 rads; ^{137}Cs source). DNA synthesis was assessed over the last 16 h of a 72-h culture by addition of 1 mCi/well ^{3}H-thymidine (Amersham, Arlington Heights, IL). Cells were harvested onto glass fiber filters and placed in scintillation fluid (Scintiverse, Fisher Chemicals, Fair Lawn, NJ) for measurement of label incorporation in a liquid scintillation counter (Beckman Coulter, Fullerton, CA). Responses were reported as mean counts per minute (cpm) ± SEM of triplicate samples. Controls included responders or stimulators alone. Background ^{3}H-thymidine incorporation as a result of stimulators alone was <500 cpm.

Immunoprecipitation

Cell monolayers were lysed in buffer containing 1% Triton X-100 and 50 mM tris(hydroxymethyl)aminomethane (Tris; pH 8.0), 100 mM NaCl, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1 μg/mL leupeptin. Lysates were precleared with protein A-Sepharose for 30 min and incubated overnight at 4°C with anti-Syk (1:80, Santa Cruz Biotechnology, CA). Protein A-Sepharose was added to each sample and incubated for 3 h with rotation at 4°C. The beads were washed briefly three times with lysis buffer without Triton X-100 and separated on 8% SDS-polyacrylamide gel electrophoresis. The entire volume recovered after boiling the beads was loaded onto the gel; lysates are derived from equal numbers of cells, but total Syk per lane was subject to variation. The proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) overnight at 100 amps and for 3 h at 200 mA.

Immunoblotting

The membrane was blocked with 5% fat-free milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h, washed three times, and then probed with antiphosphotyrosine (anti-PY; 1:900, PY20, Transduction Laboratories, Lexington, KY) for 1.5 h. After that, the membrane was washed and incubated with a horseradish-peroxidase (HRP)-conjugated sheep anti-mouse secondary antibody (1:15,000, Amersham Pharmacia Biotech, Piscataway, NJ). Phosphorylated bands were visualized using the enhanced chemiluminescence (ECL) system (Amersham). The membranes were then stripped, blocked, and reprobed with anti-Syk (1:800) for 1 h, followed by an incubation with HRP-conjugated donkey anti-rabbit secondary antibody (1:20,000, Amersham Pharmacia Biotech). The bands were visualized using the ECL system. Relative band densities were determined by densitometric analysis using National Institutes of Health Image software, and the ratios were calculated. The results were expressed as normalized Syk-PY/Syk, which represents the value of density obtained with the anti-PY blot divided by the value obtained with the anti-Syk blot. In all instances, density values of bands were corrected by subtraction of the background values.

Analysis of calcium mobilization

Adherent cells were harvested using the protease cocktail Accutase™ (eBioscience, San Diego, CA) according to the manufacturer's instructions, centrifuged for 5 min at 750 g, and then resuspended in HBSS containing 1.6 mM $CaCl_2$. The warmed cell suspension (37°C, 10^7 cells/ml) was treated with 10 μM Fura-2-acetoxymethyl ester (AM) for 30 min, then washed twice with HBSS containing 1.6 mM $CaCl_2$, and finally resuspended at a density of 10^7 cells/ml and transferred into the magnetically stirred cuvette of the luminescence spectrometer (Perkin Elmer LS50B, Perkin Elmer, Wellesley, MA). Calcium mobilization was monitored using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Data are presented as the ratio of fluorescence obtained from 340 and 380 (340/380) nm.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol® reagent, following the manufacturer's instructions (Invitrogen, Carlsbad, CA), and quantified by spectrophotometry. The PCR master mix was made from a Promega (Madison, WI) access RT-PCR system kit. It is a two-enzyme system, which allows an avian myloblastosis virus RT to process the RNA into cDNA in the first step and a Tfl DNA polymerase to facilitate the amplification over the number of desired cycles. The primers were designed to anneal at ~55°C. The primers used for the amplification of mouse high-affinity LTB_4 receptor BLT1 cDNA were (sense) 5′-GCAGTGGCCCGCCCCTTTATGTC-3′ and (antisense) 5′-CACCGGGT-TCACGCTGCTGCTC-3′ and for BLT2, were (sense) 5′-GTAGTATGGAGCT-TAGCGGGC-3′ and (antisense) 5′-GGGTCTCCAGGCTCAGATG-3′. Following the amplification, the DNA product was loaded on a 1.5% agarose gel. The DNA was then transferred to a nitrocellulose membrane in NaOH (to separate the DNA strands) and probed with a ^{32}P-labeled probe made specifically for the primer product. After hybridization, the membrane was exposed to photographic film.

Statistical analysis

The data are reported as a representative blot from two or three different experiments. Graphs represent the mean ± SEM from two or three different experiments. The means from different treatments were compared by ANOVA. When significant differences were identified, individual comparisons were subsequently made with the Bonferroni test for unpaired values. Statistical significance was set at a P value less than 0.05.

RESULTS

Surface marker profiles of BM-MØ and BM-DCs

To determine whether BM-MØ and BM-DCs are phenotypically distinct subpopulations, we used flow cytometry to analyze the surface markers expressed on both cell types (**Fig. 1A**). BM-MØ showed a moderate expression of the MØ marker F4/80, whereas BM-DCs expressed this weakly. Both populations exhibited FcγR expression, although BM-DCs expressed slightly higher levels than BM-MØ. BM-DCs expressed high levels of CD11c and CD80 and also exhibited slightly higher levels of costimulatory molecules CD86 and MHC class II antigen compared with BM-MØ. In summary, the results confirm that BM-MØ and BM-DCs are phenotypically distinct populations based on their surface antigen expression patterns.

FITC-dextran endocytosis of BM-MØ and BM-DCs

To verify the functional phenotype of the isolated cells, we evaluated the capacity of BM-MØ and BM-DCs to perform mannose receptor-mediated phagocytosis of FITC-dextran. Flow cytometric analysis showed that BM-DCs exhibited slightly better capacity to internalize FITC-dextran than did BM-MØ. Approximately 87% of BM-DCs became FITC-dextran-positive, and MFI of the cells incorporated FITC-dextran of 51.2 after 1 h of incubation; by comparison, 79% of BM-MØ were FITC-positive, and MFI of the cells was 37.7 (Fig. 1B).

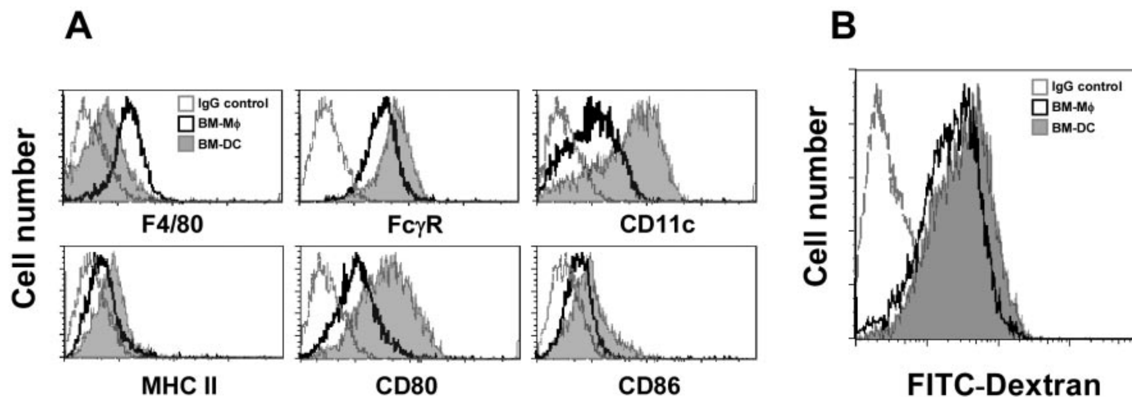

Fig. 1. (A) Cell surface phenotypes of BM-MØ and BM-DCs. Light gray lines in the histogram show nonspecific fluorescence by subclass control mAb. Fluorescence for the indicated antigens on BM-MØ and BM-DCs is shown by bold lines and shaded areas, respectively. This experiment was repeated three times with similar results, and representative results are shown. (B) Endocytosis of FITC-dextran by BM-MØ and BM-DC cells, which were incubated with FITC-dextran (1 mg/mL) for 1 h. Data are representative of three independent experiments.

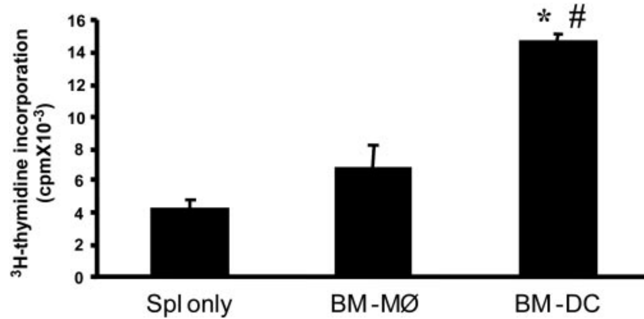

Fig. 2. Allogeneic MLR using BM-MØ and BM-DCs. Cells were irradiated and cultured for 3 days with allogeneic spleen cells, and ^{3}H-thymidine incorporation was measured. *, $P < 0.05$, compared with spleen cell alone; #, $P < 0.05$, compared with BM-MØ. Values are expressed as the mean ± SEM of triplicate samples. Results are representative of three independent experiments.

Antigen-presenting ability of BM-DCs

MLR was assessed to examine the differential antigen-presenting activity of BM-MØ and BM-DCs (**Fig. 2**). As expected, BM-DCs significantly stimulated allogeneic splenocyte proliferation, whereas BM-MØ provoked a slight proliferative response, which did not reach statistical significance.

LTs do not modulate phagocytosis of IgG-sRBC by BM-DCs

In previous reports, we demonstrated that phagocytosis of IgG-coated particles by alveolar MØ was enhanced by the 5-LO products LTC_4 and LTB_4 [8, 9, 18]. By contrast, preincubation of BM-DCs with LTC_4 or LTB_4 (each at 10 nM) did not affect FcγR-mediated phagocytosis (**Fig. 3**). Higher concentrations of the compounds (up to 100 nM) also failed to alter phagocytosis (not shown). Confirming that the effects of exogenous LTs on alveolar MØ are true for other MØ cell types, we found that LTC_4 and LTB_4 stimulated FcγR-mediated ingestion by BM-MØ (Fig. 3). The concentrations of LTs used in these studies were shown to enhance FcγR-mediated phagocytosis by alveolar MØ in parallel experiments (data not shown).

FcγR-mediated Syk activation is not modulated by LTB_4 or by other 5-LO-derived products

Ligation of FcγR by IgG is known to elicit activation (phosphorylation) of Syk in MØ [3] and DCs [19]. In MØ, this process is amplified by LTB_4 [11]. We used pharmacological and genetic approaches to investigate whether Syk activation is likewise modulated by LTB_4 in BM-DCs. As can be seen in **Figure 4A**, challenge of BM-DCs with IgG-opsonized sRBCs, but not unopsonized sRBCs, elicited Syk phosphorylation. However, treatment of BM-DCs with exogenous LTB_4 (100 nM) failed to amplify FcγR-induced Syk activation. Moreover, Syk activation was not influenced by endogenously produced LTB_4, as revealed by the use of a LTB_4 receptor antagonist (LY 292476; Fig. 4B), agents that inhibit LT synthesis by inhibition of 5-LO (zileuton) or of 5-LO-activating protein (MK 886; Fig. 4C), and cells from 5-LO$^{-/-}$ mice (**Fig. 5**). As noted in Figure 5, there is no difference in Syk phosphorylation between BM-DCs obtained from WT mice versus 5-LO$^{-/-}$ mice upon stimulation with several doses of IgG-coated RBCs.

BM-DCs express a functional LTB_4 receptor

To evaluate whether the lack of effect of LTB_4 on phagocytosis and Syk activation in BM-DCs was a result of the absence of the G protein-coupled BLT receptors, we first evaluated BLT1 and BLT2 messenger mRNA by RT-PCR. mRNA levels for BLT1 and BLT2 were similar to those obtained using rat alveolar MØ, and expression of the high-affinity BLT1 receptor was also confirmed by Western blot analysis (data not shown).

Next, we examined calcium influx in BM-DCs in response to LTB_4 challenge, as it is well known that calcium mobilization occurs immediately after LTB_4 interaction with Gαq-coupled BLTs [20]. Stimulation of BM-DCs with LTB_4 evoked a substantial increase in intracellular Ca^{2+} concentration, as can be observed in **Figure 6**, suggesting the presence of a functional receptor in BM-DCs. Confirming the presence of BLT1 on

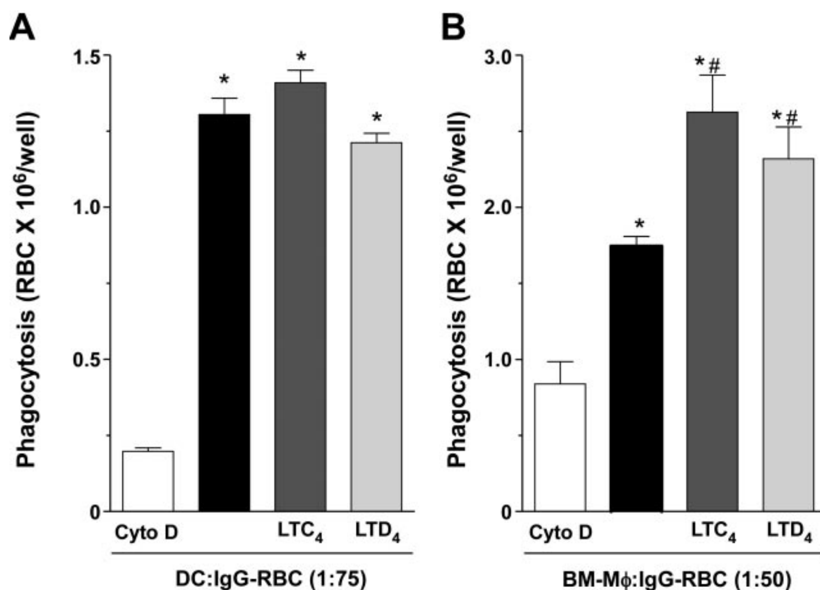

Fig. 3. FcγR-mediated phagocytosis is not affected by LTs in BM-DCs. Mouse BM-DCs (A) or BM-MØ (B) were obtained as described in Materials and Methods. Cells were pretreated with the phagocytosis inhibitor Cyto D (5 μg/ml) for 45 min or LTC_4 or LTB_4 (each at 10 nM) for 2 min and then challenged with IgG-opsonized RBCs. Phagocytosis (ingested RBCs) was calculated based on a standard curve. *, $P <$ 0.05, compared with Cyto D-treated cells; #, $P < 0.05$, compared with untreated control, as determined by ANOVA followed by Bonferroni's multiple comparison test (n=6–8).

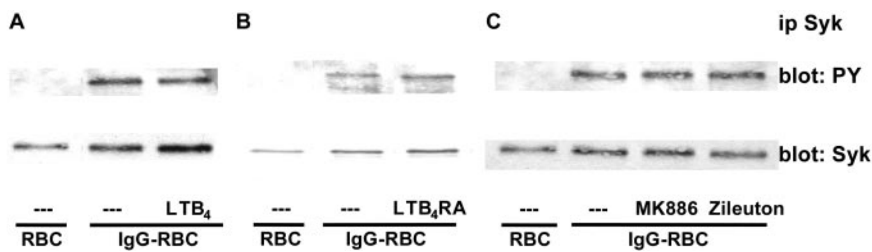

Fig. 4. FcγR-mediated Syk activation is not modulated by LTB_4 or by other 5-LO-derived products. (A) LTB_4 does not amplify FcγR-mediated Syk activation. BM-DCs were pretreated with LTB_4 (100 nM) for 2 min prior to the addition of IgG-RBCs (1:33 ratio). (B) A LTB_4 receptor antagonist (RA) does not change FcγR-mediated Syk activation. BM-DCs were pretreated with the BLT1 receptor antagonist LY 292476 (10 μM) for 10 min prior to the addition of IgG-RBCs (1:3 ratio). (C) Inhibition of LT synthesis does not change FcγR-mediated Syk activation. BM-DCs were pretreated with the FLAP inhibitor MK 886 (1 μM) or the 5-LO inhibitor zileuton (10 μM) for 20 min prior to the addition of IgG-RBCs (1:100). Seven minutes after RBCs or IgG-RBC challenge at 37°C, the incubations were terminated by addition of lysis buffer, and lysates were subjected to immunoprecipitation (ip) and immunoblotting (blot) as described in Materials and Methods. Immunoblots in upper panels represent phosphorylated Syk detected with anti-PY antibody and those in lower panels, the amounts of Syk protein evaluated with anti-Syk antibody. Results are representative of two separate experiments.

BM-DCs, the use of a BLT1 receptor antagonist (CP 105,696; 1 μM) prior to LTB_4 administration impaired Ca^{2+} mobilization (data not shown).

FcγR-mediated Syk activation is not Ca^{2+}-dependent in BM-DCs

We have demonstrated previously that enhancement of Syk activation by LTB_4 in MØ is dependent on increases in intracellular Ca^{2+} [11]. Having established that LTB_4 interaction with its receptors indeed increases intracellular Ca^{2+} concentration in BM-DCs, we considered the possibility that FcγR-mediated Syk activation is not a Ca^{2+}-dependent process in BM-DCs, which were pretreated with the extracellular Ca^{2+} chelator EGTA and/or the intracellular chelator 1,2-bis(*O*-aminophenyl-ethane-ethane)-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA)-AM for 30 min before IgG-RBC challenge. As observed in **Figure 7A**, FcγR-mediated Syk activation in BM-DCs was not inhibited by treatment with EGTA, BAPTA-AM, or the combination. By contrast, pretreatment with BAPTA-AM inhibited FcγR-mediated Syk activation in rat alveolar MØ (Fig. 7B), in BM-MØ (Fig. 7C), and in rat peritoneal MØ (Fig. 7D). The ability of LTB_4 treatment to amplify FcγR-mediated Syk activation in BM-MØ and in rat peritoneal MØ is also illustrated in Figure 7, C and D, respectively. As an alternative approach to comparing the participation of Ca^{2+} in Syk activation evoked by FcγR engagement in BM-DCs versus BM-MØ, we examined the effect of the addition of the calcium ionophore A23187 on FcγR-mediated Syk activation. As observed in **Figure 8A**, the treatment of BM-DCs with A23187 immediately before the addition of IgG-coated RBCs did not change Syk phosphorylation. In contrast, the same procedure performed in BM-MØ amplified FcγR-mediated Syk activation (Fig. 8B). Together, these results indicate that the process of Syk activation in BM-DCs is Ca^{2+}-independent, in contrast to that in MØ.

DISCUSSION

DCs avidly ingest IgG-coated targets, and activation of the nonreceptor tyrosine kinase Syk is a pivotal, early step in this process [2], as it is for MØ [1]. Syk activation has also been implicated in maturation and antigen presentation of DCs [2]. In the present study, we have, for the first time, assessed the role of LTs, important immunostimulatory lipid mediators, in modulating FcγR-induced phagocytosis as well as Syk activation in DCs. We examined the effects of exogenously supplied LTs and also used genetic and pharmacologic approaches to assess the role of endogenously generated LTs in these processes. Our data demonstrate two substantial differences between murine BM-DCs and MØ. First, neither FcγR-mediated phagocytosis nor Syk activation in DCs was influenced by exogenous or endogenous LTs. Second, FcγR-mediated Syk activation in DCs is not Ca^{2+}-regulated, as neither Ca^{2+} chelators nor Ca^{2+} ionophore treatment exerted any effect. As our previous report indicated that the ability of LTB_4 in particular to enhance FcγR-induced phagocytosis and Syk activation in MØ was dependent on receptor-mediated increases in intracellular Ca^{2+} [11], it is likely that the Ca^{2+} independence of Syk activation in DCs accounts for the lack of modulation by LTs.

We first verified that the two cell populations of interest generated in vitro from BM-derived cells exhibited the flow cytometric characteristics of BM-MØ and BM-DC phenotypes (Fig. 1A). We used adherence to yield a population of imma-

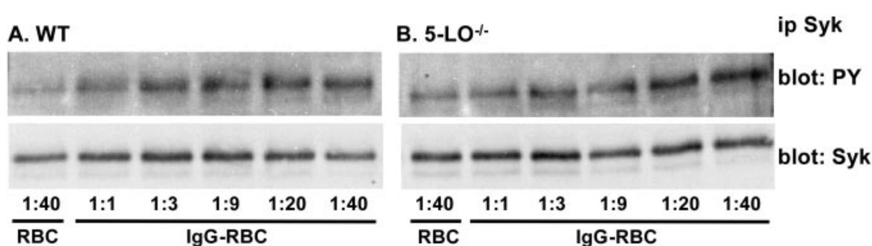

Fig. 5. Effect of 5-LO gene KO in FcγR-mediated Syk activation. BM-DCs obtained from WT (A) and 5-LO KO (B) mice were challenged with RBC (1:40) or with increasing amounts of IgG-sRBC, as indicated in the figure, and then incubated for 7 min at 37°C. Incubations were terminated by addition of lysis buffer, and lysates were subjected to immunoprecipitation and immunoblotting as described in Materials and Methods. Immunoblots in upper panels represent phosphorylated Syk detected with anti-PY antibody and those in lower panels, the amounts of Syk protein evaluated with anti-Syk antibody. Results are representative of two separate experiments.

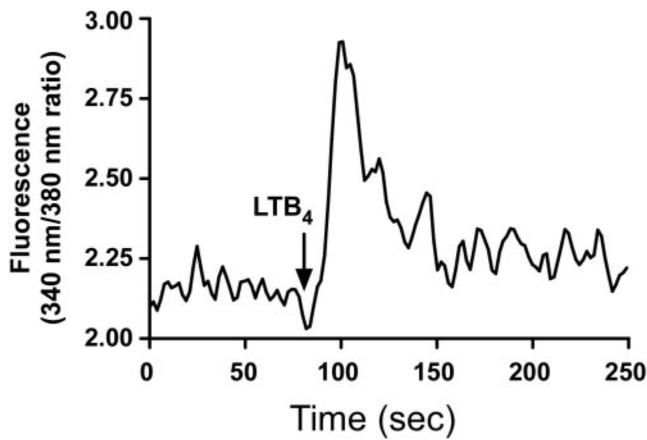

Fig. 6. LTB_4 evokes Ca^{2+} mobilization in BM-DCs, which were incubated with 10 μM Fura-2-AM for 30 min, washed, and resuspended at a density of 10^7 cells/ml into the magnetically stirred cuvette of the luminescence spectrometer. Calcium mobilization was monitored for ~80 s (stabilization), and then the cells were treated with LTB_4 (100 nM). Calcium mobilization is expressed as a fluorescence ratio obtained from 340 and 380 (340/380) nm. Results are representative of two separate experiments.

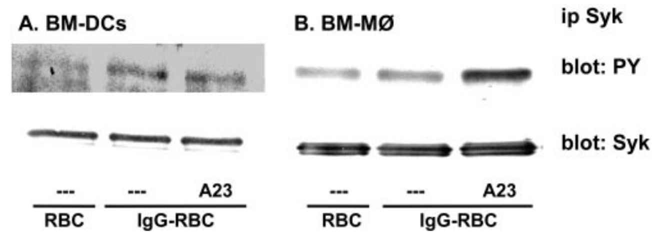

Fig. 8. Effect of Ca^{2+} ionophore on FcγR-mediated Syk activation. (A) Ca^{2+} ionophore does not modify FcγR-mediated Syk activation in BM-DCs, which were treated with A23187 (1 μM) immediately before the addition of IgG-RBCs (1:3 ratio). (B) Ca^{2+} ionophore amplifies FcγR-mediated Syk activation in BM-MØs, which were treated with A23187 (1 μM) immediately before the addition of IgG-RBCs (1:25 ratio). Seven minutes after RBCs or IgG-RBC challenge at 37°C, the incubations were terminated by addition of lysis buffer, and lysates were subjected to immunoprecipitation and immunoblotting as described in Materials and Methods. Immunoblots in upper panels represent phosphorylated Syk detected with anti-PY antibody and those in lower panels, the amounts of Syk protein evaluated with anti-Syk antibody. Results are representative of two separate experiments.

ture DCs known to actively capture and process antigens and migrate to the draining lymph nodes, wherein they increase their ability to stimulate resting T cells, thereby initiating various immune responses [21–23]. This fact was confirmed by flow cytometric analysis, in which the adherent cell population showed lower expression of MHC class II and higher expression of CD11c and FcγR compared with the nonadherent cell population. Also as expected, BM-MØ and BM-DCs exhibited comparable capacity for mannose-dependent ingestion of dextran, demonstrating the efficiency of the phagocytic machinery for both cell types (Fig. 1B).

BM-DCs have been reported to produce LTB_4 and cysLTs upon stimulation with lipopolysaccharide or antigen [24, 25] and to express the biosynthetic proteins necessary to initiate LT synthesis from AA, namely 5-LO and FLAP [25]. Furthermore, DCs have been shown to express receptors for cysLTs [25, 26], which have been implicated in DC migration to [26] and from [6] tissue sites of antigen challenge. Although LTB_4 has been reported to stimulate production of IL-6 as well as BM-DC generation when added to BM cultures [21], the receptors for LTB_4 have not been examined previously in DCs. Certainly, the inability of endogenous or exogenous LTB_4 to modulate FcγR-induced phagocytosis and Syk activation in BM-DCs could be the consequence of lack of expression of the high-affinity receptor BLT1. However, RT-PCR and immunoblot analyses revealed expression of BLT1 in BM-DCs. It has previously been reported that in contrast to epidermal MØ or peripheral blood monocytes, epidermal DCs from humans fail to mobilize intracellular Ca^{2+} in response to a variety of proinflammatory stimuli, including interleukin-1, bradykinin,

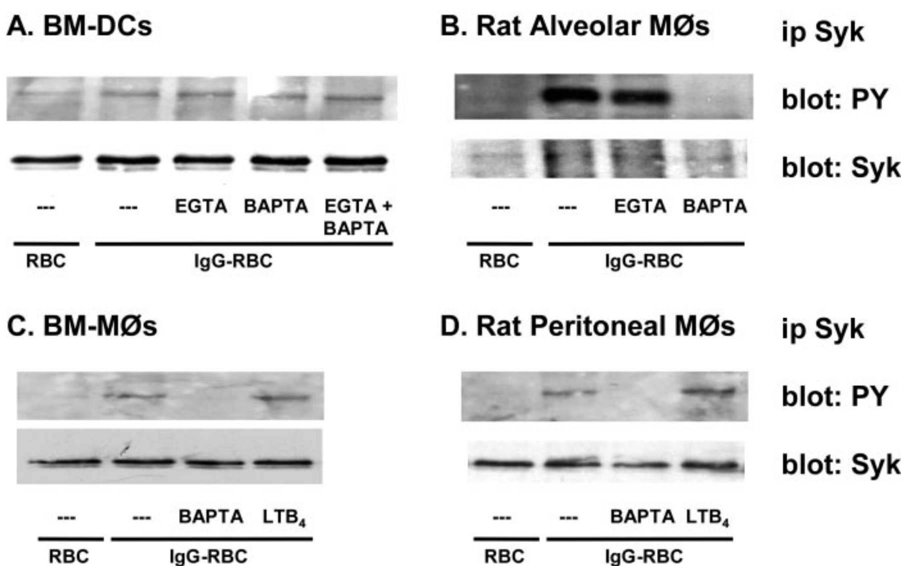

Fig. 7. Effect of Ca^{2+} chelators on FcγR-mediated Syk activation. (A) Ca^{2+} chelators do not modify FcγR-mediated Syk activation in BM-DCs, which were pretreated with EGTA (1 mM), BAPTA-AM (10 μM), or with both in the same doses for 30 min prior to the addition of IgG-RBCs (1:3 ratio). (B) Inhibition of FcγR-mediated Syk activation by BAPTA-AM in rat alveolar MØ, which were pretreated with EGTA (10 mM) or BAPTA-AM (50 μM) for 30 min prior to the addition of IgG-RBCs (1:100 ratio). (C) Inhibition of FcγR-mediated Syk activation by BAPTA-AM in BM-MØ, which were pretreated with BAPTA-AM (50 μM) for 30 min or with LTB_4 (100 nM) 2 min prior to the addition of IgG-RBCs (1:30 ratio). (D) Inhibition of FcγR-mediated Syk activation by BAPTA-AM in rat peritoneal MØ, which were pretreated with BAPTA-AM (50 μM) for 30 min or with LTB_4 (100 nM) 2 min prior to the addition of IgG-RBCs (1:30 ratio). Seven minutes after RBCs or IgG-RBC challenge at 37°C, the incubations were terminated by addition of lysis buffer, and lysates were subjected to immunoprecipitation and immunoblotting as described in Materials and Methods. Immunoblots in upper panels represent phosphorylated Syk detected with anti-PY antibody and those in lower panels, the amounts of Syk protein evaluated with anti-Syk antibody. Results are representative of two separate experiments.

and formyl-Met-Leu-Phe [27]. This raised the possibility that LTB_4 ligation of BLT1 might be incapable of triggering an intracellular Ca^{2+} flux in the BM-DCs under investigation here. It is important, however, that the functional competence of this receptor was established by demonstrating intact LTB_4-induced Ca^{2+} mobilization, which was inhibitable by a BLT1-selective antagonist. Thus, the refractoriness of BM-DCs to LTB_4 cannot be explained by the absence of its high-affinity receptor BLT1 or its inability to signal appropriately via Ca^{2+} mobilization.

Like BLT1, the high-affinity receptor for cysLTs (cysLT1) is also a Gαq-coupled receptor. That ligation of these two discrete Ca^{2+}-coupled LT receptors in BM-DCs failed to modulate FcγR-mediated phagocytosis and Syk activation suggested the alternative possibility that such processes were independent of Ca^{2+} in these cell types. Experiments using Ca^{2+} chelators and ionophores demonstrated that this was indeed the case for Syk activation in BM-DCs. This contrasts with parallel findings in murine BM-MØ as well as rat alveolar and peritoneal MØ, in which Syk activation was clearly modulated by changes in intracellular Ca^{2+}. As other DC functions are well-recognized to be regulated by Ca^{2+} [28], the Ca^{2+} independence of Syk regulation reflects a selective property of murine BM-DCs.

Little is known about how increased intracellular Ca^{2+} enhances Syk activation in MØ. Nevertheless, this mode of regulation might be expected to provide a means by which many proinflammatory ligands for Gαq-coupled receptors could amplify FcγR-mediated phagocytosis essential for antimicrobial defense. The fact that this mechanism is not operative in DCs, at least in the immature murine BM-DCs under investigation here, reveals cell-specific differences in the means by which these two types of monocyte-derived phagocytic cells are regulated. Although the teleologic explanation for distinct regulatory mechanisms in DCs is at this point unknown, one can speculate that it may reflect differences in the milieu in which these cells encounter IgG-coated targets or in the functional significance of the phagocytic process. Certainly, the functional consequences of FcγR-mediated ingestion are widely divergent between MØ (microbial killing) and DCs (antigen processing for its eventual presentation), and these downstream responses may conceivably be differentially regulated by Ca^{2+}. Consistent with this possibility is the fact that in contrast to monocytes or MØ, DCs fail to mobilize Ca^{2+} [27] or generate reactive oxygen intermediates [19] in response to FcγR ligation. It remains to be determined whether the differences we have identified in the regulation of Syk activation contribute to divergent, functional consequences of FcγR ligation in the two cell types.

ACKNOWLEDGMENTS

This work was supported by Conselho Nacional de Pesquisa (CNPq–Brazil; to C. C.), R01-HL058897 (to M. P-G.), R01-HL51082 (to G. B. T.), K08-HL078727 (to D. M. A.), and a Department of Veterans Affairs Merit Grant (to G. B. T.).

REFERENCES

1. Crowley, M. T., Costello, P. S., Fitzer-Attas, C. J., Turner, M., Meng, F., Lowell, C., Tybulewicz, V. L., DeFranco, A. L. (1997) A critical role for Syk in signal transduction and phagocytosis mediated by Fc γ receptor on macrophages. *J. Exp. Med.* **186,** 1027–1039.
2. Sedlik, C., Orbach, D., Veron, P., Schweighoffer, E., Colucci, F., Gamberale, R., Ioan-Facsinay, A., Verbeek, S., Ricciardi-Castagnoli, P., Bonnerot, C., Tybulewicz, V. O., Di Santo, J., Amigorena, S. (2003) A critical role for Syk protein tyrosine kinase in Fc receptor-mediated antigen presentation and induction of dendritic cell maturation. *J. Immunol.* **170,** 846–852.
3. Greenberg, S., Grinstein, S. (2002) Phagocytosis and innate immunity. *Curr. Opin. Immunol.* **14,** 136–145.
4. Daeron, M. (1997) Fc receptor biology. *Annu. Rev. Immunol.* **15,** 203–234.
5. Lewis, R. A., Austen, K. F., Soberman, R. J. (1990) Leukotrienes and other products of the 5-lipoxygenase pathway: biochemistry and relation to pathobiology in human disease. *N. Engl. J. Med.* **323,** 645–655.
6. Robbiani, D. F., Finch, R. A., Jager, D., Muller, W. A., Sartorelli, A. C., Randolph, G. J. (2000) The leukotriene C(4) transporter MRP1 regulates CCL19 (MIP-3β, ELC)-dependent mobilization of dendritic cells to lymph nodes. *Cell* **103,** 757–768.
7. Irvin, C. G., Tu, Y. P., Sheller, J. R., Funk, C. D. (1997) 5-Lipoxygenase products are necessary for ovalbumin-induced airway responsiveness in mice. *Am. J. Physiol.* **272,** L1053–L1058.
8. Bailie, M. B., Standiford, T., Laichalk, L., Coffey, M., Strieter, R., Peters-Golden, M. (1996) Leukotriene-deficient mice manifest enhanced lethality from *Klebsiella pneumonia* in association with decreased alveolar macrophage phagocytic and bactericidal activities. *J. Immunol.* **157,** 5221–5224.
9. Mancuso, P., Marshall, T., Standiford, T., Peters-Golden, M. (1998) 5-Lipoxygenase reaction products modulate alveolar macrophage phagocytosis of *Klebsiella pneumonia*. *Infect. Immun.* **66,** 5140–5146.
10. Mancuso, P., Nana-Sinkam, P., Peters-Golden, M. (2001) Leukotriene B_4 augments neutrophil phagocytosis of *Klebsiella pneumonia*. *Infect. Immun.* **69,** 2011–2016.
11. Canetti, C., Hu, B., Curtis, J. L., Peters-Golden, M. (2003) Syk activation is a leukotriene B_4-regulated event involved in macrophage phagocytosis of IgG-coated targets but not apoptotic cells. *Blood* **102,** 1877–1883.
12. Asavaroengchai, W., Kotera, Y., Koike, N., Pilon-Thomas, S., Mule, J. J. (2004) Augmentation of antitumor immune responses after adoptive transfer of bone marrow derived from donors immunized with tumor lysate-pulsed dendritic cells. *Biol. Blood Marrow Transplant.* **10,** 524–533.
13. Yang, S. D., Schook, L. B., Rutherford, M. S. (1995) Differential expression of novel genes by bone marrow-derived macrophage populations. *Mol. Immunol.* **32,** 733–742.
14. Peters-Golden, M., Thebert, P. (1987) Inhibition by methylprednisolone of zymosan-induced leukotriene synthesis in alveolar macrophages. *Am. Rev. Respir. Dis.* **135,** 1020–1026.
15. Smacchia, C., Rebulla, P., Drago, F., Morelati, F., Pappalettera, M., Sirchia, G. (1997) A micro colorimetric assay using cryopreserved monocytes to evaluate antibody-mediated red cell-monocyte interaction. *Haematologica* **82,** 526–531.
16. Araki, N., Johnson, M. T., Swanson, J. A. (1996) A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J. Cell Biol.* **135,** 1249–1260.
17. Sallusto, F., Cella, M., Danieli, C., Lanzavecchia, A. (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J. Exp. Med.* **182,** 389–400.
18. Mancuso, P., Peters-Golden, M. (2000) Modulation of alveolar macrophage phagocytosis by leukotrienes is Fc receptor-mediated and protein kinase C dependent. *Am. J. Respir. Cell Mol. Biol.* **23,** 727–733.
19. Fanger, N. A., Voigtlaender, D., Liu, C., Swink, S., Wardwell, K., Fisher, J., Graziano, R. F., Pfefferkorn, L. C., Guyre, P. M. (1997) Characterization of expression, cytokine regulation, and effector function of the high affinity IgG receptor Fc γ RI (CD64) expressed on human blood dendritic cells. *J. Immunol.* **158,** 3090–3098.
20. Tager, A. M., Luster, A. D. (2003) BLT1 and BLT2: the leukotriene B(4) receptors. *Prostaglandins Leukot. Essent. Fatty Acids* **69,** 123–134.
21. Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, U. J., Pulendran, B., Palucka, K. (2000) Immunobiology of dendritic cells. *Annu. Rev. Immunol.* **18,** 767–811.
22. Lambrecht, B. N., Pauwels, R. A., Fazekas de St. Groth, B. (2000) Introduction of rapid T cell activation, division and recirculation by

intratracheal injection of dendritic cells in TCR-transgenic model. *J. Immunol.* **164,** 2937–2946.

23. Vermaelen, K. Y., Carro-Muino, I., Lambrechit, B. N., Pauwels, R. A. (2001) Specific migratory dendritic cells rapidly transport antigen from airways to the thoracic lymph nodes. *J. Exp. Med.* **193,** 51–60.
24. Harizi, H., Juzan, M., Pitard, V., Moreau, J. F., Gualde, N. (2002) Cyclooxygenase-2-issued prostaglandin E(2) enhances the production of endogenous IL-10, which down-regulates dendritic cell functions. *J. Immunol.* **168,** 2255–2263.
25. Machida, I., Matsuse, H., Kondo, Y., Kawano, T., Saeki, S., Tomari, S., Obase, Y., Fukushima, C., Kohno, S. (2004) Cysteinyl leukotrienes regulate dendritic cell functions in a murine model of asthma. *J. Immunol.* **172,** 1833–1838.
26. Parameswaran, K., Liang, H., Fanat, A., Watson, R., Snider, D. P., O'Byrne, P. M. (2004) Role for cysteinyl leukotrienes in allergen-induced change in circulating dendritic cell number in asthma. *J. Allergy Clin. Immunol.* **114,** 73–79.
27. Shibaki, A., Ohkawara, A., Cooper, K. D. (1995) Differential extracellular signaling via FcγR and fMLP in functionally distinct antigen-presenting cell subsets: ultraviolet-induced epidermal macrophages versus Langerhans cells. *J. Invest. Dermatol.* **105,** 383–387.
28. Do, Y., Hegde, V. L., Nagarkatti, P. S., Nagarkatti, M. (2004) Bryostatin-1 enhances the maturation and antigen-presenting ability of murine and human dendritic cells. *Cancer Res.* **64,** 6756–6765.