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The Reduced Bactericidal Function of Complement C5-Deficient Murine Macrophages Is Associated with Defects in the Synthesis and Delivery of Reactive Oxygen Radicals to Mycobacterial Phagosomes

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Complement C5-deficient (C5−/−) macrophages derived from B.10 congenic mice were found to be defective in killing intracellular Mycobacterium tuberculosis (MTB). They were bacteriostatic after activation with IFN-γ alone but bactericidal in the combined presence of IFN-γ and C5-derived C5a anaphylatoxin that was deficient among these macrophages. Reduced killing correlated with a decreased production of reactive oxygen species (ROS) in the C5−/− macrophages measured using fluorescent probes. Furthermore, a lack of colocalization of p47phox protein of the NADPH oxidase (phox) complex with GFP-expressing MTB (gfpMTB) indicated a defective assembly of the phox complex on phagosomes. Reconstitution with C5a, a known ROS activator, enhanced the assembly of phox complex on the phagosomes as well as the production of ROS that inhibited the growth of MTB. Protein kinase C (PKC) isoforms are involved in the phosphorylation and translocation of p47phox onto bacterial phagosomes. Western blot analysis demonstrated a defective phosphorylation of PKC (α, β, δ) and PKC-ζ in the cytosol of C5−/− macrophages compared with C5 intact (C5+/+) macrophages. Furthermore, in situ fluorescent labeling of phagosomes indicated that PKC-β and PKC-ζ were the isoforms that are not phosphorylated in C5−/− macrophages. Because Fe receptor-mediated phox assembly was normal in both C5−/− and C5+/+ macrophages, the defect in phox assembly around MTB phagosomes was specific to C5 deficiency. Reduced bactericidal function of C5−/− macrophages thus appears to be due to a defective assembly and production of ROS that prevents effective killing of intracellular MTB. The Journal of Immunology, 2006, 177: 4688–4698.

Tuberculosis is the leading cause of death due to a single infectious agent in mankind. Nearly a third of the global population is infected with Mycobacterium tuberculosis (MTB), 1 and 2 million people die of active disease every year. MTB multiplies in naive macrophages, which leads to the secretion of cytokines such as IL-12. IL-12 induces the T cells to produce IFN-γ that is essential for the feedback activation of macrophages leading to killing of MTB. Although CD4+ T cells protect against acute tuberculosis, CD8+ T cells have been found to confer protection against chronic tuberculosis and reactivation. Investigators have used various strains of immunocompetent and gene knockout mice to define the role of macrophages, T cells, and cytokines like IL-12, IFN-γ, and TNF-α during antituberculosis immunity (1). There is evidence that susceptibility to human tuberculosis is regulated by similar cells and cytokines. The high prevalence of tuberculosis in AIDS patients is evidence that CD4+ T cells play a defensive role. Mutations in the receptors for IL-12 and IFN-γ are related to the enhanced susceptibility for mycobacterial infections in humans. An interesting addition to the genetic basis of susceptibility in humans is the polymorphism associated with the gene regulating the “natural resistance-associated macrophage protein” (Nrampl) (2). Nrampl is expressed on the lysosomal membranes of macrophages and has been proposed to transport metal ions into the lumen of phagosomes (3). Mutations in Nrampl gene are associated with either an increased risk or severity of tuberculosis (4, 5). However, the role of Nrampl in susceptibility of mice to tuberculosis remains unclear. Mice of the BCG-type phenotype (DBA/2) that express Nrampl are more susceptible to tuberculosis than BCG+ strains of mice (C57BL/6, BALB/c), which lack Nrampl expression (6). Because Nrampl may affect the growth of mycobacteria within phagosomes, additional studies appear necessary at the level of mouse macrophages to resolve this paradox.

We described earlier that the BCG+ phenotype A/J mice were more susceptible to tuberculosis than the BCG− phenotype C57BL/6 mice (7). Using selected routes and doses of infection with MTB, we demonstrated that acute, chronic, and reactivation tuberculosis disease patterns were more severe in A/J compared with C57BL/6 mice. During the course of these investigations, we observed that A/J was naturally deficient in Complement C5 with
a 2-bp deletion in the gene encoding C5. B10-derived congenic mice with an identical deletion have also been described previously (8). We performed additional studies to confirm that congenic C5<sup>-/-</sup> mice were more susceptible to tuberculosis (9). C5-deficient DBA/2 and SWR strains were subsequently found to be hyper susceptible to tuberculosis (10, 11). Because MTB has adapted to survive and replicate within macrophages, we hypothesized that C5 deficiency could affect macrophage function. Indeed, unlike C5-sufficient C57BL/6 mice, macrophages of C5-deficient A/J were unable to restrict the intracellular growth of MTB. Furthermore, unlike A/J, C57BL/6-derived macrophages showed a strong secretion of C5 after MTB infection, which was cleaved by extracellular proteases into the active C5a fragment (anaphylatoxin). Alternatively, macrophages can secrete a variety of complement components leading to the alternative C3/C5 convertase. C5a, in turn, activated macrophages through the C5a-receptor (C5a-R) to restrict the growth of MTB. This activation involved TNF-α through a synergistic interaction (7). Finally, increased growth of MTB in the lungs of C5-deficient mice was found associated with both reduced cytokine (IL-12, TNF-α, IL-1β, and IL-6) and chemokine (KC, MIP-2, and MIP-1α) secretion from their macrophages, which led to a defective granuloma formation. Our studies therefore identified C5a as a novel susceptibility factor for tuberculosis in mice (7).

In this study, we have investigated the molecular basis of the defective intracellular killing of MTB by C5<sup>-/-</sup> macrophages focusing on reactive oxygen species (ROS) (12). C5a is a known activator of the NADPH oxidase (‘phox’ complex) that assembles at either the plasma membrane or phagosomal membrane to produce superoxide (O<sub>2</sub><sup>-</sup>) (13, 14). The latter either spontaneously dismutates or is aided by the superoxide dismutase to form hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). H<sub>2</sub>O<sub>2</sub> can be converted into the hydroxyl radical (OH<sup>-</sup>) through the iron-catalyzed Fenton-Haber-Weiss reaction. These species can interact with each other or with others to generate other bactericidal species like hypochlorous acid (HOCl), thus extending the various elements of the ROS cascade (15). Because ROS are major mediators of antimicrobial killing, cellular apoptosis, and inter- and intracellular signaling, C5a-initiated ROS production could influence intracellular and extracellular killing mechanisms of macrophages (16–19). C5a is known to bind to a C5a-R that belongs to the G-protein family to activate protein kinase C (PKC). Activated PKCs are known to phosphorylate p47phox protein, initiating the assembly of the phox complex on phagosomes (20). We demonstrate in this study that, C5 deficiency in macrophages is associated with a defective phosphorylation of some PKC isoforms that leads to a defective ROS synthesis and consequently decreased killing of MTB.

Finally, Nramp1-mediated iron transport into the phagosome can generate the ‘OH radical, enhancing the efficacy of the ROS cascade. Because A/J and DBA/2 are wild-type Nramp1 but have defective ROS due to C5 deficiency, we propose herein that Nramp1 expression in ROS-deficient mice can adversely affect resistance to tuberculosis.

Materials and Methods

**Bacteria**

MTB strain H37Rv (27294) was obtained from the American Type Culture Collection repository and cultured to log phase in Dubos 7H9 broth. Suspensions were washed in saline, matched to McFarland number one in turbidity, and gently sonicated at 4 W for 15 s. The suspension was centrifuged at 500 rpm for 5 min to settle clumps, and the supernatants containing single CFU at 10<sup>3</sup>-<sup>10</sup> per ml were used for infections. GFP-producing BCG (gfpBCG) or MTB (gfpMTB) were prepared as described before (21), grown to log phase in 7H9 broth with 20 μg/ml kanamycin and processed as described above.

**Mice**

Six to 8-week-old female B10.D2-H2<sup>3</sup>H2-T18c Hco/oSnJ (C5-deficient, C5<sup>-/-</sup>), B10.D2-H2<sup>3</sup>H2-T18c Hc/oSnJ (C5 sufficient, C5<sup>+/+</sup>) mice, A/J, C57BL/6 and DBA/2 mice were obtained from Jackson ImmunoResearch Laboratories. They were housed within a BSL-3 vivarium and fed food and water ad libitum. All manipulations were approved by the Institutional Animal Care and Use Committee.

**Experimental infections**

Mice were infected with a log phase, highly viable single-cell suspension of H37Rv via aerosol using a Glasc-Col aerosol apparatus, calibrated to deliver 100–500 CFU per mouse lungs over 30 min of nebulization. Mice were sacrificed at weekly intervals, and lungs were homogenized and plated on 7H11 agar to determine the CFUs.

**Macrophages**

Macrophages were derived from 6- to 7-day-old cultures of femoral bone marrow (BM) cells in McCoy’s medium (iron free) with 10% heat-inactivated FBS, penicillin (100 U/ml), gentamicin (50 μg/ml), and 10 ng/ml rGM-CSF (growth medium). After macrophages were grown, they were maintained subsequently in McCoy’s medium with 5–10% heat-inactivated mouse serum, which maintained the viability of macrophages at >90% over 10 days. Adherent macrophages were washed with warm medium, refreshed for 3 days in antibiotic and GM-CSF-free medium containing 5% autologous mouse serum, and used as required. Such macrophages do not show evidence of an activated state as evaluated by the lack of cytokines and chemokines (e.g., TNF-α, KC) in culture supernatants. ROS secretion measured by dihydro-dichloro-fluorescine diacetate (DCFDA) fluorescent probe, and low nitrite in supernatants measured using the Greiss reagent. Four- or 8-well slide chambers were used for macrophage immunostains, 6-well plates were used for membrane vs cytosol phox studies, 24-well plates were used for evaluation of growth curves, and 48-well, flat-bottom wells plates were used for analysis of ROS secretion after MTB infection and C5a or Ab supplementation. In all formats, macrophages were infected at MOI of 1:1 to 1:10 as indicated. Macrophages cultures were supplemented with either affinity-purified mouse C5a (AFMC5a) or recombinant human C5a (Hu-recC5a) as indicated.

**C5a peptide**

Affinity-purified mouse C5a (AFM C5a). Monolayers of rested C5<sup>+/+</sup> macrophages were infected with MTB at a MOI of 1:1, washed extensively with serum-free McCoy’s medium, and incubated in the same medium for 24 h at 37°C and 5% CO<sub>2</sub>. As reported before, such medium contains abundant secreted and cleaved C5a. The medium was filter sterilized through a 0.22 μm filter and concentrated 10-fold in an Amicon ultraconcentrator (molecular mass cutoff 5000 kDa). A protein G-Sepharose column cross-linked with glutaraldehyde to a mono-specific Ab mouse C5a was used to bind the complex from the concentrate. The Ab was produced in rabbits to the C-terminal peptide of mouse C5a (CTIANKIRKESPHKFVQGR) linked to KLH (titer 1/240,000 vs C5a peptide in ELISA; Bethyl Laboratories). The captured C5a was then eluted with an elution buffer consisting of 50 mM HCl (pH 2.8) with 0.1% Triton X-100 and 0.15 M NaCl, neutralized with solid Tris, dialyzed, and concentrated. It was analyzed by Western blotting using the same Ab as well as a commercially available porcine anti-mouse C5a. A single band at 14 kDa and a smaller comigrating band (C5a<sub>medium</sub>) were observed with both Abs. The protein content was estimated, and aliquots were frozen at −70°C. Hu-recC5a was obtained from Sigma Chemicals and contained <100 pg of endotoxin per 100 ng of protein, as tested by the Limulus amoebocyte test.

**Effect of activation of macrophages on the intracellular viability of MTB**

Growth of MTB in naive BMs was analyzed using an MOI of 1:1, and macrophage lysates were plated for CFU counts as described before (7). A dose of 50 μ/ml IFN-γ has been used by some investigators to induce phagosome-lysosome fusion without induction of inducible NO synthase (iNOS), whereas 100-1000 U/ml has been used to induce iNOS and kill MTB in macrophages. BMs were activated with three doses of IFN-γ or 400 U of IFN-γ plus 100 ng/ml AFMC5a or Hu-recC5a for 18 h before infection. Treated and untreated macrophages were infected with MTB, and growth curves were determined over 7 days, with supplements replaced every 2 days. Growth of intracellular MTB was determined by plating macrophage lysates on 7H11 agar.
ROS and NO response to MTB infection of macrophages

ROS synthesis. IFN-γ was not used in this assay. When macrophages or neutrophils are primed by soluble agonists such as PMA or C5a peptide, the phox complex assembles against the plasma membrane and secretes O₂⁻ that is measurable by the HRP-dependent extracellular conversion of isoluminol (22, 23). Because PMA is toxic in vivo and thus a nonphysiological stimulant, we tested C5a as an agonist BM in 48-well plates were infected at a MOI of 1:1 with MTB at 37°C and 5% CO₂, washed, and incubated for 4 h. To study the effects of reconstituting C5a, AFM C5a or Hu-recC5a were added at varying concentrations using triplicate wells for each concentration. The mouse mAb to C5a-R has been well characterized before and was a gift from Dr. J. Zwierner (University ofGottingen, Germany) (24). C5a-R mAb was used at 1:100 dilution of goat Abs to mouse IgG at room temperature for 1 h (conjugates from Jackson Immunoresearch Laboratories). After further washing, cells were mounted in Elvanol for up to 72 h postinfection, all further studies were conducted with C5a-R mAb. C5a-R mAb was used as a positive control.

Membrane translocation of phox complex on phagosomes of macrophages

Cytosol and membrane fractions of macrophages infected with MTB in the presence or absence of added C5a or IFN-γ were prepared as described below, and the fractions were incubated with anti-p47phox for 1 h at 4°C. The immunocomplex was captured with protein G-Sepharose that binds goat Ab, and the sample was boiled in SDS sample buffer before electrophoretically as described below. Samples were detected with an Ab to β-actin or mouse HRP-labeled anti-phosphotyrosine for 2 h (Upstate Biotechnology). Washed blots were then developed using ECL as described below. LPS-treated macrophage fractions were used as negative controls.

Phosphorylation of PKC isoforms in macrophages

IFN-γ was not required for these assays. The Ca²⁺-dependent (classic PKC: α, β, γ), -independent (novel PKC: δ, ε, η, θ), and -atypical (ζ) PKC isoforms have been implicated in the phosphorylation of p47phox in murine macrophages (29–32). To analyze the role of PKC isoforms in phox translocation, we adapted previously described methods. Monolayers of uninfected, MTB or MTB plus C5a (100 ng/ml)-infected C5⁻/⁻ and C5⁺/⁺ macrophages were lysed at different time points by the addition of 0.4 ml of lysis buffer per 10⁶ macrophages. Lysis buffer contained 0.1% Triton X-100, 25 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 2.5 mM EDTA, and the following inhibitors: 5 mM benzamidine, 50 μg/ml leupeptin, 50 μg/ml aprotinin, 50 μg/ml trypsin inhibitor, 5 μg/ml pepstatin, 1 mM PMSF, 20 mM NaF, and 5 mM imidazole (29). The lysates were centrifuged for 45 min at 100,000 × g; supernatant was designated cytosol, the pellet was washed in lysis buffer once more, and the final pellet was solubilized in lysis buffer with 0.5% Triton X-100. The pellet was again centrifuged, and the Triton X-100 soluble supernatant was designated membrane fraction and the pellet as insoluble. Because the soluble fractions were previously found not to contain PKCs (32), only cytosol or membrane fractions were estimated for protein contents and tested in Western blots. Controls included naïve macrophages activated for 48 h with 10–100 ng/ml LPS (Sigma Chemical). Increasing protein concentrations were tested per lane to detect steady-state expression of PKC isoforms; detection of phosphorylated PKC required loading of five times more protein. Samples were separated in 4–20% gradient SDS gel and transferred to nitrocellulose, or mouse HRP-labeled anti-phosphotyrosine for 2 h (Upstate Biotechnology). Membranes were probed with primary mAbs against PKC isoforms or C5a-R mAb, and the sample was boiled in SDS sample buffer before electroblotting as described above. Western blots against lysates of MTB and were found to react with C5⁻/⁻ or C5⁺/⁺ BMs were infected with live gfpMTB as described above, but in the presence or absence of varying dilutions of homologous immune or nonimmune serum pools (1/100) for 4 h. Phox localization experiments were performed as described above. As a positive control, LPS was used as an additional activator of macrophages. It was added to macrophages at a dose of 100 ng/ml after phagocytosis, and cells were incubated up to 24 h.

Results

C5-deficient mice and macrophages permit enhanced growth of MTB

Fig. 1A illustrates the growth of MTB in the lungs of three C5-deficient strains of mice (C5⁻/⁻, A/J, DBA/2) vs two C5-sufficient strains of mice (C5⁺/⁺ and C57BL/6). C5⁻/⁻ mice showed a significant increase in CFU counts of lungs compared with C5⁺/⁺ mice (p < 0.09). Fig. 1B demonstrates that BMs from C5-deficient mice allow ~1 log₁₀ higher growth of MTB than C5-sufficient macrophages. Epifluorescence examination confirmed the intracellular localization of MTB throughout the 7-day period of incubation (data not shown).

Localization of phox proteins and PKC on phagosomes of macrophages using fluorescence

IFN-γ-activated (400 U/ml, 4 h) macrophages were allowed to phagocytose gfpBGC or gfpMTB 4 h at 37°C and 5% CO₂, washed, and incubated in fresh medium. Because gfpBGC and gfpMTB gave similar results for up to 72 h postinfection, all further studies were conducted with gfpMTB-infected macrophages. At intervals, cells were washed with warm PBS once, fixed in ice-cold methanol for 15 s, further fixed for 30 min in 2.7% paraformaldehyde, and permeabilized for 30 min with a staining buffer containing 1% saponin, 0.1% glycine, and 2% heat-inactivated autologous mouse serum in PBS. Smears were washed and incubated at 4°C for 18 h with 1/100 dilution of goat Abs to mouse p47phox or p67phox (Santa Cruz), or control goat Abs. Macrophage fractions were washed three times with the staining buffer three times, and counterstained with Texas Red-conjugated rabbit anti-goat IgG at room temperature for 1 h (conjugates from Jackson Immunoresearch Laboratories). After washing, cells were mounted in Elvanol mountant and examined using a Nikon fluorescence microscope fitted with the Metavue software to evaluate colocalization patterns using narrow band excitation filters for fluorescein and Texas Red successively on each field. Merged images were analyzed for colocalization. Selected specimens and fields were then examined under a DeltaVision laser confocal microscope to confirm colocalization patterns and analysis of the staining patterns of anti-p47phox Abs. A positive control of PMCA activated uninfected macrophages, and a negative control consisting of uninfected, untreated, and rested macrophages were stained with anti-p47phox Abs for each type of macrophage, and time point was analyzed. In addition, Ab isotype controls were included using gfpMTB-infected macrophages stained with normal goat IgG followed by Texas Red-conjugated anti-goat IgG. Unactivated or uninfected macrophages presented a uniform low background staining with this procedure. In PMA-activated C5⁻/⁻ macrophages, staining for phox complex was largely peripheral along the plasma membrane and was consistent with the immunocytochemical and electron microscopic patterns described for PMCA in mouse and human neutrophils (17, 26–28). In contrast, phox-specific staining on phagosomes was evident as dense tubular or spherical staining, strongly colocalizing with phagosomes. Similar techniques were used to identify PKC isoforms and nitrotyrosine (NT) (Ab obtained from Cell Sciences), a marker for peroxynitrite (ONOO⁻) in and around mycobacterial phagosomes. NT studies required that the BMs were either activated with 400 U/ml IFN-γ or C5a plus IFN-γ for 18 h before infection with gfpMTB and analysis.

Fig. 1A illustrates the growth of MTB in the lungs of three C5-deficient strains of mice (C5⁻/⁻, A/J, DBA/2) vs two C5-sufficient strains of mice (C5⁺/⁺ and C57BL/6). C5⁻/⁻ mice showed a significant increase in CFU counts of lungs compared with C5⁺/⁺ mice (p < 0.09). Fig. 1B demonstrates that BMs from C5-deficient mice allow ~1 log₁₀ higher growth of MTB than C5-sufficient macrophages. Epifluorescence examination confirmed the intracellular localization of MTB throughout the 7-day period of incubation (data not shown).
Effects of activation with IFN-γ and C5a on the survival of intracellular MTB in C5<sup>-/-</sup> and C5<sup>+</sup> macrophages

It is well established that IFN-γ enhances bactericidal activity of murine macrophages through the activation of iNOS. To determine the basis for reduced killing of MTB in C5<sup>-/-</sup> macrophages, we investigated the effects of IFN-γ in combination with C5a peptide on macrophages and correlated them with nitrite levels in the medium. As anticipated, IFN-γ had a bactericidal action on C5<sup>+</sup> macrophages at 250 and 400 U (left panel, Fig. 2A). In contrast, all three doses of IFN-γ had bacteriostatic effect against MTB in C5<sup>-/-</sup> macrophages (right panel, Fig. 2B). Preliminary studies showed that combination of 50 and 250 U of IFN-γ with C5a were not as effective as 400 U (data not shown). Thus, for the sake of clarity, the effect of C5a combination with 400 U has only been shown for C5<sup>-/-</sup> macrophages in Fig. 2B. Addition of C5a was synergistic and enhanced the bactericidal action of IFN-γ to bactericidal effect, reducing the day 0 baseline CFUs by >2 log<sub>10</sub>. Higher doses of IFN-γ (>400 U) were indeed capable of causing bactericidal effect (reduction of >1 log<sub>10</sub> from baseline) among C5<sup>-/-</sup> macrophages by day 5. However, due to unknown reasons, the BMs also underwent apoptosis (data not shown). Finally, nitrite as a measure of NO synthesis was determined among the supernatants of these macrophages, and the response to MTB infection was found similar among untreated C5<sup>-/-</sup> and C5<sup>+</sup> macrophages (Fig. 2C). However, C5<sup>-/-</sup> macrophages primed with IFN-γ produced more NO than C5<sup>+</sup> macrophages. These results suggest that the bactericidal activity of C5<sup>-/-</sup> macrophages depends on the concurrent presence of IFN-γ and the C5a peptide.

C5<sup>-/-</sup> macrophages have intact C5a-R but defective production of ROS

The C5a peptide binds to the C5a-R to trigger ROS in neutrophils. To confirm a similar pathway in macrophages, a surface fluorescent staining was performed to detect C5a-R, and the magnitude of ROS was measured after priming with C5a peptide and MTB infection. Because ROS synthesis readily occurred in response to peptide stimulation or infection, prior activation with IFN-γ was not necessary for these studies. C5<sup>-/-</sup> and C5<sup>+</sup> macrophages stained using an anti-C5a-R mAb to the macrophages before the addition of C5a peptide stimulation or infection, prior activation with IFN-γ; vs C5<sup>-/-</sup>, p < 0.009, Mann-Whitney U test). Affinity-purified mouse C5a (AFMC5a) had identical effects, although C5a alone had no effect on growth of MTB (data not shown). C, NO response was measured among macrophages infected with MTB or activated with 400 U/ml IFN-γ. Greiss reagent was used to detect nitrite in medium. C5<sup>-/-</sup> and C5<sup>+</sup> macrophages produced NO equally well to MTB infection; day 5 nitrite levels were higher with IFN-γ priming for both groups (*, < 0.007, Student’s t test). The horizontal dashed line shows mean levels of nitrite produced by uninfected macrophages or after addition of C5a alone (100 ng/ml).

FIGURE 1. C5-deficient mice are more susceptible to tuberculosis, and their macrophages are permissive for growth of MTB. A, Mice were infected via aerosol with ~500 CFU/mouse and sacrificed on days 0, 7, 14, and 28 for evaluation of CFU counts (4 mice/time point). C5-deficient mice (C5<sup>-/-</sup>, AJ, DBA/2) consistently contained a higher number of organisms in lungs than C5-sufficient mice (C5<sup>+</sup> and C57BL/6) (C5<sup>-/-</sup> vs C5<sup>+</sup>; **, p < 0.01, Mann-Whitney U test). B, BM-derived macrophages were infected in vitro with MTB. At the time points shown, macrophages (3 wells/mouse) were lysed and plated for CFUs on 7H11 agar. Data represent log<sub>10</sub> (mean ± SEM) CFU for triplicate wells of macrophages per mouse per time point; three mice per time point. Growth in C5-deficient (C5<sup>-/-</sup>, AJ, DBA/2) macrophages was significantly higher compared with C5-sufficient macrophages (C5<sup>+</sup>, C57BL/6; *, p < 0.004, Mann-Whitney U test).

FIGURE 2. C5<sup>-/-</sup> macrophages require both IFN-γ and C5a to kill intracellular MTB, although NO response is relatively normal. A, BMs were activated with three doses of recombinant mouse IFN-γ or a combination of 400 U/ml IFN-γ and 100 ng/ml human recombinant C5a (Hu-recC5a) for 18 h before infection. Treated and untreated macrophages were infected with MTB, and growth curves were determined over 7 days, with supplements replaced every 2 days until day 7. In experiments performed three times with similar results, each point represents the mean of log<sub>10</sub> CFU counts from plating triplicate macrophage cultures, 10<sup>6</sup> cells/well; vs C5<sup>-/-</sup>, p < 0.007, Student’s t test). The horizontal dashed line shows mean levels of nitrite produced by uninfected macrophages or after addition of C5a alone (100 ng/ml). B, C5<sup>-/-</sup> macrophages are permissive for growth of MTB. C5-deficient mice are more susceptible to tuberculosis, and their macrophages are permissive for growth of MTB.
its inhibition by the Ab suggested that, despite C5 deficiency, C5a-R was intact among C5+/− macrophages. The PKC inhibitor, Go6850 completely inhibited O$_2^•−$ production of ROS. A, Naive C5−/− or C5+/+ macrophages or C5a-treated C5−/− macrophages were stained for C5a-R using a mAb followed by FITC conjugate and analyzed by flow cytometry using a BD FACScan (BD Biosciences). All macrophages show a comparable expression of C5a-R (US, unstained cells). B, Uninfected C5−/− or C5+/+ macrophages pulsed in vitro with AFMC5a (as shown) or Hu-recC5a (similar; data not shown), respond by a transient release of superoxide (O$_2^•−$) detected using chemiluminescence, indicating that the C5a-R is intact among both types of macrophages. Addition of an anti-C5a-R Ab (1/100 dilution) reduces the luminescence, indicating that the C5a-R is intact among both types of macrophages. Addition of an anti-C5a-R Ab (1/100 dilution) reduces the superoxide response in both (C5+/−ROS vs C5+/+; * * p < 0.006, Student’s t test). Horizontal dashed line shows the background RLU of unprimed macrophages or those treated with C5a plus the PKC inhibitor Go6850. A positive control of 10 nM PMA induced a superoxide response similar to C5a after a 5-min exposure (data not shown). C, Intracellular ROS (H$_2$O$_2$ from O$_2^•−$) response was measured after MTB infection in the presence or absence of AFMC5a using DCFDA and chemiluminescence. C5+/+ macrophages show a significant ROS response with or without added C5a. C5−/− macrophages infected with MTB show reduced intracellular ROS, whereas reconstitution with C5a during infection restores significant levels of ROS ( * * p < 0.01, Student’s t test). Untreated macrophages show background fluorescence levels of <100 AFUs. The PKC inhibitor Go6850 plus C5a-primed macrophages also yielded AFUs 1 <100. Both are indicated by the horizontal dashed line.

The assembly of phox complex on mycobacterial phagosomes of C5+ and C5−/− macrophages

The various components of the phox complex assemble on bacterial phagosomes to generate O$_2^•−$ and Abs to p47phox and p67phox components have been used to study the assembly (33–36). Preliminary studies indicated that in the absence of IFN-γ activation, phox complex did not translocate onto gfpMTB phagosomes (data not shown). Thus, C5+/+ and C5−/− macrophages were activated with IFN-γ (400 U/ml) or IFN-γ plus C5a peptide (for C5−/− macrophages) for 18 h before infection with gfpMTB and Ab stains. It is pertinent to note here that we anticipated differences between gfpBCG and gfpMTB phagosomes because of their difference in virulence. However, gfpBCG showed a staining pattern nearly identical with that found for live gfpMTB containing phagosomes. Thus, fluorescent profiles are illustrated only with gfpMTB. Staining with p47phox has been illustrated in Fig. 4A. In C5−/− macrophages, there was a lack of vesicular staining near phagosomes or in the cytosol (Fig. 4A, top panels). In contrast, there was a consistent and uniform colocalization of p47phox around the mycobacterial phagosomes in C5+ macrophages (Fig. 4A, middle panels). Addition of C5a to C5−/− macrophages restored the colocalization of phox proteins with gfpMTB phagosomes (Fig. 4A, bottom panels). Although p47phox Ab mostly stained in a spherical or vesicular pattern, p67phox Ab stained cylindrical or tubular structures. Both, however, stained structures closely overlapping phagosomes. Phagosomes scored positive or negative for colocalization with p47phox in quadruplicate experiments are shown in Fig. 4B. It was of interest to determine whether other C5-deficient mice had defects comparable to C5−/− mice. Thus, macrophages from A/J and DBA/2 were also probed for phox localization. Fig. 4C confirms that these mice also had similar defects in colocalization of phox on MTB phagosomes. It was interesting to observe by immunostains that unactivated naive macrophages did not express phox proteins, whereas those with phagosomes expressed strong staining. This suggested that either naive macrophages required activation to make enough phox proteins or that their levels in naive cells were undetectable by immunostains.

Membrane translocation of phosphorylated p47phox

Naive macrophages have low levels of phox proteins in the cytosol. Investigators have analyzed the membrane translocation of phosphorylated phox proteins that occurs upon macrophage activation (31, 32). Membrane translocation studies are not required when phagosomes are identifiable (as in this study) and colocalization can be demonstrated by microscopy. However, we found that IFN-γ was required for colocalization of phox proteins with gfpMTB. Also, it was difficult to demonstrate phosphorylated p47phox on gfpMTB phagosomes.

Therefore, we tested the hypothesis that IFN-γ could enhance the translocation of phosphorylated phox from cytosol to membranes. Preliminary studies showed that fractionated membranes contained intact bacteria by electron microscopy (data not shown). In an experiment performed three times, macrophage membranes and cytosol were labeled with Abs for p47phox. Fig. 5 illustrates the profile, where the upper lanes show membrane localization for p47phox while the bottom lane shows the β-actin control. Naive uninfected macrophages did not show detectable levels of phosphorylated p47phox. MTB infection induced accumulation of abundant phosphorylated p47phox in the cytosol but not the membranes.
Similar staining was observed with macrophages show a strong staining for infected C5
FIGURE 4. effects. The conjugate. AFMC5a or Hu-recC5a were added where indicated with similar
jugated with goat Abs to p47phox protein, followed by rabbit anti-goat IgG-Texas Red conjugate. The left vertical panel shows gfpMTB, and the right vertical panels show identical fields, demonstrating the localization of red staining complex of the p47phox component of NADPH oxidase on MTB phagosomes. Similar staining was observed with p67phox (data not shown). Only C5+/+ macrophages show a strong staining for phox proteins around gfpMTB phagosomes (arrows), whereas addition of C5a to C5−/− restores p47phox labeling of gfpMTB. B, Percentage of positive gfpMTB phagosomes were scored for colocalization with p47phox 24 h postinfection in four independent experiments. (*, p < 0.008 vs C5−/−, **, NS vs −/+; Student’s t test). At least 200 microscopic fields were scored per mouse macrophage preparation, three mice per experiment per strain and mean percentage (±SEM) from four independent experiments are shown. Each microscopic field contained 5–6 macrophages, of which 3–4 contained at least 1–2 gfpMTB. Complementation was performed in the presence of 100 ng/ml AFMC5a (or Hu-recC5a) and 400 U/ml of IFN-γ. Cytosol and membrane fractions collected at 24 h were analyzed by Western blot using Abs specific to either p47phox or p67phox (both showed same pattern; p47phox illustrated). MTB induces p47phox in the cytosol of C5+/+ macrophages but requires IFN-γ to translocate to membranes. A combination of IFN-γ with C5a is required to induce p47phox and translocation in C5−/− macrophages. Actin lanes are shown to control for protein loads. One of three independent experiments with similar results is shown.

Phosphorylation of PKC isoforms during C5 deficiency

Several isoforms of PKC are involved in the phosphorylation of the p47phox protein that initiates the assembly of the phox complex (31, 32). However, previous studies suggest that the isoforms vary depending upon the type of macrophages and their activation. Because the assembly of phox proteins was defective in C5−/− macrophages, we tested the hypothesis that there may be a defect in the phosphorylation of PKC consequent to the lack of signaling via C5a-R. C5−/− and C5+/+ macrophages were tested naive and infected with MTB with or without C5a peptide. For each PKC isoform shown in Fig. 6, macrophage lysates were probed with Abs to native PKC (upper lane, native PKC) and phosphorylated isoform shown in Fig. 6, macrophage lysates were probed with Abs to native PKC (upper lane, native PKC) and phosphorylated PKC (lower lane, phos-PKC). Uninfected C5−/− and C5+/+ macrophages demonstrated steady-state expression of several PKC isoforms as reported before for murine macrophages (32). Various

A
C

FIGURE 5. Differential translocation of p47phox onto membranes in C5−/− and C5+/+ macrophages. C5−/− and C5+/+ macrophages were infected with MTB in the presence or absence of 100 ng/ml AFMC5a (or Hu-recC5a) and 400 U/ml of IFN-γ. Cytosol and membrane fractions collected at 24 h were analyzed by Western blot using Abs specific to either p47phox or p67phox (both showed same pattern; p47phox illustrated). MTB induces p47phox in the cytosol of C5+/+ macrophages but requires IFN-γ to translocate to membranes. A combination of IFN-γ with C5a is required to induce p47phox and translocation in C5−/− macrophages. A, Percentage of positive gfpMTB phagosomes were scored for colocalization with p47phox 24 h postinfection in four independent experiments. (*, p < 0.008 vs C5−/−, **, NS vs −/+; Student’s t test). At least 200 microscopic fields were scored per mouse macrophage preparation, three mice per experiment per strain and mean percentage (±SEM) from four independent experiments are shown. Each microscopic field contained 5–6 macrophages, of which 3–4 contained at least 1–2 gfpMTB. Complementation was performed in the presence of 100 ng/ml AFMC5a (10 ng/ml) or Hu-recC5a (100 ng/ml). C. Macrophages from C5-deficient A/J and DBA/2 mice have comparable defects in p47phox localization on MTB phagosomes compared with C5 intact C5BL/6 mice (*, p < 0.007 vs C57BL/6, **, p < 0.09 vs C57BL/6 for 4 and 24 time points, respectively; Student’s t test).
protein loads were then tested and, at protein concentrations between 1 and 5 µg per lane, detection of all isoforms was found reproducible. Replicate blots loaded with five times more protein were then probed with Abs specific for phosphorylated PKC. Fig. 6 illustrates one of three experiments performed with similar results. C5+/+ and C5−/− macrophages showed comparable phosphorylation of PKC-ε, PKC-η, and PKC-θ after infection with MTB. However, C5−/− macrophages strikingly lacked the PKC-ζ phosphorylation that was restored through complementation with C5a peptide. A polyclonal Ab for PKC (α, β, δ) shows reduced phosphorylation in C5−/− after MTB infection that is restored after C5a reconstitution. One of three independent experiments is shown with similar results.

**Phagosomal translocation of PKC-β in C5+/+ and C5−/− macrophages**

Because the polyclonal Ab detected differential phosphorylation in PKC (α, β, δ) but was unable to discriminate between them, we tested another approach. Besides being involved in the phosphorylation of p47phox, PKC-β has been found to translocate onto zymosan bead phagosomes in neutrophils (37). This suggested that its physical localization on MTB phagosomes could be used to identify which PKC isoform was nonfunctional in C5−/− macrophages. C5+/+ and C5−/− macrophages were infected and stained individually with mAbs specific for PKC-α, -β, -δ, and -ζ and counterstained with Texas Red conjugates. Staining of phagosomes for PKC-α, -δ and -ζ was poor (data not shown). Fig. 7A shows that PKC-β was absent on gfpMTB phagosomes of unactivated C5−/− macrophages, but prominently identifiable in C5a-reconstituted macrophages. Fig. 7B represents quantitative scoring of PKC-β distribution before and after reconstitution. These studies strongly suggest that, of the four isoforms of PKC that are defective in C5−/− macrophages, PKCβ is likely to play a major role during the phox assembly.

**NT staining on phagosomes**

It is well established that IFN-γ activates iNOS and induces NO. Studies described above showed that phox localizes to phagosomes after IFN-γ activation. We therefore hypothesized that NO and O$_2^-$ generated locally could synergize to produce ONOO that could become detectable as NT. C5−/− macrophages lacked NT after IFN-γ activation (Fig. 8A, top panel) but acquired NT after coinubcation with C5a and IFN-γ (Fig. 8A, middle panel). C5+/+ macrophages stained positive for NT after IFN-γ alone (Fig. 8A, bottom panel). The percentage of positive cells increased with time (4 h post infection; Student’s t test, p < 0.01) (**). Fig. 8B shows that C5a enhances PKC-β localization C5−/− macrophages.

**FIGURE 6.** Defective phosphorylation of PKC isoforms in C5−/− and C5+/+ macrophages. C5−/− and C5+/+ macrophages were tested for the presence of naive or phosphorylated cytosolic PKC isoforms in the cytosolic fractions of naive or MTB-infected macrophages in the presence or absence of 100 ng/ml AFMC5a (or Hu-recC5a) harvested 60 min after phagocytosis. PKC isoforms were analyzed by Western blot using Abs specific for naive forms of PKC or their phosphorylated forms. Upper lanes show the steady-state expression of naive PKCs in C5−/− and C5+/+ macrophages. Differences exist at the level of phosphorylation. C5−/− as well as C5+/+ macrophages show the phosphorylation of PKC-ε, -η, and -θ. However, PKC-ζ is not phosphorylated in C5−/−, even after supplementation with C5a. A polyclonal Ab for PKC (α, β, δ) shows reduced phosphorylation in C5−/− after MTB infection that is restored after C5a reconstitution. One of three independent experiments is shown with similar results.

**FIGURE 7.** Localization of protein kinase-β on MTB phagosomes. A. Macrophages were infected with gfpMTB, fixed at 4 h after phagocytosis, permeabilized, and stained with an Ab specific for mouse PKC-β, followed by a Texas Red conjugate. PKC-β is not demonstrable in C5−/− macrophages (top panels) but clearly visible within C5+/+ macrophages. B. Percentage of positive gfpMTB phagosomes were scored for colocalization with PKC-β in C5−/− and C5+/+ macrophages before and after reconstitution with C5a peptide. C5a enhances PKC-β localization C5−/− macrophages (*, p < 0.01 vs C5+/+; **, NS vs C5+/+; Student’s t test).
were negative for NT, even after IFN-γ activation. Macrophages were fixed and stained for NT, a marker for ONOO synthesis, and analyzed for colocalization using laser confocal microscopy. gfpMTB phagosomes in C5−/− macrophages are negative for NT staining, even after IFN-γ plus C5a (middle panels). gfpMTB within C5−/− macrophages show a staining for NT after IFN-γ activation (bottom panels). One of three independent experiments is shown. B, Percentage of positive gfpMTB phagosomes were scored for staining for NT. C5a complementation enhances NT staining in C5−/− macrophages (*, p < 0.009 vs C5 C5−/− macrophages with IFN-γ without C5a; Student’s t test).

Bottom panel). Unactivated macrophages of either phenotype did not show NT staining (data not shown). Macrophages were then scored for NT staining, and percentage of positive phagosomes was determined in quadruplicate experiments. Fig. 8B shows that complementation with C5a was able to restore NT staining in a significant proportion of gfpMTB phagosomes. In contrast with phox protein stains, however, nearly 50% of MTB phagosomes were negative for NT, even after IFN-γ and C5a stimulation. Although the sensitivity of immunostaining could be a limiting factor for decreased frequency of staining, these studies do suggest that C5a may help during the synergistic synthesis of ONOO from NO and O2−.

Fc receptor-mediated translocation of phox

Foregoing studies strongly suggested that ROS synthesis was defective due to C5a deficiency. To rule out a global deficiency, we further analyzed Fc receptor-dependent induction of ROS. C5−/− macrophages showed a strong localization of p47phox after phagosytosis in the presence of immune but not preimmune serum in a pattern similar to that shown earlier for MTB phagosomes. The colocalization data suggest that Fc receptor-mediated mechanism of phox localization was comparable in C5−/− and C5+/+ macrophages (Fig. 9). Finally, we tested the possibility that minor LPS contamination in C5a peptide could affect ROS. Incubation of macrophages with LPS, however, failed to induce phox localization to membranes in C5−/− macrophages (data not shown). These studies together suggest that ROS defect was specific to C5a deficiency in C5−/− macrophages.

Discussion

It has been well established that C5-deficient mice like A/J are more susceptible to diverse intracellular pathogens like Listeria monocytogenes and MTB (7, 38). This study describes a novel intracellular defect that appears to be common for C5-deficient mice like A/J, DBA/2, and B.10-derived C5 congenics (7). Although all three strains had enhanced susceptibility to tuberculosis, we selected the C5 congenic mice for in-depth studies, because their genetic background was the same except for a single 2-bp deletion within the C5 gene for the C5−/− strain (8).

The bactericidal activity of murine macrophages is generally dependent on the induction of NO because IFN-γ induces iNOS, and iNOS inhibitors neutralize the effects of IFN-γ. This study initially demonstrated that the IFN-γ had an incomplete activation effect on macrophage bactericidal function in the absence of C5a peptide. This suggested that either IFN-γ induced NO-dependent mechanism of killing was defective in C5−/− macrophages or that C5a induced additional bactericidal mechanisms. However, IFN-γ induces significant NO in both but lesser peak levels among C5−/− macrophages, whereas MTB-induced NO responses were comparable. Although absence of C5a did not seem to affect the NO response, the synergy between C5a and IFN-γ suggests that a molecular connection existed between these two macrophage activators that facilitated killing of MTB. It is pertinent to recall here that, although IFN-γ has been extensively reported to induce iNOS and phagosome-lysosome fusion, it has also been reported to activate phox activity in murine macrophages (39). We therefore examined the hypothesis that C5a is an innate regulator of bactericidal function in macrophages that may synergize with the activation effects of IFN-γ that is usually an acquired immunity mediator. Our studies expanded on the observation that C5a is a known activator of respiratory burst in neutrophils as well as cells of the monocytic lineage, and its absence could lead to defects in the synthesis of ROS (40–42). C5−/− macrophages indeed secreted significant levels of O2− after in vitro priming with C5a peptide (Fig. 3A). ROS response could be blocked by an Ab to C5a-R, confirming that C5a triggered ROS via C5a-R. After phagocytosis in both neutrophils and macrophages, the phox complex assembles on phagosomal membranes to generate O2− that is in turn converted to H2O2. Depending upon the availability of iron, H2O2 is subsequently converted to hydroxyl radical (OH·). Consistent with this, C5−/− macrophages containing MTB phagosomes produced significant levels of intracellular ROS, whereas MTB-infected C5−/− macrophages showed markedly reduced intracellular ROS. The fact that the lack of C5a could have affected ROS production was evidenced by the restoration of ROS response after reconstitution with C5a.

These observations led us to propose that the partial efficacy of IFN-γ and C5a in C5−/− macrophages was due to the activation...
of the NO and ROS pathway, respectively, and that they synergized against MTB (Fig. 2, A and B). Mechanisms of phox targeting were investigated, and initial studies showed that live virulent MTB excluded phox complex in naive macrophages. Because the nonpathogen *Mycobacterium smegmatis* readily acquired phox in macrophages (43, 44), this suggested that MTB is able to prevent the acquisition of phox. Separate studies in progress have indicated that MTB inhibits PKC activation in macrophages, and this effect could be overcome with IFN-γ in macrophages (our unpublished observations). The exclusion of phox by MTB was unexpected. However, it was consistent with the propensity of live virulent MTB organisms to localize within phagosomes that avoid fusion with lysosomal hydrolyses but selectively fuse with some endocytic compartments (45–48). Next, macrophages are activated by IFN-γ, and we reasoned that if C5a-driven ROS and IFN-γ-mobilized NO pathways synergized, they may target phagosomes to kill MTB within. The assembly of phox complex on MTB phagosomes therefore provided a mechanism of targeting ROS. This appeared logical because previous studies showed that the phox complex assembled around the phagosomes of *gfp*-expressing *Salmonella* mutants that were susceptible to the action of ROS (33, 36). The latter studies used immunostains to localize *p47phox* and *p67phox* proteins of the phox complex. Our recent studies showed that phox proteins assembled around *M. smegmatis* phagosomes, and their assembly was enhanced on mutant phagosomes that were more susceptible to oxidants (44). We used a similar strategy to colocalize phox in and around MTB phagosomes. Interestingly, the phox complex rapidly assembled around MTB phagosomes in C5−/− macrophages as early as 4–8 h postinfection and was found on nearly all phagosomes by 72 h (Fig. 4A). In striking contrast, C5−/− macrophages lacked such colocalization. Complementation of the C5−/− macrophages with C5a peptide restored phox colocalization with phagosomes, supporting its regulatory role (Fig. 4B).

Subsequent studies addressed the mechanism underlying defective assembly. The phox complex can be triggered by a number of ligand-receptor interactions in macrophages. However, the PKC isoforms occupy a pivotal position in signal transduction events that precede phox assembly (17, 29, 49–51). Although both C5−/− and C5+/+ macrophages showed a steady-state expression for several isoforms of PKC (α, β, δ, ε, η, θ, and ζ), anti-phospho-PKC indicated that C5−/− macrophages had defective phosphorylation localized to α, β, δ, and ζ (Fig. 6). PKC-β was then found on MTB phagosomes (Fig. 7A), suggesting that PKC-β and PKC-ζ were the major isoforms that were not phosphorylated among C5−/− macrophages. However, the participation of PKC-α, -δ, -ζ, or other as yet unidentified isoforms in the phosphorylation of *p47phox* cannot be ruled out due to limitations in detection methods and approaches.

Incidentally, activation of PKC isoforms may also be critical for other manifestations of macrophage activation that can lead to control of mycobacteria. PKC-α and PKC-β have been implicated in regulation of phagocytosis, release of O2−, and TNF-α during the differentiation of human THP1 macrophages. PKC-β was also reported to be a key mediator during the differentiation of promonocytic HL60 cells into monocytes (52). Nonetheless, events leading to defective phosphorylation of PKC in C5−/− macrophages remain somewhat unclear. C5a binding to the rhodopsin family of G protein-coupled receptors elicits a pertussis toxin-sensitive activation of phospholipase C, which in turn activates PKC (53). Perhaps, absence of C5a may cause lack of activation of phospholipase C and thereby PKC.

Because C5-deficient macrophages had more growth of MTB, it is pertinent to discuss the role of ROS in defense against mycobacteria. Although a number of in vitro studies have shown that ROS, like H2O2, can kill MTB under various conditions, the rather high concentrations of ROS required to significantly kill MTB in vitro has led to a speculation that such concentrations may not be achievable within macrophages. However, previous studies in neutrophils have demonstrated that as much as 5–10 nM of O2− per second could be generated by the phox complex (15). Thus, in a confined space like that of phagosomes, phox complex can generate ROS (O2−; H2O2) that can attain very high levels, potentially achieving mycobacterial capacity. Of course, a combination of O2− with NO can lead to ONOO− formation, and more effective killing and evidence for the formation of ONOO was found in this study. In addition, H2O2 can combine with iron to form the lethal OH− radical or other derivatives that are a part of the ROS cascade. However, tuberculosis is a disease where the lethal effects of host-produced ROS are neutralized by the pathogen. Thus, MTB is known to shed a variety of lipidaceous oxygen scavengers and secrete prolific quantities of catalase, superoxide dismutase, and peroxidase enzymes that can negate the lethal effects of ROS (54, 55). Mycobacterial sulfatides were earlier shown to interfere with the expression of PKC and ROS expression of infected monocytes (56). Furthermore, live virulent but not dead MTB down-regulated the mitochondrial cytochrome oxidase gene in murine macrophages (57). These observations suggest that the ultimate survival of MTB within macrophages is decided both by the ability of the pathogen to counter the host response and defects in the ability of the host to produce an oxidant response. This concept is supported by the recent observation that the katG mutant of MTB survived better in *gfp*phox-deficient mice (58).

However, it is important to stress here that C5 deficiency alone may not account for the hypersusceptibility of C5-deficient mice to tuberculosis. For example, restoring C5 did not fully confer resistance to *Listeria in A/J* mice (38, 59). Furthermore, *phox*-deficient mice that have an absolute defect in the ROS production showed only an early susceptibility to tuberculosis (60). Thus, C5-deficient mice may have other defects that contribute to susceptibility. C5a is a known chemoattractant for monocytes, dendritic cells, and T cells, and has been found to induce maturation of DCs (24, 61, 62). We demonstrated earlier that C5-deficient mice had defective formation of lung granulomas during tuberculosis (9). C5-deficient SWR mice have also been found to have a defective influx of T cells into the lungs after MTB infection (11). Together, these observations suggest that the defect in phox targeting may combine with other defects in containment of tuberculosis to result in the susceptibility phenotype of the C5-deficient mice.

Interestingly, C5 deficiency may in part explain the enhanced susceptibility of wild-type Nram1 mice. Iron that has been pumped into the phagosomal lumen by Nramp1 may not be used by defective ROS cascade in these mice. As a result, iron may promote the growth of mycobacteria instead of generating the bacterial ·OH radical in wild-type Nram1 mice like A/J and DBA/2. Clearly, additional studies are needed to further define the role of metal ions in the context of defective macrophage function.

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**Disclosures**

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