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Complement Receptor Type 3 Mediates Phagocytosis and Killing of *Listeria monocytogenes* by a TNF-α- and IFN-γ-Stimulated Macrophage Precursor Hybrid[1]

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Previous work demonstrated that engagement of complement receptor type 3 (CR3) was required for inflammatory peritoneal macrophages to phagocytose and kill the facultative intracellular bacterium *Listeria monocytogenes*. The experiments described here tested the role of CR3 in phagocytosis and killing of *Listeria* by a clonal population of TNF-α/IFN-γ-stimulated macrophage precursor hybrids. Stimulation with TNF-α and IFN-γ increased CR3 expression 20-fold and induced a big increase in phagocytic activity. Phagocytosis and killing of *Listeria* by these cells were inhibited when bacteria were opsonized with complement-depleted serum or by incubation of the macrophages with anti-CR3 mAb. Furthermore, cytokine-stimulated macrophages could not kill *Listeria* opsonized with heat-inactivated anti-*Listeria* antiserum, indicating that macrophage receptors which mediate phagocytosis do not necessarily promote bactericidal activity. These data suggest that upregulation of CR3 and CR3-mediated phagocytosis are mechanisms by which TNF-α and IFN-γ stimulate nonphagocytic, nonbactericidal macrophage precursors to kill intracellular bacterial pathogens.

INTRODUCTION

Macrophages are important effector cells for resolving infection by the facultative intracellular bacterium *Listeria monocytogenes*. Whereas many macrophages can phagocytose *Listeria*, the ability to kill these bacteria is not a common characteristic (1, 2). Current models suggest that macrophage listericidal activity can be induced by certain cytokines (reviewed in 3). *In vivo* studies using mice depleted of specific cytokines by antibodies or genetically altered mice that are deficient in production of specific cytokines or their receptors have shown that tumor necrosis factor-α (TNF-α)[4] and interferon gamma (IFN-γ) are required for host defense during primary *Listeria* infection (4–8). Despite this body of evidence the mechanisms by which these cytokines induce host resistance are not well-understood.

Previous work from this laboratory showed that complement receptor type 3 (CR3) is important for phagocytosis and killing of *Listeria* by bactericidal mouse peritoneal exudate cells (9–11). Nonbactericidal macrophages appear to use alternative receptors for phagocytosis (10). Recently we described a macrophage precursor hybrid, W1C3, that requires sequential stimulation with TNF-α and IFN-γ for induction of phagocytic and bactericidal activity against *Listeria* (12). This model indicates that these cytokines induce macrophage bactericidal activity by acting directly at the level of the macrophage, rather than acting through other cells. Because this is a clonal population of cells stimulated by a defined regimen of cytokines, it offers a unique system in which to ask mechanistic questions about how TNF-α and IFN-γ stimulate macrophage bactericidal activity. In the experiments reported here we used these cells to test our hypothesis that TNF-α and IFN-γ induce phagocytosis through CR3, and that

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[4] Abbreviations used: TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ, CR3, complement receptor type 3; TPB, tryptose phosphate broth; NMS, normal mouse serum; HIMS, heat-inactivated mouse serum; BSS, balanced salt solution; mAb, monoclonal antibody; RNI, reactive nitrogen intermediates.

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this is one mechanism required for the induction of listericidal activity by these cells.

MATERIALS AND METHODS

Bacteria. *L. monocytogenes* strain EGD, originally obtained from G. B. Mackaness, was kept frozen at −70°C in tryptose phosphate broth (TPB) with 15% glycerol. Prior to use, an aliquot was thawed and 30 μl was inoculated into 4 ml TPB and incubated overnight at 37°C with shaking.

Serum. Normal mouse serum (NMS) was obtained from anesthetized C57Bl/6 mice by cardiac puncture. Following separation from whole blood, serum was frozen at −70°C until used. Heat-inactivated mouse serum (HIMS) was made by incubating normal serum at 56°C for 30 min. We have previously shown by immunofluorescence and fluorimetry that *Listeria* incubated in HIMS do not have C3 deposited on their surface (9). Mouse anti-*Listeria* anti-serum was made by repeated injections of 1×10^3–1×10^4 live *L. monocytogenes*, strain EGD, into BDF_1 mice with a 2-week rest period between injections. The presence of anti-*Listeria* antibody was demonstrated by immunofluorescence as previously described (9).

Cells and culture conditions. The W1C3 macrophage precursor hybrid cell line was clonally derived after fusion of M-CSF-stimulated bone marrow-derived macrophages with HAT-sensitive variants of the WEHI-3B myeloid cell line (13). Cells were maintained in logarithmic phase in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% FCS (HyClone Laboratories, Ogden, UT), 2 m*M* L-glutamine, and 20 μM 2-ME, or DMEM (Gibco) with 10% FCS. No antibiotics were added to the culture medium (14). Cytokine stimulation was performed as described previously (12). Briefly, 3×10^6 W1C3 cells/ml were cultured in 100-mm tissue culture dishes at 2×10^5 cells/ml. Then 400 U/ml recombinant murine TNF-α was added on the 1st day followed by 100 U/ml recombinant murine IFN-γ 24 hr later. The W1C3 cells were cultured for an additional 48 hr, then their phagocytic and bactericidal activities were measured.

Phagocytosis. Phagocytosis of *Listeria* by cytokine-stimulated and unstimulated W1C3 cells was measured as previously described (9, 12). Briefly, cells and bacteria were mixed together at a 1:10 ratio in BSS containing 5% NMS or HIMS or without serum in the diluent, BSS (15), and incubated with continuous rotation for 30 min. The cells were washed and cytocentrifuge preparations were made and stained with Diff-Quik (Baxter Healthcare Corp., Hialiah, FL), then the cells were evaluated using light microscopy under oil immersion (1000×). Phagocytosis was quantified by counting 150–200 cells per slide and is represented by a phagocytic index calculated as (% cells with $\geqslant$ one bacterium) × (mean bacteria/positive cell). Where indicated, the cells were incubated with 3.0 μg/ml (final concentration) mAb prior to incubation in NMS; this mAb concentration was found previously to cause maximal inhibition of CR3-dependent phagocytosis.

Bactericidal assay. To test the effect of inhibition of CR3-mediated phagocytosis on bacterial killing, a bactericidal assay was used as previously described (11). Briefly, 1×10^6 cytokine-stimulated W1C3 cells were incubated without or with 3.0 μg/ml mAb (final concentration) for 45 min at 7°C. The cells were then mixed in the cold for 20 min with 1×10^6 *Listeria* which had been opsonized by incubation in BSS plus 5% NMS or HIMS, then cooled. Next, unbound bacteria were removed by washing the cells twice, then centrifuging them through a 1-ml layer of 30% sucrose. The cells were resuspended in medium with 5% NMS or HIMS and 100 μl was removed for serial dilution and plating on agar to determine CFU *Listeria* initially present (T-0). The remaining cells were incubated at 37°C for 90 min to allow internalization of bound bacteria and subsequent intracellular growth or killing. A second 100-μl aliquot was removed at the end of 90 min for plating as above. In experiments in which mouse anti-*Listeria* antiserum was used, all reagents were mixed together at Time 0, and samples were taken at both T-0 and T-90. Bacterial growth was detected by an increase in CFU from T-0 to T-90, bacterial killing resulted in a decrease in CFU.

Flow cytometry. Cytokine-stimulated or unstimulated W1C3 cells were incubated for 30 min at room temperature in round-bottom 96-well plates (10^6 cells in 25 μl) with the indicated mAb. The cells were washed and stained with the optimum dilution of FITC-labeled $F(ab')_2$ fragments of rabbit anti-rat IgG (Cappel, Organon Teknika, Durham, NC). After each labeling step, the cells were washed three times with PBS containing 1% bovine serum albumin and 0.1% azide. Cellular fluorescence was assessed using a FACScan flow cytometer (Becton–Dickinson, Sunnyvale, CA). Antibody binding to the cells is semiquantitatively expressed as molecules of equivalent soluble fluorochrome based on interpolation of median fluorescence on a standard calibration curve generated using microspheres with known fluorescence intensity according to the manufacturer's instructions (Flow Cytometry Standards Corp., San Juan, Puerto Rico). Since the clonal population of W1C3 cells showed homogeneous antigen-expression profiles, antibody binding to the whole population is reported rather than percentage of positive cells.

Antibodies. The hybridomas producing M1/70 (anti-Mac-1/CR3, IgG2b), M3/38 (anti-Mac-2, IgG2a), and M5/114 (anti-MHC class II, IgG2b) mAbs were obtained from the American Type Culture Collection

TABLE 1

CR3 Expression on W1C3 Macrophage Hybrids Is Increased by Cytokine Stimulation

Marker	Cytokine stimulation			
	None	TNF-α 72 hr	IFN-γ 48 hr	TNF-α/IFN-γ
CR3 (CD11b)	3.3 ± 0.3[a]	7.3 ± 2.0	11.0 ± 2.8	68.5 ± 28.6
MHC class II	0.0 ± 0[b]	0.1 ± 0.1[b]	40.2 ± 15.4	24.2 ± 5.3
Mac-2	0.4 ± 0.4	1.8 ± 1.0	0.6 ± 0.4	20.8 ± 9.6

[a] Mean ± SEM molecules of equivalent soluble fluorochrome × 10^4 from four separate experiments.
[b] Below detectable limits.

(Rockville, MD). The rat anti-mouse CR3 mAb 5C6 (IgG2b) was purchased from Serotec (Oxford, UK) (18). The rat anti-mouse CR1 mAb 8C12 was a generous gift from T. Kinoshita (19). The rat anti-mouse tissue macrophage mAb BM8 (IgG2a) (20) was kindly provided by BMA Biomedicals Ag (Augst, Switzerland). Finally, the hybridoma-producing rat anti-mouse transferrin receptor, CD71 mAb, ER-MP21 (IgG2a), was generated as described before (21). For bacterial phagocytosis and killing experiments M1/70 was used as $F(ab')_2$ fragments made from protein G-purified IgG; all other antibodies were used in these experiments as intact, purified IgG. For flow cytometry experiments, mAbs were either applied as hybridoma culture supernatants or as optimally titrated purified IgG.

RESULTS

Cytokine Treatment Increases CR3 Expression and CR3-Mediated Phagocytosis

Previous experiments showed that stimulation of W1C3 macrophage hybrid cells with TNF-α and IFN-γ induced these nonphagocytic macrophage precursors to phagocytose *Listeria* (12). Thus we tested the effect of these cytokines on cellular CR3 expression and whether this receptor was involved in phagocytosis of *Listeria*. Table 1 shows the results of flow cytometry measurements of CR3 expression on W1C3 cells following stimulation with either TNF-α or IFN-γ alone or following sequential stimulation with both cytokines. Stimulation with either TNF-α or IFN-γ alone resulted in a modest increase in CR3 expression, approximately 2-fold for TNF-α and 3-fold for IFN-γ. In contrast, CR3 expression increased 20-fold in cells sequentially stimulated with both cytokines. In addition to increased CR3 expression, increased expression of the mature macrophage marker Mac-2 was evidence that macrophage maturation was caused by cytokine stimulation. As expected, upregulation of MHC class II antigens by IFN-γ also occurred.

Next we tested the role of serum complement and macrophage CR3 in phagocytosis of *Listeria* by cytokine-stimulated W1C3 cells. For this we measured phagocytosis of *Listeria* in the presence and absence of complement or anti-CR3 mAb by W1C3 cells stimulated with TNF-α, IFN-γ, or both (Table 2). As reported earlier, phagocytosis by unstimulated cells was negligible, but was increased by stimulation with either cytokine alone and synergistically by both (12). The important role of complement in phagocytosis of *Listeria* by these stimulated W1C3 cells was demonstrated by the finding that phagocytosis in the absence of serum or in heat-inactivated serum was only 7–17% of the level observed in the presence of normal serum. When CR3 was blocked by the anti-CR3 mAb M1/70, phagocytosis of NMS-opsonized *Listeria* was significantly inhibited whether the cells were stimulated with either cytokine or with both TNF-α and IFN-γ. Moreover, a second mAb, 5C6, which binds to a different epitope on

TABLE 2

Roles of Complement and CR3 in Phagocytosis of *Listeria* by W1C3 Macrophage Hybrids

Cytokine stimulation	Phagocytosis conditions[a]	Phagocytic index[b]
None	NMS	7 ± 2
TNF-α 72 hr	NMS	273 ± 55
	HIMS	16 ± 7
	BSS	5 ± 4
	NMS + anti-CR3 mAb[c]	50 ± 6
IFN-γ 48 hr	NMS	220 ± 34
	HIMS	12 ± 7
	BSS	6 ± 3
	NMS + anti-CR3 mAb	16 ± 6
TNF-α/IFN-γ	NMS	1765 ± 261
	HIMS	300 ± 203
	BSS	119 ± 98
	NMS ± anti-CR3 mAb	477 ± 150

[a] Cells and bacteria were incubated 1:10 in 5% NMS or HIMS or without serum in BSS alone for 30 min at 37°C.
[b] Cytocentrifuge preparations of washed cells were stained with Diff-Quik and phagocytosis was quantified using light microscopy. Phagocytic index (PI) was calculated by counting bacteria associated with 200 cells/slide as (% phagocytic cells) × (mean bacteria/positive cell). Results are the mean PI ± SEM of three separate experiments.
[c] Cells were incubated in 3.0 μg/ml (final conc) M1/70 before mixing with bacteria.

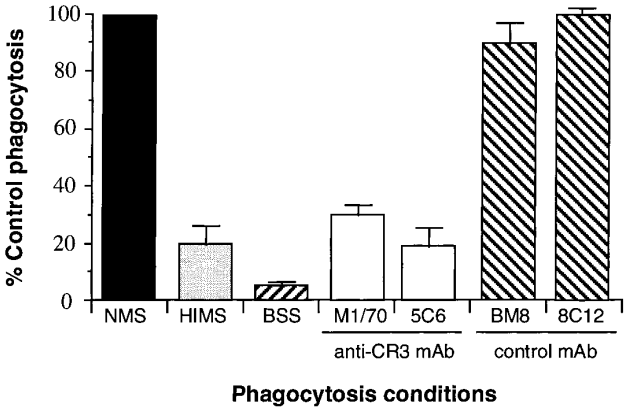

FIG. 1. CR3-mediates phagocytosis of *Listeria* by TNF-α and IFN-γ-stimulated W1C3 macrophage hybrids. W1C3 macrophage hybrids were stimulated with 400 U/ml TNF-α for 24 hr then 100 U/ml IFN-γ was added for another 48 hr. Cells and bacteria were mixed 1:10 for 30 min in 5% NMS (positive control), or HIMS, or no serum (BSS), or with NMS plus 3.0 μg/ml anti-CR3 or control mAb. Cytocentrifuge preparations of washed cells were stained with Diff-Quik. Phagocytosis was quantified by counting 150–200 cells per slide by light microscopy. Phagocytic index (PI) was calculated as the (% phagocytic cells) $\times$ (mean bacteria/positive cell). Results are the mean % control PI $\pm$ SEM from three separate experiments.

CR3 than does M1/70, inhibited phagocytosis slightly better than did M1/70 (Fig. 1). By comparison, the anti-CR1 mAb 8C12, and a control anti-mouse macrophage mAb, BM8, had essentially no effect on uptake of *Listeria* by TNF-α/IFN-γ-stimulated W1C3 cells. Taken together these data demonstrate that the dramatic increase in phagocytic activity of W1C3 cells caused by sequential stimulation with TNF-α and IFN-γ was primarily caused by increased CR3 expression and CR3-mediated phagocytosis.

Role of CR3-Mediated Phagocytosis in Bactericidal Activity

The second series of experiments tested whether CR3-mediated phagocytosis was important for bactericidal activity of TNF-α/IFN-γ-stimulated W1C3 macrophage hybrid cells. First we measured killing of *Listeria* in the presence of NMS, HIMS, and NMS plus anti-CR3 mAb or control mAb. Figure 2 shows that stimulated W1C3 cells killed NMS-opsonized *Listeria* very well as shown by a mean $\pm$ SEM decrease in $\log_{10}$ CFU *Listeria* of 0.62 $\pm$ 0.09 (n = 3). Bacterial killing was decreased by 50% when these cells phagocytosed *Listeria* which had been opsonized in complement-depleted serum. Killing was also inhibited when the cells were incubated with the anti-CR3 mAb M1/70. A different anti-CR3 mAb, 5C6, inhibited killing even more than did M1/70 and in fact induced bacterial growth. Bactericidal activity was not affected by control mAbs 8C12 or BM8. These data indicate that phagocytosis of bacteria through CR3 is required for optimal listericidal activity by TNF-α and IFN-γ-stimulated macrophage hybrids.

An alternative explanation to these experiments could be that, when the anti-CR3 mAb bound to macrophage CR3, this event triggered an "inhibitory signal" which abrogated cellular bactericidal activity. Thus the final experiments tested whether these macrophage hybrids could kill *Listeria* phagocytosed through a receptor other than CR3, in the absence of anti-CR3 mAb. For this we measured the ability of TNF-α/IFN-γ-stimulated W1C3 macrophage hybrids to kill *Listeria* opsonized with heat-inactivated mouse anti-*Listeria* antiserum as a source of IgG to promote FcR-mediated phagocytosis in the absence of complement. In these experiments there was very little difference in uptake of bacteria by the macrophage hybrids opsonized with antiserum or with NMS (Time 0 values in Fig. 3), indicating that cytokine-stimulated W1C3 cells possess alternative means to phagocytose *Listeria*. However, the macrophage hybrids did not kill *Listeria* opsonized with antiserum, and bacterial CFU actually increased in this group. Thus, these data show that in the absence of anti-CR3 mAb, TNF-α/IFN-γ-stimulated macrophage hybrids, which are bactericidal for *Listeria* opsonized in NMS and phagocytosed through CR3, cannot kill *Listeria* phagocytosed via anti-*Listeria* IgG/FcR interaction. These data support the concept that phagocytosis of bacteria through different receptors may lead to different intracellular fates.

DISCUSSION

Macrophage killing of intracellular bacterial pathogens is required for host defense against this class of

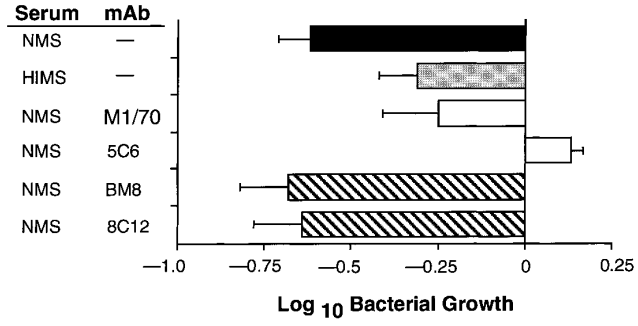

FIG. 2. Complement depletion and anti-CR3 mAb inhibit killing of *Listeria* by cytokine-stimulated macrophage hybrids. W1C3 macrophage hybrids were stimulated with TNF-α and IFN-γ as before and incubated without or with the indicated mAb at a final concentration of 3.0 μg/ml. *Listeria* were incubated with 5% NMS or HIMS, then mixed 1:1 with macrophages and incubated for 20 min at 7°C. The cells were washed to remove unbound bacteria, and initial CFU *Listeria* associated with the cells was determined by serial dilution and plating. After 90 min at 37°C to allow bacterial killing or growth, CFU *Listeria* remaining were measured as before. Net bacterial growth (increased CFU) or killing (decreased CFU) was determined as ($\log_{10}$ CFU *Listeria* T-0) − ($\log_{10}$ CFU *Listeria* T-90). Results are the mean bacterial growth $\pm$ SEM from three separate experiments.

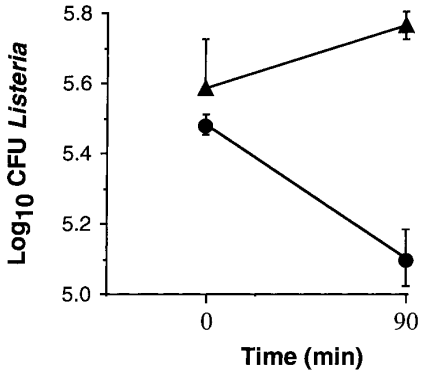

FIG. 3. Cytokine-stimulated macrophage hybrids kill *Listeria* phagocytosed through CR3, but not *Listeria* phagocytosed through FcR. W1C3 macrophage hybrids were stimulated with TNF-α and IFN-γ as before. They were mixed 1:1 with *Listeria* in the presence of 5% NMS (circle) or 2.5% HIMS plus 2.5% heat-inactivated mouse anti-*Listeria* antiserum (triangle). CFU *Listeria* were determined at the beginning and after a 90-min incubation. The mean CFU *Listeria* ± SEM from three separate experiments are shown.

pathogens. The current paradigm for this event holds that cytokine stimulation of mononuclear phagocytes is necessary for these cells to acquire microbicidal activity. In this respect, the inflammatory cytokines TNF-α and IFN-γ are thought to be important stimulators of macrophage bactericidal activity against intracellular bacteria. Nevertheless, the mechanism(s) by which these cytokines induce macrophages to kill these bacteria are not yet clear.

Experiments from this laboratory have been testing the hypothesis that the receptors which mediate phagocytosis of *L. monocytogenes* by inflammatory macrophages are important factors determining whether a phagocytosed bacterium is killed or survives intracellularly. These experiments demonstrated that CR3 (CD11b/CD18) mediated most phagocytosis of *Listeria* by bactericidal peptone-elicited peritoneal macrophages, but not by macrophages which cannot kill *Listeria* (9, 10). Moreover, normally bactericidal peritoneal macrophages lost their ability to kill phagocytosed *Listeria* when CR3-mediated phagocytosis was inhibited (11). These experiments suggest that CR3-mediated phagocytosis leads to macrophage bactericidal activity against *Listeria,* whereas phagocytosis through other receptors does not (11). These results led to the hypothesis that the intracellular fate of bacteria is influenced by the receptor through which they are phagocytosed.

The experiments reported here tested whether this event was part of the mechanism by which a clonal population of cytokine-stimulated macrophages acquired bactericidal activity. For this we used a recently described macrophage precursor hybrid which, when incubated sequentially in TNF-α and IFN-γ, acquires both phagocytic and bactericidal activity against *L. monocytogenes* (12). We show here that stimulation with either cytokine alone induced a modest increase in CR3 expression and CR3-mediated phagocytosis, but sequential stimulation with both TNF-α and IFN-γ resulted in much greater surface expression of CR3 and CR3-mediated phagocytosis. Another marker of macrophage maturation, Mac-2, was also increased on these cells following incubation with TNF-α and IFN-γ, indicating a general maturation of W1C3 cells by treatment with these cytokines. These data clearly demonstrate the importance of CR3 in phagocytosis of *Listeria* by TNF-α and IFN-γ-stimulated cells. The fact that total inhibition of phagocytosis was not achieved supports the idea that multiple mechanisms for microbe:macrophage binding are operative (24).

Other experiments tested more directly the relationship between CR3-mediated phagocytosis and bactericidal activity in TNF-α/IFN-γ-stimulated cells. They demonstrated that killing was decreased when CR3-mediated phagocytosis was inhibited by using complement-depleted serum as the opsonin or following incubation with anti-CR3 mAb. Inhibition of killing was not as great as in the elicited peritoneal cells (11) and probably reflects the use of a clonal population of cells or perhaps intrinsic differences between the two populations of phagocytes. That the mAb 5C6 inhibited both phagocytosis and killing of *Listeria* more than did M1/70 is interesting. Both of these antibodies bind CD11b, but they bind to different epitopes. Both block iC3b binding to CR3, but neither bind the iC3b-binding site (18). This differential effect of the mAbs on killing is compatible with the notion that different epitopes on CR3 may lead to different intracellular consequences for ingested microbes (24).

Finally, we compared the listericidal activity of cells that had phagocytosed *Listeria* opsonized in normal serum versus *Listeria* opsonized in complement-depleted mouse anti-*Listeria* antiserum, containing opsonizing antibodies which mediate phagocytosis by binding to Fc receptors on macrophages. These experiments showed that TNF-α/IFN-γ-stimulated macrophages did phagocytose these antiserum-opsonized *Listeria* about as well as did complement-opsonized bacteria, but could not kill them. Interestingly, the bacteria internalized through FcR replicated during incubation with these normally bactericidal macrophages. In addition, because no anti-CR3 mAb was used, the loss of macrophage bactericidal activity was not due to possible inhibitory signaling initiated by the mAb. Thus phagocytosis of *Listeria* via FcR does not permit killing of *Listeria* by these cytokine-stimulated macrophages. Control experiments showed that antiserum- and NMS-opsonized bacteria bound to the macrophages and were internalized equally (not shown), similar to many other reports that IgG-opsonized targets are constitutively internalized by macrophages after binding (25, 26).

In summary, this study demonstrates two main

points regarding macrophage killing of facultative intracellular bacteria. First, upregulation of CR3 and CR3-mediated phagocytosis are important mechanisms by which TNF-α and IFN-γ stimulate macrophages to become efficient killers of *Listeria*. This does not, however, exclude other events involved in macrophage-mediated anti-*Listeria* activity. It is possible, for instance, that multiple mechanisms may be required for a cell to exhibit listericidal activity. Examples of such mechanisms include sequestration of intracellular iron away from bacterial pathogens and generation of reactive nitrogen intermediates (RNI) by the interferon-inducible nitric oxide synthase (reviewed in 27–29). Modulation of intracellular iron has been shown to influence the survival of several facultative intracellular bacteria, including *Legionella, Listeria,* and *Francisella* (30–32). A number of studies have provided evidence that RNI play an important, but sometimes controversial, role in host defense to *Listeria* infection *in vivo* (33–36). However, in these situations, RNI may not necessarily operate as a macrophage effector molecule. This idea is suggested by previous work from this laboratory showing that the listericidal activity of TNF-α/IFN-γ-stimulated macrophage hybrids is RNI-independent (12) and by a study demonstrating that mice deficient in NF-IL-6 demonstrate an *in vitro* defect in macrophage listericidal activity despite normal RNI production (37).

The second main point of the present study is that different receptors, perhaps even on the same macrophage, may lead a phagocytosed bacterium to different intracellular fates (9–11, 24, 38). A reasonable explanation for this lies in the observation that specific phagocytic receptors may be coupled to specific intracellular signaling events and trafficking pathways (38). In summary, our data support the hypothesis that the receptors that mediate phagocytosis of intracellular pathogens by macrophages are important in determining the fate of the ingested microbe.

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