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Alveolar Macrophage Priming by Intravenous Administration of Chitin Particles, Polymers of *N*-Acetyl-D-Glucosamine, in Mice

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Intravenous (i.v.) administration of phagocytosable chitin particles (1 to 10 μm) in C57BL/6 mice and SCID mice primed alveolar macrophages (Mϕ) within 3 days to yield up to a 50-fold increase in their oxidative burst when elicited in vitro with phorbol myristate acetate (PMA). C57BL/6 mice pretreated with monoclonal antibodies (MAbs) against mouse gamma interferon (IFN-γ) or NK1.1 showed a markedly decreased level of alveolar Mϕ priming following injection of chitin particles. To confirm IFN-γ production in vitro, spleen cells isolated from normal C57BL/6 mice and SCID mice were cultured with chitin particles. Significant IFN-γ production was observed following stimulation with chitin but not with chitosan or latex beads. When spleen cells were treated with anti-NK1.1 MAb, IFN-γ production was significantly inhibited. Another set of experiments showed that when C57BL/6 mice were pretreated i.v. with a small dose IFN-γ, a higher level of priming was induced with not only phagocytosable chitin particles but also phagocytosable chitosan and even latex beads. Likewise, the spleen cell cultures preconditioned with IFN-γ provided an up-regulation of IFN-γ production by these phagocytosable particles. Taken together, the in vivo and in vitro results suggest that (i) the alveolar Mϕ priming mechanism is due, at least in part, to direct activation of Mϕ by IFN-γ, which is produced by NK1.1$^+$ CD4$^-$ cells; (ii) IFN-γ would have an autocrine-like effect on Mϕ and make them more responsive to particle priming; and (iii) phagocytosis of particulates, probably by a postmembrane event such as interiorization, appears to be important for the up-regulation of alveolar Mϕ priming and IFN-γ production.

It is well established that endogenous gamma interferon (IFN-γ) produced by natural killer (NK) cells and activated T cells is an essential cytokine involved in protective cell-mediated immunity (CMI) against facultative intracellular bacteria, including *Listeria monocytogenes* and *Mycobacterium tuberculosis* (7, 9, 13), fungi (19), and parasites (31). IFN-γ-primed macrophages (Mϕ) are known to become microbicidal by massively releasing toxic agents including reactive oxygen intermediates (ROI) and reactive nitrogen intermediates. It is also known that IFN-γ up-modulates the early development of cell-mediated immune (Th1) responses and down-modulates humoral immune (Th2) responses in these infectious models (31).

Myrvik et al. have observed recently that the intravenous (i.v.) administration of phagocytosable-size particles in New Zealand White rabbits primes alveolar Mϕ within 1 to 3 days to yield up to a 100-fold increase in their oxidative burst in vitro when elicited with phorbol myristate acetate (PMA) or opsonized zymosan (21). In this up-regulation, it appears that bacteria (heat-killed [HK] *Mycobacterium bovis* BCG) and microbial particulate components (zymosan) are more effective than phagocytosable-size polystyrene latex beads (21). The magnitude of the elicited oxidative responses of alveolar Mϕ primed nonspecifically with HK BCG was comparable to the maximum priming achieved (up to 100-fold) 2 to 3 weeks after specific immunization with HK BCG in oil (8, 14).

Alveolar Mϕ phagocytosing these particles in vitro, however, do not express such up-regulation of superoxide anion release, which suggests that additional cell populations or immunoregulatory mediators are involved in the in vivo particle-priming response (21). In this regard, current publications (17, 31, 32, 40) indicate that the initial phagocytosis of bacteria such as *L. monocytogenes* results in the production of mediators such as interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α) which induce the maturation of NK and Th1 cells and their production of IFN-γ. This CMI response appears to be highly regulated by various additional immunoregulatory mediators, including IL-4, IL-10, transforming growth factor (TGF-β), and prostaglandin E_2 (PGE_2).

Based on this information, we hypothesized that (i) appropriate biodegradable and nontoxic particles induce local Mϕ to become bactericidal, which could be beneficial in the prophylaxis and treatment of infection; (ii) the selected particles administered initiate CMI responses along with IFN-γ production, which primes Mϕ; and (iii) endogenous IFN-γ, as an endogenous adjuvant, further amplifies the particle-induced MØ priming.

Chitin and chitosan, polymers of *N*-acetyl-D-glucosamine and deacetylated glucosamine, respectively, are essential components of fungal cell walls and have been the targets of antifungal drugs (11, 22). Chitin and chitosan are similar in structure to cellulose and are the second most abundant polysaccharides in nature, comprising the horny substance in the exoskeletons of crabs, shrimp, and insects as well as fungi. Because some of their derivatives are proved to be nontoxic, biodegradable, and biocompatible, prostheses (artificial skin [matrix], contact lens, and surgical stitch) produced from the chitin derivatives have been developed and widely used in medical practice. In addition, a series of studies by Azuma and colleagues suggest that some chitin/chitosan derivatives are

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able to up-regulate Mϕ functions (measured by TNF, IL-1, and colony-stimulating factor production) and induce immunologic adjuvant effects (2, 24–26). This information has led us to test the effects of chitin/chitosan particles (mimetic microbes) on alveolar Mϕ activation and to further determine the mechanisms of the particle-induced Mϕ priming.

The specific aims of this study were to (i) establish mouse models for alveolar Mϕ priming induced by phagocytosable-size chitin/chitosan particles, using C57BL/6 and SCID mice; (ii) determine the roles of NK cells and endogenous IFN-γ in the chitin/chitosan-induced alveolar Mϕ priming in C57BL/6 mice, using selective inactivation of IFN-γ and depletion of $NK1.1^+$ cells by the administration of monoclonal antibodies (MAbs) specific for IFN-γ and NK1.1, respectively; (iii) examine chitin/chitosan particle-induced IFN-γ production in vitro by selected spleen cell populations; and (iv) assess the effects of exogenous IFN-γ on the in vivo chitin/chitosan-induced alveolar Mϕ priming response and on the in vitro pattern of IFN-γ production.

MATERIALS AND METHODS

Mice. Nonpregnant, 7- to 9-week-old female C57BL/6 and CB-17/IRC (SCID) mice (both from Charles River Laboratories, Wilmington, Mass.) were maintained in barrier-filtered cages and fed Purina Laboratory Chow and tap water ad libitum.

Preparations of chitin/chitosan particles. Chitin powders (Sigma Chemical Co., St. Louis, Mo.) and chitosan powders (Kyowa Technos, Chiba, Japan) were suspended at 10 mg/ml in saline and sonicated at 25% output power two times for 5 min each with a Branson sonicator (Sonifier 450; Branson Ultrasonics, Danbury, Conn.). Following a light centrifugation ($50 \times g$, 10 min), the supernatants mainly contained small particles (1 to 10 μm in diameter) which were removed, filtered through a 400/2,800 stainless steel mesh, and further centrifuged to collect the particles at $1,400 \times g$ for 10 min. One milligram of chitin/chitosan particles (1 to 10 μm) contained 2×10^8 to 3×10^8 particles (data not shown). On the other hand, the ($50 \times g$)-centrifuged pellets containing large particles (50 to 100 μm) were resuspended in saline, filtered through a 200/600 stainless steel mesh, and centrifuged at $1,400 \times g$ for 10 min to obtain the large particles. Chitin oligosaccharides and chitosan oligosaccharides were kindly provided by Aihara, Kyowa Technos. Particle sizes and size distribution were flow cytometrically determined by using several sizes of latex bead controls (1.1, 10.0, and 47.8 μm in diameter; Polysciences, Warrington, Pa.). Figure 1 shows chitin particles with typical size distributions of the particles.

In vivo neutralization of IFN-γ. Endogenously produced IFN-γ was neutralized by injecting mice intraperitoneally (i.p.) with 2×10^5 neutralizing units of anti-IFN-γ MAb R4-6A2 (specific activity, 2×10^5 neutralizing units per mg of immunoglobulin G [IgG]; a generous gift from E. A. Havell, Trudeau Institute, Saranac Lake, N.Y.) 1 day before the chitin injection. An equivalent amount of normal rat IgG (Sigma) was used to control for the nonspecific effects of injecting foreign immunoglobulin.

In vivo $NK1.1^+$ cell depletion. In vivo depletion of $NK1.1^+$ cells was achieved 1 day before chitin particle administration by i.p. injection of 5 mg of anti-NK1.1 (IgG2a; clone PK 136 from the American Type Culture Collection [ATCC], Rockville, Md.) which was purified from mouse ascites fluid by ammonium sulfate precipitation with a 50% saturated solution. The extent of $NK1.1^+$ cell depletion was determined by flow cytometry as described below. Control mice received saline.

Alveolar Mϕ and spleen cells. Alveolar Mϕ were obtained by repeated pulmonary lavage with a total 5 ml of sterile Hanks' balanced salt solution (pH 7.2). Mϕ enrichment was done by the plastic adherence method (37°C, 1 h) in the presence of 10% heat-inactivated fetal bovine serum (FBS). Spleens were gently minced with scissors and filtered through a stainless steel sieve. Removal of adherent Mϕ from spleen cell suspensions was done by passage through a Sephadex G-10 column (33). Plastic-adherent spleen Mϕ were prepared as described previously (33). In certain experiments, $NK1.1^+$ cells and $CD4^+$ cells in the spleen cells were eliminated with MAbs against NK1.1 (clone PK 136; ATCC) and CD4 (clone GK 1.5; ATCC), respectively, followed by treatment with rabbit serum (1:10; Sigma) as a source of complement. The effect of the treatment was confirmed by indirect immunofluorescence staining with the MAbs against F4/80, NK1.1, and CD4 as described below.

Cell enumeration. Plasma membranes of spleen cells and alveolar Mϕ which were freshly isolated and cultured were lysed with Zapoglobin (Coulter Diagnostics, Hialeah, Fla.). Nuclei were counted with an electronic particle counter (Coulter Counter model D2). Viable cell counts were performed by trypan blue dye exclusion. Differential cell counts were made on cytocentrifuged preparations and stained with May-Gruenwald-Giemsa stain.

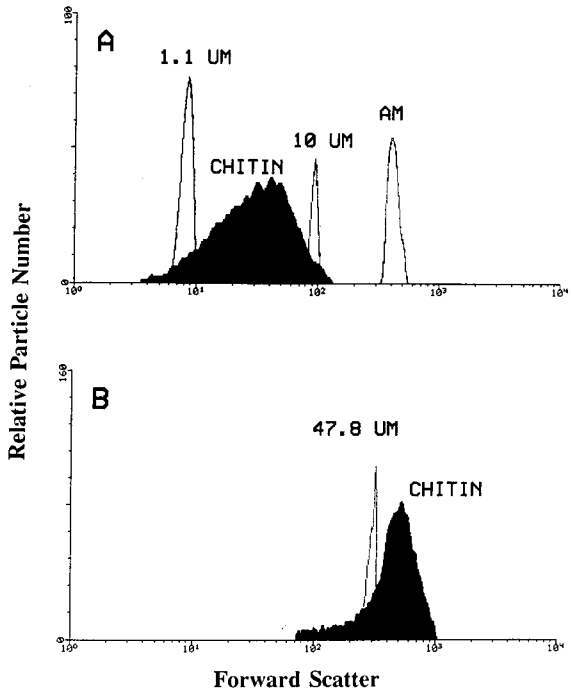

FIG. 1. Determination of size distribution of chitin particles. The size distributions of two different preparations of chitin particles (1- to 10-μm [phagocytosable-size] [A] and 50- to 100-μm [nonphagocytosable size] [B] particles) as described in Materials and Methods were determined by a FACScan cell sorter. Alveolar Mϕ (AM) and latex beads at diameters of 1.1, 10.0, and 47.8 μm were used as comparison controls. The *x* and *y* axes indicate particle sizes defined by forward scatter and particle numbers, respectively.

Flow cytometric cell characterization. The expression of surface antigens on the Mϕ, T cells, and NK cells was determined by indirect immunofluorescence in the presence of 5% heat-inactivated newborn calf serum (GIBCO, Grand Island, N.Y.), pH 7.2 (34). Serum-free culture supernatants containing MAbs against F4/80, NK1.1, and CD4 were prepared from hybridoma cell lines F4/80, PK 136, and GK 1.5, respectively (all from ATCC). MAbs in the culture supernatants were purified by an ammonium sulfate precipitation with a 50% saturated solution. Fluorescein isothiocyanate-conjugated donkey anti-mouse IgG and donkey anti-rat IgG (both from Jackson ImmunoResearch, West Grove, Pa.) were used as secondary antibodies for indirect immunofluorescence. Fluorescence of 10^4 stained cells was quantitated with a FACScan flow cytometer using the FACScan research program (Becton Dickinson, Mountain View, Calif.). All tested cells as defined by forward and sideward scatter pattern were gated; only debris was excluded from analysis. Cells stained with the second antibody alone were used as negative controls in all experiments.

Protocol for in vivo priming of mice. To prime alveolar MØ in vivo, mice were injected i.v. with 0.1 or 1 mg of chitin/chitosan particles suspended in 0.2 ml of endotoxin-free saline. The alveolar Mϕ were harvested 1 to 7 days postinjection. In comparison controls, mice received i.v. 1 mg of HK *Corynebacterium parvum* vaccine (33) or 1.1-μm latex beads.

Superoxide anion release assay. Superoxide dismutase (SOD)-inhibitable superoxide anion levels released by alveolar Mϕ were measured by a cytochrome *c* reduction assay. Adherent Mϕ were placed in a 24-well plate (Corning, Corning, N.Y.) with HEPES-bicarbonate buffer containing 50 μM ferricytochrome *c* (Sigma). Mϕ were incubated at 37°C for 1 h in the presence or absence of PMA (10^{-6} M). SOD (700 U/ml; Sigma) was also added as a negative control. The amount of reduced ferricytochrome *c* was measured by using a molecular extinction coefficient of 21.1 $mM^{-1}\ cm^{-1}$ from the change in absorbance at 550 nm against a cell-free blank (3, 18). Superoxide formation was expressed as nanomoles/10^6 cells.

Production of IFN-γ by spleen cell cultures stimulated with chitin. Spleen cells isolated from normal C57BL/6 and SCID mice were resuspended (2×10^6 cells/ml) in RPMI 1640 plus 10% heat-inactivated FBS and incubated with chitin/chitosan particles at various doses at 37°C. After 24 h of incubation, the culture supernatants were harvested, filtered (0.22-μm-pore-size filter), and stored at −80°C for later assays for IFN-γ. In some experiments, chitin particle-stimulated spleen cell cultures were further treated with cytokines (recombinant mouse IFN-γ [Sigma], recombinant mouse IL-4 [Pepro Tech, Rocky Hill, N.J.], recombinant mouse IL-10 [Pepro Tech], and natural human TGF-β1 [Genzyme,

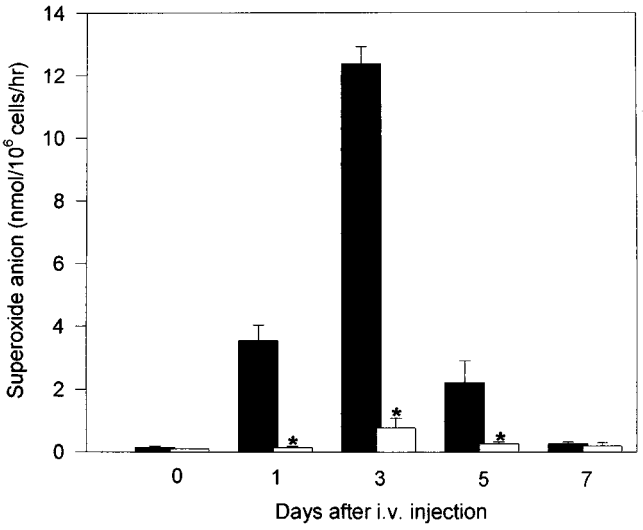

FIG. 2. Kinetics of superoxide anion release by alveolar Mϕ in C57BL/6 mice given chitin particles i.v. C57BL/6 mice (three/group) received 1 mg of chitin particles (1 to 10 μm) i.v. After the indicated days, alveolar Mϕ in each mouse were assayed in vitro for PMA-elicited superoxide anion release in the presence (open bars) or absence (solid bars) of 700 U of SOD. Results are expressed as means ± SD ($n = 3$). *, $P < 0.05$ compared with groups without SOD treatment.

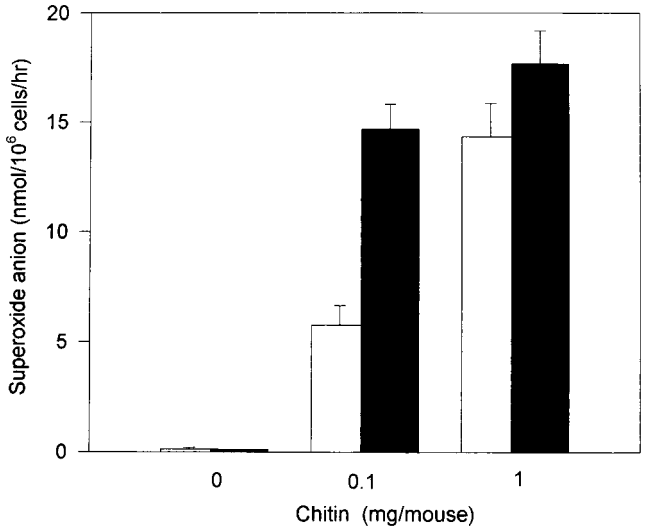

FIG. 3. Superoxide anion release by alveolar Mϕ in C57BL/6 mice and SCID mice given chitin particles i.v. at different doses. Groups of C57BL/6 mice (open bars) and SCID mice (solid bars) received chitin particles (1 to 10 μm) at 0 (saline), 0.1, and 1 mg per mouse (three mice/group). After 3 days, alveolar MØ in each mouse were assayed in vitro for PMA-elicited superoxide anion release. Results are expressed as means ± SD ($n = 3$).

Cambridge, Mass.]), PGE_2 (Sigma), and antibodies (rat anti-mouse IL-12 [clone 17.8; Genzyme] and polyclonal rabbit anti-mouse TNF-α [Genzyme]). To remove possibly contaminated endotoxin, all tissue culture media and soluble reagents were passed through a polymyxin B column (Sigma), followed by concurrent filtration and sterilization through 0.22-μm-pore-size Zetapore filters (Cuno, Meriden, Conn.) manufactured for this purpose (34, 35).

IFN-γ assay. The levels of mouse IFN-γ in the spleen cell cultures were measured with specific two-site enzyme-linked immunosorbent assay (ELISA) using monoclonal anti-mouse/rat IFN-γ (clone DB-1; Biosource, Camarillo, Calif.) as a capture antibody and rabbit polyclonal anti-mouse IFN-γ (Biosource) as a detector antibody. Briefly, Immunlon II plates (Nunc) were coated with the MAb overnight. The following day, the plates were blocked with 5% heat-inactivated newborn calf serum for 1 h and then washed. Samples from in vitro spleen cell cultures were added to the wells. The plates were incubated overnight at 4°C and then washed four times with 0.05% Tween-Tris buffer (pH 7.2). The secondary antibodies were added for 1 h, followed by extensive washing. Finally, horseradish peroxidase conjugates were added for 30 min and washed, and the peroxide substrates were mixed with *o*-phenylenediamine dihydrochloride (Sigma). After the reaction was stopped with 3 N HCl, the plates were read spectrophotometrically at 492 nm. IFN-γ levels were quantitated based on a standard curve obtained by using recombinant mouse IFN-γ (Sigma).

Statistics. Difference between mean values were analyzed by Student's *t* test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Enhancement of superoxide anion release by alveolar Mϕ following i.v. administration of chitin/chitosan particles. A 1-mg dose of chitin particles (1 to 10 μm [Fig. 1], 2×10^8 to 3×10^8 particles) was administered i.v. to C57BL/6 mice. After selected periods, alveolar lavage cells were collected and incubated in the presence of 10% FBS for 1 h. The adherent cells (>90% of Mϕ determined morphologically [data not shown]) were isolated. ROI release by PMA-elicited alveolar Mϕ was detected as described in Materials and Methods. As shown in Fig. 2, normal alveolar Mϕ generated superoxide anion at a level less than 0.2 nmol/10^6 cells/h when elicited with PMA. In contrast, the ROI levels were greatly enhanced 1 to 3 days after the injection of chitin particles; the levels returned to normal by day 7. SOD at 700 U/ml completely inhibited the ROI release by alveolar Mϕ (Fig. 2).

As shown in Fig. 3, SCID mice receiving chitin particles also showed a massive ROI release by alveolar Mϕ in vitro equal to or greater than that of C57BL/6 mice. These data suggest that neither T nor B lymphocytes appear to be essential for particle-induced Mϕ activation.

In contrast, chitosan particles (1 to 10 μm) at the same dose (1 mg/mouse) showed significantly less capacity to prime alveolar Mϕ to release superoxide anion (Table 1). There was no effect of chitin oligosaccharides and chitosan oligosaccharides (Table 1).

Effect of treatments with anti-NK1.1 or anti-IFN-γ in C57BL/6 mice. To further assess the obligatory role of NK cells for Mϕ priming, C57BL/6 mice were pretreated with anti-NK1.1 MAb i.p. 1 day before chitin particle injection. The MAb treatment resulted in a significant reduction of splenic $NK1.1^+$ cells but not $CD4^+$ cells detected by flow cytometric monitoring (Fig. 4) as well as the priming effect on alveolar Mϕ as determined by PMA-elicited ROI release (Fig. 5).

It has been reported that neutralization of IFN-γ in mice

TABLE 1. Alveolar Mϕ priming by i.v. injection of chitin particles at selected sizes in C57BL/6 mice[a]

Particle	Size	Dose (mg)	O_2^- (nmol/10^6 cells/h)
Expt 1			
Saline			0.5
Chitin	1–10 μm	1	20.3*
Chitosan	1–10 μm	1	7.2*
Chitin	Oligosaccharide	1	0.5
Chitosan	Oligosaccharide	1	0.5
Expt 2			
Saline			0.5
Chitin	1–10 μm	1	17.9*
HK *C. parvum*		1	24.5*
Latex beads	1.1 μm	1	0.7

[a] Groups of 3 C57BL/6 mice were injected i.v. with the particle preparations indicated. Bronchoalveolar lavage cells were collected day 3. Superoxide anion released by alveolar Mϕ was determined as described in Materials and Methods. Results are expressed as means ($n = 3$). *, $P < 0.05$ compared with the control (saline) group.

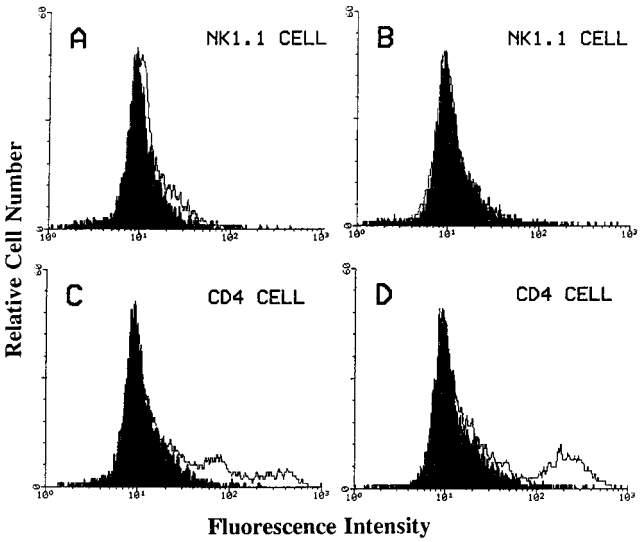

FIG. 4. Splenic NK1.1$^+$ cells and CD4$^+$ cells in C57BL/6 mice treated with anti-NK1.1 MAb. C57BL/6 mice were treated with anti-NK1.1 MAb (B and D) or with saline (A and C) as described for Fig. 5, and NK1.1$^+$ cells (A and B) and CD4$^+$ cells (C and D) in the spleen cells were determined cytometrically. The histogram obtained by staining with either anti-NK1.1 or anti-CD4 was shown by a solid line which was superimposed with a shaded background (NSB) obtained by staining with the second antibody alone.

infected with *L. monocytogenes* by using anti-murine IFN-γ MAbs causes a lethal exacerbation of a sublethal infection (12, 40). To determine whether endogenous IFN-γ was produced during the particle priming, we treated C57BL/6 mice with 2×10^5 neutralizing units of rat anti-IFN-γ MAb 1 day before inoculating chitin particles. Figure 5 shows that the treatments with the MAb resulted in a significant reduction of the priming effect on alveolar Mϕ, while there was little or no effect of control IgG.

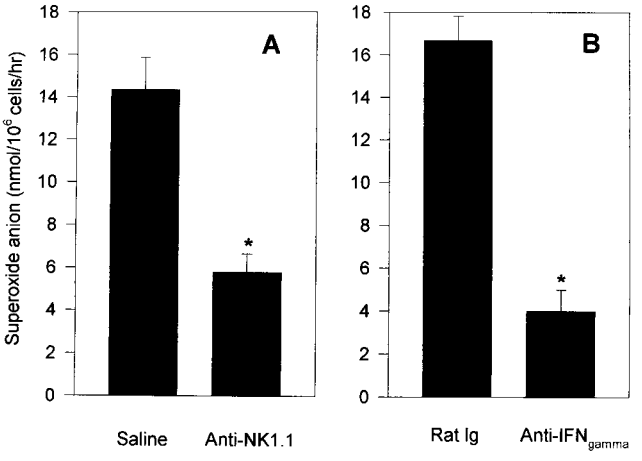

FIG. 5. In vivo priming of mouse alveolar Mϕ induced by i.v. injection of chitin particles in C57BL/6 mice pretreated with MAbs against IFN-γ or NK1.1. C57BL/6 mice (three mice per group) were injected i.p. with anti-NK1.1 mouse IgG at 5 mg (A) or anti-IFNγ rat IgG at 1 mg (B) 1 day before i.v. injection of chitin particles at 1 mg/mouse. Control mice in panels A and B received saline and rat IgG (1 mg), respectively, before the chitin particle (1 to 10 μm) injection. Three days after the particle injection, alveolar lavage Mϕ in each mouse were assayed in vitro for superoxide anion release with PMA (1 μM) as the eliciting agent. Results are expressed as means ± SD ($n = 3$). *, $P < 0.05$ compared with the control (saline) or (rat Ig) group.

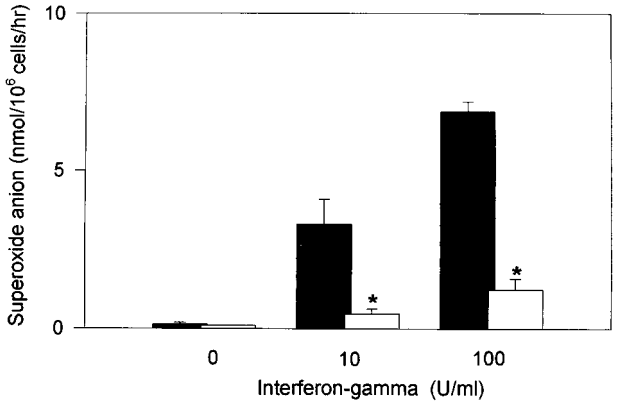

FIG. 6. In vitro alveolar Mϕ primed with IFN-γ. Alveolar Mϕ isolated from normal C57BL/6 mice were incubated with IFN-γ at 0 (medium alone), 10, and 100 U/ml for 24 h at 37°C. Superoxide anion levels released by the IFN-γ-primed Mϕ were measured in the presence (open bars) or absence (solid bars) of SOD at 700 U/ml following PMA elicitation. Results are expressed as means ± SD ($n = 3$). *, $P < 0.05$ compared with groups without SOD treatment.

Figure 6 shows that when alveolar Mϕ isolated from normal C57BL/6 mice were treated with exogenous IFN-γ (10 and 100 U/ml) in vitro for 24 h, IFN-γ primed alveolar Mϕ in a dose-dependent manner.

Preadministration of IFN-γ in C57BL/6 mice modulates particle-induced alveolar Mϕ priming. To further assess the role of IFN-γ in particle-induced alveolar Mϕ priming, we injected mouse recombinant IFN-γ (10,000 U) i.v. in C57BL/6 mice 4 h before the particle priming with chitin and chitosan. Although IFN-γ alone at this dose did not prime alveolar Mϕ at day 3, the additional injection of chitin particles (1 to 10 μm) primed alveolar Mϕ to a level in which they released significantly more ROI release when elicited by PMA in vitro (Table 2) compared to mice that did not receive an injection of IFN-γ. Surprisingly, chitosan particles and even 1.1-μm latex beads also induced significant alveolar Mϕ priming in mice that were treated with exogenous IFN-γ (Table 2). Oligosaccharides of chitin and chitosan, however, did not show any priming effects (Table 2).

In vitro chitin/chitosan particle-induced IFN-γ production in spleen cell cultures. It has been reported that mouse spleen cells incubated with HK bacteria (for example, *L. monocytogenes*, BCG, and *C. parvum*) produce IFN-γ (16, 17, 39, 40).

TABLE 2. Effect of administration of IFN-γ before the injection of chitin/chitosan particles on the alveolar Mϕ priming in C57BL/6 mice[a]

Particle	Size	Dose (mg)	O_2^- (nmol/10^6 cells/h)	
			No treatment	IFN-γ treatment
Saline			0.5	0.5
Chitin	1–10 μm	1	14	20*
Chitosan	1–10 μm	1	4.3	14*
Chitin	Oligosaccharide	1	0.5	0.5
Chitosan	Oligosaccharide	1	0.5	0.5
Latex beads	1.1 μm	1	0.7	12*

[a] C57BL/6 female mice (three per group) were injected i.v. with recombinant mouse IFN-γ (10,000 U; Sigma) or saline (control) 4 h before i.v. injection of chitin particles, chitosan particles, or 1.1-μm latex beads at 1 mg/mouse. Alveolar lavage Mϕ in each mouse were assayed in vitro for superoxide anion release with 1 μM PMA as the eliciting agent. Results are expressed as means ($n = 3$). *, $P < 0.05$ compared with the control (no treatment) groups.

TABLE 3. IFN-γ production by mouse spleen cells cultured with chitin or chitosan[a]

Particle	Size	Dose (mg/ml)	IFN-γ (U/ml)	
			C57BL/6	SCID
Saline			<5	<5
Chitin	1–10 μm	0.1	54*	65*
Chitosan	1–10 μm	0.1	<5	<5
Chitin	50–100 μm	0.1	<5	8
Chitosan	50–100 μm	0.1	<5	<5
Chitin	Oligosaccharide	0.1	<5	<5
Chitosan	Oligosaccharide	0.1	<5	<5
HK *C. parvum*		0.1	110*	127*
Latex beads	1.1 μm	0.1	<5	<5
	47.8 μm	0.1	<5	<5

[a] Spleen cells isolated from C57BL/6 mice or SCID mice were suspended in RPMI 1640 plus 10% FBS (4×10^6 cells/ml) and incubated with the particles indicated. After 24 h of incubation, the culture supernatants were filtered with a 0.22-μm-diameter filter, and IFN-γ levels in the filtrates were measured by ELISA. Results are expressed as means ($n = 3$). *, $P < 0.05$ compared with the control (saline) group.

TABLE 4. Effects of C57BL/6 mouse spleen cells depleted of NK cells or $CD4^+$ cells on the production of IFN-γ[a]

Treatment	IFN-γ (U/ml)
Spleen cells	78
Nonadherent spleen cells	7*
Adherent spleen cells	8*
Nonadherent spleen cells + adherent cells	85
Spleen cells treated with:	
Rabbit serum	74
Anti-NK1.1 + rabbit serum	9**
Anti-CD4 + rabbit serum	83

[a] C57BL/6 mouse spleen cells, nonadherent spleen cells, adherent spleen cells, and spleen cells pretreated with either anti-NK1.1 (PK 136) plus rabbit serum (1:10 dilution; Sigma), anti-CD4 (GK 1.5) plus rabbit serum, or rabbit serum alone were suspended in RPMI 1640 plus 10% FBS at 4×10^6 cells/ml and incubated with chitin particles (1 to 10 μm) at 0.1 mg/ml for 24 h. In some experiments, nonadherent spleen cells (2×10^6 cells/ml) and nonadherent spleen cells (2×10^6 cells/ml) were mixed before chitin particle stimulation. The culture supernatants were filtered, and IFN-γ levels in the filtrates were measured by ELISA. Data are presented as means ($n = 3$). Without chitin, IFN-γ levels in the six groups were <5 U/ml. * and **, $P < 0.05$ compared with spleen cell group and rabbit serum-treatment group, respectively.

Based on this information, we incubated normal spleen cells isolated from C57BL/6 and SCID mice in the presence of various forms of chitin and chitosan.

Our initial studies using C57BL/6 mouse spleen cells showed that IFN-γ production was detected (7 ± 3 U/ml [mean ± standard deviation {SD}, $n = 3$]) 4 h after 1- to 10-μm chitin particle stimulation (100 μg/ml), became maximum (64 ± 10 U/ml) at 24 h, and declined slightly (49 ± 8 U/ml) after 48 h (data not shown). Table 3 shows that the chitin particles induced significant amounts of IFN-γ production 24 h after the stimulation, at a level approximately 50% of the level induced by HK *C. parvum* in spleen cells isolated from both the strains of normal mice. Unlike HK *C. parvum*, either 1.1-μm or 47.8-μm latex beads as comparison controls did not induce IFN-γ production (Table 3). Neither the large chitin particles (50 to 100 μm) nor chitin oligosaccharides induced IFN-γ production. Furthermore, it is notable that none of the chitosan preparations used in this study induced detectable amounts of IFN-γ production in either C57BL/6 or SCID mouse spleen cells.

IFN-γ production by the C57BL/6 mouse spleen cells depleted of $NK1.1^+$ cells or adherent cells. To determine the key cell populations in the spleen cell cultures that were responsible for IFN-γ production, several spleen cell subpopulations were fractionated and incubated with chitin particles (1 to 10 μm). As shown in Table 4, neither nonadherent spleen cells prepared by passing cell suspensions through Sephadex G-10 columns nor plastic-adherent spleen cells produced significant amounts of IFN-γ. When the two cell populations were mixed, chitin particle-induced IFN-γ production was restored (Table 4). $F4/80^+$ Mϕ in the nonadherent cell and adherent cell populations amounted to 0.5 and 84%, respectively (data not shown).

Furthermore, spleen cells depleted of $NK1.1^+$ cells showed a significant reduction of chitin particle-induced IFN-γ levels, whereas the elimination of $CD4^+$ cells resulted in a slight enhancement of IFN-γ production (Table 4).

Effects of neutralizing antibodies against IL-12 and TNF-α on chitin particle-induced IFN-γ production. To test a role of selected IFN-γ-inducing cytokines (IL-12 and TNF-α) for chitin particle-induced IFN-γ production, spleen cell cultures (C57BL/6 mice) stimulated with 1- to 10-μm chitin particles were pretreated with neutralizing antibodies against IL-12 (bioactive IL-12p70) and TNF-α. As shown in Fig. 7, both treatments resulted in significantly blocking IFN-γ production, suggesting that the two cytokines play obligatory roles in chitin particle-induced IFN-γ production.

Exogenous inflammatory mediators (IFN-γ, IL-4, IL-10, TGF-β1, and PGE_2) modulate chitin particle-induced IFN-γ production in C57BL/6 mouse spleen cell cultures. To assess the effect of exogenous IFN-γ on particle-induced IFN-γ production, the spleen cells were preincubated with IFN-γ at 100 U/ml for 12 h before particle stimulation. Table 5 showed that phagocytosable-size particles (chitin, chitosan, latex beads, and HK *C. parvum*) but not large (50- to 100-μm) particles (chitin, chitosan, and latex beads) or oligosaccharides (chitin and chitosan) further induced significantly higher levels of IFN-γ in the exogenous IFN-γ-treated spleen cells than in control

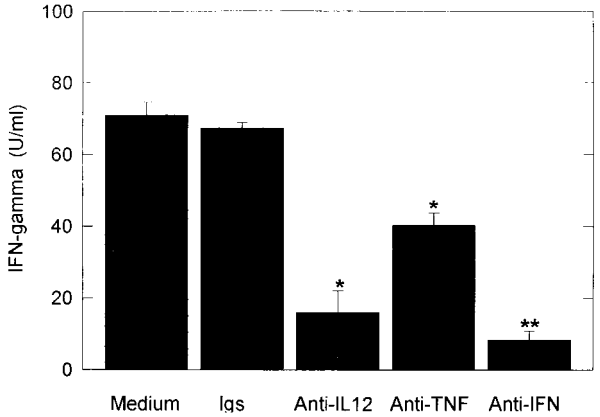

FIG. 7. Effects of neutralizing antibodies against IL-12 and TNF-α on chitin particle-induced IFN-γ production. Spleen cells isolated from C57BL/6 mice were incubated with 1- to 10-μm chitin particles at 100 μg/ml in the presence of either rat anti-IL-12 (40 μg/ml; clone 17.8), polyclonal rabbit anti-mouse TNF-α (40 μg/ml), or rat anti-IFN-γ (40 μg/ml; clone R4-6A2, as a comparison control) for 24 h at 37°C. Control group had a mixture of rat immunoglobulin (40 μg/ml; Sigma) and rabbit immunoglobulin (40 μg/ml; Sigma). The levels of IFN-γ in the culture supernatants were measured as described in Materials and Methods. Results are expressed as means ± SD ($n = 3$). * and **, $P < 0.005$ and $P < 0.001$, respectively, compared with the immunoglobulin control group.

TABLE 5. Particle-induced IFN-γ production by mouse spleen cells pretreated with exogenous IFN-γ[a]

Particle	Size	Dose (mg/ml)	IFN-γ (U/ml)	
			C57BL/6	SCID
Saline			<5	<5
Chitin	1–10 μm	0.1	102*	116*
Chitosan	1–10 μm	0.1	45*	53*
Chitin	50–100 μm	0.1	9	12
Chitosan	50–100 μm	0.1	6	8
Chitin	Oligosaccharide	0.1	<5	<5
Chitosan	Oligosaccharide	0.1	<5	<5
HK *C. parvum*		0.1	142*	163*
Latex beads	1.1 μm	0.1	84*	88*
	47.8 μm	0.1	<5	<5

[a] Spleen cells isolated from C57BL/6 mice and SCID mice were suspended in RPMI 1640 plus 10% FBS (4×10^6 cells/ml) and incubated with mouse IFN-γ at 100 U/ml for 12 h. Following washing, cells were further incubated with the particles indicated. After another 24 h of incubation, the culture supernatants were filtered with a 0.22-μm-diameter filter, and IFN-γ levels in the filtrates were measured by ELISA. These procedures except for the IFN-γ pretreatment were comparable to those for Table 3. Results are expressed as means ($n = 3$). *, $P < 0.05$ compared with the control (saline) group.

spleen cells. In sharp contrast, the chitin particle-induced IFN-γ production was significantly inhibited by the presence of selected inflammatory mediators (IL-4, IL-10, and TGF-β1, and PGE_2) (Table 6).

To determine whether chitin/chitosan preparations used in this study induce such factors which could inhibit IFN-γ production, the culture supernatants of particle-induced spleen cells were added to the chitin particle-induced spleen cell cultures. As shown in Table 6, the supernatants which did not contain IFN-γ (Table 3) failed to inhibit IFN-γ production. Our data suggest that the failure of IFN-γ production by chitin/chitosan preparations other than 1- to 10-μm chitin particle preparations is not due to the presence of factors which are known to down-modulate IFN-γ production. Our preliminary study, in addition, indicated that there was no detectable level of IL-4 (<20 pg/ml, determined by ELISA [Genzyme]) in any particle-stimulated spleen cell cultures used in this study (data not shown).

It is of special interest that control spleen cell cultures incubated in the absence of chitin particles produced IFN-γ when costimulated with the culture supernatants of chitin particle-stimulated spleen cells (Table 6). These data suggest that, in addition to IFN-γ, chitin particles induce other factors, including IL-12 and TNF-α, which augment IFN-γ production.

TABLE 6. Modulation of chitin particle-induced IFN-γ production in C57BL/6 spleen cells[a]

Treatment	Dose	IFN-γ (U/ml)	
		No chitin particle	Chitin particle
Saline		<5	88
IL-4	5 ng/ml	<5	7**
IL-10	5 ng/ml	<5	15**
TGF-β1	5 ng/ml	<5	18**
PGE_2	1 μM	<5	25**
24-h culture supernatants of spleen cells stimulated with:			
Chitin (1–10 μm)	50%	88*	160**
Chitosan (1–10 μm)	50%	<5	83
Chitin (50–100 μm)	50%	<5	91
Chitosan (50–100 μm)	50%	<5	81
Chitin (Oligo)	50%	<5	87
Chitosan (Oligo)	50%	<5	92
HK *C. parvum*	50%	103*	166**
Latex beads (1.1 μm)	50%	<5	93
Latex beads (40 μm)	50%	<5	88

[a] Spleen cells isolated from C57BL/6 mice were suspended in RPMI 1640 plus 10% FBS (4×10^6 cells/ml) and incubated with selected cytokines (IL-4, IL-10, and TGF-β1) all at 5 ng/ml and PGE_2 at 1 μM. In some experiments, spleen cell suspensions were mixed with an equal volume of the culture supernatant of particle-stimulated spleen cells (C57BL/6) prepared as for Table 3 (50% dose). These exogenous mediator-treated spleen cell suspensions were incubated in the presence or absence of chitin particles (1 to 10 μm) at 0.1 mg/ml. After 24 h of incubation, the culture supernatants were filtered through a 0.22-μm-diameter filter, and IFN-γ levels in the filtrates were measured by ELISA. Results are expressed as means ($n = 3$). *, $P < 0.05$ compared with control (saline) treatment in the no-chitin particle treatment groups; **, $P < 0.05$ compared with control (saline) treatment in the chitin particle treatment groups.

DISCUSSION

When chitin/chitosan particles, structures with no mammalian counterpart, are administered i.v. to mice, they become bound to Mϕ plasma membrane mannose/fucose receptors (19, 28, 38, 41) that mediate the interiorization of the chitin/chitosan particles if they are in the phagocytosable range. These particles are eventually degraded by lysozyme and *N*-acetyl-β-D-glucosaminidase, which are present in the phagocytic Mϕ and sera in human and experimental animals (6, 15). The present study clearly indicates that the i.v. injection of C57BL/6 mice with phagocytosable chitin particles primes alveolar Mϕ within 3 days to give a large oxidative burst when elicited in vitro with PMA. Phagocytosable chitosan also primes alveolar Mϕ but at a significantly lower level of the elicited oxidative response than the phagocytosable chitin. Soluble chitin oligosaccharides and chitosan oligosaccharides, in sharp contrast, are unable to prime alveolar Mϕ. The magnitude of the elicited oxidative responses of alveolar Mϕ primed by i.v. injection of chitin particles is comparable to the maximum priming achieved by HK *C. parvum*. Our data strongly suggest that initial phagocytosis of chitin particles or bacteria but not latex beads is the key event for alveolar Mϕ priming.

Another important finding is that the mechanism for alveolar Mϕ priming by the chitin particle involves, at least in part, the production of endogenous IFN-γ. We also found that a comparable level of alveolar Mϕ priming is induced in SCID mice receiving chitin particles i.v., indicating that neither T nor B lymphocytes are required for the particle priming response in this animal model. We also established that the administration of monoclonal anti-NK1.1 antibodies to C57BL/6 mice caused depletion of their $NK1.1^+$ cells as well as the priming response of alveolar Mϕ. It has been reported that the resistance of mice to *L. monocytogenes* infection involves an early, (1 to 2 days) nonspecific phase of resistance, mediated by IFN-γ produced by NK cells, which appears to be an essential immune mechanism to eliminate bacteria in SCID mice as well as in immunocompetent mice (12, 40). In our study, the injection of C57BL/6 mice and SCID mice with chitin particles also induced this nonspecific host defense system, which is generally seen at the early stages of the microbial infections.

To further dissect the mechanisms of IFN-γ production during the chitin particle treatment, we have modified the spleen cell culture models established by Tripp et al. (39, 40). Our data clearly show that phagocytosable-size chitin particles induce significant amounts of IFN-γ production and strongly suggest that the chitin particle-induced IFN-γ production appears to be due to cell-to-cell interactions, especially Mϕ-to-$NK1.1^+$ cell interaction. $CD4^+$ helper T cells or other T cells ($CD8^+$) known as IFN-γ producers in various immune re-

sponses are not involved. Since other bacterial particles such as HK *C. parvum*, but not 1.1-μm latex beads, also induced comparable levels of IFN-γ, these responses might be common with microbial particulate components (30, 44). Taken together, the capacities of particles to prime alveolar Mϕ in vivo closely parallel the responses of our in vitro cell system.

Recent publications suggest that various steps of CMI responses (measured by IFN-γ production and microbicidal Mϕ formation) are regulated by a variety of cells and their inflammatory mediators (31), including IL-4 (16, 29, 42), IL-10 (16, 29), TGF-β1 (43), and PGE_2 (4). We confirmed here that the chitin particle-induced IFN-γ production in vitro is significantly inhibited when costimulated with IL-4, IL-10, TGF-β1, or PGE_2. One could speculate, therefore, that low levels of IFN-γ production by the particles and oligosaccharides other than the phagocytosable-size chitin particles indicated in Table 3 are due to the production of some negative mediators. However, our results (Table 6) suggest that there are no such inhibitory activities produced by these particles. It is more likely that the particle priming of alveolar Mϕ is dependent on the production of interdependent factors up-regulating IFN-γ production, such as IL-12 (17, 32, 40), TNF-α (40), and an 18-kDa IFN-γ-inducing factor (IGIF) (27). Our results clearly demonstrated obligatory roles of IL-12 and TNF-α in this mechanism. In this regard, it has been reported that TNF-α alone does not induce IFN-γ production but enhances IL-12-induced IFN-γ production (40). The contribution of IGIF, however, remains to be elucidated.

Consistent with earlier observations (5, 20, 23), in vitro, exogenous IFN-γ can prime normal mouse alveolar Mϕ which release massive amounts of SOD-sensitive reactive oxygen intermediates. In addition to direct Mϕ activation by IFN-γ, our present study made a surprising discovery that if C57BL/6 mice are preconditioned with a small dose of IFN-γ, a higher level of priming is induced by not only phagocytosable chitin particles but also phagocytosable chitosan and even latex beads. Furthermore, the in vitro study using spleen cell cultures preconditioned with IFN-γ provides an up-regulation of IFN-γ production by these phagocytosable particles, suggesting increases in endogenous IFN-γ (nonspecific CMI) in vivo by the particles. However, nonphagocytosable chitin/chitosan/latex particles or soluble oligosaccharides of chitin and chitosan fail to induce significant IFN-γ production in these cultures. Phagocytosis of particulates, probably by a postmembrane event such as interiorization, appears to be important for the up-regulation of nonspecific CMI.

Because the chitin particles do not induce detectable levels of IL-4 in the spleen cell cultures, the inhibition of Th2-derived cytokine production, unlike in Scott's *Leishmania* infectious models (31), may not be major mechanisms for the effects of exogenous IFN-γ treatment. We speculate that the formation of Mϕ-derived immunoregulatory mediators such as IL-12, IGIF, and TNF-α, which up-regulate CMI (IFN-γ production), is enhanced transcriptionally and translationally when Mϕ are exposed to IFN-γ and elicited by phagocytosis (36, 37). Although the exact mechanisms remain to be elucidated, circumstantial evidence for the phagocytosis-mediated elicitation of inflammatory mediators has been reported in various Mϕ activation studies (1, 10, 35).

Finally, this observation provides many new avenues of study that could lead to new methodologies for the up-regulation of nonspecific CMI that might have important clinical utility. It is a particularly intriguing idea that IFN-γ might have an autocrine-like effect on Mϕ and make them more responsive to particle priming.

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REFERENCES

1. **Allen, L.-A. H., and A. Aderem.** 1995. A role of MARCKS, the α isozyme of protein kinase C and myosin I in zymosan phagocytosis by macrophages. J. Exp. Med. **182:**829–840.
2. **Azuma, I.** 1992. Synthetic immunoadjuvants: application to non-specific host stimulation and potentiation of vaccine immunogenicity. Vaccine **10:**1000–1006.
3. **Bautista, A. P., A. Schuler, Z. Spolarics, and J. J. Spitzer.** 1992. In vivo late phagocytosis primes the Kupffer cells and hepatic neutrophils to generate superoxide anion. J. Leukocyte Biol. **51:**39–45.
4. **Betz, M., and B. S. Fox.** 1991. Prostaglandin E_2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. J. Immunol. **146:**108–113.
5. **Black, C. M., J. R. Catterall, and J. S. Remington.** 1987. In vivo and in vitro activation of alveolar macrophages by recombinant interferon-γ. J. Immunol. **138:**491–495.
6. **Bourbouze, R., R. Raffi, G. Dameron, H. Hali-Miraftab, F. Loko, and J.-L. Vilde.** 1991. N-Acetyl-β-D-glycosaminidase (NAG) isozymes released from human monocytes-derived macrophages in response to zymosan and human recombinant interferon-γ. Clin. Chim. Acta **199:**185–194.
7. **Buchmeier, N. A., and R. D. Schreiber.** 1985. Requirement of endogenous interferon-γ production for resolution of *Listeria monocytogenes* infection. Proc. Natl. Acad. Sci. USA **82:**7404–7408.
8. **Chida, K., Q. N. Myrvik, E. S. Leake, M. R. Gordon, P. H. Wood, and M. J. Ricardo Jr.** 1987. Chemiluminescent responses of alveolar macrophages from normal and *Mycobacterium bovis* BCG-vaccinated rabbits as a function of age. Infect. Immun. **55:**1476–1483.
9. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1994. Disseminated tuberculosis in interferon γ gene-disrupted mice. J. Exp. Med. **178:**2243–2247.
10. **Corradin, S. B., and J. Mauel.** 1991. Phagocytosis of *Leishmania* enhances macrophage activation by IFN-γ and lipopolysaccharide. J. Immunol. **146:**279–285.
11. **Debono, M., and R. S. Gordee.** 1994. Antibiotics that inhibit fungal cell wall development. Annu. Rev. Microbiol. **48:**471–497.
12. **Dunn, P. L., and R. J. North.** 1991. Early gamma interferon production by natural killer cells is important in defense against murine listeriosis. Infect. Immun. **59:**2892–2900.
13. **Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom.** 1994. An essential role for interferon γ in resistance to *Mycobacterium tuberculosis* infection. J. Exp. Med. **178:**2249–2254.
14. **Hayakawa, H., K. Umehara, and Q. N. Myrvik.** 1988. Oxidative responses of rabbit alveolar macrophages: comparative priming activities of MIF/MAF, sera, and serum components. J. Leukocyte Biol. **45:**231–238.
15. **Hirano, S., M. Iwata, K. Yamanaka, H. Tanaka, T. Toda, and H. Inui.** 1991. Enhancement of serum lysozyme activity by injecting a mixture of chitosan oligosaccharides intravenously in rabbits. Agric. Biol. Chem. **55:**2623–2625.
16. **Hsieh, C. S., A. B. Heimberger, J. S. Gold, A. O'Garra, and K. M. Murphy.** 1992. Differential regulation of T helper phenotype development by interleukin 4 and 10 in an alpha-beta T-cell-receptor transgenic system. Proc. Natl. Acad. Sci. USA **89:**6065–6069.
17. **Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy.** 1993. Development of T_H1 $CD4^+$ through IL-12 produced by *Listeria* induced macrophages. Science **260:**547–549.
18. **Johnston, R. B., C. A. Godzik, and Z. A. Cohn.** 1978. Increased superoxide anion production by immunologically activated and chemically elicited macrophages. J. Exp. Med. **148:**115–127.
19. **Marodi, L., S. Schreiber, D. C. Anderson, R. P. MacDermott, H. M. Korchak, and R. B. Johnston, Jr.** 1993. Enhancement of macrophage candidacidal activity by interferon-γ. Increased phagocytosis, killing, and calcium signal mediated by a decreased number of mannose receptors. J. Clin. Invest. **91:**2596–2601.
20. **Murray, H. W.** 1988. Interferon-gamma, the activated macrophage, and host defense against microbial challenge. Ann. Intern. Med. **108:**595–608.
21. **Myrvik, Q. N., A. G. Gristina, G. Giridhar, and H. Hayakawa.** 1993. Particle-induced in vivo priming of alveolar macrophages for enhanced oxidative responses: a novel system of cellular immune augmentation. J. Leukocyte Biol. **54:**439–443.
22. **Nafsik, H. G., and J. S. Tkacz.** 1995. The fungal cell wall as a drug target. Trends Microbiol. **3:**98–104.
23. **Nathan, C. F., H. W. Murray, M. E. Wiebe, and B. Y. Rubin.** 1983. Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J. Exp. Med. **158:**670–689.
24. **Nishimura, K., S. Nishimura, H. Seo, N. Nishi, S. Tokura, and I. Azuma.** 1986. Macrophage activation with multi-porous beads prepared from par-

tially deacetylated chitin. J. Biomed. Mater. Res. **20:**1359–1372.
25. **Nishimura, K., C. Ishihara, S. Ukei, S. Tokura, and I. Azuma.** 1986. Stimulation of cytokine production in mice using deacetylated chitin. Vaccine **4:**151–156.
26. **Nishimura, K., S. Nishimura, H. Seo, N. Nishi, S. Tokura, and I. Azuma.** 1987. Effect of multiporous microspheres derived from chitin and partially deacetylated chitin on the activation of mouse peritoneal macrophages. Vaccine **5:**136–140.
27. **Okumura, H., H. Tsutsui, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori, K. Akita, M. Namba, F. Tanabe, K. Konishi, S. Fukuda, and M. Kurimoto.** 1995. Cloning of a new cytokine that induces IFNγ production by T cells. Nature **378:**88–91.
28. **Pontow, S. E., V. Kery, and P. D. Stahl.** 1992. Mannose receptor. Int. Rev. Cytol. **137B:**221–244.
29. **Powrie, F., S. Menon, and R. L. Coffman.** 1993. Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity *in vivo*. Eur. J. Immunol. **23:**3043–3049.
30. **Schafer, R., and T. K. Eisenstein.** 1992. Natural killer cells mediated protection induced by a *Salmonella aroA* mutant. Infect. Immun. **60:**791–797.
31. **Scott, P.** 1991. IFNγ modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. J. Immunol. **147:** 3149–3155.
32. **Scott, P.** 1993. IL-12: initiation cytokine for cell-mediated immunity. Science **260:**496–497.
33. **Shibata, Y., and A. Volkman.** 1985. The effect of bone marrow depletion on prostaglandin E producing suppressor macrophages in mouse spleen. J. Immunol. **135:**3897–3904.
34. **Shibata, Y., D. R. Bjorkman, M. Schmidt, Y. Oghiso, and A. Volkman.** 1994. Macrophage colony stimulating factor-induced bone marrow macrophages do not synthesize or release prostaglandin E_2. Blood **83:**3316–3323.
35. **Shibata, Y.** 1995. Prostaglandin E_2 release triggered by phagocytosis of latex beads. A distinct association with prostaglandin synthase isozymes in bone marrow macrophages. J. Immunol. **154:**2878–2887.
36. **Skeen, M. J., M. A. Miller, T. M. Shinnick, and H. K. Ziegler.** 1996. Regulation of murine macrophage IL-12 production. Activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines. J. Immunol. **156:**1196–1206.
37. **Snijders, A., C. M. U. Hilkens, C. T. M. van der Pouw Kraan, M. Engel, L. A. Aarden, and M. L. Kapsenberg.** 1996. Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. J. Immunol. **156:**1207–1212.
38. **Stepherd, V. L., Y. C. Lee, P. H. Schlesinger, and P. D. Stahl.** 1981. L-Fucose-terminated glycoconjugates are recognized by pinocytosis receptors on macrophages. Proc. Natl. Acad. Sci. USA **78:**1019–1022.
39. **Tripp, C. S., M. K. Gately, J. Hakimi, P. Ling, and E. R. Unanue.** 1994. Neutralization of IL-12 decreases resistance to *Listeria* in SCID and C.B-17 mice. J. Immunol. **152:**1883–1887.
40. **Tripp, C. S., S. F. Wolf, and E. R. Unanue.** 1993. Interleukin 12 and tumor necrosis factor α are costimulators of interferon γ production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. Proc. Natl. Acad. Sci. USA **90:** 3725–3729.
41. **Warr, G. A.** 1980. A macrophage receptor for (mannose/glucosamine)-glycoproteins of potential importance in phagocytic activity. Biochem. Biophys. Res. Commun. **93:**737–745.
42. **Wong, H. L., M. T. Lotze, L. M. Wahl, and S. M. Wahl.** 1992. Administration of recombinant IL-4 to humans regulates gene expression, phenotype, and function in circulating monocytes. J. Immunol. **148:**2118–2125.
43. **Yamamoto, N., J. P. Zou, X. F. Li, H. Takenaka, S. Noda, T. Fujii, S. Ono, Y. Kobayashi, N. Mukaida, K. Matsushima, H. Fujiwara, and T. Hamaoka.** 1995. Regulatory mechanisms for production of IFN-γ and TNF by antitumor T cells or macrophages in the tumor-bearing state. J. Immunol. **154:** 2281–2290.
44. **Yoshihara, R., S. Shiozawa, T. Fujita, and K. Chihara.** 1993. Gamma interferon is produced by human natural killer cells but not T cells during *Staphylococcus aureus* stimulation. Infect. Immun. **61:**3117–3122.

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