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Impaired Phagocytosis of Apoptotic Cells by Macrophages in Chronic Granulomatous Disease Is Reversed by IFN- γ in a Nitric Oxide-Dependent Manner

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Abstract

Immunodeficiency in chronic granulomatous disease (CGD) is well characterized. Less understood are exaggerated sterile inflammation and autoimmunity associated with CGD. Impaired recognition and clearance of apoptotic cells resulting in their disintegration may contribute to CGD inflammation. We hypothesized that priming of macrophages (M ϕ s) with IFN- γ would enhance impaired engulfment of apoptotic cells in CGD. Diverse M ϕ populations from CGD (gp91^{phox}^{-/-}) and wild-type mice, as well as human M ϕ s differentiated from monocytes and promyelocytic leukemia PLB-985 cells (with and without mutation of the gp91^{phox}), demonstrated enhanced engulfment of apoptotic cells in response to IFN- γ priming. Priming with IFN- γ was also associated with increased uptake of Ig-opsonized targets, latex beads, and fluid phase markers, and it was accompanied by activation of the Rho GTPase Rac. Enhanced Rac activation and phagocytosis following IFN- γ priming were dependent on NO production via inducible NO synthase and activation of protein kinase G. Notably, endogenous production of TNF- α in response to IFN- γ priming was critically required for inducible NO synthase upregulation, NO production, Rac activation, and enhanced phagocytosis. Treatment of CGD mice with IFN- γ also enhanced uptake of apoptotic cells by M ϕ in vivo via the signaling pathway. Importantly, during acute sterile peritonitis, IFN- γ treatment reduced excess accumulation of apoptotic neutrophils and enhanced phagocytosis by CGD M ϕ s. These data support the hypothesis that in addition to correcting immunodeficiency in CGD, IFN- γ priming of M ϕ s restores clearance of apoptotic cells and may thereby contribute to resolution of exaggerated CGD inflammation.

Chronic granulomatous disease (CGD) is traditionally characterized by immunodeficiency due to the lack of a functioning NADPH oxidase (1–4). Additionally, this disorder is characterized by autoimmunity and over-exuberant sterile inflammation (1–3, 5). It is

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hypothesized that inflammatory complications of CGD result from the lack of inhibitory signals downstream of the NADPH oxidase. The nature of such anti-inflammatory signals is not fully elucidated (2, 6, 7). However, both a critical absence of proper signaling by apoptosing CGD neutrophils with inadequate exposure and modification of phosphatidylserine (7–10), as well as the inability of improperly programmed CGD macrophages (Mφs) to recognize and engulf apoptosing cells, have been documented (11). Apoptosing neutrophils, in particular, if not cleared, degrade and release injurious intracellular constituents (e.g., proteases and cationic proteins) that further spur inflammation and can lead to autoimmunity (12). Conversely, apoptotic cell recognition is potentially anti-inflammatory, driving the production of anti-inflammatory mediators, such as TGF-β, that actively suppress production of inflammatory cytokines, chemo-kines, and eicosanoids (13). Diminished production of these aforementioned anti-inflammatory mediators accompanying Mφ phagocytosis of apoptotic cells has been shown in CGD (6).

Recombinant human IFN-γ has been used therapeutically for CGD patients for a number of years since it was demonstrated to reduce the frequency and severity of infections (14, 15). The mechanisms for its efficacy are likely multifactorial (16–19) and remain controversial (20, 21). For instance, IFN-γ enhancement of antimicrobial activity includes upregulation of Fc and complement receptor expression and enhanced phagocytosis of opsonized pathogens, as shown in some (22, 23), but not all, studies (24). In addition to antimicrobial effects, anti-inflammatory effects downstream of IFN-γ treatment are also recognized (25). Recently, it was shown that IFN-γ, when administered with the tryptophan metabolite kynurenine, suppressed inflammation and enhanced survival in fungal infection in CGD mice by a mechanism theorized to include enhanced metabolism of kynurenine and generation of regulatory T cells (2). Additionally, IFN-γ priming has been shown to enhance clearance of apoptotic cells by several Mφ populations although the mechanisms have not been fully elucidated (26, 27) (see *Discussion*). The effects of IFN-γ on apoptotic cell clearance have not been investigated in CGD. Accordingly, we hypothesized that IFN-γ priming of CGD Mφs would enhance apoptotic cell clearance, reversing the contribution of this defect to exaggerated CGD inflammation.

In this study, we demonstrate that IFN-γ priming enhances the uptake of apoptotic cells by both CGD and wild-type (WT) Mφs, reversing the impaired uptake of apoptotic cells of the former in vitro and in vivo. The IFN-γ-driven intracellular pathway leading to enhanced engulfment is defined. Investigation showed that the effects of IFN-γ priming were not isolated to the engulfment of apoptotic cells; that is, IFN-γ priming enhanced the uptake of fluid phase markers (pinocytosis), Ig-opsonized targets, and latex beads, all thought to be dependent on activation of the Rho GTPase Rac (28, 29). Mechanistic studies utilizing both murine and human Mφs demonstrated that the IFN-γ-enhanced phagocytic capacity was dependent on the sequential production of TNF-α leading to upregulation of inducible NO synthase (iNOS), NO production, and the activation of protein kinase G (PKG); these events led ultimately to the activation of Rac. We hypothesize that IFN-γ treatment in CGD aids in the removal of apoptotic inflammatory cells before their disintegration, and it thereby may help to overcome pervasive CGD Mφ impairment (11) in apoptotic cell clearance that results in proinflammatory consequences.

Materials and Methods

Reagents

Recombinant mouse IFN- γ was purchased from R&D Systems (Minneapolis, MN), and PMA and 1 α ,25-dihydroxycholecalciferol (vitamin D3) were from Sigma-Aldrich (St. Louis, MO). *N*⁶-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL) from Cayman Chemical (Ann Arbor, MI) was used to inhibit iNOS. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) from Cal-biochem (San Diego, CA) was used as a spontaneous NO donor. Texas Red dextran from Invitrogen (Carlsbad, CA) and Lucifer yellow (LY) from Molecular Probes (Eugene, OR) were used as indicators of fluid phase uptake. Rabbit anti-LY and isotype rabbit IgG (Molecular Probes) were used to amplify the signal and were detected using FITC-labeled anti-rabbit Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). A546 phalloidin (Molecular Probes) was used to demonstrate assembled actin. KT5823 from Calbiochem was used to inhibit PKG. TNF- α -neutralizing Ab and goat IgG isotype Ab (10 μ g/ml) were obtained from R&D Systems. Latex beads (5 μ m) came from Bangs Laboratories (Fishers, IN). Anti-human CD3 Ab (1 μ g/10⁶ cells) from eBioscience (San Diego, CA) was used to opsonize viable Jurkat T cells.

Animals

All mice received care in accordance with the guidelines of the Institutional Animal Care and Use Committee at National Jewish Health (Denver, CO). C57BL/6, X-CGD (gp91^{phox}^{-/-}), TNFR1^{-/-}, and iNOS^{-/-} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained on food and water ad libitum in the Animal Care Facility at National Jewish Health. Except where otherwise indicated, mice were used between 8 and 16 wk of age and strains were age and gender matched for each experiment. The mice provided a source of bone marrow-derived M ϕ s (BMDM ϕ s), resident and thioglycollate-elicited peritoneal M ϕ s, and thymocytes (see below for cell culture conditions). Thioglycollate-elicited peritoneal M ϕ s were obtained according to previously established methods (30). Briefly, mice were injected i.p. with 1.0 ml of a 4% sterile and aged (1 mo) solution of Brewer thioglycollate medium (Difco Laboratories, Detroit, MI). At 3 d postinjection, mice were euthanized with CO₂, and the peritoneal cavity was lavaged with 5–10 ml of sterile HBSS (Cellgro, Kansas City, MO) supplemented with 1 mM EDTA and 10 mM HEPES (pH 7.2). Peritoneal cells were collected, centrifuged at 1000 rpm for 10 min at 4°C, and resuspended in DMEM (Cellgro) supplemented with 10% heat-inactivated FBS (American Type Culture Collection [ATCC], Manassas, VA), 2 mM L-glutamine, 100 μ g/ml streptomycin, and 100 U/ml penicillin in humidified 10% CO₂ at 37°C. After 1 h of culture, nonadherent cells were aspirated and prewarmed fresh media was added to each well. For resident peritoneal M ϕ isolations, naive mice were used and the harvested cells handled as above.

Where indicated, mice were treated with IFN- γ (500 ng, 42,000 U i.p.) versus PBS on a single occasion, and resident peritoneal M ϕ s were harvested 24 h later, plated, and tested ex vivo for phagocytosis (see below). In the acute, sterile peritonitis model, mice were treated with IFN- γ or PBS at time -24 h, then zymosan (1 mg i.p.) at time 0 h, and then IFN- γ or PBS at time 24 h. Mice were euthanized and peritoneal cells were harvested at either time 6

or 48 h after zymosan instillation. Peritoneal cells were harvested by lavage as indicated above, and absolute cell numbers were obtained using a Coulter counter (Coulter Channelyzer 256). Cytospins of cells from peritoneal lavages were prepared, fixed, and stained with modified Wright-Giemsa (Fisher Scientific, Pittsburgh, PA) and read in a blinded fashion to determine cell differentials, phagocytic indices, and apoptosis of neutrophils by nuclear morphology from microscopic examinations.

Human monocyte-derived Mφs

Healthy subjects were the source of whole blood obtained by venipuncture in accordance and with the approval of the National Jewish Health Institutional Review Board. Written informed consent was obtained from each subject. Monocytes were isolated from whole blood using a Percoll gradient centrifugation, as previously described (31). Monocytes (5×10^5) were plated in 24-well tissue culture plates (BD Biosciences, San Jose, CA) and matured to Mφs by culturing in X-Vivo medium (BioWhittaker, Walkersville, MD) containing 10% human serum at 37°C in 10% CO₂ for 6 d. Medium was changed on days 3 and 6.

Cell culture

Peritoneal resident and thioglycollate-elicited Mφs were cultured in DMEM supplemented with 10% heat-inactivated FBS (ATCC), 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin in humidified 10% CO₂ at 37°C for 24 h before use. Mouse BMDMφs were prepared and cultured in DMEM containing 10% (v/v) FBS and 10% (v/v) L cell-conditioned medium as a source of M-CSF for 5 d as previously described (32). Murine J774 Mφ-like cells (ATCC) were cultured as described for peritoneal Mφs. Murine RAW 264.7 Mφ-like cells (ATCC) were cultured in the same medium as above in humidified 5% CO₂ at 37°C. Promyelocytic leukemia PLB-985 cell lines, both functionally sufficient for, and devoid of, NADPH oxidase activity, were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. These cells have been described previously, and the latter cells carry an in-frame deletion (exon 3) within gp91^{phox}, rendering them with a nonfunctional NADPH oxidase (33). Differentiation into Mφ-like cells was done by plating 3.0×10^5 cells/ml in a 24-well tissue culture dish and stimulated using 30 nM PMA and 200 nM vitamin D3 for 3 d prior to use. Differentiation into Mφ-like cells was determined by assessment of morphological features (34). The human leukemia Jurkat T cell line was obtained from ATCC and was cultured in RPMI 1640 (MediaTech, Herndon, VA) containing 10% heat-inactivated FBS supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich) in humidified 5% CO₂ at 37°C.

Induction of apoptosis

Jurkat T cells were exposed to UV irradiation at 254 nm for 10 min and cultured for 2.5 h in 5% CO₂ at 37°C. Apoptosis was quantified by evaluation of nuclear morphology at the light microscopic level. By these methods, these cells were typically 70–90% apoptotic. Thymocytes isolated from 4-wk-old C57BL/6 mice were exposed to UV radiation for 5 min in RPMI 1640 with 10% FBS and cultured in 5% CO₂ at 37°C for × h. Apoptosis of these cells was confirmed by Alexa Fluor 488-annexin V (Invitrogen) and propidium iodide

(Sigma-Aldrich) staining. Thymocytes were 50–70% apoptotic (annexin V⁺, propidium iodide⁻) by these methods. PKH26 (10 μ M; Sigma-Aldrich) was used to label thymocytes for 15 min in a 37°C water bath. The reaction was stopped with 10% FBS and cells were washed twice in RPMI 1640 and resuspended in PBS (20 \times 10⁶ cells/ml).

Phagocytosis assays

In vitro phagocytosis assays were performed as previously described (35). For these assays, M ϕ cultures were as follows (number per well in 24-well plates): BMDM ϕ s (1 \times 10⁵) for 5 d, thioglycollate-elicited M ϕ s (3 \times 10⁵) overnight, resident peritoneal M ϕ s (5 \times 10⁵) overnight, human monocyte-derived M ϕ s (HMDM ϕ s; 5 \times 10⁵) for 6 d, and PLB-985 cells (1 \times 10⁵) for 2 d. Where pretreated, the M ϕ s were washed with warm media before treatment with IFN- γ (50 ng/ml for 24 h), L-NIL (0.5 mM for 15 min), SNAP (0.05 mM for 24 h), anti-TNF- α (10 μ g/ml for 30 min before IFN- γ), or KT5823 (10 μ M for 30 min before IFN- γ). The cells were washed three times before adding apoptotic or opsonized Jurkat T cells at a ratio of 4:1 (Jurkat T cells to phagocytes), or latex beads at a ratio of 1:4 resuspended in phagocyte media. The cells were cocultured with targets for 90 min at 37°C in 5% CO₂, washed three times with PBS, and stained with a modified Wright-Giemsa stain (Fisher Scientific). Wells were read without knowledge of treatments. The phagocytic index was calculated by multiplying the percentage of M ϕ s that phagocytosed a target by the average number of targets engulfed per M ϕ (30). A minimum of 200 M ϕ s were counted and each condition was tested in duplicate and results averaged and repeated at least three times. Expressing the phagocytic index as a percentage of control allowed for comparison of experiments from various murine strains, M ϕ sources, and cell line passage numbers.

In vivo phagocytosis was assessed in sterile peritonitis following examination of uptake by M ϕ s on cytopins. Alternatively, and where indicated, in vivo phagocytosis by resident M ϕ s of the peritoneum was assessed by injecting 10 \times 10⁶ PKH-labeled apoptotic thymocytes (see above) i.p. for 1 h, and lavages were performed after mice were euthanized. The phagocytic index was calculated as above from cytopsin slides read without knowledge of mouse genotype or treatment (PBS or IFN- γ).

Uptake of fluid phase markers

RAW 264.7 M ϕ s were plated at 1.0 \times 10⁵ for 24 h and treated with IFN- γ (50 ng/ml) for 24 h. LY was added at a final concentration of 0.5 mg/ml for the last 10 min of incubation. The cells were then washed three times with cold PBS and fixed with 4% paraformaldehyde (Electron Microscopy Services, Ft. Washington, PA) on ice for 20 min. The cells were washed again three times with PBS and the nuclear stain DAPI (Invitrogen) was added at 1/1000 in PBS for 10 min at room temperature. After three further washes with PBS, the cells were permeabilized in PBS 0.5% Triton X-100, blocked in PBS plus 2% BSA before being incubated with a 15/00 dilution of anti-LY Ab, washed, and counterstained with FITC anti-rabbit IgG Ab. The cells were then analyzed by fluorescent microscopy (Leica Microsystems, Wetzlar, Germany) with imaging software Slidebook (Intelligent Imaging Innovations, Denver, CO).

NO detection

NO was detected in the supernatants after stimulation using a modified colorimetric assay based on the assay described by Green et al. (36). The colorimetric assay is a two-step process. The first step involves chemical or enzymatic reduction of nitrate to nitrite; the second step requires reaction of nitrite with the Griess reagent, diazotization of sulfanilamide, and coupling to naphthylene diamine. The samples are compared with a standard curve and measured at an absorbance of 550 nm in an ELISA reader.

ELISA measurements

For cytokine measurements, BMDMφs were cultured and treated with the various reagents as described above, and the supernatants were carefully removed and immediately frozen at -70°C . ELISAs were performed according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). OptEIA reagents were used for these ELISAs and sensitivity was ~ 7 pg/ml.

Western blot analysis

Cell lysates (10 μg of protein) were analyzed using 10% SDS-PAGE gel, transferred onto polyvinylidene difluoride membrane, blocked for 1 h (PBS plus 5% nonfat milk), and incubated with Ab (anti-mouse iNOS from Calbiochem; 1/1000) for 20 h followed by addition of secondary Ab (1/1000) for a half hour. Detection was performed using ECL substrate (Amersham Biosciences, Piscataway, NJ) following the manufacturer's instructions.

Rac activity assays

Rac activity assays were performed according to the manufacturer's indications (Upstate, Charlottesville, VA). Briefly, 2.5×10^6 BMDMφs were plated for 5 d and, where indicated, they were stimulated with IFN- γ (50 ng/ml for 24 h). Samples were lysed and active Rac was isolated using Sepharose-bound PAK. Lysates were incubated for 1 h, washed, boiled, and run on a 12% SDS-PAGE gel. To ensure equal loading, 50 μl of whole-cell lysate were run on the gel for each condition, and total Rac levels were evaluated after being transferred onto polyvinylidene difluoride membrane, blocked for 1 h (H_2O plus 5% nonfat milk), and incubated with Ab (anti-mouse Rac provided with kit; 1/1000) for 20 h followed by addition of secondary Ab (1/1000) for a half hour. Detection was performed using ECL substrate (Amersham Biosciences) following the manufacturer's instructions.

Densitometry analysis

Western blots were scanned and analyzed using ImageJ analysis from the National Institutes of Health. The scans were inverted and the background was subtracted before measuring the relative protein amounts. All conditions were expressed as a percentage of the untreated control. For Rac activity, the amount of protein was expressed as a ratio of active Rac to total Rac.

Statistical analysis

All experiments were performed at least three times. Statistical analysis and *p* value calculations were conducted using ANOVA (JMP statistical program [SAS Institute, Cary, NC]). The Dunnett's and Tukey-Kramer nonparametric tests were used for single and multiple comparisons, respectively.

Results

IFN- γ priming enhances uptake of apoptotic cells by both CGD and WT M ϕ s in vitro and in vivo

To test the hypothesis that IFN- γ priming enhances clearance of apoptotic cells by CGD M ϕ s, BMDM ϕ s, and harvested peritoneal M ϕ s from gp91^{phox}^{-/-} and WT mice were incubated with or without recombinant IFN- γ (50 ng/ml) for 24 h before assaying uptake of apoptotic cells in vitro. Initial pilot experiments indicated that these were the optimal dose and time for assessment of uptake (Supplemental Fig. 1). As described previously, uptake of apoptotic cells by CGD BMDM ϕ s and peritoneal M ϕ s, both resident and thioglycollate-elicited, was impaired relative to the respective WT M ϕ populations (Fig. 1A–C) (11). For example, phagocytic indices were consistently 30% or lower for CGD versus WT BMDM ϕ s (*p* = 0.05). Significantly, overnight priming with IFN- γ in vitro enhanced apoptotic cell engulfment by all CGD M ϕ populations such that uptake was “normalized” or surpassed that of respective control WT M ϕ s. These same findings were demonstrated for phagocytosis of apoptotic cells in vivo (Fig. 1D). In this study, fluorescently labeled apoptotic cells were injected into the peritonea of CGD and WT mice, cells were harvested 1 h later, and uptake of apoptotic cells into M ϕ s was determined (see *Materials and Methods*). The phagocytic index for CGD peritoneal M ϕ s was significantly less than for WT.

Whereas a single i.p. injection of either IFN- γ or PBS did not induce obvious peritoneal inflammation based on harvested cell numbers or differentials (data not shown), following IFN- γ treatment, in vivo uptake of apoptotic cells into CGD peritoneal M ϕ s was markedly enhanced. Notably, treatment of WT M ϕ populations with IFN- γ , both in vitro and in vivo, significantly enhanced uptake of apoptotic cells over the respective baselines as well, demonstrating a generalized effect of IFN- γ priming for apoptotic cell uptake that was independent of the NADPH oxidase. Furthermore, just as with primary M ϕ s, overnight priming of RAW 264.7 and J774 cells with IFN- γ enhanced uptake of apoptotic cells by 500 and 160%, respectively (data not shown).

To determine whether IFN- γ enhanced phagocytosis in human cells, monocytes were isolated from whole blood and matured into M ϕ s over a period of 6 d. These HMDM ϕ s also showed enhanced engulfment of apoptotic cells after overnight priming with IFN- γ (Fig. 1E). Finally, the effect of IFN- γ was investigated using human promyelocytic leukemia PLB-985 cells, with and without a functional gp91^{phox}, following their differentiation into M ϕ -like cells. As reported recently by Sanmun et al. (34), and consistent with the other populations shown, the PLB-985 cells with functionally deficient gp91^{phox} (X-CGD PLB cells) demonstrated diminished uptake of apoptotic cells at baseline in comparison with nonmutated PLB-985 cells. As in the other M ϕ s, IFN- γ priming enhanced uptake

significantly in both cell types (Fig. 1F). Taken together, these data demonstrated the pervasiveness of the deficit in apoptotic cell uptake in CGD Mφs relative to normal Mφs, and they showed that IFN-γ enhanced uptake of apoptotic cells in all of the populations tested.

Notably, in the literature, IFN-γ treatment has been associated with both enhancement and suppression of phagocytic responses depending on the Mφs, target for phagocytosis, or conditions investigated (22–24, 37–41). Under conditions of this investigation, IFN-γ priming of RAW 264.7 cells resulted in obvious uptake of LY (Fig. 2B–D) or Texas Red dextran (molecular mass, 10,000 kDa) (not shown), staining that largely colocalized to areas of ruffling and assembled actin (Fig. 2B–D). These observations suggested the possibility that enhanced uptake of apoptotic cells into IFN-γ-primed Mφs might represent a more generalized enhancement of phagocytosis. Phagocytosis of Ig-opsonized cells and unopsonized 5 μm latex beads were next investigated in BMDMφs from CGD and WT mice to test for specificity of the target engulfed. IFN-γ priming resulted in enhancement of phagocytosis of Ig-opsonized viable Jurkat cells by both WT and CGD Mφs (Fig. 2E). Similarly, the uptake of unopsonized latex beads was also enhanced by IFN-γ priming, demonstrating clearly that under these conditions, IFN-γ enhancement of phagocytosis was not specific for apoptotic cells (Fig. 2F).

IFN-γ priming enhances phagocytosis of apoptotic cells by a mechanism dependent on NO production

Previous reports have demonstrated that IFN-γ stimulation frequently results in de novo synthesis of iNOS and increased NO production (19) implicated in IFN-γ-associated host defense (e.g., increased ability to kill ingested microbes that is ablated by inhibitors of iNOS) (18, 42, 43). Furthermore, NO production itself has been associated with enhanced phagocytosis by LPS-stimulated Mφs (44). We proposed that IFN-γ-driven enhancement of phagocytosis was mediated by NO. As shown, overnight priming of CGD and WT BMDMφs with IFN-γ resulted in increased levels of iNOS detected in cell lysates and detectable NO in culture supernatants (Fig. 3A). Provision of the NO donor SNAP in place of IFN-γ resulted in similar enhancement of engulfment (Fig. 3B). Furthermore, a requirement for NO at the time of engulfment was demonstrated in that addition of an iNOS inhibitor, L-NIL, for 15 min prior to uptake assays abrogated enhancement due to IFN-γ priming, while having no effect on the constitutive ability of either cell type to ingest apoptotic cells. Additional support that iNOS-dependent NO production was required for IFN-γ-enhanced engulfment was obtained using BMDMφs from iNOS^{-/-} mice. Following priming of these cells with IFN-γ, neither increased NO levels (Fig. 3C) nor enhanced apoptotic cell engulfment was demonstrated (Fig. 3D). On the contrary, in the absence of NO production, IFN-γ priming of these cells resulted in diminished uptake of apoptotic cells.

NO was originally recognized as an endogenous vasodilator, and in vascular endothelium PKG is the principal effector of this response. To explore the role of PKG in IFN-γ-primed/NO-mediated phagocytosis, an inhibitor of PKG, KT5823, was added to CGD and WT BMDMφs before IFN-γ priming and subsequent uptake was assayed. The PKG

inhibitor significantly abolished the effects of priming by IFN- γ (Fig. 3E). Similarly, enhancement of uptake mediated by the NO donor, SNAP, was inhibited by KT5823. These results suggest that IFN- γ /iNOS/NO-cGMP-PKG signaling is required in the IFN- γ -dependent enhancement of apoptotic cell phagocytosis. To test whether this same pathway was used by human M ϕ populations, which are thought to produce less NO in response to IFN- γ than to murine M ϕ s (45), we investigated HMDM ϕ s and the PLB-985 cells. As shown in Fig. 3F, these M ϕ s responded identically, suggesting that the IFN- γ /iNOS/NO-cGMP-PKG signaling is operant in these human cells as well.

Endogenous production of TNF- α is required for NO-dependent enhancement of apoptotic cell engulfment following IFN- γ priming

There is growing evidence for TNF- α mediating IFN- γ -induced NO production. IFN- γ -induced expression of iNOS and production of NO is enhanced by addition of TNF- α (46), and, more importantly, IFN- γ is known to upregulate endogenous TNF- α production in M ϕ s, including BMDM ϕ (47). Furthermore, IFN- γ -mediated NO production after trypanosome infection requires signaling via the TNF- α p55 receptor (48), and endogenous production of TNF- α was necessary for NO-driven uptake of opsonized yeast by phagocytes (49). In light of these data, the role of TNF- α in IFN- γ -primed M ϕ s and the enhanced uptake of apoptotic cells was investigated. Enhancement in the level of secreted TNF- α was observed for both CGD and WT BMDM ϕ s following IFN- γ priming (Fig. 4A). To establish that TNF- α production was not downstream of PKG activation, BMDM ϕ s were pretreated with KT5823 and TNF- α levels were determined. As shown, inhibition of PKG had no effect on TNF- α production. Conversely, upregulation of iNOS and production of NO were dependent on endogenous TNF- α production as demonstrated by pretreatment with a neutralizing Ab to TNF- α (but not isotype control) in both CGD and WT BMDM ϕ s (Fig. 4B). Although TNF- α itself has variable effects on apoptotic cell uptake depending on conditions (see *Discussion*), given the role of TNF- α in the upregulation of iNOS, we hypothesized that phagocytic enhancement associated with IFN- γ priming would be TNF- α dependent. To determine this, BMDM ϕ s were primed with IFN- γ overnight in the presence or absence of either anti-TNF- α Ab or isotype control Ab. As predicted, neutralization of TNF- α prevented enhancement of apoptotic cell uptake by IFN- γ -primed CGD and WT BMDM ϕ s, whereas isotype control Ab had no effect (Fig. 4C). Identical responses were demonstrated in IFN- γ -primed HMDM ϕ s and M ϕ -like PLB-985 cells (Fig. 4E, 4F). Additionally, BMDM ϕ s from TNFR1^{-/-} mice did not show enhanced uptake of apoptotic cells following IFN- γ priming (Fig. 4D). Further confirmation of this sequential relationship was obtained when provision of the NO donor SNAP resulted in enhanced phagocytosis that was not inhibited by TNF- α neutralization (data not shown). These data establish that IFN- γ priming enhances the uptake of apoptotic cells by the sequential production of TNF- α , resulting in iNOS upregulation, NO production, and activation of PKG.

IFN- γ priming for enhanced uptake of Ig-opsonized cells is also dependent on the TNF- α -driven, NO-dependent pathway

Generally, uptake of apoptotic cells involves uptake of fluid phase constituents into spacious phagosomes (but see Ref. 50) via unique signaling pathways (35), whereas phagocytosis of latex beads (5 μ m) and Ig-opsonized cells is thought to occur by a tighter “zipper”

mechanism (51). To test the specificity of the described pathway, IFN- γ priming for enhanced phagocytosis of Ig-opsonized cells was also investigated. Just as in the case of uptake of apoptotic cells, TNF- α neutralization, inhibition of NO production, or inhibition of PKG activation also inhibited the IFN- γ -enhanced phagocytosis of these targets in CGD and WT BMDMs (Fig. 5). Thus, the same pathway for IFN- γ enhancement was required regardless of particle phagocytosed and, by inference, was independent of the surface receptors employed to engage the particle.

Rac activation results from IFN- γ priming and is dependent on TNF- α , NO production, and PKG activation

Activation of the Rho GTPase Rac and consequent actin assembly are necessary for lamellipodia formation and membrane ruffling crucial to the uptake of apoptotic cells (52), pinocytosis (29), Ig-opsonized targets (28), and likely fungal conidia (53) and latex beads (54). Furthermore, NO and PKG have been shown to mediate effects on the cytoskeleton, and iNOS has been shown to localize to cortical actin and to physically interact with Rac in M ϕ s (55). Given the data, as well as the ruffled appearance of IFN- γ -primed cells (Fig. 2), we hypothesized downstream activation of Rac following IFN- γ priming. Specifically, NO activation of Rac was hypothesized to be a likely effector mechanism underlying the enhanced phagocytic phenotype. As shown in Fig. 6, IFN- γ priming or provision of the NO donor SNAP strongly activated Rac in both CGD and WT BMDMs. As predicted, pretreatment with KT5823 prior to IFN- γ , or addition of L-NIL 30 min before assay, inhibited Rac activation. Finally, concurrent neutralization of TNF- α was effective in inhibiting Rac activation following IFN- γ priming, but not after provision of NO by SNAP, demonstrating that TNF- α acts upstream of NO production leading to Rac activation, just as in apoptotic cell uptake.

IFN- γ treatment of CGD mice primes M ϕ s via the TNF- α /NO-dependent pathway

Because IFN- γ treatment of mice significantly enhanced the *in vivo* uptake of apoptotic cells by CGD and WT resident peritoneal M ϕ s as shown in Fig. 1D, we investigated the *in vitro* mechanism as elucidated above for its role following *in vivo* IFN- γ treatment. Mice were treated as before with a single dose of IFN- γ or PBS, and 24 h later, M ϕ s lavaged from the peritoneum were plated and treated *in vitro* with inhibitors as indicated for an additional 24 h (Fig. 7A). Capacity for uptake of apoptotic cells was then determined and shown in Fig. 7B. Forty-eight hours following injection of PBS *in vivo*, deficient uptake was again evident for CGD peritoneal M ϕ relative to WT M ϕ s (indicated by the dashed line). Conversely, 48 h after IFN- γ treatment of CGD mice, enhanced apoptotic cell engulfment by CGD M ϕ was demonstrated with the level of uptake “normalized” to that of M ϕ s from PBS-treated WT mice. Incubation of CGD M ϕ s *ex vivo* with neutralizing Ab to TNF- α (but not isotype control) or KT5823 for 24 h prior to assay of apoptotic cell uptake inhibited the enhancement associated with *in vivo* IFN- γ treatment (Fig. 7B). As with *in vitro* priming with IFN- γ , the addition of the iNOS inhibitor L-NIL for 30 min before uptake assay inhibited the effect of *in vivo* IFN- γ treatment as well. For WT M ϕ s, the enhancing effect of *in vivo* treatment with IFN- γ appeared to wane somewhat at 48 h, in that *ex vivo* apoptotic cell engulfment at this time point was only slightly increased in comparison with M ϕ s harvested following PBS treatment (Fig. 7C and compare with 24 h *in vivo* data in Fig. 1D).

Nonetheless, WT Mφs assayed ex vivo demonstrated the same responses to inhibitors. Taken together, these data confirm that the IFN- γ /TNF- α /iNOS/NO-PKG-dependent pathway (Fig. 7D), demonstrated initially in vitro for Mφs, was also operant for peritoneal Mφ following IFN- γ treatment of mice.

IFN- γ treatment of CGD mice results in reduced accumulation of apoptotic neutrophils and their enhanced phagocytosis by Mφs during acute inflammation

These data demonstrated that IFN- γ treatment of mice resulted in enhanced uptake of apoptotic cells, both in vivo (Fig. 1D) and ex vivo (Fig. 7A–C), by resident Mφs obtained from the uninflamed peritoneum, and established the mechanism (Fig. 7D). Next, it was important to determine whether IFN- γ treatment of CGD mice would enhance uptake of apoptotic cells in vivo during inflammation and potentially alter its exaggerated course. To answer this question, we used a well-described model of zymosan-induced peritonitis in which CGD mice demonstrate exaggerated neutrophilia and have accumulation of apoptotic neutrophils during the resolution of inflammation (56). Mice were treated with IFN- γ or PBS (i.p.) on the day before (–24 h) and the day after (24 h) i.p. zymosan injection (time 0) (Fig. 8A). Peritoneal cells were harvested for analysis at 6 and 48 h following zymosan. As shown, at 6 h after zymosan treatment, neutrophil and Mφ numbers were not significantly altered by IFN- γ (versus PBS) treatment of mice of either genotype, suggesting that treatment had little effect on early recruitment of inflammatory cells (Fig. 8B). Apoptotic neutrophil numbers (as determined by nuclear morphology) were also not different between the treatment groups. However, a small but significant enhancement in the phagocytic indices for Mφs from both WT and CGD mice treated with a single dose of IFN- γ (versus PBS) was observed. In a second group of mice receiving two doses of either PBS or IFN- γ on alternate days, peritoneal cell harvests at 48 h following zymosan treatment demonstrated striking differences during resolution of inflammation. Considering first mice treated with PBS, exaggerated neutrophilia was observed in the CGD mice compared with WT mice, in keeping with previous reports (7, 56, 57). Additionally, greater accumulations of apoptotic neutrophils and reduced phagocytic indices for Mφs were observed in harvests from CGD compared with WT mice (Fig. 8C). These data are in accordance with a recent report by Rajakariar et al. (56), and they were consistent with defective clearance. In contrast to PBS treatment, treatment with IFN- γ resulted in significantly fewer neutrophils present in both CGD and WT mice, with near resolution of neutrophilia noted in the latter mice. Furthermore, the data from CGD mice supported enhanced clearance following IFN- γ treatment; accumulation of apoptotic neutrophils was significantly reduced and phagocytosis of apoptotic cells was significantly increased following IFN- γ treatment. Importantly, the clearance of cells by CGD Mφs was nearly normalized to levels observed for WT Mφs (Fig. 8C).

Discussion

Uptake of apoptotic cells occurs by a unique process called efferocytosis (“to carry to the grave”) (58, 59). In efferocytosis, ligands on the apoptotic cell (e.g., phosphatidylserine and calreticulin) signal through various receptors or bridge molecule/receptor combinations on the Mφs (59–61), and this signaling activates Rac1, leading to uptake into spacious

phagosomes in a process akin to macropinocytosis (35). Impaired efferocytosis has been demonstrated in murine CGD M ϕ populations, as well as in M ϕ -like cells derived from human PLB-985 cells lacking a functional gp91^{phox}, relative to corresponding M ϕ s with sufficient gp91^{phox} function (Fig. 1) (11, 34). Impaired efferocytosis is hypothesized to contribute to exaggerated inflammation and autoimmunity characteristic of this disease (11). We have previously shown that efferocytosis (but not other phagocytic pathways) is restored in CGD with IL-4, a cytokine deficient in CGD M ϕ s (11). Notably, heightened phagocytic capacity for various targets, and in various settings, has also been associated with IFN- γ (exogenous or endogenously produced), for example, the uptake of pathogens (23, 62) and apoptotic cells (27). Given that IFN- γ is used as a prophylactic treatment in CGD (see below), we sought to determine whether IFN- γ priming reversed impairment of CGD M ϕ s in the uptake of apoptotic cells and, if so, to determine the mechanism.

As shown, IFN- γ priming, in vitro and in vivo, normalized the uptake of apoptotic cells by gp91^{phox}-deficient M ϕ s to levels equal to or surpassing gp91^{phox}-sufficient M ϕ s (Fig. 1). Uptake by gp91^{phox}-sufficient M ϕ populations was also significantly enhanced over baseline levels by IFN- γ treatment. Importantly, however, priming of M ϕ s with IFN- γ led to Rac activation and heightened phagocytosis for various particles tested in addition to apoptotic cells (Fig. 2). Notably, these effects of IFN- γ priming were unlike the effect of IL-4, which specifically restored efferocytosis in CGD M ϕ s while having no effect on the uptake of these nonapoptotic targets or on WT M ϕ s (11). These findings extend those of Ren and Savill (27) who showed that IFN- γ priming increased both the percentage of M ϕ s (differentiated from human monocytes after 4 d of culture) capable of taking up apoptotic cells and the number of cells ingested per M ϕ . In this earlier work, phospho-L-serine did not block this IFN- γ -enhanced uptake, suggesting independence of the lipid ligand, phosphatidylserine, exposed on the surface of almost all apoptotic cells (but reportedly deficient on apoptosing CGD neutrophils) (8, 9). Uptake of nonapoptotic targets was not reported in this previous work (27), and we hypothesize that IFN- γ treatment may have enhanced phagocytosis nonspecifically as described in this investigation, rather than having simply enhanced efferocytosis.

Mechanistically, TNF- α production, iNOS induction, NO production, and PKG activation (Fig. 6D) were essential for Rac activation and the enhanced phagocytic state associated with IFN- γ priming (Fig. 1). As such, priming with IFN- γ appears to result in enhancement of existing mechanisms for phagocytosis by acting downstream of various receptor-mediated pathways, including those used in efferocytosis (see above). Such might be an important advantage in CGD where apoptosis, phosphatidylserine exposure, and its modification are reportedly deficient (8, 9). TNF- α is well known to act synergistically with IFN- γ for the production of iNOS (46), and this effect was shown to be critically required for NO production (Figs. 3, 4) necessary for the activation of Rac (Fig. 6). As proof of concept, it is hypothesized that M ϕ s from CGD mice crossed with TNFR1- or iNOS-deficient mice would not demonstrate IFN- γ -dependent normalization of apoptotic cell uptake, and these experiments are proposed for future investigations. In contrast to the combined effects of TNF- α and IFN- γ , TNF- α alone in the absence of IFN- γ has very different and variable effects on efferocytosis, depending on conditions and context (27, 63). For instance, treatment of mature (but not immature) BMDM ϕ s with TNF- α induced

transient inhibition of efferocytosis lasting 6–8 h due to production of oxidants downstream of cytosolic phospholipase A₂ (63). Conversely, Ren and Savill (27) showed that, similar to IFN- γ , TNF- α enhanced engulfment of apoptotic cells by an unknown, but phosphatidylserine-independent, mechanism in relatively immature monocyte-derived human M ϕ s. Whether NO production may have mediated enhanced uptake was not investigated in these studies.

IFN- γ has protean effects, including induction of antimicrobial pathways, differentiation of cells, and upregulation of Ag processing and presentation. More recently, its appearance in Th2 cells, so-called Th2+1 cells, has been associated with curtailment of inflammation, and the absence of these cells is associated with chronic inflammation (64, 65). Although it is not clear which of the many effects of IFN- γ play a role in its clinical efficacy in CGD, prophylactic treatment with IFN- γ (three times weekly) significantly (although only partially) prevents serious infection in CGD, and it has been used therapeutically in this disease in the United States since the mid-1990s (5, 14). Whether IFN- γ treatment is associated with either increased or decreased inflammatory aspects of this disease is unclear (2). Marciano et al. (66) in a recent review of patients with gastrointestinal involvement in CGD concluded that IFN- γ treatment was not associated with increased inflammatory manifestations. Clinical trials to formally answer whether IFN- γ treatment is associated with less inflammation in CGD have not been conducted and would likely be confounded by its antimicrobial effects. Additionally, given these observations, it is hypothesized that IFN- γ treatment may be useful in other inflammatory disorders where M ϕ phagocytosis is thought to be impaired (e.g., malakoplakia).

It is hypothesized that one effect of continuous treatment with IFN- γ in CGD may be the priming of M ϕ s for enhanced phagocytosis. Such could contribute to necessary clearance of recruited and effete inflammatory cells as was demonstrated in CGD mice during acute sterile peritonitis (Fig. 8) in addition to enhanced clearance of pathogens (5, 14). While enhanced phagocytosis of apoptotic cells is associated with IFN- γ priming, anti-inflammatory signaling characteristic of efferocytosis is quite possibly altered, and diminished production of anti-inflammatory TGF- β has been reported following IFN- γ treatment of M ϕ s (67). Nonetheless, the stimulated removal of apoptotic inflammatory cells, especially dying neutrophils and other inflammatory cells, by a mechanism bypassing the need for phosphatidylserine exposure, before these cells become necrotic, may nevertheless be critically important for CGD inflammation and maintenance of tissue function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

| | |
|--------------|-----------------------------------|
| BMDMφ | bone marrow-derived macrophage |
| CGD | chronic granulomatous disease |
| HMDMφ | human monocyte-derived macrophage |
| iNOS | inducible NO synthase |

| | |
|--------------|--|
| L-NIL | <i>N</i> ⁶ -(1-iminoethyl)-L-lysine dihydrochloride |
| LY | Lucifer yellow |
| Mφ | macrophage |
| PI | phagocytic index |
| PKG | protein kinase G |
| RPMφ | resident peritoneal macrophage |
| SNAP | <i>S</i> -Nitroso- <i>N</i> -acetylpenicillamine |
| WT | wild-type |

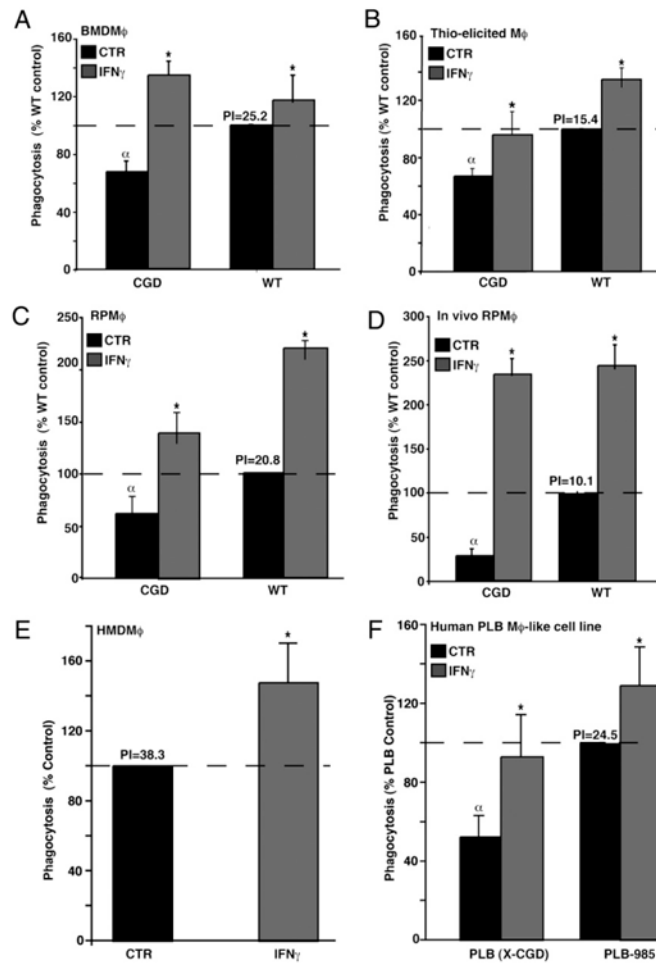


Figure 1.

IFN- γ priming of CGD and WT M ϕ populations in vitro and in vivo enhances uptake of apoptotic cells. BMDM ϕ s (A), thioglycollate-elicited M ϕ s (B), RPM ϕ s (C), HMDM ϕ s (E), and M ϕ -like differentiated PLB-985 and mutated PLB-985 (X-CGD) cell lines (F) were primed overnight with IFN- γ (50 ng/ml, 4200 U) or PBS in vitro and uptake of apoptotic Jurkat cells was assayed ($n = 5$ for each condition). D, Mice were treated with IFN- γ (500 ng, 42,000 U) or PBS i.p. and 24 h later, PKH-labeled apoptotic thymocytes were instilled into peritonea and in vivo uptake by M ϕ s was assessed (see *Materials and Methods*) ($n = 4$). Dashed line represents baseline uptake by normal or WT M ϕ s, and PI is shown for each cell type; *p < 0.05 compared with control M ϕ s of each cell type, respectively, and α p < 0.05 compared with uptake by normal or WT M ϕ s of each cell type, respectively, at baseline. PI, phagocytic index; RPM ϕ , resident peritoneal M ϕ .

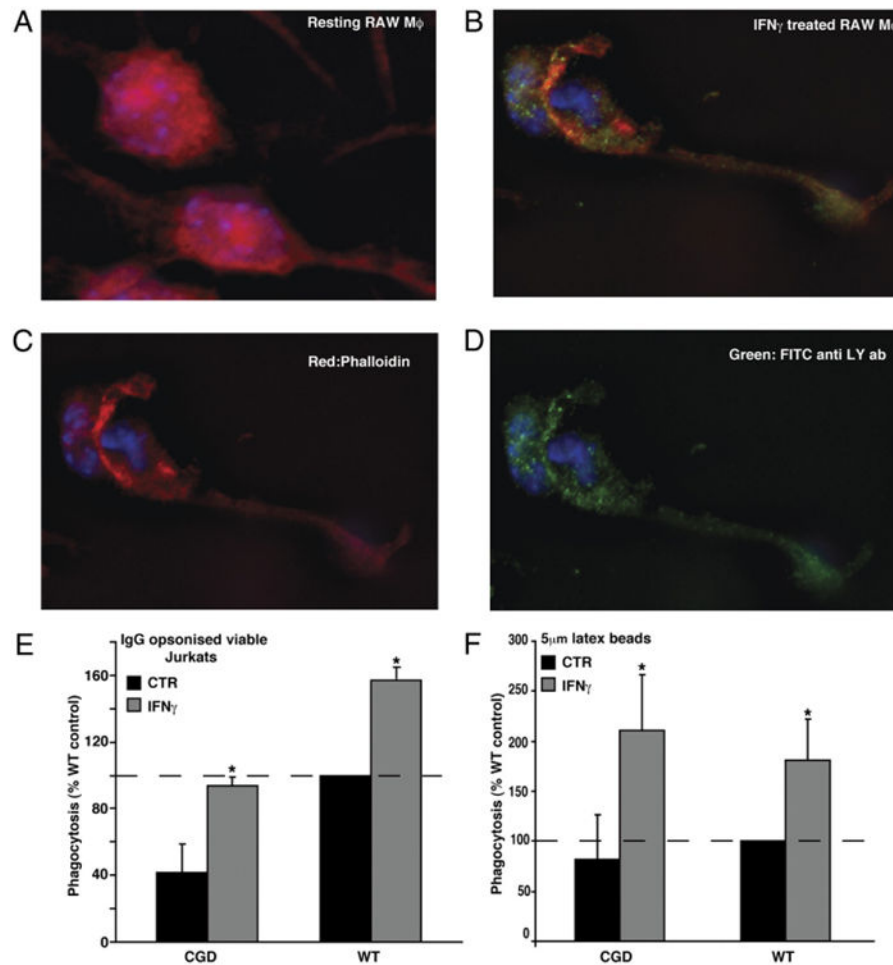
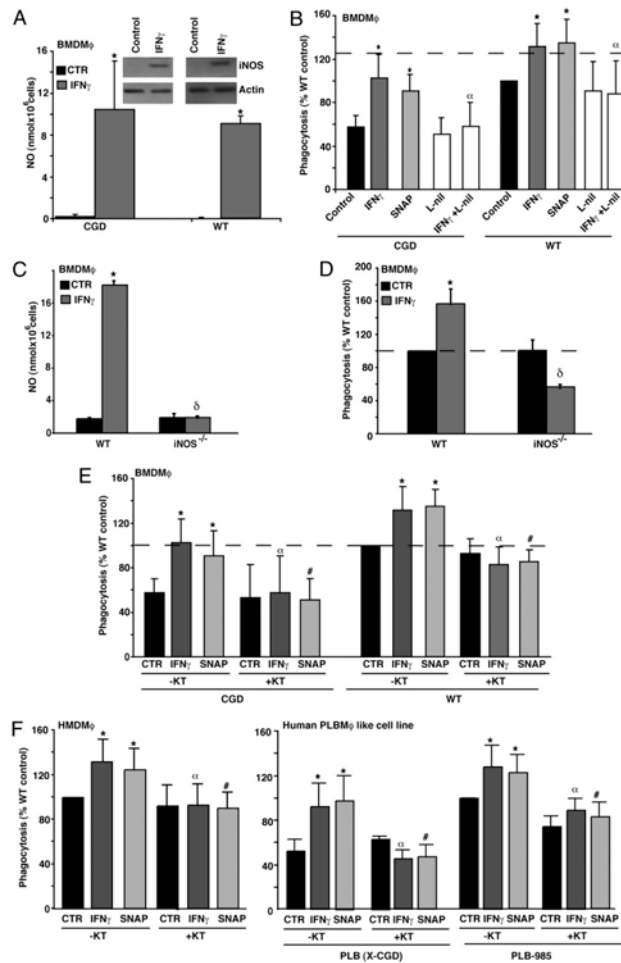
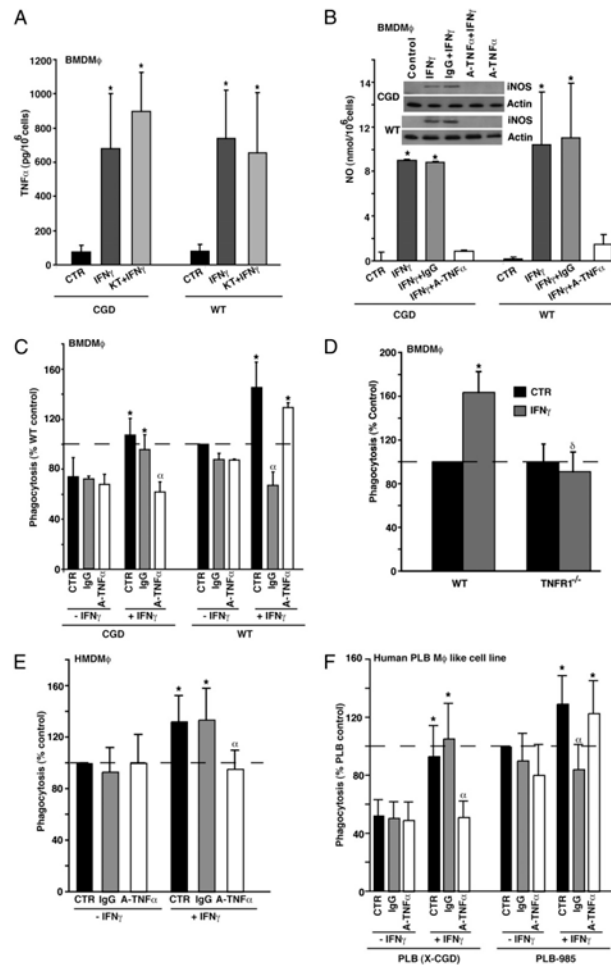


Figure 2.

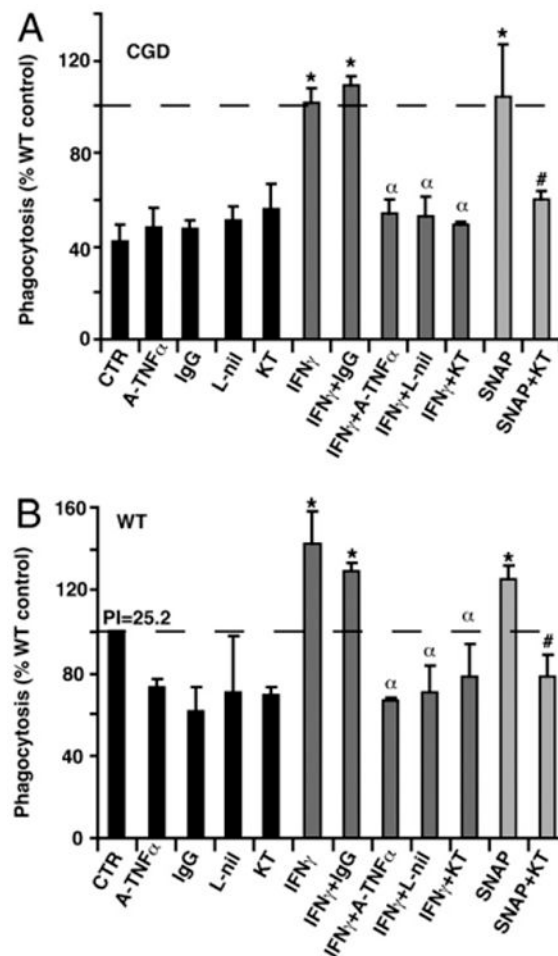
IFN- γ priming enhances ruffling, fluid phase uptake, and phagocytosis of opsonized viable cells and latex beads by M ϕ s. Resting (A) and IFN- γ primed (50 ng/ml, overnight) (B–D) RAW 264.7 cells were analyzed for actin polymerization (red, C) and uptake of LY dye (green, D) at $\times 100$ magnification. Yellow indicates colocalization of markers (B). BMDM ϕ s were primed with IFN- γ or not as in Fig. 1, and the uptake of Ig-opsonized Jurkat cells (E) ($n = 3$) or latex beads (5 μ m) (F) ($n = 3$) was assayed. * $p < 0.05$ compared with respective control cells.

**Figure 3.**

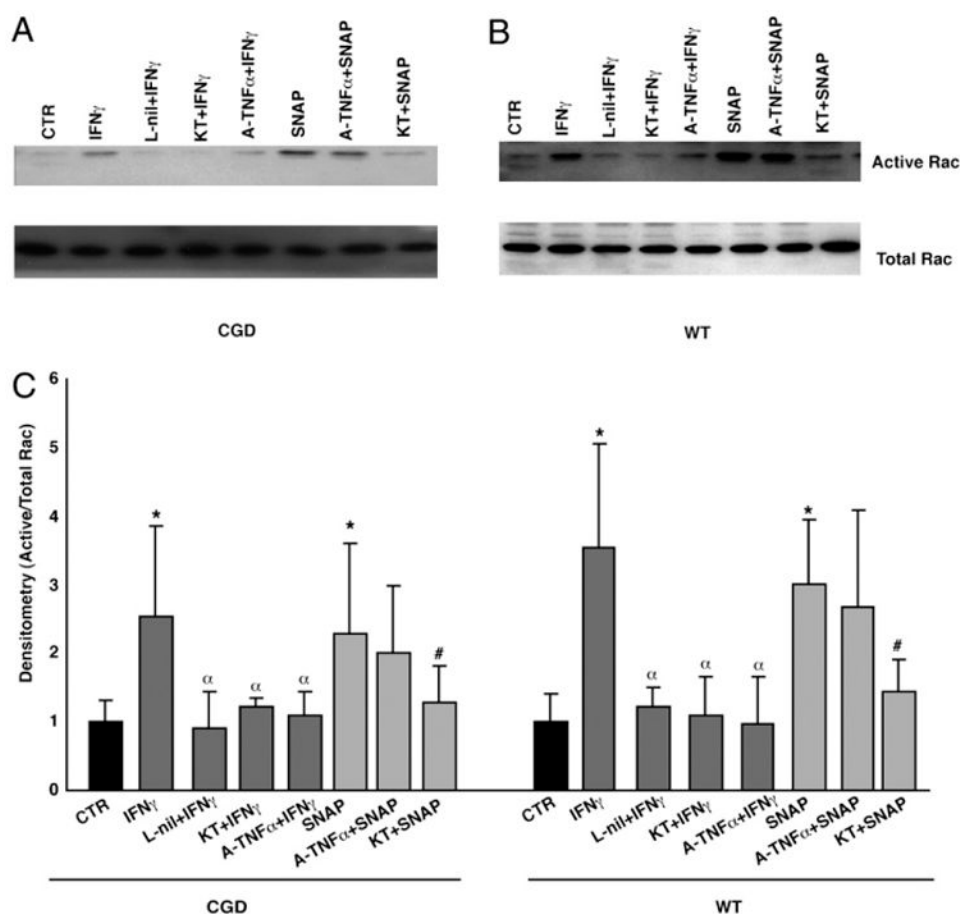
Enhanced phagocytosis of apoptotic cells by IFN- γ -primed CGD and WT M ϕ s is dependent on NO production. IFN- γ priming of BMDM ϕ s was performed as in Fig. 1. A, iNOS was detected by immunoblotting (*inset*) and NO was measured in supernatants following overnight culture ($n = 3$). B, Cells were IFN- γ primed (or not) or SNAP treated (50 μ M for 24 h) and, where indicated, cells were treated with L-NIL (0.5 mM) for 30 min before phagocytosis assayed ($n = 3$). BMDM ϕ s from WT and iNOS^{-/-} mice were primed with IFN- γ and NO production (C) or uptake of apoptotic cells (D) was determined ($n = 5$). E, Cells were treated with the PKG inhibitor, KT5823 (10 μ M for 30 min) before IFN- γ priming or treated with SNAP as in B, and uptake of apoptotic cells was determined for BMDM ϕ s ($n = 5$), HMDM ϕ s ($n = 10$), and the PLB-985 M ϕ cell lines ($n = 4$) (F). * $p < 0.05$ compared with the untreated control M ϕ s for each cell type, respectively; ^a $p < 0.04$ compared with IFN- γ -primed M ϕ s of each cell type, respectively; [#] $p < 0.04$ compared with SNAP-treated M ϕ s of each cell type, respectively; ^{δ} $p < 0.03$ compared with IFN- γ -primed WT M ϕ s.

**Figure 4.**

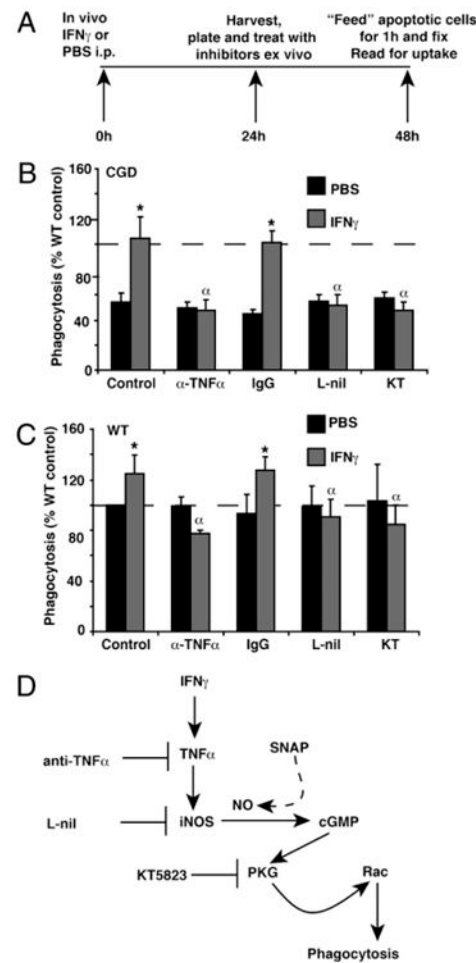
TNF- α production and action in response to IFN- γ priming is required for iNOS induction and NO production. **A**, TNF- α was measured in the supernatants of BMDM ϕ s following IFN- γ priming with and without treatment with the PKG inhibitor KT5823 as in Fig. 3 ($n = 5$). **B**, BMDM ϕ s were primed with IFN- γ in the presence of A-TNF- α or isotype control and NO was measured in supernatants and iNOS was detected by immunoblotting (*inset*, one representative experiment). Following treatment as in **B**, phagocytosis of apoptotic cells was determined in M ϕ s: BMDM ϕ s from WT and CGD mice ($n = 5$) (**C**), WT and TNFR1^{-/-} mice (**D**) ($n = 5$), HMDM ϕ s ($n = 10$) (**E**), and PLB-X-CGD and PLB-985 cell lines ($n = 4$) (**F**). * $p < 0.04$ compared with the untreated control M ϕ s for each cell type, respectively; ^a $p < 0.04$ compared with IFN- γ -primed M ϕ s of each cell type, respectively; [#] $p < 0.04$ compared with SNAP-treated M ϕ s of each cell type, respectively; ^δ $p < 0.02$ compared with IFN- γ -primed WT M ϕ s. A-TNF- α , neutralizing Ab to TNF- α .

**Figure 5.**

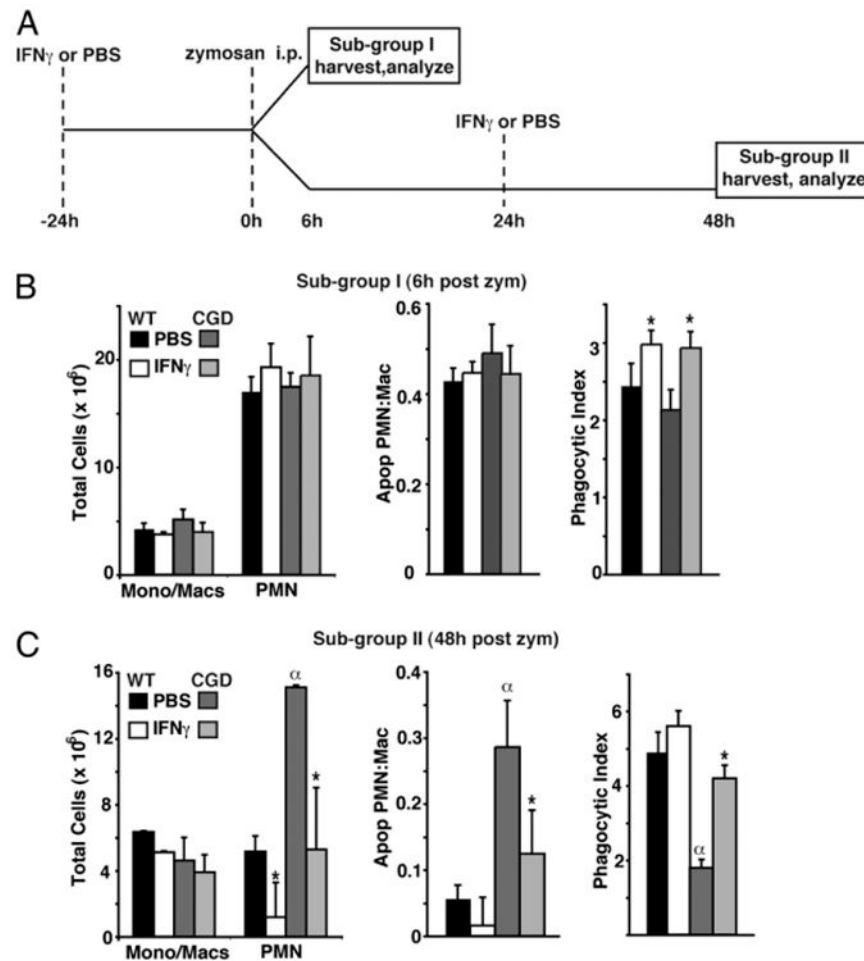
IFN- γ priming enhances phagocytosis of Ig-opsonized Jurkat cells by the TNF- α /iNOS/NO-PKG-dependent mechanism. BMDM ϕ s from CGD (A) and WT (B) mice were treated with the agents/inhibitors as previously described, and phagocytosis of CD45-opsonized Jurkat cells was determined ($n = 4$). * $p < 0.05$ compared with the control M ϕ s for each cell type, respectively; $\alpha p < 0.05$ compared with IFN- γ -primed M ϕ s of each cell type, respectively; # $p < 0.02$ compared with SNAP-treated M ϕ of each cell type, respectively.

**Figure 6.**

IFN- γ priming leads to Rac activation by the TNF- α /NO/PKG-dependent pathway. BMDM ϕ s from CGD (A) and WT (B) mice were tested for Rac activation using the agents/inhibitors as in Fig. 3, and representative immunoblots are shown. C, Densitometry was used to determine the ratio of active Rac to total Rac ($n = 3$). * $p < 0.05$ compared with the control M ϕ s for each cell type, respectively; $^{\alpha}p < 0.05$ compared with IFN- γ -primed M ϕ s of each cell type, respectively; $^{\#}p < 0.05$ compared with SNAP-treated M ϕ s of each cell type, respectively.

**Figure 7.**

In vivo treatment with IFN- γ enhances ex vivo phagocytosis of apoptotic cells by resident peritoneal M ϕ s via the TNF- α /NO/PKG pathway. A, Experimental design is shown. CGD (B) and WT (C) mice were injected i.p. with IFN- γ or PBS as in Fig. 1D; 24 h later peritoneal M ϕ s were lavaged, plated, rested for 24 h in the presence or absence of the specified inhibitors/agents, and then phagocytosis of apoptotic Jurkat cells was assessed ($n = 5$). Dashed line represents uptake in WT M ϕ s following PBS treatment. * $p < 0.05$ compared with control mice of each genotype injected with PBS, respectively; # $p < 0.05$ compared with mice of each genotype injected with IFN- γ , respectively. D, Mechanism of IFN- γ enhancement of uptake operant both in vitro and ex vivo.

**Figure 8.**

IFN- γ treatment of CGD mice enhances phagocytosis of apoptotic cells by M ϕ s during resolution of inflammation. **A**, Experimental design is shown. CGD and WT mice were injected i.p. with IFN- γ (500 ng, 42,000 U) or PBS and 24 h later all mice were injected with 1 mg of zymosan i.p. **B**, Mice in subgroup I (6 h after zymosan treatment) were euthanized and peritoneal cells lavaged and analyzed for differentials, apoptotic neutrophils, and phagocytic indices. **C**, Mice in subgroup II were treated with a second dose of PBS or IFN- γ at 24 h after zymosan treatment, and peritoneal cells were analyzed at 48 h after zymosan treatment. * p = 0.03 compared with mice treated with PBS for each genotype, respectively; $^{\alpha}p$ = 0.04 compared with WT mice treated with PBS.