

FcεRI- and Fcγ Receptor-Mediated Production of Reactive Oxygen Species by Mast Cells Is Lipoxygenase- and Cyclooxygenase-Dependent and NADPH Oxidase-Independent¹

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We investigated the enzymes responsible for FcεRI-dependent production of reactive oxygen species (ROS) and the influence of ROS on mast cell secretory responses. 5-Lipoxygenase (5-LO) was the primary enzyme involved in ROS production by human mast cells (huMC) and mouse bone marrow-derived mast cells (mBMMC) following FcεRI aggregation because incubation with 5-LO inhibitors (AA861, nordihydroguaiaretic acid, zileuton) but not a flavoenzyme inhibitor (diphenyleneiodonium) completely abrogated Ag-induced dichlorodihydrofluorescein (DCF) fluorescence. Furthermore, 5-LO-deficient mBMMC had greatly reduced FcεRI-dependent DCF fluorescence compared with wild type mBMMC or those lacking a functional NADPH oxidase (i.e., gp91^{phox}- or p47^{phox}-deficient cells). A minor role for cyclooxygenase (COX)-1 in FcεRI-dependent ROS production was demonstrated by inhibition of Ag-mediated DCF fluorescence by a COX-1 inhibitor (FR122047) and reduced DCF fluorescence in COX-1-deficient mBMMC. Complete abrogation of FcεRI-dependent ROS production in mast cells had no effect on degranulation or cytokine secretion. In response to the NADPH oxidase-stimulating agents including PMA, mBMMC and huMC produced negligible ROS. IgG-coated latex beads did stimulate ROS production in huMC, and in this experiment 5-LO and COX again appeared to be the enzymatic sources of ROS. In contrast, IgG-coated latex bead-induced ROS production in human polymorphonuclear leukocytes occurred by the NADPH oxidase pathway. Thus mBMMC and huMC generate ROS by 5-LO and COX-1 in response to FcεRI aggregation; huMC generate ROS upon exposure to IgG-coated latex beads by 5-LO and COX; and ROS appear to have no significant role in FcεRI-dependent degranulation and cytokine production. *The Journal of Immunology*, 2007, 179: 7059–7071.

Reactive oxygen species (ROS)³ are classically associated with bacterial killing through activation of the enzyme NADPH oxidase to produce high levels of toxic species including superoxide anion (O₂^{•−}) and hydrogen peroxide (H₂O₂). This pathway is primarily present in phagocytes, including polymorphonuclear leukocytes (PMN) and macrophages, and is part of the innate immune response toward infection (1, 2). ROS are also generated at low levels through enzymatic or nonenzymatic mechanisms, where they act as signaling molecules (3). These alternative pathways are found in a variety of cells including T cells and B cells and smooth muscle cells. TCR cross-linking induces both O₂^{•−} and H₂O₂ (4, 5), which participate in cytokine secretion, proliferation, and Fas ligand-mediated cell death. BCR cross-linking induces ROS, which participate in a calcium feedback loop to am-

plify early signaling events (6, 7). In smooth muscle cells, H₂O₂ is involved in platelet-derived growth factor (8), epidermal growth factor (9), and angiotensin II (10) mediated signal transduction.

Mast cells are central effector cells in both specific and innate immunity (11–13). In specific IgE-mediated responses they are activated by multivalent Ag to release both preformed and newly synthesized mediators including histamine, leukotriene (LT), PG, cytokine, and chemokines (11, 14). In innate immunity they promote PMN recruitment and phagocytosis (15–17) and lymph node hyperplasia (18, 19). However, there is much debate about the role and enzymes responsible for ROS production in mast cells. For example, we have shown that mast cells generate ROS but not NO following FcεRI aggregation (20) and these ROS are retained inside the cell, in contrast to macrophages that export ROS (21). However, others (22) have reported blockade of Ag-mediated ROS production in mast cells by the flavoenzyme inhibitor diphenyleneiodonium (DPI) or the antioxidant ebselen. DPI inhibited Ag-induced phospholipase Cγ and LAT phosphorylation, calcium influx, degranulation, and LT release, although very high concentrations of DPI were used (22). In addition, PMA-induced ROS generation in the HMC-1 mast cell line was implicated in IL-8 and TNF-α secretion, as the antioxidants dimethylthiourea, catalase and L-N-acetylcysteine inhibited cytokine secretion (23). These publications suggested but did not prove conclusively that the enzyme responsible for ROS production in mast cells was an isoform of NADPH oxidase.

We therefore sought to determine the precise mechanism by which ROS are produced in mast cells and to expand on the modulatory role of ROS on other mast cell secretory responses, such as LT, PG, and cytokine production. In addition, we investigated the extent of ROS production following classic NADPH oxidase stimulation to determine whether mast cells

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³ Abbreviations used in this paper: ROS, reactive oxygen species; AUC, area under the curve; COX, cyclooxygenase; mBMMC, mouse bone marrow-derived mast cell; PMN, polymorphonuclear leukocyte; LT, leukotriene; DCF, dichlorofluorescein; HSA, human serum albumin; DPI, diphenyleneiodonium; huMC, human mast cell; 5-LO, 5-lipoxygenase; DHE, dihydroethidium; DHR, dihydrorhodamine; IgG-LB, IgG-coated latex bead; PHGP, phospholipid hydroperoxide glutathione peroxidase; WT, wild type.

generated appreciable levels of ROS compared with PMN via this pathway.

As our results will show mouse bone marrow-derived mast cells (mBMMC) and human mast cells (huMC) generate ROS in response to Fc ϵ RI aggregation enzymatically through 5-lipoxygenase (5-LO) and cyclooxygenase (COX)-1 but not NADPH oxidase. Inhibition of ROS production by 5-LO and COX-1 inhibitors had negligible effects on degranulation and cytokine secretion. Following stimulation with IgG-coated latex beads (IgG-LB), ROS production in huMC was dependent on 5-LO and COX-1; this was not the case in PMN that used primarily NADPH oxidase in this response.

Materials and Methods

Animals and materials

Wild-type (WT) C57BL/6 mice (< 6-mo-old, 20 g) and 5-LO-deficient mice were obtained from The Jackson Laboratory. Aged-matched WT, gp91^{phox}-deficient or p47^{phox}-deficient mice were a gift from Dr. S. M. Holland (Laboratory of Clinical Infectious Diseases, National Institutes of Health, Bethesda, MD). WT (C57BL/6/6-129/Ola mixed genetic background) mice and COX-1-deficient mice were a gift of Dr. C. Toscano and Dr. F. Bosetti (National Institute on Aging, National Institutes of Health, Bethesda, MD). Mice were maintained and sacrificed in accordance with the National Institutes of Health guidelines on animal care and use, which were reviewed and approved by the National Institute of Allergy and Infectious Diseases (NIAID) review committee.

BSA, DNP₃₀-human serum albumin (HSA), dihydroascorbic acid (antioxidant), dihydroethidium (DHE), DPI, fMLP, monoclonal anti-DNP clone SPE-7 (IgE anti-DNP), PMA, LPS, sodium azide, toluidine blue, trypan blue, and zymosan were purchased from Sigma-Aldrich. Dichlorofluorescein (DCF) diacetate, nordihydroguaiaretic acid, and Trolox (antioxidant) were obtained from Calbiochem (EMD Biosciences). AA861 was purchased from Biomol and BAY-u9773, FR122047, LY2552833, MK571, U-75302, and zileuton were obtained from Cayman Chemicals. Cell culture media and supplements were from Invitrogen Life Technologies or BioSource International. Human GM-CSF and IFN- γ were obtained from PeproTech and murine GM-CSF and IFN- γ were purchased from (R&D Systems). Latex beads were from Polysciences (Warrington, PA) and rabbit IgG-HSA Ab was from MP Biomedicals. For PMN isolation, dextran (T-500) and Ficoll-Hypaque were obtained from Amersham Biosciences.

Cell isolation and mast cell culture

The mBMMC collected were cultured from femoral marrow cells in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 25 mM HEPES, 1.0 mM sodium pyruvate, nonessential amino acids, 0.0035% 2-ME, and 30 ng/ml mouse IL-3 as previously described (19). mBMMC were >99% pure by toluidine blue staining of cytospin preparations and were used after 4–6 wk of culture. HuMC were grown from CD34⁺ PBMC obtained following informed consent from healthy volunteers using a protocol for human subjects approved by the NIAID Institutional Review Board at the National Institutes of Health (Bethesda, MD). CD34⁺ cells were cultured in IL-3 (week 1 only), stem cell factor, and IL-6 as described (20). HuMC were >95% pure by toluidine blue staining of cytospin preparations and were used after 6–10 wk of culture.

PMN isolation and culture

PMN were isolated from human peripheral blood donated by healthy volunteers by standard methods (24) using a protocol approved by the NIAID Institutional Review Board at National Institutes of Health for human subjects. PMN were kept on ice in PBS containing glucose (10 mM) until required for functional assays.

IgG-LB preparation

Latex beads (2.0 μ m) were pelleted at 20,000 $\times$ g for 2 min, resuspended in borate buffer (0.1 M (pH 8.5)) containing 5% HSA, and incubated overnight at 4°C with rotation. After three washes in PBS, the resulting HSA-coated Latex beads were resuspended in double-distilled H₂O containing rabbit IgG anti-HSA (5 mg/ml) and incubated overnight at 4°C with rotation. Following three washes in PBS the resulting IgG-LB were resuspended in PBS containing 0.02% sodium azide and stored at 4°C. The density of IgG-LB was determined by 1/10,000 dilution of the solution and

counted using a hemocytometer and a phase-contrast microscope. The binding of IgG to latex beads was confirmed by SDS-PAGE.

Cell activation

HuMC or mBMMC optimally sensitized with mouse IgE anti-DNP or biotinylated human IgE, respectively (both 100 ng/ml, overnight) were washed to remove excess IgE and resuspended in either mBMMC or huMC medium at the required cell density for a specific assay. After stimulation of mBMMC with DNP₃₀-HSA (30 ng/ml) or huMC with streptavidin (100 ng/ml) cell-free supernatants were removed after 20 min for LTC₄ and PGD₂ analysis, after 4 h for mouse TNF- α and IL-6 analysis or after 10 h for human GM-CSF and IL-8 determination.

Cell degranulation assay

IgE-sensitized mast cells were seeded at 1×10^4 (huMC) or 5×10^4 (mBMMC) per well in 80 μ l of HEPES plus 0.04% BSA buffer (HEPES buffer) in a 96-well cell culture plate. Enzyme inhibitors or ROS scavengers (10 μ l) were added for 10 min before addition of DNP-HSA (mBMMC) or streptavidin (huMC), and degranulation was measured as the percentage of release of β -hexosaminidase as described (20).

Intracellular ROS detection by microfluorimetry

Intracellular ROS were measured in a 96-well plate assay using the fluorescent probe DCF (the intracellular product of DCF diacetate that fluoresces in the presence of ROS that include H₂O₂, hydroperoxides, and NO) (25). Mast cells or PMN (1×10^6 /ml) were incubated with DCF diacetate (20 μ M) in cell culture medium for 15–20 min at 4°C with rotation. Cells were then washed in HEPES buffer (10 ml) and seeded at $1\text{--}2 \times 10^5$ cells per well in a black opaque 96-well microplate in the presence or absence of enzyme inhibitors or ROS scavengers (10 μ l). For Fc ϵ RI aggregation experiments, DCF fluorescence was monitored at 37°C in 10-s intervals for 5 min before and 10 min after the addition of DNP-HSA (mBMMC) or streptavidin (huMC). For IgG-LB experiments, huMC or PMN were kept at 4°C and added to the microplate at 2×10^5 cells per well on ice containing enzyme inhibitors and stimulants. The microplate was then synchronized by centrifugation (170 $\times$ g, 5 min at 4°C) before monitoring of DCF fluorescence at 30-s intervals for 60 min at 37°C. In all experiments, DCF fluorescence was monitored using a GENios fluorescent plate reader (ReTiSoft) at 37°C with an excitation wavelength of 492 nm and emission wavelength of 535 nm. Fluorescence was expressed as relative fluorescent units. The kinetic data were collected using an XFluor4 macro within Microsoft Excel. For confocal microscopy, cells were visualized and photographed using an Axiophot microscope (Zeiss) at a lens objective of $\times 63$.

Intracellular ROS detection by flow cytometry

To determine whether O₂^{•−} was produced following Fc ϵ RI aggregation on mast cells, we used DHE, which is an O₂^{•−}-specific intracellular probe, and when used in combination with flow cytometry is a sensitive method for cell-specific O₂^{•−} production. For analysis of ROS production by flow cytometry, we used the nonspecific ROS probes DCF and dihydrorhodamine (DHR)123. Briefly, mast cells were incubated with DCF diacetate (2 μ M), DHR123 diacetate (2 μ M), or DHE (2 μ M) for 15 min at 37°C. Following stimulation of the cells with either DNP-HSA (mBMMC) or streptavidin (huMC) over various time points (0–30 min), samples were cooled on ice until analyzed by flow cytometry. Cell fluorescence was analyzed (10,000 events) on a gated forward light scatter and side light scatter area previously determined as mast cell-specific using a FACSCaliber flow cytometer (BD Biosciences) and associated CellQuest software (AppleMAC).

LTC₄ and PGD₂ measurements

IgE-sensitized mast cells were seeded at 1×10^3 (huMC) or 1×10^4 (mBMMC) cells per well in 80 μ l of HEPES buffer in a 96-well cell culture plate. Enzyme inhibitors or ROS scavengers (10 μ l) were added for 20 min before the addition of 10 μ l of DNP-HSA (mBMMC) or streptavidin (huMC). Following 20 min of stimulation, cell-free supernatants were removed and analyzed for LTC₄ and PGD₂ by competitive enzyme immunoassay (Cayman Chemicals), according to the manufacturer's instructions. Ag-stimulated mBMMC supernatants were diluted 1/1000 before analysis of LTC₄ release, and mBMMC cell number was increased to 2×10^5 per well before analysis of PGD₂ release in order for samples to be read within the range of the respective standard curve.

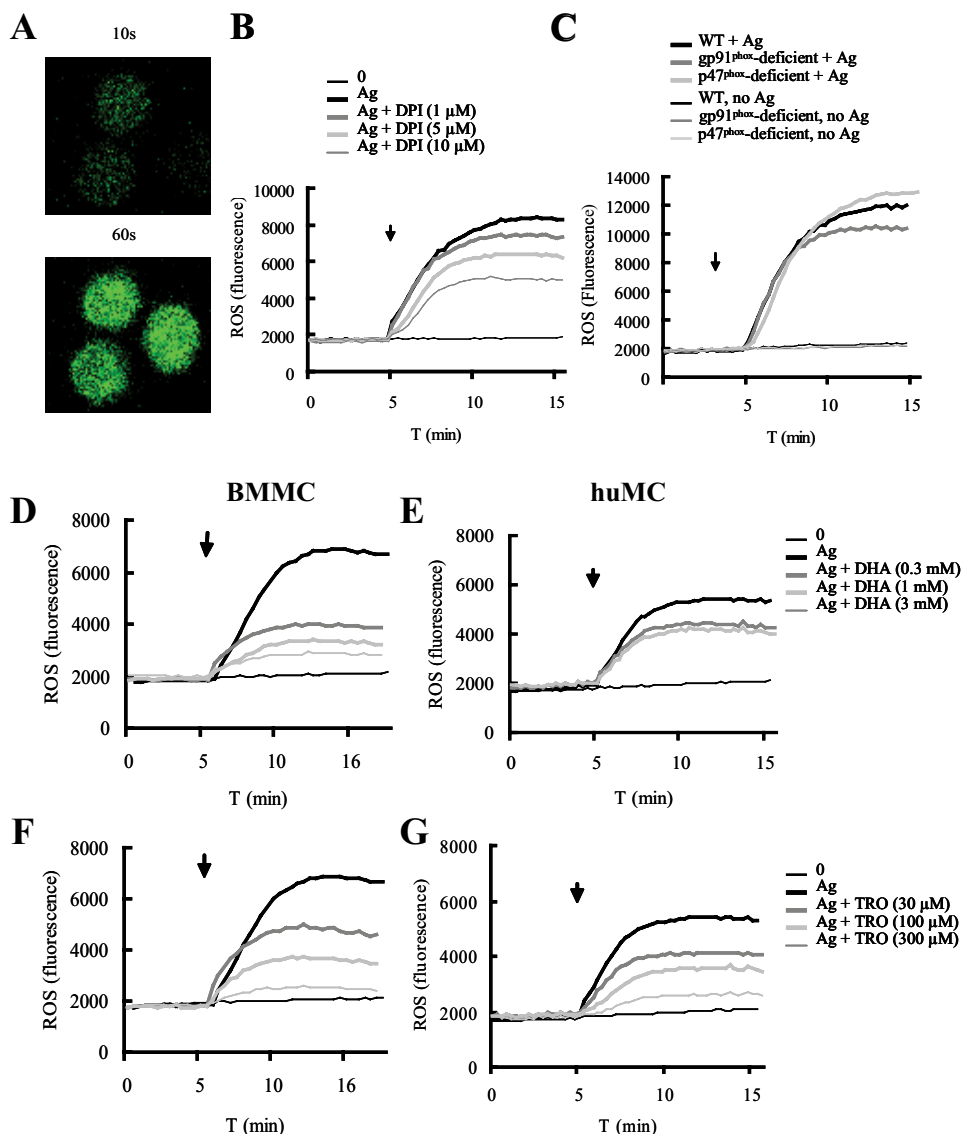


FIGURE 1. WT, gp91^{phox}- or p47^{phox}-deficient mBMMC have comparable levels of Ag induced ROS production, whereas antioxidants reduce ROS in both mBMMC and huMC. IgE-sensitized WT mBMMC (A–D and F) or huMC (E and G) were incubated with increasing concentrations of DPI (1–10 μM), or the antioxidants dihydroascorbic acid (DHA, 0.3–3 mM) or Trolox (TRO, 30–300 μM) for 5 min before addition of DNP-HSA (30 ng/ml), streptavidin (100 ng/ml) (thick black line), or HEPES buffer as a control (thin black line) and DCF fluorescence was monitored for up to 10 min at 10-s intervals. For comparison, WT (thick black line), gp91^{phox}- (thick dark gray line), or p47^{phox}-deficient (thick light gray line) mBMMC were stimulated with DNP-HSA (30 ng/ml) and the kinetic generation of ROS was monitored by DCF fluorescence over time (B). Results are shown as single experiments and are representative of three to five separate experiments performed in duplicate for BMMC and three separate donors performed in duplicate for huMC.

Cytokine measurements

IgE-sensitized huMC and mBMMC were seeded at 2×10^5 cells per well in 160 μl of huMC or mBMMC medium (medium contained 0.04% FCS for mBMMC cultures) in a 96-well cell culture plate. Enzyme inhibitors or ROS scavengers (20 μl) were added for 20 min before addition of 20 μl of streptavidin (100 ng/ml) for huMC and of DNP-HSA (100 ng/ml) for mBMMC (medium contained 20% FCS for mBMMC cultures). Cell-free supernatants were removed 4 h poststimulation for analysis of mouse TNF-α and IL-6 by ELISA (BioSource International) and 24 h poststimulation for human IL-8 and GM-CSF by ELISA (R&D Systems). ELISA was done according to the manufacturer's instructions.

Data presentation and statistical analysis

Results shown are for real-time kinetic readings and as grouped data from a minimum of three separate experiments performed in duplicate. Area under the curve (AUC) data for total ROS production was determined after baseline subtraction by Prism (GraphPad Software). Differences between individual concentration response curves for inhibitors or Ag was tested for statistical significance using ANOVA. If statistical significance was found by ANOVA ($p < 0.05$), differences between groups within concentration response curves or between responses of WT and 5-LO- or COX-1-deficient mBMMC were determined by Student's *t* test. Significant difference at a value of $p < 0.05$ was displayed at +, $p < 0.05$, ++, $p < 0.01$, or +++, $p < 0.001$ when comparing responses to Ag alone or control unstimulated cells or was displayed as *, $p < 0.05$, **, $p < 0.01$, or ***, $p < 0.001$ when comparing WT to 5-LO- and COX-1-deficient responses.

Results

ROS production in mast cells following FcεRI aggregation is NADPH oxidase-independent

We initially visualized ROS production following FcεRI aggregation of DCF-loaded mBMMC using confocal microscopy. As shown in Fig. 1A, FcεRI-dependent ROS production was observed as soon as 60 s poststimulation. To determine the enzymes responsible for ROS generation in mast cells, we initially examined the effect of DPI, an inhibitor of flavoenzymes including NADPH oxidase and NO synthase, on the DCF signal generated following FcεRI aggregation. IgE-sensitized, DCF-loaded mBMMC were incubated with increasing concentrations of DPI, and DCF fluorescence was monitored for 5 min before (baseline reading) and 10 min after FcεRI aggregation induced by the addition of Ag. FcεRI-dependent ROS production rose steadily over 5 min after cell stimulation and reached a plateau after 10 min (Fig. 1B). Incubation of mBMMC with DPI caused a concentration-dependent inhibition in FcεRI-dependent ROS production ($p < 0.05$ by ANOVA), which was significant at 10 μM (data not shown, $n = 5$ experiments, $p < 0.01$ by Student's *t* test). These data suggest a NADPH oxidase enzyme could be partially responsible for FcεRI-dependent

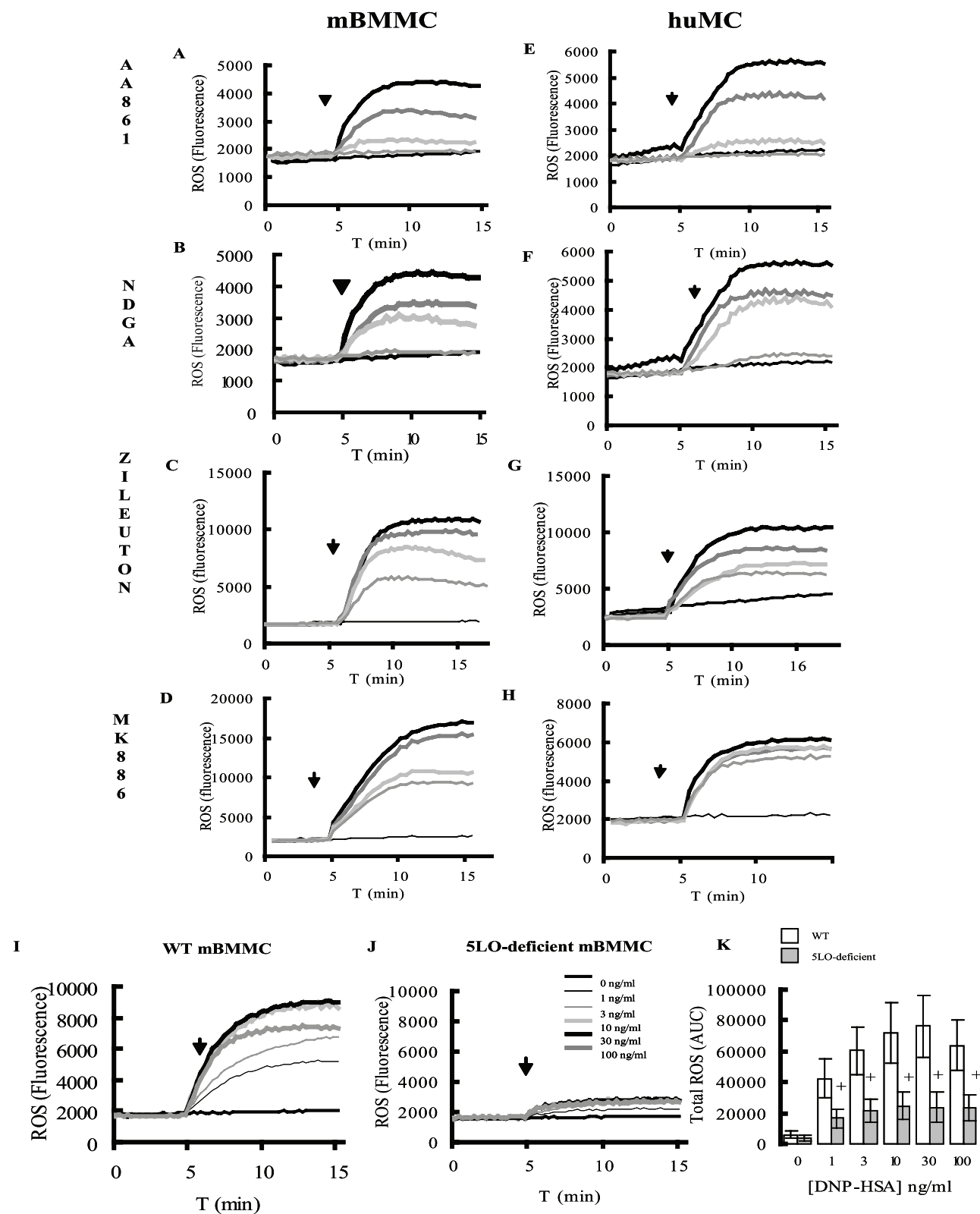


FIGURE 2. 5-LO as the primary source of Ag induced intracellular ROS production in mast cells. Experiment was conducted as in Fig. 1 except mBMMC (A and B) and huMC (C and D) were incubated with the 5-LO inhibitors AA861 at 100 nM (thick dark gray line), 1000 nM (thick light gray line), or 10,000 nM (thin light gray line) (A and E) or nordihydroguaiaretic acid (NDGA) at 100 nM (thick dark gray line), 1000 nM (thick light gray line), 10,000 nM (thin light gray line) (B and F) or zileuton at 2 μ M (thick dark gray line), 10 μ M (thick light gray line), 20 μ M (thin light gray line) (C and G), or the 5-LO-activating protein inhibitor MK886 at 10 nM (thick dark gray line), 100 nM (thick light gray line), 1000 nM (thin light gray line) (D and H) followed by addition of DNP-HSA (mBMMC) or streptavidin (huMC) (thick black line) or HEPES plus 0.04% BSA as control (thick black line). DCF fluorescence was monitored for a total of 15 min at 10-s intervals throughout the entire experiment. ROS production was also monitored in WT (I and K) and 5-LO-deficient (J and K) mBMMC following stimulation with increasing concentrations of

ROS production in mast cells. To further examine this possibility, DCF fluorescence was monitored in mBMMC that were deficient in the major NADPH oxidase subunits gp91^{phox} or p47^{phox} following FcεRI aggregation. However, no differences in the kinetic generation of ROS over the 10-min stimulation period were observed (Fig. 1C). The inhibitory effect of DPI (10 μM) on FcεRI-dependent DCF fluorescence was retained in WT, gp91^{phox}- or p47^{phox}-deficient mBMMC, indicating that the effect of this metabolic inhibitor on mast cells at these concentrations is not related to inhibition of NADPH oxidase (data not shown). In similar experiments, FcεRI-mediated ROS production was completely abrogated by the antioxidants dihydroascorbic acid (Fig. 1, D and E) or Trolox (Fig. 1, F and G) in both mBMMC (Fig. 1, D and F) and huMC (Fig. 1, E and G) further supporting the hypothesis that ROS are produced as a consequence of Ag stimulation in mast cells. These data taken together are consistent with the conclusion that ROS production by mast cells following FcεRI aggregation is NADPH oxidase-independent.

5-LO is the major enzyme responsible for FcεRI-dependent generation of ROS in mast cells, a minor role for COX-1

Because ROS production following FcεRI aggregation was NADPH oxidase-independent, we turned our attention to other ROS-producing enzymes present in mast cells, namely those involved in arachidonic acid metabolism. In both mBMMC and huMC, the 5-LO inhibitors AA861 (Fig. 2, A and E, respectively), nordihydroguaiaretic acid (Fig. 2, B and F, respectively), and zileuton (Fig. 2, C and G, respectively) reduced FcεRI-mediated DCF fluorescence in a concentration-dependent manner ($p < 0.05$ by ANOVA). This reduction by AA861 and nordihydroguaiaretic acid (1000 and 10,000 nM) and zileuton (20 μM) was statistically significant when total ROS production was calculated from AUC (data not shown, $n = 3$ –6 donors per group, $p < 0.05$ by Student's *t* test). The 5-LO-activating protein inhibitor MK886 was also used to investigate the requirement of 5-LO-activating protein in ROS production by mast cells. In mBMMC, MK886 caused a partial reduction in Ag-mediated ROS production (Fig. 2D), whereas it was ineffective in huMC (Fig. 2H). Therefore ROS generation following FcεRI aggregation was primarily lipoxygenase-dependent.

To confirm these data regarding the effect of 5-LO inhibitors on FcεRI-mediated ROS production, we obtained mBMMC from 5-LO-deficient and WT mice and compared the DCF signal in response to increasing concentrations of Ag. The concentration-dependent increase in DCF fluorescence typically associated with WT mBMMC following FcεRI aggregation (Fig. 2I) was absent in 5-LO-deficient mBMMC (Fig. 2J). These reduced ROS levels in 5-LO-deficient mBMMC were significant when total ROS production was compared with WT mBMMC (Fig. 2K with statistics). However, the DCF response in 5-LO-deficient mBMMC to Ag was never completely abrogated to baseline levels. These data demonstrate the production of ROS following FcεRI aggregation is primarily 5-LO-dependent.

We next investigated which enzyme was responsible for the residual ROS present in 5-LO-deficient mBMMC following FcεRI aggregation. Incubation of 5-LO-deficient mBMMC with FR122047 (COX-1 inhibitor) caused a concentration-dependent

reduction ($p < 0.05$ by ANOVA) in FcεRI-mediated DCF fluorescence (Fig. 3A). Participation of COX-1 was further demonstrated by the ability of FR122047 to partially reduce FcεRI-dependent DCF fluorescence ($p < 0.05$ by ANOVA) in WT mBMMC and huMC (Fig. 3, B and C, respectively). To clarify the contribution of 5-LO and COX-1, WT mBMMC and huMC were incubated with 5-LO (zileuton or AA861) and COX-1 (FR122047) inhibitors alone or in combination before Ag stimulation. Incubation of WT mBMMC with zileuton (Fig. 3D), AA861 (Fig. 3, E and F), or FR122047 (Fig. 3, D–F) alone caused a partial reduction in FcεRI-dependent ROS production, whereas zileuton or AA861 in combination with FR122047 completely abrogated the ROS response (Fig. 3, D–F). Similar data were observed for Ag-stimulated huMC incubated with zileuton, AA861, and FR122047 alone or in combination (Fig. 3, G–I). Incubation of mBMMC or huMC with cysteinyl LT antagonists (MK571 and BAY-u9773) or LTB₄ receptor antagonists (LY2552833 and U-75302) did not alter the ROS response induced by Ag suggesting no autocrine effect of released LTs on the DCF signal (data not shown).

To confirm the data regarding the effect of COX-1 inhibitor on FcεRI-mediated ROS production we obtained mBMMC from COX-1-deficient and WT mice and compared the DCF signal in response to increasing concentrations of Ag. The concentration-dependent increase in DCF fluorescence typically associated with WT mBMMC following FcεRI aggregation (Fig. 3J) was reduced ($p < 0.05$ by ANOVA) in COX-1-deficient mBMMC (Fig. 3K). These reduced ROS levels in COX-1-deficient mBMMC were significant when total ROS production was compared ($p < 0.05$ by Student's *t* test) between the same Ag concentrations in WT mBMMC (Fig. 3L). However, the DCF response in COX-1-deficient mBMMC to Ag was never completely abrogated to baseline levels, suggesting the residual ROS was 5-LO-specific. These data unequivocally show that FcεRI aggregation leads to ROS production in mast cells, which is primarily 5-LO-dependent and partially COX-1-dependent.

FcεRI-dependent superoxide production by mast cells

DCF reacts with several oxidant species including H₂O₂ and hydroperoxides but not O₂^{•−}. Thus to determine whether O₂^{•−} were produced following FcεRI aggregation on mast cells we used DHE, which is a O₂^{•−}-specific intracellular probe, in combination with flow cytometry to provide a sensitive method for cell-specific O₂^{•−} production. We used this probe in addition to the nonspecific ROS probes DCF and DHR123, which detect hydroperoxides H₂O₂ and NO (not produced by mast cells). As shown in Fig. 4A following FcεRI aggregation on huMC and mBMMC (WT), a statistically significant increase in DHE fluorescence was observed ($p < 0.05$ by Student's *t* test). This FcεRI-dependent increase in DHE fluorescence in mBMMC was absent in 5-LO-deficient mBMMC ($p < 0.05$ by Student's *t* test) and partially reduced in COX-1-deficient mBMMC ($p < 0.01$ by Student's *t* test), suggesting O₂^{•−} was derived from 5-LO and COX-1. Following FcεRI aggregation of DCF and DHR123 loaded cells, an increase in DCF and DHR123 fluorescence was observed in huMC and WT mBMMC (Fig. 4B), which is consistent with data obtained by microfluorimetry (Figs. 2 and 3). Furthermore, both the DCF and DHR123 signals were greatly reduced in the 5-LO-deficient

DNP-HSA. AUC data were calculated for each kinetic curve and averaged (A–K). Results are shown as either kinetic data of single experiments performed in duplicate and are representative of three to six independent experiments for mBMMC (A–D) or three separate donors for huMC (E–H) or two to three experiments performed in duplicate from three different mice for WT and 5-LO-deficient mBMMC (I and J). Data are mean ± SE for AUC data calculated for each kinetic curve, in $n = 3$ experiments in duplicate (K). AUC data was calculated for inhibitor (A–H) or Ag (I–K) concentration response curves and statistical significance was determined by ANOVA ($p < 0.05$). Following ANOVA, differences between individual groups within inhibitor curves (WT mBMMC) or between WT and 5-LO-deficient mBMMC Ag responses was determined by Student's *t* test. +, $p < 0.05$ for comparison with Ag between WT and 5-LO-deficient mBMMC.

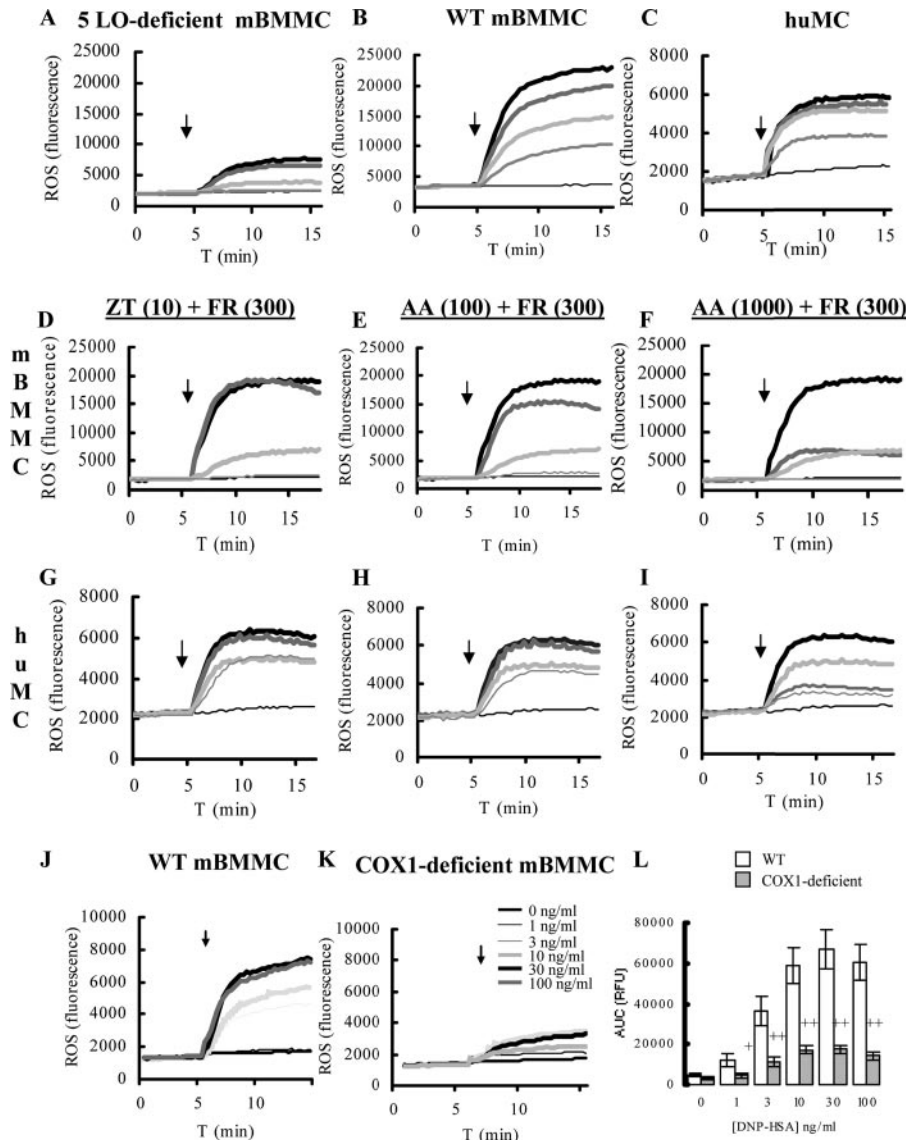


FIGURE 3. Contribution of 5-LO and COX-1 in the production of ROS induced by Ag-stimulated mBMMC and huMC. Experiments were conducted as in Fig. 1 for measurement of ROS. mBMMC (A and B, D–F, and J–L) or huMC (C, and G–I) were incubated with increasing concentrations of FR122047 at 3 nM (thick dark gray line), 30 nM (thick light gray line), 300 nM (thin light gray line) (A–C) or FR122047 alone at 300 nM (thick light gray line) (D–I), zileuton alone at 10 μ M (thick dark gray line) (D and G), AA861 alone at 100 nM (E and H) or 1000 nM (F and I) (thick dark gray line) or FR122047 (300 nM) in combination with zileuton (10 μ M) or AA861 (100–1000 nM) (thin light gray line) for 5 min before stimulation with DNP-HSA or streptavidin, respectively (thick black line), or HEPES plus 0.04% BSA as control (thin black line) for a further 10 min. DCF fluorescence was monitored for a total of 15 min at 10-s intervals throughout the entire experiment. Results shown are representative of three independent experiments performed in duplicate for WT mBMMC, 5-LO-deficient mBMMC, and huMC (A–I). ROS production was also monitored in WT and COX-1-deficient mBMMC following stimulation with increasing concentrations of DNP-HSA and shown as representative kinetic curves of six independent experiments performed in duplicate (J and K) or averaged AUC data calculated from individual kinetic curves and shown as mean $\pm$ SE ($n = 6$ experiments in duplicate) (L). Statistical significance for inhibitor (A–I) or Ag (J–L) concentration response curves was determined by ANOVA from calculated AUC data. If statistical significance was achieved following ANOVA ($p < 0.05$), differences between individual groups within inhibitor curves (WT or 5-LO-deficient mBMMC and huMC) or between WT and COX-1-deficient mBMMC Ag responses was determined by Student's t test. +, $p < 0.05$ and ++, $p < 0.01$ for comparison with Ag between WT and COX-1-deficient mBMMC.

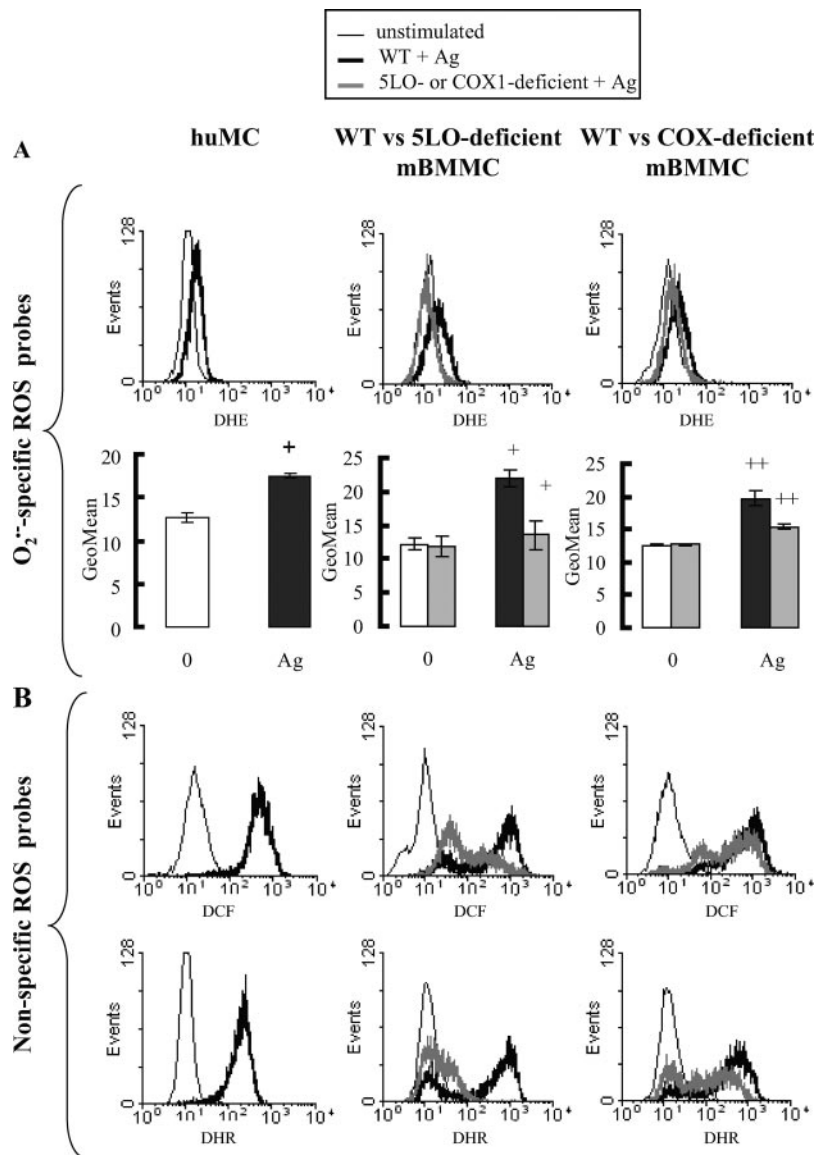
mBMMC and partially reduced in the COX-1-deficient mBMMC compared with the WT mBMMC (Fig. 4B). These data are consistent with the conclusion that ROS produced by mast cells following Fc ϵ RI aggregation, in addition to hydroperoxides and H₂O₂, include O₂^{•−} generated through 5-LO and COX-1.

Complete abrogation of ROS in WT and 5-LO-deficient mBMMC does not alter Fc ϵ RI-dependent degranulation and cytokine secretion

Once the enzymes responsible for ROS production had been identified and the resulting ROS production could be fully inhibited,

the next step was to determine the effect of ROS inhibition on mast cell secretory responses following Fc ϵ RI aggregation because previously published data suggested a positive role in some responses (21). Initially, mBMMC were incubated with increasing concentrations of the COX-1 inhibitor FR122047 before Ag stimulation and levels of β -hexosaminidase, LTs, PGs, and cytokines released into cell-free supernatants were determined. For WT mBMMC, a concentration-dependent partial reduction in ROS ($p < 0.05$ by ANOVA) was observed following incubation with FR122047 before Fc ϵ RI aggregation (Fig. 5A, open histogram), but this had little effect on β -hexosaminidase, LTC₄, TNF- α , or IL-6 release

FIGURE 4. FcεRI-dependent superoxide generation by mBMMC and huMC is mediated through 5-LO and COX-1. IgE-sensitized huMC or WT 5-LO-deficient mBMMC and COX-1-deficient mBMMC were incubated with DHE (2 μM) (A) or DCF (2 μM) or DHR123 (2 μM) (B) for 15 min before stimulation with streptavidin (huMC) (thick black line), DNP-HSA (mBMMC) (thick black line) or HEPES plus 0.04% BSA as control (thin black line) for 5 min. After which cells were placed on ice and FL1 (DCF and DHR123) or FL2 (DHE) was monitored by flow cytometry of 10,000 events within a gated area defined previously as IgE-positive mast cells. Results shown are representative histograms or geometric mean from three independent experiments performed in duplicate of Ag-mediated ROS generation. Geometric mean for individual histograms was determined, and statistical significance between individual groups was analyzed by Student's *t* test. +, *p* < 0.05 and ++, *p* < 0.01).



(Fig. 5, B, C, E, and F, respectively). As expected, FR122047 caused a complete inhibition (*p* < 0.001 by ANOVA) of Ag-dependent PGD₂ release (Fig. 5D). In 5-LO-deficient mBMMC incubated with FR122047, complete abrogation of Ag-mediated ROS production was observed (*p* < 0.001 by ANOVA) at the highest concentration tested (300 nM; *p* < 0.01 by Student's *t* test) (Fig. 5A, gray histogram). However, no effect on β-hexosaminidase, TNF-α or IL-6 release was observed (Fig. 5, B, E, and F, respectively). As expected, LTC₄ could not be detected in 5-LO-deficient mBMMC (Fig. 5C), whereas PGD₂ levels were enhanced compared with WT mBMMC; furthermore, FR122047 incubation caused complete blockade of PGD₂ levels (Fig. 5D). When directly comparing WT and 5-LO-deficient mBMMC secretory responses, complete abrogation of ROS production in 5-LO-deficient mBMMC caused no change in β-hexosaminidase, TNF-α, or IL-6 release, whereas differences in LTC₄ and PGD₂ levels were observed even though cells had comparable levels of FcεRI bound IgE (data not shown). Taken together, these data suggest that even though ROS are generated following FcεRI aggregation in mBMMC, they are not required for degranulation or cytokine secretion.

To support our conclusion that ROS have a minimal participation in mast cell secretory responses, we next incubated WT mBMMC or huMC with 5-LO and COX-1 inhibitors alone or in combination to assess the

effect of varied levels of ROS on mast cell secretory responses including β-hexosaminidase, LTs, PGs, and cytokines. As shown in Fig. 6, WT mBMMC incubated with the 5-LO inhibitors zileuton, AA861 or the COX-1 inhibitor FR122047 (Fig. 6A) caused partial reduction in ROS production, whereas a combination of 5-LO and COX-1 inhibition completely abrogated ROS levels (Fig. 6A). However, when ROS production was absent no effect on β-hexosaminidase release (Fig. 6B) and TNF-α or IL-6 secretion was observed (Fig. 6, E and F, respectively) but LTC₄ and PGD₂ secretion was fully inhibited (Fig. 6, C and D, respectively). Note that the mean TNF-α production reported in Fig. 6E is lower than TNF-α mean presented in Fig. 5E, but within the range of TNF-α data averaged for Fig. 5E. We attribute this variation to reported differences in measurements of TNF-α levels possibly due to differences in mast cell cultures, which has also been noted by others (26–28). These data were similar for huMC (*n* = 3 donors) (data not shown). These results further demonstrate that ROS generated as a consequence of FcεRI aggregation do not participate in mast cell secretory responses.

Selective phagocytic stimuli induce ROS production in mast cells

Because 5-LO and COX-1 were responsible for ROS generation following FcεRI aggregation, we next assessed whether mast cells exclusively used these enzymes in response to stimuli known to

induce ROS in other cell types. Phagocytic cells such as PMN primarily use NADPH oxidase to generate ROS and this enzyme can be activated by soluble and/or particulate stimuli including PMA, fMLP, zymosan, and IgG-LB. Therefore, we tested the ability of mast cells to generate ROS in response to these stimuli and assessed whether the resultant ROS were derived from the 5-LO and COX-1 pathway.

mBMMC or huMC were incubated with the ROS-sensitive probe DCF before stimulation with PMA (1000 nM), fMLP (1 μ M), zymosan (1/20), IgG-LB (1/20), or ionomycin (300 nM) as a positive control. ROS generation was then monitored for 60 min. As shown in Fig. 7, mBMMC generated low levels of ROS in response to zymosan and IgG-LB, whereas PMA and fMLP were ineffective (Fig. 7A). The lack of responsiveness to fMLP was not surprising because mast cells do not express the fMLP receptor (29, 30). However, the response to zymosan and IgG-LB was variable and overall negligible ROS were detected. In contrast, huMC stimulated with IgG-LB but not zymosan, fMLP or PMA generated low levels of ROS (Fig. 7B) and this phenomenon was reproducible with cells from several donors (data not shown). The levels of ROS generated by huMC were less than observed in PMN, which generated high levels in response to PMA or IgG-LB and low levels in response to fMLP and ionomycin (Fig. 7C). However, the rate of ionomycin-induced ROS production in PMN was reduced compared with huMC (Fig. 7, B and C).

Because it is well documented that NADPH oxidase activity can be enhanced following exposure of PMN to various cytokines and bacterial products including IFN- γ , GM-CSF, TNF- α , and LPS, we next investigated whether ROS generation could be induced in mBMMC by the same stimuli following pre-exposure to IFN- γ , GM-CSF, and LPS for 1–24 h. However, no ROS production was observed with stimulation alone or following pre-exposure to these cytokines or LPS ($n = 2$) (data not shown). When huMC were pre-exposed to IFN- γ or LPS for 1–24 h no increase in ROS production was observed following stimulation with PMA, fMLP, or zymosan ($n = 2$) (data not shown). However, when huMC were preincubated with IFN- γ (24 h) before IgG-LB stimulation, an increase in ROS production was observed (Fig. 8A). When AUC data were calculated over replicate donors, a significant increase in ROS production was observed in the presence ($p < 0.01$ by Student's t test) or absence ($p < 0.05$ by Student's t test) of IFN- γ following IgG-LB stimulation and the enhancing effect of IFN- γ was also significant ($p < 0.01$ by Student's t test) (Fig. 8B).

We next sought to determine whether the enzyme responsible for the enhanced ROS generation following IgG-LB stimulation was NADPH oxidase, 5-LO, or COX-1. HuMC were pretreated with IFN- γ for 24 h, preloaded with DCF and added to a plate containing increasing concentrations of the flavoenzyme inhibitor DPI (1–10 μ M), 5-LO inhibitors zileuton (2–20 μ M), or AA861 (0.1–10 μ M), or the COX-1 inhibitor FR122047 (3–300 nM) before IgG-LB stimulation. PMN were used as a positive control in all experiments and were also pretreated with inhibitors. As shown in Fig. 9, there was no effect of DPI on IFN- γ -primed IgG-LB induced ROS production in huMC (Fig. 9A), whereas FR122047 (Fig. 9D) or zileuton (Fig. 9B) partially inhibited and AA861 completely abrogated ($p < 0.05$ by ANOVA) IgG-LB-induced ROS production (Fig. 9C). As expected, DPI completely abrogated IgG-LB-dependent ROS production in PMN at all concentrations tested (Fig. 9E), whereas the 5-LO and COX-1 inhibitors were without effect (Fig. 9, F–H). These data demonstrate that mast cells use 5-LO and COX-1, whereas PMN use NADPH oxidase in the generation of ROS following IgG-LB stimulation.

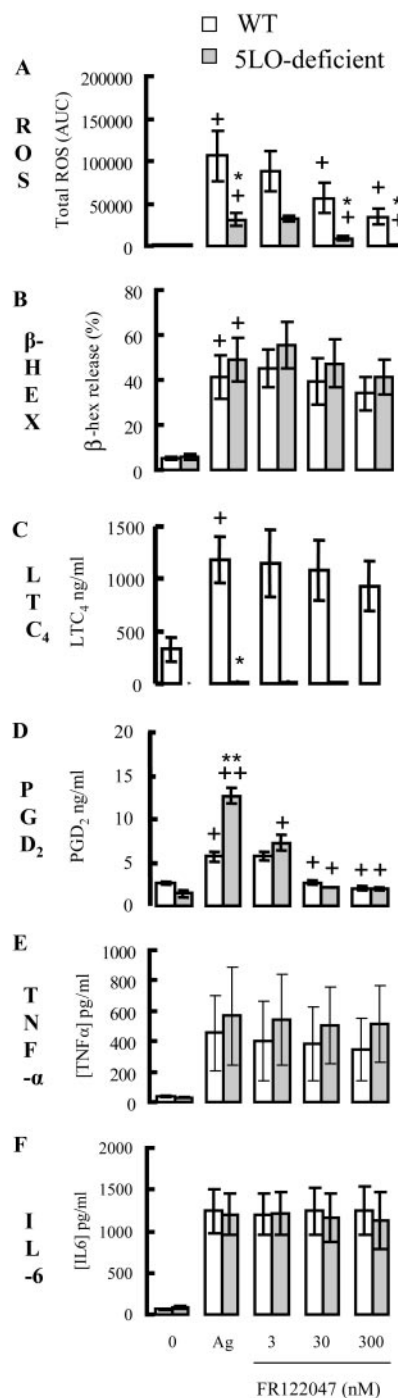
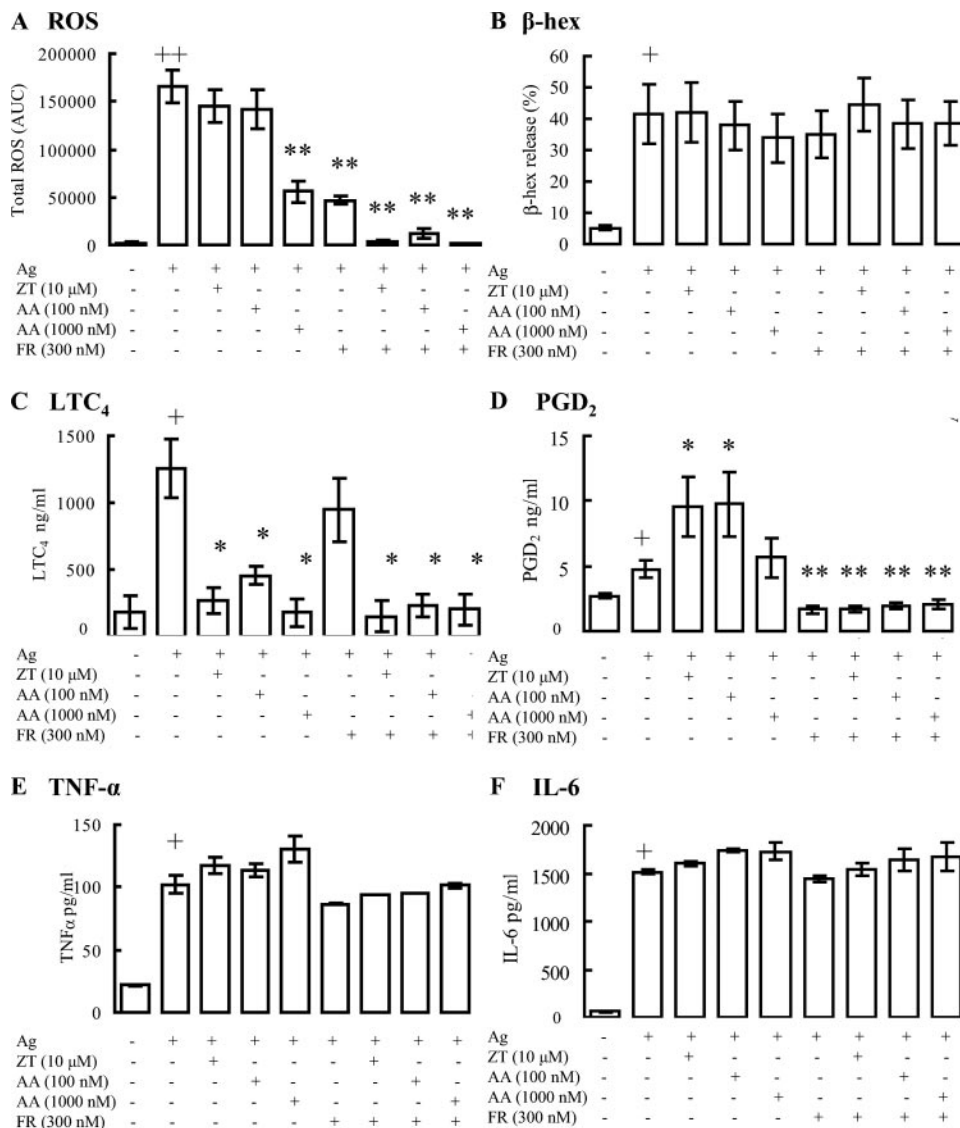


FIGURE 5. Effect of a COX-1 inhibitor on Fc ϵ RI-dependent secretory responses in WT and 5-LO-deficient mBMMC. IgE-sensitized WT or 5-LO-deficient mBMMC were pretreated with FR122047 (3–300 nM) before stimulation with DNP-HSA (30 ng/ml). Total ROS production was determined by DCF fluorescence before calculation of AUC over 10 min (A). Cell-free supernatants were collected and quantified for net β -hexosaminidase after 30 min (B), LTC₄ (C) or PGD₂ (D) after 20 min by competitive enzyme immunoassay, or TNF- α (E) or IL-6 (F) after 4 h by ELISA. Note the average measurements for TNF- α data of individual experiments in WT mBMMC shown as 455 ± 251 varied from 124 to 1440 pg/ml. Results are the mean $\pm$ SE for $n = 3-5$ experiments in duplicate. Differences in individual concentration response curves were determined by ANOVA. Following ANOVA, differences between individual groups within single concentration response curves were determined by Student's t test. +, $p < 0.05$ and ++, $p < 0.01$ for comparison with Ag alone; *, $p < 0.05$ and **, $p < 0.01$ for comparison between WT and 5-LO-deficient mBMMC.

FIGURE 6. Effect of 5-LO and COX-1 inhibitors on FcεRI-dependent secretory responses in WT mBMMC. IgE-sensitized WT mBMMC were pretreated with zileuton (10 μM), AA861 (100–1000 nM), or FR122047 (300 nM) alone or in combination before stimulation with DNP-HSA (30 ng/ml). Total ROS production was determined by DCF fluorescence before calculation of AUC over 10 min (A). Cell-free supernatants were collected and quantified for net β-hexosaminidase after 30 min (B), LTC₄ (C), or PGD₂ (D) after 20 min by competitive enzyme immunoassay or TNF-α (E) or IL-6 (F) after 4 h by ELISA. Results are the mean ± SE of *n* = 3 experiments in duplicate. Differences between individual groups within single concentration response curves were determined by Student's *t* test. +, *p* < 0.05 and ++, *p* < 0.01 for comparison with unstimulated cells; +, *p* < 0.05 and ++, *p* < 0.01 for comparison with Ag alone.



Discussion

In this study we have demonstrated that FcεRI aggregation on both human and mouse mast cells leads to ROS generation derived from the 5-LO and COX enzymes. Blockade of FcεRI-dependent ROS generation had negligible effects on mast cell secretory responses including degranulation and cytokine secretion but, as expected, completely abrogated PG and LT secretion. This is consistent with the hypothesis that 5-LO and COX-1 are the enzymatic sources of ROS in mast cells. In addition, mast cells also generated ROS following IgG-LB stimulation, which was enhanced by incubation with IFN-γ. IgG-LB dependent ROS production in huMC was derived from 5-LO and COX-1 and not NADPH oxidase. This was in sharp contrast to PMN where IgG-LB mediated ROS generation was exclusively through the activity of NADPH oxidase.

Our previous results (20) and those of others (22, 31–33) have revealed an inhibitory effect of the flavoenzyme inhibitor DPI on FcεRI-dependent ROS production, suggesting the involvement of a NADPH oxidase isoform. At concentrations of 10–30 μM, DPI partially reduced ROS production in mast cells (22, 33), which is in agreement with data presented in this study. However, complete inhibition of ROS by DPI was only observed at 100–200 μM in these other studies (22, 33). These

DPI concentrations are one hundred times higher than those required to inhibit NADPH oxidase activity in PMN (1–2 μM) (Fig. 8E) and as such may reflect nonspecific effects. In support of a minimal role for NADPH oxidase in FcεRI-dependent ROS production, WT, gp91^{phox}- and p47^{phox}-deficient mBMMC had negligible differences in ROS production following Ag stimulation. Furthermore, residual ROS levels in 5-LO-deficient mBMMC were not affected by DPI incubation. In support of a role for 5-LO and COX-1 in FcεRI-dependent ROS production, other studies (22, 33) showed that this response was inhibited by the phospholipid hydroperoxide glutathione peroxidase (PHGP) mimetic ebselen, which is a potent inhibitor of 5-LO (34–37) and COX activity (37). In addition, overexpression of PHGP in RBL-2H3 cells reduced hydroperoxide, LTC₄, and PGD₂ release following Ag stimulation (38, 39), and arachidonic acid pretreatment enhanced FcεRI-dependent ROS production (40). On the basis of the inhibitory effect of DPI and the ROS scavenging activity of ebselen, previous studies speculated the enzyme responsible for ROS production in mast cells was a NADPH oxidase isoform (22, 33). Data presented in this study are consistent with the conclusion that the enzyme primarily responsible for the production of ROS following FcεRI aggregation in mast cells is 5-LO with a partial role of COX-1.

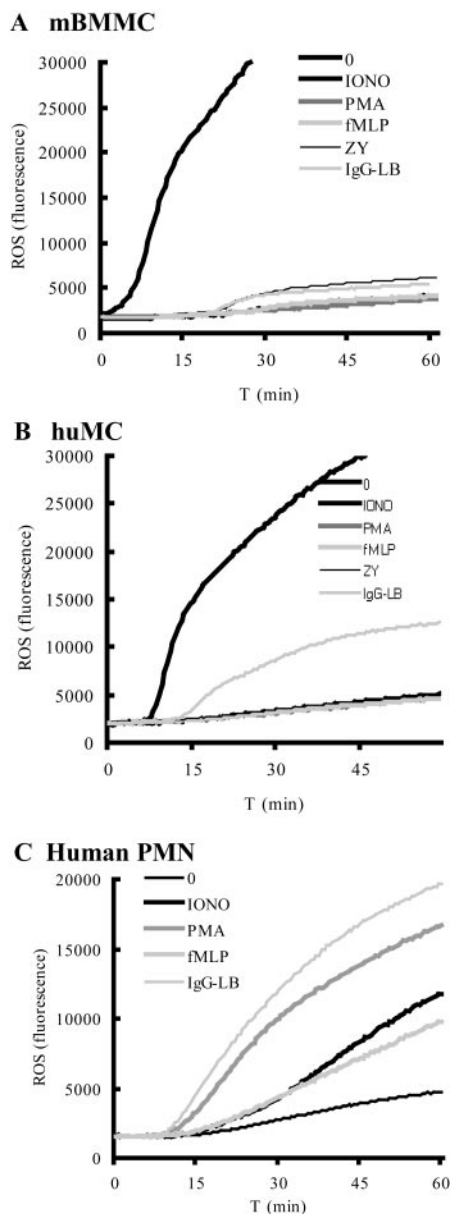


FIGURE 7. Effect of NADPH oxidase stimulants on mBMMC, huMC and PMN ROS generation. WT mBMMC (A), huMC (B), or PMN (C) were incubated with PMA (1000 nM) (thick dark gray line), fMLP (1000 nM) (dotted light gray line), zymosan (1/20) (thin black line), IgG-LB (1/20) (thick light gray line), ionomycin (300 nM) (thick black line) as a positive control or HEPES buffer as a negative control (thick black line). ROS production was then measured by DCF fluorescence for 60 min at 30-s intervals at 37°C. Results are shown as a single experiment and are representative of three separate experiments performed in duplicate.

There are several possible species of oxidant generated following FcεRI aggregation in mast cells, including most notably hydroperoxides, H_2O_2 , and $\text{O}_2^{\bullet-}$. The enzyme 5-LO can generate free radical intermediates including hydroperoxides (41–44) and $\text{O}_2^{\bullet-}$ (45–47) during the production of LT, and COX-1 can generate hydroperoxides (48–49) and $\text{O}_2^{\bullet-}$ (45) during the course of PG synthesis. In the presence of superoxide dismutase, 5-LO- and COX-1-dependent $\text{O}_2^{\bullet-}$ production could yield H_2O_2 and therefore both hydroperoxides and H_2O_2 could contribute equally to ROS levels in mast cells. In support of this conclusion, the probe used to detect ROS, namely DCF diacetate, can detect both H_2O_2 (25) and hydroperoxides (50). Also antioxidant enzymes including

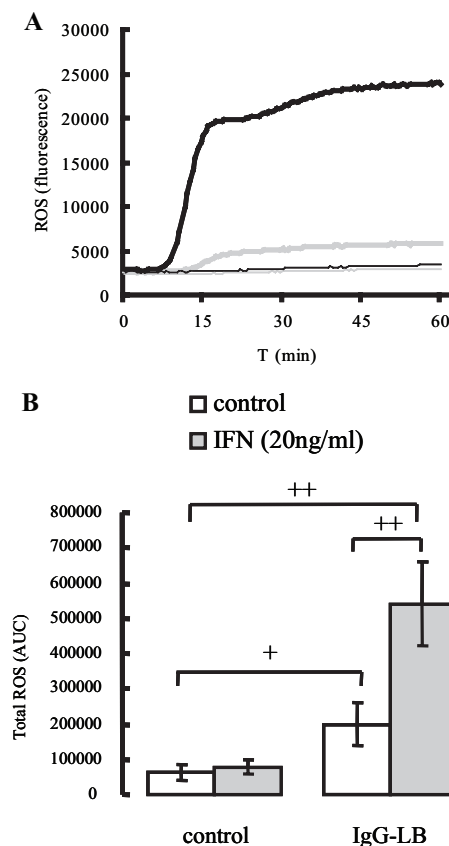


FIGURE 8. Effect of IFN- γ on IgG-LB induced ROS generation in huMC. HuMC were incubated in the presence or absence of IFN- γ (20 ng/ml) for 24 h before stimulation with IgG-LB (1/20) (thick black line) or HEPES buffer as a negative control (thin black line). ROS production was then measured by DCF fluorescence for 60 min at 30-s intervals at 37°C. Results are shown as single kinetic curves and are representative of two experiments performed in duplicate on five different huMC donors (A) or AUC data calculated for each kinetic curve after baseline subtraction and expressed as the mean $\pm$ SE of $n = 5$ donors (B). Differences between individual groups were determined by Student's t test. +, $p < 0.05$ and ++, $p < 0.01$.

PHGP can scavenge both H_2O_2 and hydroperoxides (51). The increase in DHE fluorescence by huMC and mBMMC following FcεRI aggregation is consistent with the conclusion that $\text{O}_2^{\bullet-}$ was produced. Additionally, analysis of intracellular H_2O_2 by the amplex red assay yielded little production of H_2O_2 by mast cells following Ag stimulation. In summary we propose that the principal oxidants generated by mast cells following FcεRI aggregation are $\text{O}_2^{\bullet-}$, hydroperoxides and H_2O_2 .

The probe used to detect intracellular oxidants in this study was DCF diacetate, which is known to react with H_2O_2 , hydroperoxides and NO. There are concerns associated with this probe despite its universal use to detect ROS in that the reaction with oxidants can be accelerated in the presence of peroxidases and fluorescence can be observed in the absence of ROS production. In our study the primary enzymes responsible for FcεRI-dependent ROS production (detected by DCF fluorescence) were primarily 5-LO with a minor role for COX-1. Because the activity of these enzymes and subsequent hydroperoxide generation is dependent on peroxidases the likelihood of DCF fluorescence monitoring both reactions is a possibility. However, using enzyme preparations of 5-LO no increase in DCF fluorescence was observed by others (52) consistent with the conclusion that the enzyme itself does not induce DCF

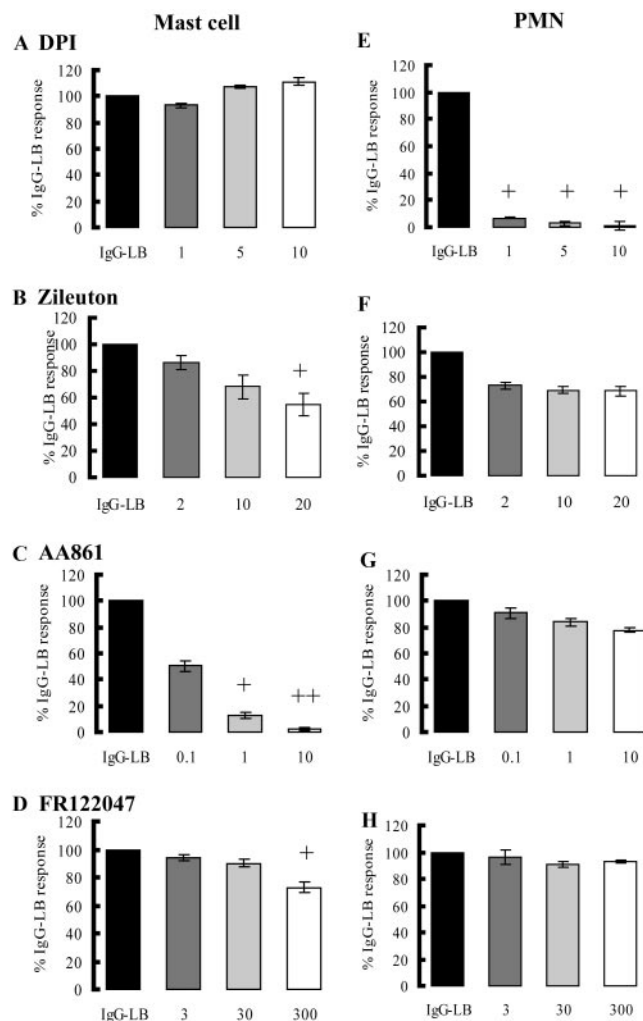


FIGURE 9. Effect of enzyme inhibitors on IgG-LB induced ROS generation in huMC primed with IFN- γ and PMN. HuMC pretreated with IFN- γ (20 ng/ml) for 24 h (A–D) or PMN (E–H) were stimulated with IgG-LB (1/20) (■) or HEPES buffer as a negative control in the presence of increasing concentrations of DPI (1–10 μ M) (A and E), zileuton (2–20 μ M) (B and F), AA861 (100–10,000 nM) (C and G), or FR122047 (3–300 nM) (D and H). DCF fluorescence was monitored for 60 min at 30-s intervals at 37°C. Results shown are the mean $\pm$ SE of $n = 3$ –4 donors. Differences in individual concentration response curves were determined by ANOVA. Following ANOVA, differences between individual groups within single concentration response curves were determined by Student's t test. +, $p < 0.05$ and ++, $p < 0.01$ for comparison with IgG-LB alone.

fluorescence. Arachidonic acid-mediated COX-1 activity does induce DCF fluorescence in presence of phenol, to enhance the signal, but this could be an indication of hydroperoxide and peroxidase activity (52). Incubation of BMMC with the peroxidase inhibitor sodium azide before incorporation in the DCF assay caused no reduction in ionomycin-dependent DCF fluorescence (data not shown), suggesting the DCF signal is not a result of peroxidases activity but of ROS production. These data are consistent with the conclusion that DCF fluorescence occurred independent of peroxidase activity.

Intracellular ROS production following Ag stimulation in mast cells does not influence subsequent degranulation and cytokine secretion. Incubation of mBMMC or huMC with 5-LO and COX-1 inhibitors at concentrations that completely abrogated ROS generation had no effect on degranulation and cytokine secretion following stimulation with Ag. As expected LT and PG production

were reduced, supporting the hypothesis that the enzymes involved in arachidonic acid metabolism are the source of ROS in mast cells. Results presented in this study support our previous data (20) showing no effect of intracellular ROS on degranulation of mBMMC but does not support the huMC data. This may be due to donor-to-donor variability of CD34⁺ blood cells. In data not shown, incubation of mBMMC and huMC with DPI before Ag stimulation had no effect on degranulation. Previous work by others (22, 31–33) showed DPI (3–33 μ M) partially reduced Fc ϵ RI-dependent degranulation (22, 31–33), but complete inhibition was only achieved at higher concentrations of 100–200 μ M (22, 31, 32). These higher concentrations may have nonspecific effects with global inhibition of enzymes involved in many aspects of mast cell biology. Also the sources of mast cells and culture conditions may explain apparent differences between our results and those of others regarding the role of ROS in Ag-mediated secretory responses (22, 31–33). By specifically reducing Fc ϵ RI-dependent ROS production in mast cells using a combination of 5-LO and COX-1 inhibitors or genetic deletion of 5-LO, no effect of intracellular ROS on mast cell degranulation was observed in the current study. Reduction of 5-LO activity by the PHGP mimetic ebselen (22, 33) or using specific 5-LO and COX-1 inhibitors (53) has minimal effects on mast cell degranulation. In another study using mBMMC deficient in the antioxidant enzyme thioredoxin, Ag mediated degranulation was reduced compared with WT mBMMC (54). These data appear to contradict our current data on degranulation and may involve differences in localization of the antioxidant enzyme. However, the same study found no differences in Fc ϵ RI-dependent TNF- α or IL-6 secretion, which supports our hypothesis regarding the lack of a role for ROS in cytokine secretion. ROS derived from 5-LO are involved in physiological responses in other cell types, including TNF- α mediated ROS generation (55) and senescence-like growth arrest (56) in fibroblasts and mitogenic signaling in keratinocytes (57). Although 5-LO- and COX-1-dependent ROS did not modulate mast cell secretory responses investigated in this study, we cannot rule out the possibility that other responses of mast cells will be altered. Generation of arachidonic acid metabolites by the action of 5-LO and other enzymes occurs at the inner nuclear membrane in mast cells (58) and this suggests novel roles of these mediators and ROS derived from 5-LO at this level. Future studies will elucidate the functional role of 5-LO-mediated ROS production in mast cells.

In this study, we demonstrate that huMC generate ROS following stimulation with IgG-LB which was enhanced by pre-exposure to IFN- γ . The production of ROS was 5-LO- and COX-1-dependent and NADPH oxidase-independent. This was in sharp contrast to PMN, in which the primary enzyme responsible for ROS production was NADPH oxidase. In addition, huMC failed to generate appreciable levels of ROS following stimulation with other NADPH oxidase activating agents including fMLP, PMA, and zymosan. Previous studies have shown mast cells are capable of generating ROS following activation with other phagocytic stimuli including bacteria (17) and silica (59). In the case of silica 5-LO was partially responsible for the observed ROS (59). With regard to the enhancing effect of IFN- γ on IgG-LB-dependent ROS generation, previous work in our laboratory demonstrated the induction of Fc γ RI expression in huMC pretreated with IFN- γ and the resulting production of LTC₄ and PGD₂ in response to Fc γ RI cross-linking (60, 61). Furthermore, agents that are known to prime NADPH oxidase activity in PMN, including LPS and IFN- γ , are also known to increase 5-LO expression and activity (43). These studies support the hypothesis that IFN- γ induces the expression of Fc γ R and 5-LO in huMC, which results in increased

responsiveness to IgG-LB thereby leading to enhanced ROS production derived from 5-LO and COX-1. We have preliminary unpublished data that demonstrate phagocytosis of bacteria by mast cells also generates ROS via a 5-LO- and COX-dependent mechanism.

In PMN the primary ROS producing enzyme activated by phagocytic stimuli is NADPH oxidase (1), but there are reports of phospholipase A₂ and 5-LO activation leading to subsequent LT and PG generation in these cells (62, 63). In addition, IFN- γ pretreatment can enhance 5-LO-dependent product formation in macrophages (64, 65). This suggests both the NADPH oxidase and 5-LO pathways are activated by similar stimuli in phagocytes and there are several reports of arachidonic acid modulating NADPH oxidase dependent ROS production in PMN (66–68). Using phospholipase A₂ inhibitors, a reduction in NADPH oxidase-dependent ROS production was observed with no direct effect on NADPH oxidase enzyme activity itself (67). However, a critical study using a specific phospholipase A_{2 α} inhibitor pyrrolidine-1 in PMN and phospholipase A_{2 α} -deficient mouse neutrophils showed no differences ROS production induced by opsonized zymosan, fMLP, or PMA (68–70). Therefore, in professional phagocytes both NADPH oxidase and phospholipase A_{2 α} can be activated but ROS are only generated by NADPH oxidase. However, mast cells appear to exclusively derive all ROS from the 5-LO and COX-1 pathways following a phagocytic stimulus, and the role played by these ROS in host defense remains to be determined.

In summary, mBMMC and huMC generate ROS in response to Fc ϵ RI aggregation and IgG-LB stimulation through the activity of 5-LO and COX-1 enzymes but not NADPH oxidase. In terms of Fc ϵ RI aggregation inhibition of ROS had negligible effects on degranulation and cytokine secretion while having concurrent inhibition on LTC₄ and PGD₂ production consistent with the hypothesis that 5-LO and COX-1 were the enzymes responsible for ROS production in mast cells. The source of IgG-LB induced ROS production in huMC was in sharp contrast to that of PMN, which primarily use NADPH oxidase for ROS generation. Overall our data demonstrate that mast cells exclusively use 5-LO and COX-1 for the generation of ROS.

Disclosures

The authors have no financial conflict of interest.

References

- Hampton, M. B., A. J. Kettle, and C. C. Winterbourn. 1998. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* 92: 3007–3017.
- Babior, B. M. 2004. NADPH oxidase. *Curr. Opin. Immunol.* 16: 42–47.
- Thannickal, V. J., and B. L. Fanburg. 2000. Reactive oxygen species in cell signaling. *Am. J. Physiol.* 279: L1005–L1028.
- Jackson, S. H., S. Devadas, J. Kwon, L. A. Pinto, and M. S. Williams. 2004. T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. *Nat. Immunol.* 5: 818–827.
- Devadas, S., L. Zaritskaya, S. G. Rhee, L. Oberley, and M. S. Williams. 2002. Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: selective regulation of mitogen-activated protein kinase activation and Fas ligand expression. *J. Exp. Med.* 195: 59–70.
- Reth, M. 2002. Hydrogen peroxide as second messenger in lymphocyte activation. *Nat. Immunol.* 3: 1129–1134.
- Singh, D. K., D. Kumar, Z. Siddiqui, S. K. Basu, V. Kumar, and K. V. S. Rao. 2005. The strength of receptor signaling is centrally controlled through a cooperative loop between Ca²⁺ and an oxidant signal. *Cell* 121: 281–293.
- Sundaresan, M., Z. X. Yu, V. J. Ferrans, K. Irani, and T. Finkel. 1995. Requirement for generation of H₂O₂ for platelet-derived growth factor signal transduction. *Science* 270: 296–299.
- Bae, Y. S., S. W. Kang, M. S. Seo, I. C. Baines, E. Tekle, P. B. Chock, and S. G. Rhee. 1997. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide: role in EGF receptor-mediated tyrosine phosphorylation. *J. Biol. Chem.* 272: 217–221.
- Ushio-Fukai, M., R. W. Alexander, M. Akers, Q. Yin, Y. Fujio, K. Walsh, and K. K. Griendling. 1999. Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. *J. Biol. Chem.* 274: 22699–22704.
- Metcalfe, D. D., D. Baram, and Y. A. Mekori. 1997. Mast cells. *Physiol. Rev.* 77: 1033–1079.
- Marshall, J. S. 2004. Mast-cell responses to pathogens. *Nat. Immunol.* 4: 787–799.
- Galli, S. J., J. Kalesnikoff, M. A. Grimaldeston, A. M. Piliponsky, C. M. M. Williams, and M. Tsai. 2005. Mast cells as “tunable” effector and immunoregulatory cells: recent advances. *Annu. Rev. Immunol.* 23: 749–786.
- Bingham, C. O., and K. F. Austen. 2000. Mast-cell responses in the development of asthma. *J. Allergy Clin. Immunol.* 105: S527–S534.
- Malaviya, R., T. Ikeda, E. Ross, and S. N. Abraham. 1996. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF- α . *Nature* 381: 77–80.
- Echtenacher, B., D. N. Mannel, and L. Hultner. 1996. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature* 381: 75–77.
- Malaviya, R., E. A. Ross, J. I. MacGregor, T. Ikeda, J. R. Little, B. A. Jakschik, and S. N. Abraham. 1994. Mast cell phagocytosis of FimH-expressing enterobacteria. *J. Immunol.* 152: 1907–1914.
- McLachlan, J. B., J. P. Hart, S. V. Pizzo, C. P. Shelburne, H. F. Staats, M. D. Gunn, and S. N. Abraham. 2003. Mast cell-derived tumour necrosis factor induces hypertrophy of draining lymph nodes during infection. *Nat. Immunol.* 4: 1199–1205.
- Jawdat, D. M., G. Rowden, and J. S. Marshall. 2006. Mast cells have a pivotal role in TNF-independent lymph node hypertrophy and the mobilization of langerhans cells in response to bacterial peptidoglycan. *J. Immunol.* 177: 1755–1762.
- Swindle, E. J., D. D. Metcalfe, and J. W. Coleman. 2004. Rodent and human mast cells produce functionally significant intracellular reactive oxygen species but not nitric oxide. *J. Biol. Chem.* 279: 48751–48759.
- Swindle, E. J., J. A. Hunt, and J. W. Coleman. 2002. A comparison of reactive oxygen species generation by rat peritoneal macrophages and mast cells using the highly sensitive real-time chemiluminescent probe pholasin: inhibition of antigen-induced mast cell degranulation by macrophage-derived hydrogen peroxide. *J. Immunol.* 169: 5866–5873.
- Suzuki, Y., T. Yoshimaru, T. Matsui, T. Inoue, O. Niide, S. Nunomura, and C. Ra. 2003. Fc ϵ RI signaling of mast cells activates intracellular production of hydrogen peroxide: role in the regulation of calcium signals. *J. Immunol.* 171: 6119–6127.
- Kim, J. Y., and J. Y. Ro. 2005. Signal pathway of cytokines produced by reactive oxygen species generated from phorbol myristate acetate-stimulated HMC-1 cells. *Scand. J. Immunol.* 62: 25–35.
- Clark R. A., and W. M. Nauseef. 2003. Isolation and functional analysis of neutrophils. In *Current Protocols in Immunology*. J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, eds. Wiley and Sons, Hoboken, N.J. p. 7.23.1.
- Keston, A. S., and R. Brandt. 1965. The fluorometric analysis of ultramicro quantities of hydrogen peroxide. *Anal. Biochem.* 11: 1–5.
- Ali, H., J. Ahamed, C. Hernandez-Munain, J. L. Baron, M. S. Krangel, and D. D. Patel. 2000. Chemokine production by G protein-coupled receptor activation in a human mast cell line: roles of extracellular signal-regulated kinase and NFAT. *J. Immunol.* 165: 7215–7223.
- Vines, C. M., M. Xue, D. C. Maestas, D. F. Cimino, and E. R. Prossnitz. 2002. Regulation of N-formyl peptide-mediated degranulation by receptor phosphorylation. *J. Immunol.* 169: 6760–6766.
- Iwaki, S., C. Tkaczyk, A. B. Satterthwaite, K. Halcomb, M. A. Beaven, D. D. Metcalfe, and A. M. Gilfillan. 2005. Btk plays a crucial role in the amplification of Fc ϵ RI-mediated mast cell activation by kit. *J. Biol. Chem.* 280: 40261–40270.
- Klein, M., S. Klein-Hessling, A. Palmethofer, E. Serfling, C. Tertilt, T. Bopp, V. Heib, M. Becker, C. Taube, H. Schild, E. Schmitt, and M. Stassen. 2006. Specific and redundant roles for NFAT transcription factors in the expression of mast cell-derived cytokines. *J. Immunol.* 177: 6667–6674.
- Hernandez-Hansen, V., J. D. J. Bard, C. A. Tarleton, J. A. Wilder, C. A. Lowell, B. S. Wilson, and J. M. Oliver. 2005. Increased expression of genes linked to Fc ϵ RI signalling and to cytokine and chemokine production in Lyn-deficient mast cells. *J. Immunol.* 175: 7880–7888.
- Matsui, T., Y. Suzuki, K. Yamashita, T. Yoshimaru, M. Suzuki-Karasaki, S. Hayakawa, M. Yamaki, and K. Shimizu. 2000. Diphenyleneiodonium prevents reactive oxygen species generation, tyrosine phosphorylation, and histamine release in RBL-2H3 mast cells. *Biochem. Biophys. Res. Commun.* 276: 742–748.
- Yoshimaru, T., Y. Suzuki, T. Matsui, K. Yamashita, T. Ochiai, M. Yamaki, and K. Shimizu. 2002. Blockade of superoxide generation prevents high-affinity immunoglobulin E receptor-mediated release of allergic mediators by rat mast cell line and human basophils. *Clin. Exp. Allergy* 32: 612–618.
- Yoshimaru, T., Y. Suzuki, T. Inoue, O. Niide, and C. Ra. 2006. Silver activates mast cells through reactive oxygen species production and a thiol-sensitive store-independent Ca²⁺ influx. *Free Radic. Biol. Med.* 40: 1949–1959.
- Schewe, C., T. Schewe, and A. Wendel. 1994. Strong inhibition of mammalian lipoxygenases by the antiinflammatory seleno-organic ebelen in the absence of glutathione. *Biochem. Pharmacol.* 48: 65–74.
- Safayhi, H., G. Tiegs, and A. Wendel. 1985. A novel biologically active seleno-organic compound-V: inhibition by ebelen (PZ51) of rat peritoneal neutrophil lipoxygenase. *Biochem. Pharmacol.* 34: 2691–2694.
- Weitzel, F., and A. Wendel. 1993. Selenoenzymes regulate the activity of leukocyte 5-lipoxygenase via the peroxide tone. *J. Biol. Chem.* 268: 6288–6292.
- Huang, H.-S., C.-J. Chen, H. Suzuki, S. Yamamoto, and W.-C. Chang. 1999. Inhibitory effect of phospholipid hydroperoxide glutathione peroxidase on the activity of lipoxygenases and cyclooxygenases. *Prostaglandins Other Lipid Mediat.* 58: 65–75.

38. Imai, H., K. Narashima, M. Arai, H. Sakamoto, N. Chiba, and Y. Nakagawa. 1998. Suppression of leukotriene formation in RBL-2H3 cells that overexpressed phospholipid hydroperoxide glutathione peroxidase. *J. Biol. Chem.* 273: 1990–1997.
39. Sakamoto, H., H. Imai, and Y. Nakagawa. 2000. Involvement of phospholipid hydroperoxide glutathione peroxidase in the modulation of prostaglandin D₂ synthesis. *J. Biol. Chem.* 275: 40028–40035.
40. Nakano, N., A. Nakao, T. Uchida, N. Shirasaka, H. Yoshizumi, K. Okumura, R. Tsuboi, and H. Ogawa. 2005. Effects of arachidonic acid analogs on FcεRI-mediated activation of mast cells. *Biochim. Biophys. Acta* 1738: 19–28.
41. Murphy, R. C., S. Hammarström, and B. Samuelsson. 1979. Leukotriene C: a slow-reacting substance from mouse mastocytoma cells. *Proc. Natl. Acad. Sci. USA* 76: 4275–4279.
42. Corey, E. J., and P. T. Lansbury, Jr. 1983. Stereochemical course of 5-lipoxygenation of arachidonate by rat basophil leukemic cell (RBL-1) and potato enzymes. *J. Am. Chem. Soc.* 105: 4093–4094.
43. Werz, O. 2002. 5-Lipoxygenase: cellular biology and molecular pharmacology. *Curr. Drug Targets Inflamm. Allergy* 1: 23–44.
44. Chamulitrat, W., M. F. Hughes, T. E. Eling, and R. P. Mason. 1991. Lipid peroxyl radical intermediates in the peroxidation of polyunsaturated fatty acids by lipoxygenase. *Arch. Biochem. Biophys.* 290: 153–159.
45. Kukreja, R. C., H. A. Kontos, M. L. Hess, and E. F. Ellis. 1986. PGH synthase and lipoxygenase generate superoxide in the presence of NADH or NADPH. *Circ. Res.* 59: 612–619.
46. Roy, P., S. K. Roy, A. Mitra, and A. P. Kulkarni. 1994. Superoxide generation by lipoxygenase in the presence of NADH and NADPH. *Biochim. Biophys. Acta* 1214: 171–179.
47. Ivanov, I., J. Samm, H. Kuhn, and H.-G. Holzthütter. 2005. Dual role of oxygen during lipoxygenase reactions. *FEBS J.* 272: 2523–2535.
48. Mason, R. P., B. Kalyanaraman, B. E. Tainer, and T. E. Eling. 1980. A carbon-centered free radical intermediate in the prostaglandin synthetase oxidation of arachidonic acid. *J. Biol. Chem.* 255: 5019–5022.
49. Schreiber, J., G. L. Foureman, M. F. Hugher, R. P. Mason, and T. E. Eling. 1989. Detection of glutathione thyl free radical catalyzed by prostaglandin H synthase present in keratinocytes. *J. Biol. Chem.* 264: 7936–7943.
50. Cathcart, R., E. Schwiers, and B. N. Ames. 1983. Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal. Biochem.* 134: 111–116.
51. Chance, B., H. Sies, and A. Boveris. 1979. Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* 59: 527–605.
52. Larsen, L. N., and E. D. J. Bremer. 1996. Peroxidative oxidation of leuco-dichlorofluorescein by prostaglandin H synthase in prostaglandin biosynthesis from polyunsaturated fatty acids. *Biochim. Biophys. Acta* 1299: 47–53.
53. Igarashi, Y., J. D. Lundgren, J. H. Shelhamer, M. A. Kaliner, and M. V. White. 1993. Effects of inhibitors of arachidonic acid metabolism on serotonin release from rat basophilic leukemia cells. *Immunopharmacol.* 25: 131–144.
54. Son, A., H. Nakamura, N. Kondo, Y. Matsuo, W. Liu, S. Oka, Y. Ishii, and J. Yodoi. 2006. Redox regulation of mast cell histamine release in thioredoxin-1 (TRX) transgenic mice. *Cell Research* 16: 230–239.
55. Woo, C.-H., Y.-W. Eom, M.-H. Yoo, H.-J. You, H. J. Han, W. K. Song, Y. J. Yoo, J.-S. Chun, and J.-H. Kim. 2000. Tumor necrosis factor-α generates reactive oxygen species via a cytosolic phospholipase A₂-linked cascade. *J. Biol. Chem.* 275: 32357–32362.
56. Catalano, A., S. Rodilossi, P. Caprari, V. Coppola, and A. Procopio. 2005. 5-Lipoxygenase regulates senescence-like growth arrest by promoting ROS-dependent p53 activation. *EMBO J.* 24: 170–179.
57. Sekharam, M., J. M. Cunnick, and J. Wu. 2000. Involvement of lipoxygenase in lysophosphatidic acid-stimulated hydrogen peroxide release in human HaCaT keratinocytes. *Biochem. J.* 346: 751–758.
58. Chen, X.-S., T. A. Naumann, U. Kurre, N. A. Jenkins, N. G. Copeland, and C. D. Funk. 1995. cDNA cloning, expression, mutagenesis, intracellular localization, and gene chromosomal assignment of mouse 5-lipoxygenase. *J. Biol. Chem.* 270: 17993–17999.
59. Brown, J. M., E. J. Swindle, N. M. Kushnir-Sukhov, A. Holian, and D. D. Metcalfe. 2006. Silica-directed mast cell activation is enhanced by scavenger receptors. *Am. J. Respir. Cell. Mol. Biol.* 36: 43–52.
60. Okayama, Y., D. D. Hageman, and D. D. Metcalfe. 2001. A comparison of mediators released or generated by IFN-γ-treated human mast cells following aggregation of FcγRI or FcεRI. *J. Immunol.* 166: 4705–4712.
61. Woolhiser, M. R., Y. Okayama, A. M. Gilfillan, and D. D. Metcalfe. 2001. IgG-dependent activation of human mast cells following up-regulation of FcγRI by IFN-γ. *Eur. J. Immunol.* 31: 3298–3307.
62. Satake, Y., B. L. Diaz, B. Balestrieri, B. K. Lam, Y. Kanoaka, M. J. Grusby, and J. P. Arm. 2004. Role of group V phospholipase A₂ in zymosan-induced eicosanoid generation and vascular permeability revealed by targeted gene disruption. *J. Biol. Chem.* 279: 16488–16494.
63. Balestrieri, B., V. W. Hsu, H. Gilbert, C. C. Leslie, W. K. Han, J. V. Bonventre, and J. P. Arm. 2006. Group V secretory phospholipase A₂ translocates to the phagosome after zymosan stimulation of mouse peritoneal macrophages and regulates phagocytosis. *J. Biol. Chem.* 281: 6691–6698.
64. Meslier, N., A. J. Aldrich, and T. D. Bigby. 1992. Effect of interferon-γ on the 5-lipoxygenase pathway of rat lung macrophages. *Am. J. Respir. Cell Mol. Biol.* 6: 93–99.
65. Nassar, G. M., A. Montero, M. Fukunaga, and K. F. Badr. 1997. Contrasting effects of proinflammatory and T-helper lymphocyte subset-2 cytokines on the 5-lipoxygenase pathway in monocytes. *Kidney Int.* 51: 1520–1528.
66. Maridonneau-Parini, I., and A. I. Tauber. 1986. Activation of NADPH-oxidase by arachidonic acid involves phospholipase A₂ in intact human neutrophils but not in the cell-free system. *Biochem. Biophys. Res. Commun.* 138: 1099–1105.
67. Dana, R., T. L. Leto, H. L. Malech, and R. Levy. 1998. Essential requirement of cytosolic phospholipase A₂ for activation of the phagocyte NADPH oxidase. *J. Biol. Chem.* 273: 441–445.
68. Serezani, C. H. C., D. M. Aronoff, S. Jancar, and M. Peters-Golden. 2005. Leukotriene B₄ mediates p47^{phox} phosphorylation and membrane translocation in polyunsaturated fatty acid-stimulated neutrophils. *J. Leukocyte Biol.* 78: 976–984.
69. Ozaki, Y., T. Ohashi, and Y. Niwa. 1986. A comparative study on the effects of inhibitors of the lipoxygenase pathway on neutrophil function: inhibitory effects on neutrophil function may not be attributed to inhibition of the lipoxygenase pathway. *Biochem. Pharmacol.* 35: 3481–3488.
70. Rubin, B. B., G. P. Downey, A. Koh, N. Degousee, F. Ghomashchi, L. Nallan, E. Stefanski, D. W. Harkin, C. Sun, B. P. Snart, et al. 2005. Cytosolic phospholipase A_{2α} is necessary for platelet-activating factor biosynthesis, efficient neutrophil-mediated bacterial killing, and the innate immune response to pulmonary infection: cPLA_{2α} does not regulate neutrophil NADPH oxidase activity. *J. Biol. Chem.* 280: 7519–7529.