Harboring of Particulate Allergens within Secretory Compartments by Mast Cells following IgE/FcεRI-Lipid Raft-Mediated Phagocytosis[1]

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Although much is known regarding the exocytic responses of mast cells following allergen/IgE-mediated activation, little is currently known of the fate of the activating allergens, many of which are particles. We have found that IgE-bound particulate allergens were phagocytosed by activated mast cells in a lipid raft-dependent manner. The nascent allergen-containing phagosomes were found to transform into granule compartments by acquiring VAMP7 and serotonin and exhibited the capacity to empty their contents upon mast cell activation. When allergen-harboring mast cells were stimulated, the intracellular allergens were expelled intact and shown to activate adjacent mast cells. This capacity of mast cells to phagocytose and retain whole and antigenically intact allergens could potentially contribute to the course of inflammatory diseases such as asthma. ***The Journal of Immunology,*** **2006, 177: 5791–5800.**

Mast cells (MCs)[3] are the principal effector cells of allergy because of their ability to release a wide range of pharmacologically active mediators upon contact with allergens (1–3). Activation of skin and mucosal MCs by allergens is mediated by allergen-specific IgE Abs bound on FcεRI (high-affinity IgE receptor) molecules expressed on MC surfaces (4). Cross-linking of the IgE/FcεRI complexes by allergens initiates a series of signaling events within the MCs (5, 6). These intracellular signaling events result in the release of MC granule contents into the extracellular environment, a response that is preceded by the fusion of granule membranes with the plasma membrane (7). Although much is known of MC exocytic responses and its pathologic sequelae, little is known of the fate of the allergens following MC activation. This uncharacterized aspect of allergic responses is important, as retention or degradation of allergens within MCs might have significant impact on the exacerbation of the allergic condition.

Although FcεRI molecules have been shown to mediate internalization of cross-linking agents (8, 9), internalization of activating allergens by MCs has not been reported. Recent studies of FcεRI-mediated signaling in MCs have shown that crosslinked FcεRI molecules become associated with distinct membrane domains called lipid rafts (10–12). Lipid rafts have been implicated in many signaling events, which has been attributed in part to their capacity to sequester a large pool of signaling molecules within the membranes (13). Because Lyn, a Src family protein tyrosine kinase known to be critical in initiating FcεRI-mediated signaling in MCs, was found to be associated with lipid rafts (9, 10), it was postulated that the association of IgE/FcεRI complexes with lipid rafts was for the purpose of triggering Lyn-mediated signaling leading to MC activation (14). However, the data supporting this hypothesis have remained tenuous and sometimes controversial (15). Thus, the relevance of the association of IgE/FcεRI complexes with lipid rafts following ligation by allergens remains unclear.

There is a growing body of evidence demonstrating that lipid rafts are involved in the internalization of many foreign agents. Ligands ranging from small toxins to large microbial pathogens such as SV40 and *Mycobacteria kansasii* have been shown to be internalized by a mechanism involving lipid rafts (16). In many of these cases, the internalization is triggered by the cross-linking of specific cellular receptors found within lipid rafts. Conceivably, the association of IgE/FcεRI molecules with lipid rafts following ligation may contribute to the internalization of the bound allergens. To date, most studies investigating FcεRI-mediated activation of MCs have used soluble proteins as the Ag. However, several allergens, especially those implicated in asthma, are not soluble but are particles of respirable size (17, 18). These particles include pollen, animal dander, and house dust mite fecal pellets (17). When airborne, they are of a size that allows them to reach deep into the airways following inhalation. Upon reaching these sites, they can interact with local airway cells, including MCs (18). Because of their polyvalency, these particles are likely to elicit powerful responses from inflammatory cells, including a phagocytic response. In the present study, our purpose was to investigate whether MC activation by particulate allergens involves phagocytosis of the activating allergens and, if so, to examine the role of lipid rafts in the phagocytic process as well as the intracellular fate of the allergens.

Materials and Methods

Abs and reagents

Mouse anti-trinitrophenol (TNP) IgE Abs, biotin-labeled anti-mouse IgE Abs, and mouse anti-protein kinase Cδ Abs were from BD Biosciences. Mouse anti-rat CD48 Abs were purchased from Serotec. Rabbit anti-Syk

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[3] Abbreviations used in this paper: MC, mast cell; PSG, pollen starch granule; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNP, trinitrophenol; TRITC, tetramethylrhodamine isothiocyanate.

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Abs came from Santa Cruz Biotechnology. Rabbit anti-Gab2 Abs and HRP-conjugated anti-phosphotyrosine Abs were acquired from Upstate Biotechnology. Cy3-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories. Mouse anti-serotonin Ab was purchased from DakoCytomation. HRP-conjugated Abs, HRP-conjugated ExtrAvidin, methyl-β-cyclodextrin, filipin, 2,4,6-trinitrobenzenesulfonic acid (TNBS), and calcium ionophore were from Sigma-Aldrich. Alexa Fluor 488/Alexa Fluor 633 carboxylic acid succinimidyl ester was acquired from Molecular Probes. Polyclonal goat anti-mouse c-*kit* Abs were from R&D Systems, Cy5-labeled donkey anti-goat Abs from Jackson ImmunoResearch Laboratories, and avidin-tetramethylrhodamine isothiocyanate (TRITC) from Sigma-Aldrich.

Cell culture and IgE sensitization

Primary MCs were cultured from peritoneal cells obtained from C57BL/6 mice. Peritoneal cells were incubated in DMEM supplemented with 10% FBS for 3 h at 37°C. Nonadherent cells were removed and cultured in DMEM supplemented with 10% FBS, 10 ng/ml recombinant mouse stem cell factor, 5 ng/ml mouse IL-3, and antibiotics for 3 wk. Approximately 97% of these cultured cells were determined to be MCs based on their toluidine blue staining reaction and by FACS analysis using anti-CD117(c-*kit*) and anti-IgE Abs as probes. Peritoneal macrophages were obtained by plating peritoneal cells obtained from C57BL/6 mice on plastic dishes in DMEM supplemented with 10% FBS. After 3 h of incubation at 37°C, nonadherent cells were discarded and the adherent macrophages were extensively washed and used for experiments. GFP-VAMP7-expressing RBL-2H3 cells were generated as described (19). Sensitization of MCs in vitro was performed by incubating cells with 0.5 μg/ml mouse IgE Abs overnight.

Modification of PSG and Escherichia coli

Pollen starch granules (PSGs) from rye grass pollen were purified as described (20). Coating of PSG with TNP was performed by incubating PSG with 0.005% TNBS (w/v) in borate buffer (pH 8.2) for 20 min at 37°C. All unbound TNBS molecules were washed off by repeated centrifugation. Labeling of TNP-PSGs with Alexa Fluor 488 or Alexa Fluor 633 was performed using Alexa Fluor 488 or Alexa Fluor 633 carboxylic acid succinimidyl ester, respectively. The *E. coli* strain used for these studies was ORN103(pUT2002), a nonadherent, fimbriated K-12 laboratory strain (21). TNP-*E. coli* was generated as described for TNP-PSG. Red fluorescent *E. coli* were generated by introducing a compatible plasmid pKEN-HcRed1 to ORN103(pUT2002). Plasmid pKEN-HcRed1, a derivative of plasmid pKEN-EGFP (a gift from Dr. M. Kuehn, Duke University, Durham, NC), was created by replacing the *EGFP* gene of plasmid pKEN-EGFP with the *HcRed1* gene using the restriction enzymes *Xba*I and *Hin*dIII. *HcRed1* gene fragments containing *Xba*I and *Hin*dIII enzyme sites were obtained from plasmid pHcRed1 (BD Clontech) by PCR using the primers 5′-GATTTCTAGATTTAAGAAGGAGATATACATATGGTGAGCGGCCTGCTGAAG-3′ and 5′-CCCAAGCTTTCAGTTGGCCTTCTCGGGCAG-3′.

Antibiotic protection assay

After incubation with TNP-*E. coli*, MCs or macrophages were treated with 200 μl of gentamicin (100 μg/ml) for 30 min to kill all extracellular bacteria. The cells were washed and subsequently solubilized with 100 μl of 0.1% Triton X-100 in PBS. The cell lysates were plated onto MacConkey agar plates and incubated at 37°C overnight. The number of the CFUs was counted.

Bacterial viability assay

Following exposure of 10^5 host cells to 10^7 TNP-*E. coli* bacteria for 30 min, gentamicin was treated to the cells to kill extracellular bacteria as described for the antibiotic protection assay. Cells were further incubated in their growth medium containing gentamicin. At a specified time point the cells were solubilized with Triton X-100 and the number of viable bacteria at the time point was determined by counting CFUs as described above.

Electron microscopy

For scanning electron microscopy, sensitized MCs were exposed to TNP-PSGs for 30 min and extensively washed to get rid of extracellular TNP-PSGs. Following 5 h of incubation in the medium, the cells were treated or untreated with anti-IgE Abs for 15 min on plastic coverslips and subsequently fixed in 2% glutaraldehyde in PBS. Samples were then postfixed with OsO_4, rinsed, dehydrated in ascending concentrations of ethyl alcohol, critical point dried, coated with ~150-Å gold, and examined under a scanning electron microscope. For transmission electron microscopy, MCs that were treated as described in the text were fixed in 2% glutaraldehyde in PBS and postfixed with OsO_4. Samples were dehydrated in ascending concentrations of ethyl alcohol, embedded in Epon, collected on mesh grids, and contrasted with 4% uranyl acetate. Samples were viewed under a transmission electron microscope.

Fluorescent microscopy on mouse lung sections

Alexa Fluor 488-labeled 1×10^8 TNP-PSG, TNP-PSG, or saline was intratracheally instilled with 5 μg of LPS into anesthetized mice 24 h after i.p. injection of 10 μg of anti-TNP IgE Abs. After 24 h, mice were sacrificed, and whole lungs and trachea were removed and frozen in OCT compound. Twenty-micrometer sections were prepared and fixed in acetone for 30 min. Slides were dried and then blocked in 1% BSA for 1 h at room temperature. Polyclonal anti-c-*kit* was added to each slide overnight at 4°C. Slides were washed three times in 1× PBS. Avidin-TRITC and Cy5-labeled anti-goat IgG were added to the slides for 1 h. Slides were then washed three times in 1× PBS. Confocal images of tissue sections were obtained using a Plan Fluor 20.0/.75/.35 ×20 objective and EZ-C1 Nikon software (Silver Version 2.01). Threshold values for background fluorescence were determined using sections from LPS and TNP-PSG-challenged lung stained with donkey polyclonal IgG.

Flow cytometry of peritoneal cells

Alexa Fluor-488-labeled TNP -PSGs or TNP-PSGs (1×10^7) suspended in 100 μl of PBS were instilled into the peritoneal cavities of mice passively sensitized with 10 μg of TNP-specific IgE. After 24 h, peritoneal cells from mice challenged with TNP-labeled PSGs were lavaged and stained either with allophycocyanin-labeled anti-c-*kit* Ab or allophycocyanin-labeled isotype control. Peritoneal cells from mice challenged peritoneally with Alexa Fluor 488-labeled TNP-PSGs were stained with allophycocyanin-labeled anti-c-*kit* Ab. Flow cytometry was performed on each of the peritoneal cell preparations using a FACSVantage instrument (BD Pharmingen). All c-*kit*-positive cells were sorted out and toluidine blue stained to confirm purity. For serotonin staining, the sorted mast cells were permeated and blocked in PBS containing 0.1% saponin and 1% BSA for 30 min at 4°C. Subsequently, the cells were incubated with mouse anti-human serotonin Ab for 30 min at 4°C. After washing, the cells were incubated with Cy3-conjugated goat anti-mouse IgG for 30 min at 4°C.

Hexosaminidase assay

The enzymatic activities of β-hexosaminidase in supernatants and cell pellets solubilized with 0.5% Triton X-100 in Tyrode's buffer were measured with *p*-nitrophenol-*N*-acetyl-β-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) for 30 min at 37°C. The reaction was stopped by the addition of 0.1 M carbonate buffer (pH 10.0). The release of the product 4-*p*-nitrophenol was detected by absorbance at 405 nm. The extent of degranulation was calculated by dividing 4-*p*-nitrophenol absorbance in the supernatant by the sum of absorbance in the supernatants and in cell pellets solubilized in detergent.

Expulsion of intracellular allergens and re-uptake by IgE-sensitized MCs

A group of primary mouse MCs were sensitized with equal amounts of TNP-specific IgE and dansyl-specific IgE Abs and then exposed to Alexa Fluor 488-TNP-PSGs. After TNP-IgE-mediated MC phagocytosis of the PSGs was completed, residual extracellular PSGs were completely washed off and the MCs were incubated overnight in the presence of more dansyl-specific IgE Abs. This group of MCs was then mixed with an equal concentration of a second group comprised of MCs sensitized with TNP-specific IgE Abs that were conjugated by a Cy5 fluorophore. The first group of MCs was readily distinguishable from the second group because membranes of MCs in the first group were not fluorescent, but most of them contained one or more green fluorescent PSGs. MCs from the second group were distinguishable by the red fluorescence of their Cy5-labeled IgE bound to their cell surfaces. To selectively activate the first group of MCs (allergen-harboring cells, i.e., the donor population) and induce extracellular discharge of TNP-PSGs, we added dansyl-BSA to the MC mixture. Subsequently, the mixture of cells was subjected to microscopic analysis.

Results

MC phagocytosis of particulate allergens

Because Ags found on common particulate allergens have not been characterized and the specific IgE Abs to these Ags are not readily available, we initiated our studies by generating a particulate allergen through the covalent binding of TNP moieties to an inert laboratory *E. coli* strain, ORN103(pUT2002) (21). TNP is routinely used

as an Ag for in vitro assays because of the commercial availability of specific IgE Abs. *E. coli* strain ORN103(pUT2002) possesses only 50% of the genome typically found on a clinical strain of *E. coli* and is devoid of any adhesive capabilities (21). We selected this particulate allergen model for several reasons. First, like many natural particulate allergens, bacteria are biodegradable particles and their sizes fall within the "respirable" range of particles. Second, multiple TNP molecules can be covalently coupled to the bacterial surface to create a potent polyvalent Ag. Third, the bacteria have a distinct morphology and are therefore readily distinguishable from any other intracellular substructures of MCs in morphological studies. Fourth and most importantly, MC phagocytosis of live bacteria can be accurately and conveniently quantitated using standard antibiotic protection assays. To validate this particulate allergen model, we examined whether TNP-*E. coli* was capable of evoking an exocytic response from rat basophilic leukemia (RBL)-2H3 MC lines by measuring the extracellular release of hexosaminidase, a prestored component of MC secretory compartments. As shown in Fig. 1*A*, TNP-*E. coli* induced significant hexosaminidase release from RBL-2H3 cells sensitized with anti-TNP IgE Abs but not from unsensitized cells. We investigated whether this exocytic response of RBL-2H3 cells was accompanied by the phagocytosis of TNP-*E. coli* using an antibiotic protection assay. As shown in Fig. 1*B*, an appreciable number of TNP-coated *E. coli* was internalized by the IgE-sensitized cells but not by unsensitized cells, indicating that this phagocytic response of MCs was IgE-specific. Thus, in addition to the IgE/FcεRI-mediated degranulation response, particulate allergens can trigger MC phagocytosis of the activating particles.

FcεRI-mediated MC phagocytosis is lipid raft-dependent

We sought to examine the underlying mechanism of FcεRI-mediated MC phagocytosis by focusing on the possible role of lipid rafts. Previous studies have shown that IgE/FcεRI complexes in RBL-2H3 cells become associated with lipid rafts upon cross-linking (10, 11). Therefore, we sought to investigate whether TNP-*E. coli* induced the association of IgE/FcεRI complexes with lipid rafts in RBL-2H3 cells sensitized with TNP-specific IgE Abs. Triton X-100-treated RBL-2H3 cell lysates were prepared before and after exposure to TNP-*E. coli*. To separate the lipid raft fractions, these samples were fractionated using a sucrose density gradient cell fractionation assay (22). Before the addition of TNP-*E. coli*, the majority of IgE/FcεRI complexes were found in fractions 8–10, where soluble proteins normally fractionate (Fig. 2*A*, *top panel*). However upon treatment with TNP-*E. coli*, up to 30% of the IgE/FcεRI complexes were detectable in fractions 3–5, where lipid raft components typically fractionate (Fig. 2*A*, *two middle panels*) (22). Thus, the ligation of IgE/FcεRI complexes in the particulate allergen model induces the association of these complexes with lipid rafts. To examine the possible connection between IgE/FcεRI lipid raft association and MC phagocytosis of TNP-*E. coli*, we examined the effects of methyl-β-cyclodextrin, a raft-disrupting drug, on the phagocytic process. Pretreatment of RBL-2H3 cells with this drug completely blocked TNP-*E. coli*-induced association of IgE/FcεRI complexes with lipid rafts (Fig. 2*A*, *bottom panel*) and markedly reduced phagocytosis of the activating particles in a dose-dependent manner (Fig. 2*B*). Another raft-disrupting drug, filipin, exhibited a similar inhibitory effect on the phagocytosis of TNP-*E. coli* (Fig. 2*C*). It should be noted that neither drug had any effect on bacterial viability at the doses used in this study. Taken together, our findings indicate that lipid rafts play a key role in FcεRI-mediated phagocytosis of particulate allergens by MCs.

Phagocytosed allergens avoid degradation within MCs

Lipid raft mediated uptake of particulate microbes in various cells including MCs have been associated with the intracellular persistence of the pathogen, implying that the intracellular compartment

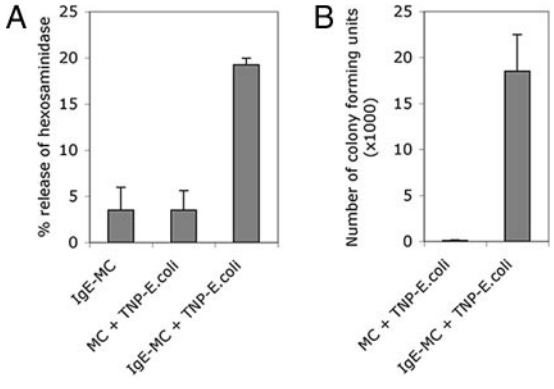

FIGURE 1. IgE-dependent degranulation of RBL-2H3 cells by TNP-*E. coli* and the accompanying phagocytosis of activating particles. IgE-sensitized or unsensitized RBL-2H3 cells, labeled IgE-MC and MC, respectively, were cultured on 96-well plates and exposed to TNP-coated *E. coli* (TNP-*E. coli*) for 30 min (*E. coli* to RBL-2H3 cell ratio was 100:1). *A*, Degranulation was assessed by measuring the percentage of hexosaminidase released from RBL-2H3 cells during the incubation period. *B*, Phagocytosis was assessed by a standard antibiotic protection assay, and number of CFUs per well is indicated.

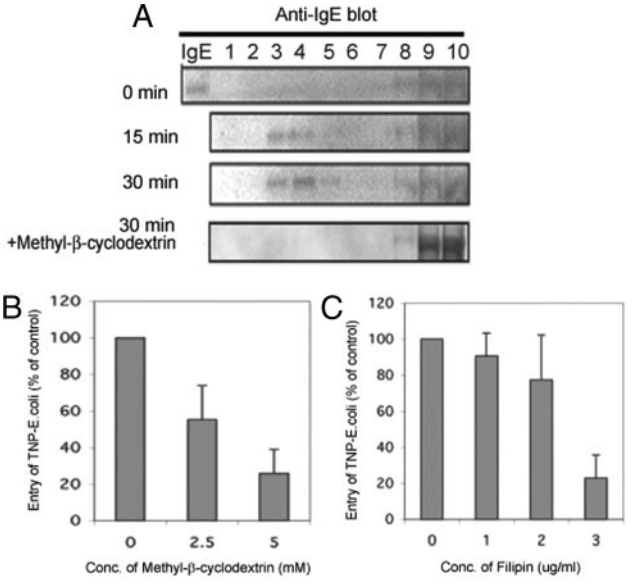

FIGURE 2. MC phagocytosis of particulate allergen, but not degranulation, is lipid raft dependent. *A*, Allergen-induced association of IgE/FcεRI complexes with lipid raft domains. Lysates of RBL-2H3 cells sensitized with TNP-specific IgE Abs were prepared before (*top panel*), 15 min after (*second panel from top*), and 30 min (*third panel from top*) after exposure to TNP-coated *E. coli*, and cell fractionation was performed as described in *Materials and Methods*. Each of the fractions was subjected to Western blot analysis using biotin-labeled anti-IgE Abs and HRP-streptavidin to detect IgE/FcεRI complexes. As a positive control for the Western blot analysis, pure IgE Ab molecules were loaded onto an extra lane during SDS-PAGE (*far left lane in the top panel*). Shown in the *bottom panel* is the effect of the pretreatment of IgE-sensitized MCs with methyl-β-cyclodextrin for 30 min on the association of IgE/FcεRI complexes with lipid raft domains. *B* and *C*, Inhibition of MC phagocytosis of allergens by raft-disrupting agents. RBL-2H3 cells sensitized with TNP-specific IgE Abs were pretreated with methyl-β-cyclodextrin for 30 min (*B*) or filipin for 15 min (*C*) before the phagocytosis assay. Neither reagent affected cell viability as determined by a dye exclusion assay. Conc., concentration.

housing the pathogens was not degradative. We examined whether the TNP-*E. coli* phagocytosed by sensitized RBL-2H3 cells was subsequently degraded. Because the TNP-*E. coli* that we used as the artificial allergen was viable, we could readily assess the intracellular degradation of TNP-*E. coli* following phagocytosis by monitoring the viability of the bacteria using standard bacterial viability assays. As shown in Fig. 3*A*, the number of viable bacteria in RBL-2H3 cells did not decrease over time. Instead, we observed a significant increase in bacterial numbers, indicating that the intracellular compartments housing TNP-*E. coli* were not degradative but even conducive to microbial growth. As a control for the biodegradability of TNP-*E. coli* following phagocytosis, we also monitored the intracellular fate of TNP-*E. coli* following phagocytosis by murine macrophages. As shown in Fig. 3*A*, up to

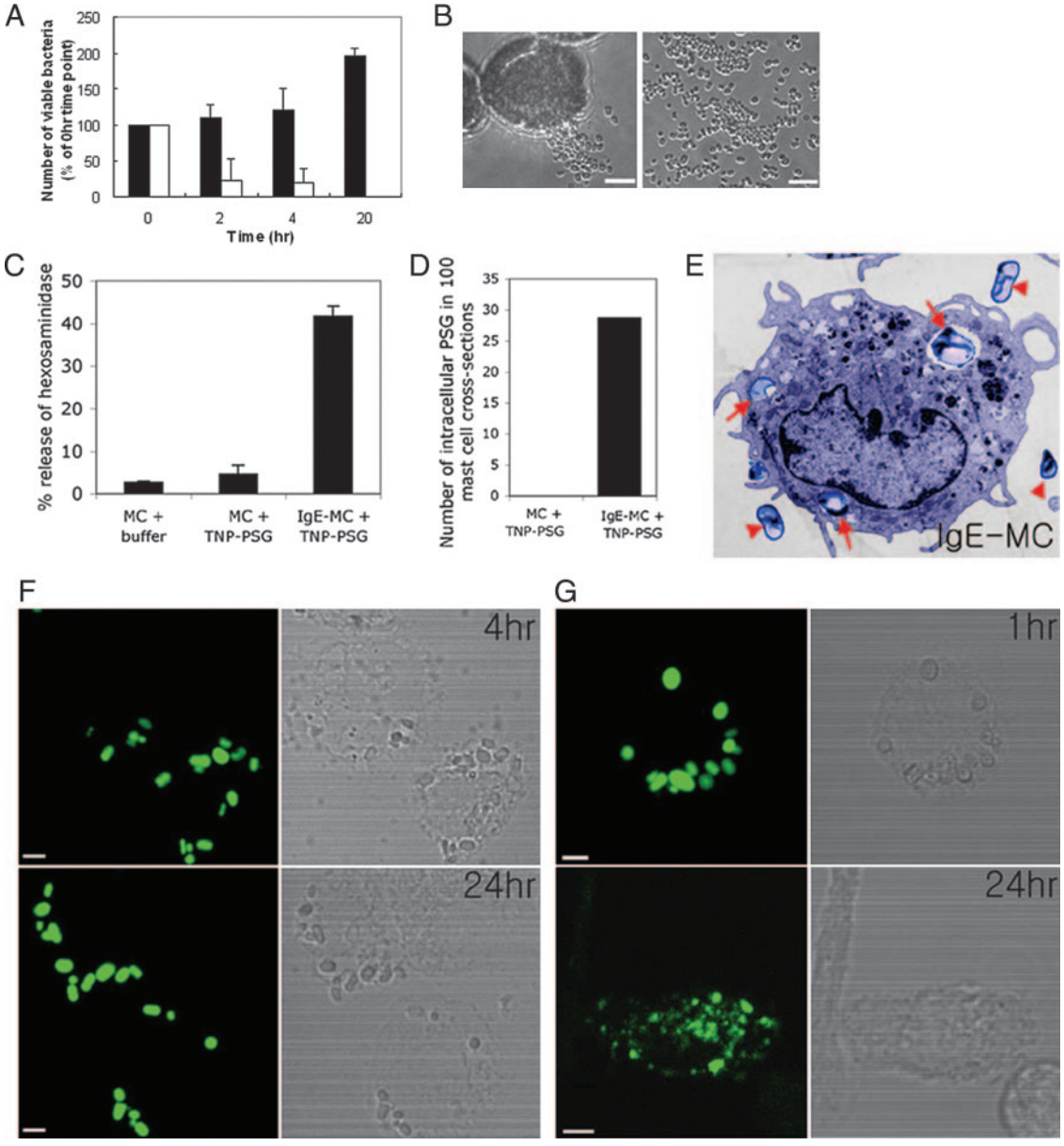

FIGURE 3. Particulate allergens remain nondegraded following MC phagocytosis. *A*, Intracellular survival of TNP-*E. coli* following phagocytosis by IgE-sensitized MCs. IgE-sensitized RBL-2H3 cells were incubated with TNP-coated *E. coli* for 30 min. Afterward, extracellular bacteria were rigorously washed off and all residual extracellular bacteria were killed by gentamicin treatment. Viability of intracellular bacteria was followed at various time points by solubilizing cells and enumerating viable bacteria by using standard bacterial colony counts on agar plates. For comparative purposes, TNP-coated *E. coli* were incubated for 30 min with mouse peritoneal macrophages before being processed as described above. The number of viable bacteria associated with RBL-2H3 cells (■) and with macrophage (□) at each time point was determined from the mean ± SE of viable bacteria at a given time point and the mean of viable bacteria at 0 h. *B*, Light microscopy of rye grass pollen suspension extruding PSGs (*left panel*; scale bar, 27 μm) and purified PSGs (*right panel*; scale bar, 5 μm). *C–E*, MC degranulation (*C*) and phagocytic (*D*) responses to TNP-PSGs. Unsensitized (MC) or IgE-sensitized MCs (IgE-MC) (10^6) were added to 5×10^7 TNP-PSGs for 30 min. Hexosaminidase was assessed from the supernatant (*C*), and the pellet of cells was processed for electron microscopy (*D* and *E*). MC cross-sections were examined by transmission electron microscopy to count the number of intracellular PSGs (*D*). Shown in *E* is a representative transmission electron microscopy image of sensitized MCs (IgE-MC) that were exposed to TNP-PSGs. Arrows and arrowheads in *E* indicate intracellular and extracellular PSGs, respectively. This figure was given false color to more readily visualize PSGs. *F* and *G*, Confocal microscopy of primary MCs (*F*) and macrophages (*G*) at different time points following phagocytosis of Alexa Fluor 488-TNP-PSGs. The ratio of cells to PSG was 1:50 in the incubation mixture. Shown to the *right* of each fluorescent image are the corresponding differential interference contrast images. Scale bars, 3 μm.

80% of bacteria phagocytosed by macrophages had lost their viability within 2 h.

Although these findings suggest that allergen particles phagocytosed by MCs do not undergo intracellular degradation, these studies were undertaken using an artificial allergen model, TNP-*E. coli*, and an immature MC line. Hence, it was important to establish the lack of intracellular degradation of allergens by using natural allergen particles and primary MCs. Therefore, we examined the intracellular fate of a natural particulate allergen, PSG, following phagocytosis by murine primary MCs. PSGs are well-known activators of MCs, triggering bronchial constriction immediately following inhalation in atopic asthma patients (18). These allergens are 0.6- to 2.5-μm-sized particles naturally released from the cytoplasm of various pollen grains following osmotic shock, typically after light rainfall (18, 24). We isolated PSGs from rye grass pollen grains following hydration-induced disruption of the pollens (Fig. 3*B*). For the experiments, we bound TNP molecules to these PSGs and used commercially available anti-TNP mouse IgE Abs to sensitize primary mouse MCs. Before examining the intracellular fate of PSGs, we demonstrated that the exocytic and phagocytic responses of primary MCs to PSG particles were comparable to those exhibited by RBL-2H3 cells upon exposure to TNP-*E. coli*. TNP-PSGs induced up to 40% release of hexosaminidase from sensitized MCs and only 5% release from unsensitized MCs or unstimulated controls (Fig. 3*C*). Electron microscopic examination of cross-sections of the MCs following exposure to TNP-PSGs revealed the presence of ~30 intracellular PSGs per 100 MC (Fig. 3*D*). Because this number reflects only the number of intracellular PSGs observed in a single cross-sectional plane, the actual numbers of PSGs per 100 whole MCs is likely to be markedly higher. In contrast, no intracellular PSGs were detected when unsensitized MCs were exposed to TNP-PSGs (Fig. 3*D*). Intracellular PSGs were distinguishable from MC organelles by their shape, size, and the presence of a distinct electron dense mark in their cross-section (Fig. *3*E). We examined the intracellular fate of PSGs following phagocytosis by primary MCs using microscopy. To facilitate visualization of these particles, TNP-PSGs were prelabeled with Alexa Fluor 488 before they were mixed with sensitized MCs. The state of degradation of the internalized allergens was monitored at an early (1 or 4 h) and a much later (24 h) period of incubation. To ensure that most of the monitored PSGs were actually intracellular, we focused exclusively on planes in the middle of each cell. At the 24-h time point no detectable changes in either the size or the shape of intracellular PSGs was observed in the MCs (Fig. 3*F*). As before, we included macrophages in this assay as a control to demonstrate the biodegradability of these allergens. Note that following 24 h of incubation most of the PSGs were detectable as fluorescent remnants within the macrophages (Fig. 3*G*). Because of the high degree of PSG degradation in macrophages after 24 h, this image was generated only after markedly increasing the gain of fluorescence during microscopy. Taken together, the data suggest that minimal degradation of allergen particles occurs following FcεRI-mediated phagocytosis by MCs.

Maturation of allergen-containing phagosomes into regulatory secretory compartments

To understand how allergens were not degraded following phagocytosis by MCs, we attempted to characterize the allergen-containing phagosomes. It has previously been shown that the secretory granule compartments of MCs sometimes intersect with the endocytic pathway (25, 26). Hence, we investigated the possibility that phagocytosed allergens were accessible to the secretory granules of MCs. We transfected RBL-2H3 cells with DNA encoding GFP-VAMP7, a specific marker of MC secretory compartments (19). As expected, GFP-VAMP7 was localized in intracellular vesicles containing serotonin, a specific constituent of MC secretory compartments (27) (data not shown). Next, we sensitized GFP-VAMP7-expressing RBL-2H3 cells with Cy5-labeled anti-TNP IgE Abs and then exposed them to TNP-*E. coli*. We monitored the nascent phagosomes and GFP-VAMP7 secretory compartments by confocal microscopy. Shown in Fig. 4, *A* and *B,* are a series of photomicrographs revealing that MC phagocytosis of TNP-*E. coli* is closely followed by the fusion of GFP-VAMP7 vesicles with the nascent phagosome. We found that internalization of TNP-*E. coli* (deduced from the encapsulation of the allergens by Cy5-labeled anti-TNP IgE Abs) occurred within 5 min of their addition to the MCs and that this activity was associated with active membrane ruffling (Fig. 4*A*). Note that there is no coassociation between the secretory compartments (labeled green) and the nascent phagosome (labeled red) at this time (Fig. 4*A*). Soon afterward, secretory compartments were found to coalesce around the nascent phagosome. Shown in Fig. 4*B* is an extraordinarily long TNP-*E. coli* bacterium in the process of being phagocytosed. Because of the increased duration of the phagocytic process involved here, we were able to simultaneously visualize different stages of the interactions between MC secretory compartments and the elongated nascent phagosome. Notice that even as the phagocytic process is being completed, already formed portions of the phagosomal membrane appear to have acquired the GFP label presumably through fusion with multiple GFP-VAMP7-labeled secretory vesicles (Fig. 4*B*). By 1 h of incubation, all of the phagosomes appeared to have acquired GFP-VAMP7 (Fig. 4*C*). This observation of phagosomal fusion with secretory compartments was also confirmed with RBL-2H3 cells exposed to TNP-PSGs. Shown in Fig. 4*D* are red fluorophore-labeled TNP-PSGs encased by GFP-VAMP7 1 h following phagocytosis by transfected RBL-2H3 cells. The presence of GFP-VAMP7 within phagosomal membranes was consistently observed for at least 18 h (data not shown). Thus, allergen-bearing phagosomes, initially formed from plasmalemmal components, appear to fuse with multiple secretory compartments.

Next, we examined whether the phagosomes that acquire the membranes of secretory compartments also acquire their secretory functions. Upon stimulation, MC secretory compartments typically fuse with the plasma membrane and partially or completely release their contents into the extracellular medium (28). During the fusion event, the contents of the secretory compartment and the extracellular environment become continuous, allowing seepage of extracellular material into the MCs. To determine whether TNP-*E. coli*-harboring phagosomes fuse with the plasma membranes during MC activation, we stimulated TNP-*E. coli*-harboring RBL-2H3 cells in the presence of antibiotics in the medium. We reasoned that if the fusion of TNP-*E. coli*-encasing phagosomes with the plasma membranes occurred by activation of the MCs, antibiotics in the medium would enter bacteria-containing phagosomes, or alternatively, bacteria might escape the phagosomes to be released into extracellular medium. In either case, a loss of viability of intracellular bacteria should result. As shown in Fig. 4*E*, the treatment of TNP-*E. coli*-harboring MCs with anti-IgE Abs and with calcium ionophore, but not with buffer alone, resulted in a marked drop in the viability of intracellular bacteria (~30 and ~70%, respectively, for each treatment), indicating fusion of allergen-containing phagosomes with the plasma membrane following MC stimulation. To confirm that this membrane fusion coincided with activation of MCs, we measured the level of MC degranulation. The amounts of hexosaminidase released after each treatment, shown in Fig. 4*F*, correlated well with the loss in intracellular bacterial viability shown in Fig. 4*F*. Because the fusion of MC

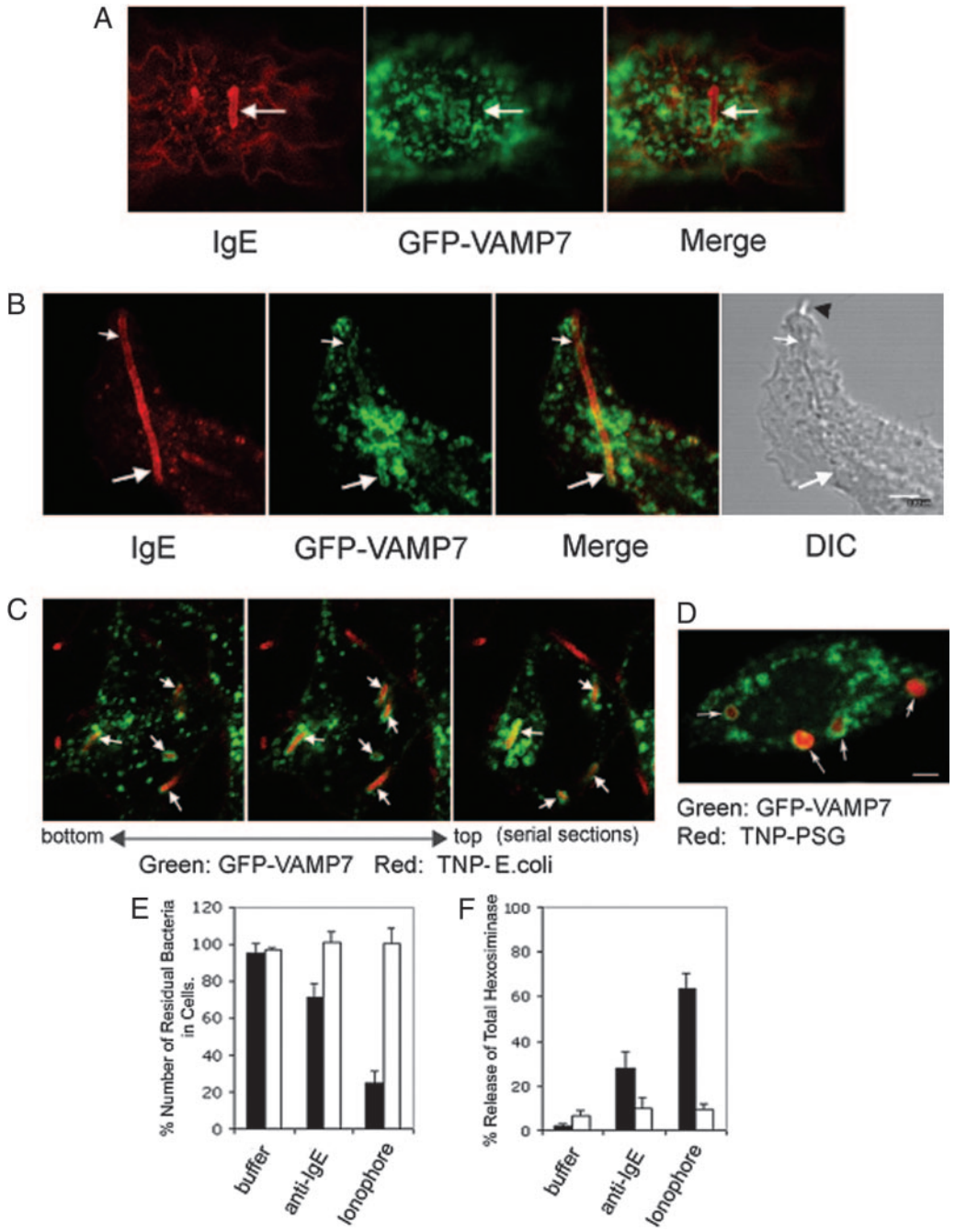

FIGURE 4. Nascent allergen-containing phagosomes mature into regulatory secretory compartments. *A* and *B*, Confocal microscopy of GFP-VAMP7-expressing RBL-2H3 cells that were exposed to TNP-*E. coli* for 5 min (*A*) and 30 min (*B*). GFP-VAMP7-expressing RBL-2H3 cells were sensitized with Cy5-conjugated IgE Abs to visualize nascent phagosomes. Note the lack of colocalization of the GFP-VAMP7 label (green) with the nascent phagosome (red) at the 5-min time point (arrows of *A*). *B*, confocal and bright field images of an elongated TNP-*E. coli* in the process of being phagocytosed. Note the various stages of recruitment and fusion of GFP-VAMP7-labeled secretory vesicles with the nascent phagosome. The large arrow indicates phagosomal membranes decorated by GFP-VAMP7. The small arrow points to phagosomal membrane regions that have not, as yet, acquired the GFP-VAMP7 label. The arrowhead points to the tip of TNP-*E. coli* that is not yet encased by MC membranes. DIC, differential interference contrast. *C*, Serial images across a GFP-VAMP7-expressing RBL-2H3 cell that had been exposed to HcRed-expressing TNP-*E. coli* for 1 h. *E. coli* expressing HcRed fluorescent protein was used in this experiment to label bacteria for visualization by confocal microscopy. Notice that all the intracellular TNP-*E. coli* (red) retains GFP-VAMP7 (green) around itself. *D*, Confocal microscopy of GFP-VAMP7-transfected RBL-2H3 harboring Alexa Fluor 633-labeled TNP-PSGs. Note that all of the phagocytosed PSGs (red) are completely encased by GFP-VAMP7 (green) (arrows). *E* and *F*, Reduction in the viability of intracellular TNP-*E. coli* following the activation of MCs. RBL-2H3 cells harboring TNP-*E. coli* were treated with buffer, anti-IgE Abs, or calcium ionophore for 30 min in the presence (■) or absence (□) of calcium in the gentamicin-containing medium. The number of live bacteria in the cells was determined before and after each treatment by standard colony counting assays (*E*). In addition, the amount of hexosaminidase released during each treatment was determined (*F*).

secretory compartments with the plasma membrane during activation is critically dependent on extracellular Ca^{2+}, we repeated the experiments described above in the absence of Ca^{2+}. We found that treatment of MCs with anti-IgE Abs or with a calcium ionophore in the absence of Ca^{2+} failed to reduce intracellular bacterial viability and also failed to induce specific MC degranulation (Fig.

4, *E* and *F*). Taken together, these findings demonstrate that allergen-containing phagosomes, like typical MC secretory compartments, fuse with the plasma membrane in a Ca^{2+}-dependent manner upon MC stimulation. Thus, allergen-containing phagosomes mature into distinct compartments exhibiting both the biochemical and the functional traits of the regulatory secretory compartments of MCs.

Allergens harbored in MCs retain their morphology and antigenicity

If particulate allergens are indeed housed within nondegradative intracellular compartments exhibiting exocytic properties, then these allergens should continue to retain both their morphology and antigenicity. We reasoned that one way of verifying this hypothesis was through the reactivation of allergen-bearing MCs resulting in the expulsion of morphologically intact allergens. If these recently released particles are antigenically active, they should be readily recognized and reinternalized by neighboring IgE-sensitized MCs. To determine whether MCs exocytosed structurally intact allergens following reactivation, primary MCs were allowed to phagocytose PSGs in a FcεRI-mediated manner and then examined for allergen expulsion upon reactivation. Shown in Fig. 5*A* is a transmission electron micrograph of a PSG-bearing MC. Transmission electron microscopy revealed that the activation of PSG-bearing MCs with anti-IgE Abs was accompanied by vigorous membrane ruffling, which is a characteristic behavior of activated MCs (Fig. 5*B*). Enumeration of intracellular PSGs revealed ~50% reduction in the number of intracellular PSGs following MC activation (Fig. 5*C*). Shown in Fig. 5*A* is the morphologic appearance of a MC previously exposed to PSG (before reactivation). Scanning electron microscopy also revealed that the expulsion of structurally intact PSGs was accompanied by vigorous membrane ruffling of MCs (Fig. 5, *E* and *F*). It is noteworthy that the escaping PSGs were often associated with a "cup" of ruffling around them (Fig. 5*F*).

To demonstrate that the released PSGs retained their antigenicity, we designed an experiment to show that recently released TNP-PSGs were capable of activating and inducing their reuptake by previously unexposed MCs sensitized with TNP-specific IgE Abs. Two groups of primary mouse MCs were prepared and coincubated. The first (donor) group comprised MCs that contained fluorescently labeled TNP-PSGs. The second (recipient) group comprised MCs sensitized with Cy5-labeled anti-TNP-IgE and was readily distinguishable from the first group in the mixed population of MCs by its red fluorescence (Fig. 5*G*). The donor MCs were selectively activated (see *Materials and Methods*) to induce

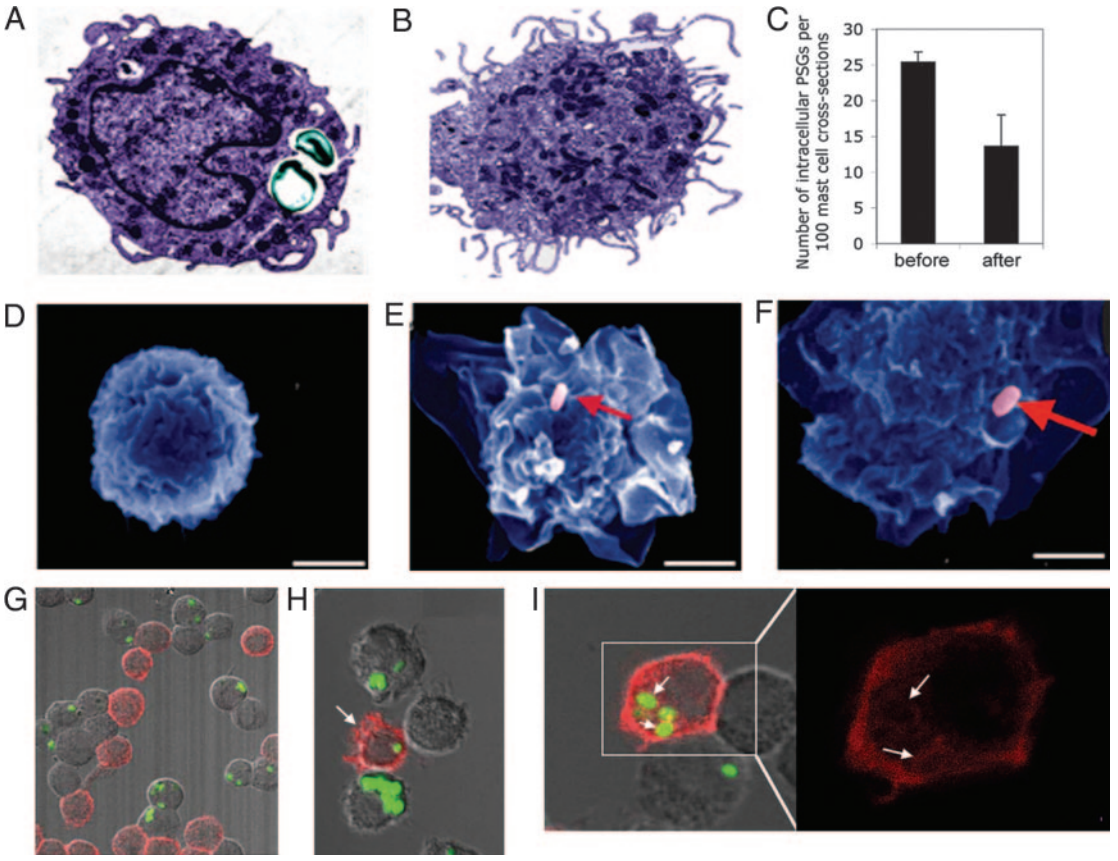

FIGURE 5. Expulsion of allergens by activation of MCs harboring allergens. Transmission electron microscopy and scanning electron microscopy of PSG-harboring MCs were performed before (*A* and *D*) and after (*B*, *E*, and *F*) stimulation of the cells by anti-IgE Abs (0.5 μg/ml). Note the membrane ruffling of the activated MCs (*B*, *E*, and *F*) and the "membrane cup" associated with the expulsion of PSGs (depicted by arrows in *E* and *F*). Scale bars, 6 μm. *C* represents the number of intracellular PSGs determined by transmission electron microscopy before and after exposure of PSG-harboring MCs to anti-IgE Abs. The electronic microscopy images presented here have been given false color to improve visualization of the allergen. *G–I*, Superimposed differential interference contrast/fluorescent images of a mixture of PSG donor and recipient MC populations. Shown in *G* is a mixture of two groups of MCs; one group (donor) is comprised of MCs harboring Alexa Fluor 488-TNP-PSGs (green) and the other group (recipient) is comprised of MCs sensitized with Cy5-conjugated anti-TNP IgE Abs (red). The images in *H* and *I* were taken following selective activation of the PSG-harboring donor MC group. Note the membrane ruffling of the recipient MCs (arrow in *H*). Shown in the right panel of *I* is an enlarged image of a recipient MC taken with a far-red laser to exclusively excite the Cy5 label. Notice the recruitment of IgE Abs to the membrane encasing the intracellular PSGs (arrows in *I*).

the expulsion of fluorescently labeled (green) TNP-PSGs. As additional controls for recipient cells, we used MCs sensitized with IgE against an unrelated Ag. Examination of the Cy5-labeled MCs (recipient population) for the presence of TNP-PSGs revealed that several of them had now acquired TNP-PSGs (Fig. 5, *H* and *I*). In several instances, the acquisition of PSGs by recipient MCs was associated with membrane ruffling, an indicator of MC activation (arrow in Fig. 5*H*). An enlarged image of another PSG-bearing recipient MC reveals encapsulation of the PSGs by Cy5-labeled TNP-specific IgE Abs (Fig. 5*I*), confirming phagocytosis of the PSGs by recipient MCs. No association with TNP-PSGs was observed in the control recipient cells, indicating that specific recognition of the TNP-PSG by IgE on recipient MCs is required for the association and subsequent uptake of recently released PSGs. Taken together, these observations indicate that, following uptake by MCs, allergens are harbored in exocytic compartments that preserve both the morphology and antigenicity of particulate allergens.

Interactions of PSGs with MCs in vivo

Whereas our studies have focused on the interactions of particulate allergens with MCs in vitro, it was not clear whether particulate allergens can interact and activate MCs in the airway following their inhalation in vivo. Because the airways of many asthmatics sensitive to various particulate allergens are known to be inflamed (29–31), we attempted to induce a similar inflammatory condition by instilling LPS, a common airway inflammogen, along with Alexa Fluor 488-labeled TNP-PSGs into the tracheas of mice that have been passively sensitized with TNP-specific IgE Abs. Twenty-four hours later the mice were sacrificed and frozen sections of the airway were prepared and stained with avidin-TRITC (a probe for heparin, a constituent of MC granules) and a c-*kit* specific Ab to localize MCs. Under these conditions, we observed significant penetration of the airway epithelium with PSGs and direct association of the PSGs with underlying MCs (Fig. 6*A*). Shown in the *lower left panel* of Fig. 6*A* is a LPS-activated mouse lung before the addition of Alexa Fluor 488-labeled PSGs. Note the presence of underlying MCs but the complete absence of PSGs. A tightly packed nondegranulated MC is shown in the *lower middle panel* of Fig. 6*A*, which contrasts with the "fluffy" appearance of a degranulated MC shown in the *lower right panel*. Note that in this *lower right panel* (Fig. 6*A*) a PSG can be seen associating with the MC. It appears yellow due to a close association with a heparin granule.

We also sought to confirm that PSGs were retained for extended periods of time within MC granule chambers under in vivo conditions. Because of the difficulty of isolating lung MCs under the above experimental conditions, we resorted to studying MCs in a more readily accessible site. MCs in the peritoneal cavities of mice were sensitized with a peritoneal injection of TNP-specific IgE. Twenty-four hours later, Alexa Fluor 488-labeled TNP-PSGs were injected into the same site. Another 24 h later, peritoneal cells were collected and examined using FACS analysis for MCs bearing PSGs. As shown in the *right panel* of Fig. 6*B*, significant portion of c-*kit*-positive cells were positive with Alexa Fluor 488 stain, indicating the MCs were harboring PSGs. Shown in the other two panels are negative controls for FACS analysis. The cells that stained positive with c-*kit* Ab (R2 regions of Fig. 6*B*) were sorted and examined. Toluidine blue staining confirmed that these cells were 99% pure MCs (data not shown). Staining with serotonin-specific Abs followed by confocal microscopy revealed that all of the Alexa Fluor 488-labeled PSGs harbored in MCs appeared to colocalize with serotonin (*inset* of the *right panel* of Fig. 6*B*). Taken together, these in vivo studies demonstrate that, under inflammatory conditions whole PSGs can penetrate the airway epithelium and directly interact with underlying MCs. Following phagocytosis by IgE-sensitized MCs, PSGs are preserved intracellularly for at least 24 h in vivo within secretory chambers.

Discussion

Using a combination of in vitro and in vivo experiments and both artificial and natural allergen particles, we have presented evidence that the cross-linking of IgE/FcεRI complexes on MCs by particulate allergens induces not only MC degranulation but also phagocytosis of the activating allergens. Following phagocytosis, the allergens avoid intracellular degradation and acquire the potential for extracellular release. This remarkable phenomenon is directly attributable to lipid raft-mediated phagocytosis of allergen followed by the conversion of allergen-containing phagosomes into secretory compartments within the cells. Evidence that the IgE/FcεRI-mediated MC phagocytosis of particulate allergens involves lipid raft entities comes from the observations that: 1) the phagocytic event is preceded by the association of IgE/FcεRI complexes with lipid rafts; and 2) the disruption of lipid rafts in MCs blocked the phagocytosis of allergens. Lipid rafts have previously been reported to be essential for initiating IgE-mediated signaling events leading to MC degranulation (12, 23). Conceivably, discrete signaling components important for IgE signaling as well as the phagocytic process are located within lipid rafts, and this could explain how lipid raft disruptors specifically blocked phagocytosis of allergens.

It was of interest to note that, unlike their fate following internalization by macrophages, the phagocytosed allergens avoided degradation within MCs, and these intact allergens were extracellularly released upon MC restimulation. We have attributed the lack of intracellular degradation of phagocytosed allergens as well as the capacity of the intracellular allergen to escape MCs to the maturation of the allergen-containing phagosomes into regulatory secretory compartments. The maturation of the nascent phagosomes appears to involve their fusion with multiple secretory granule compartments. The secretory compartments of MCs serve as organelles of storage in which various inflammatory mediators such as serotonin and histamine are harbored following synthesis until they are required. However, these compartments are also known to exhibit some of the properties of conventional lysosomes and, therefore, are sometimes referred to as secretory lysosomes (25, 32). Secretory lysosomes contain typical lysosomal proteins such as Lamp1 and CD63 and exhibit a capacity to fuse with endosomes just as conventional lysosomes (25, 32). Indeed, the fusion of the secretory compartments with nascent allergen-containing phagosomes in MCs observed in these studies is analogous to the fusion of conventional lysosomes with late endosomes. However, unlike the destructive fate of cargo processed via the classical endosome-lysosome pathway (33), our data suggest that allergens harbored within the secretory compartments of MCs fail to be degraded. This may indicate that the secretory compartments of MCs are inherently defective in their degradative potential, which is consistent with their role as organelles for the storage of inflammatory mediators. However, we cannot rule out other possibilities. For example, the secretory lysosomes of MCs may be further compartmentalized, where the degradative and storage functions occur in separate compartments. Allergen-containing phagosomes could selectively fuse with the subcompartments that are devoid of the degradative activity where regular MC secretory components are stored.

Lung MCs are normally localized underneath the epithelium of the airway and are not immediately accessible to particulate agents in healthy individuals. However, the airway epithelium of asthmatics is often severely inflamed, which is accompanied by marked damage in the barrier function of the epithelium (34, 35).

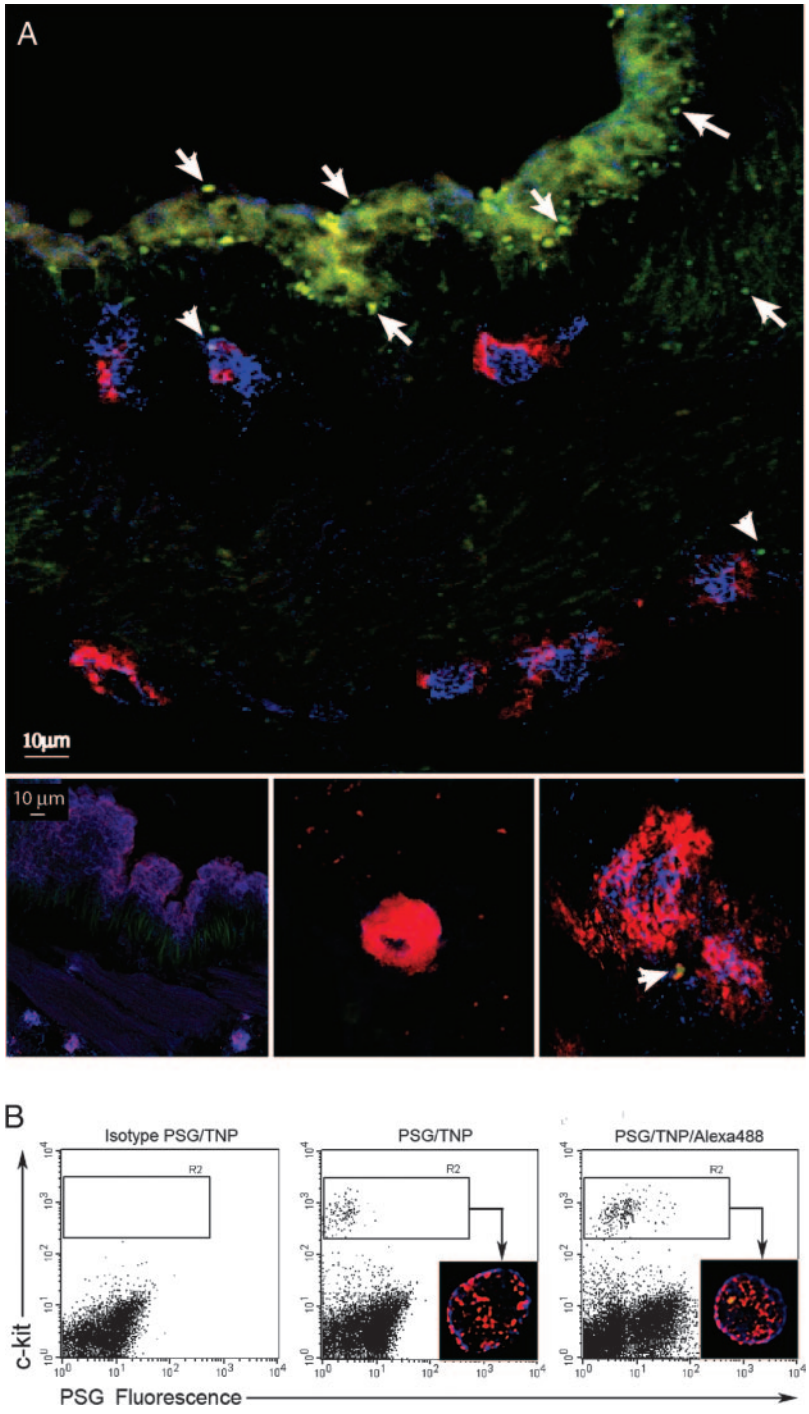

FIGURE 6. Physical interactions of PSGs with MCs in vivo. *A*, Frozen sections of mouse lung showing Alexa Fluor 488-labeled PSGs (green) penetrating the LPS-activated airway epithelium and interacting with underlying MCs costained with avidin-TRITC (red) and anti-c-*kit* (blue). PSGs in the tissue are indicated by arrows. PSGs in direct contact with MCs are indicated by arrowheads. *Lower left panel*, Frozen section of a LPS-activated mouse lung before the addition of Alexa Fluor 488-labeled PSGs. Note the presence of underlying MCs but the complete absence of PSGs. *Lower middle panel*, A tightly packed un-degranulated MC. *Lower right panel*, "Fluffy" degranulated MCs physically associated with a PSG. Notice that the PSG appears yellow, suggesting colocalization with heparin. *B*, Flow cytometry of peritoneal cells isolated from IgE-sensitized mice challenged with TNP-PSGs. The cells in the *left* and *middle panel*s were challenged with unlabeled TNP-PSG, whereas the cells in the *right panel* were challenged with Alexa Fluor 488-TNP-PSG. In the *left panel*, cells were stained with allophycocyanin-conjugated isotype control Ab. In the *middle* and *right panels*, the cells were stained with allophycocyanin-conjugated anti-c-*kit* serum. Cells contained in the R2 regions were sorted and stained for the MC granule marker serotonin (red) and subsequently examined by confocal microscopy. The *inset*s in the *middle* and *right panels* represent a cell found in each sort.

We observed that the tracheal instillation of PSG particles into mice that had been pre-exposed to LPS resulted in penetration of the epithelium by PSGs and association of the PSGs with MCs. LPS, like several other inflammogens, has been shown to enhance the paracellular permeability of the airway epithelial barrier (36, 37). Presumably, this results in increased translocation of inhaled particles from the lumen into the underlying tissue through the airway epithelium (34). Thus, in asthmatics where the airways are typically inflamed, invasion of the epithelium by particulate allergens may be a frequent occurrence.

It is distinctly plausible that the harboring of intact allergens in MCs and the extracellular release of antigenically active allergens following MC reactivation can markedly contribute to the pathogenic process. For example, recently released allergens can potentially activate the same or a proximal MC, initiating another cycle of MC activation and thereby leading to progressive intensification of the pathological responses of the MC to allergen challenge. The capacity of MCs to harbor allergens may even contribute to the high levels of allergen-specific IgE Abs (atopy) observed in the circulation of individuals suffering from allergy. It has been reported that MCs in the skin during contact hypersensitivity reactions trafficked to the regional lymph nodes where Ag-specific immunity is developed (33, 38). Conceivably, MCs that phagocytosed allergens in peripheral body sites also traffic to regional lymph nodes and directly release allergens to APCs in the nodes, thereby facilitating the development of allergen-specific immune responses, including the generation of allergen-specific IgE Abs.

In conclusion, it is well known that interactions of allergens, including several particulate Ags, with MCs in the skin or mucosae trigger robust MC degranulation responses, resulting in chronic inflammatory diseases. The fate of the allergen following MC activation, however, has not been addressed until now. We have observed that particulate allergens are phagocytosed by MCs and harbored in nondegradative secretory compartments for extended periods of time or until they are re-released in a morphologically and antigenically intact state by reactivation of the MCs. There is currently no clinical or experimental evidence pointing to the persistence of allergens in the body of allergic subjects and to their possible contribution to the inflammatory condition. Therefore, efforts are currently under way to address this important issue by using various in vivo models of allergic disease. Because of the prominent role played by MCs as effectors of a wide range of inflammatory diseases other than allergic diseases (39), their distinct capacity to harbor for extended periods of time and subsequently release antigenically active Ags could have a much broader relevance than is indicated from this particular investigation.

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Disclosures

The authors have no financial conflict of interest.

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