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Nonopsonic and Opsonic Association of *Mycobacterium tuberculosis* with Resident Alveolar Macrophages Is Inefficient[1]

Richard W. Stokes,[2]*†‡§ Lisa M. Thorson,*‡ and David P. Speert*†‡§¶

The association of *Mycobacterium tuberculosis* with alveolar macrophages (Mϕ) in a serum-free environment is a crucial first step in the pathogenesis of this facultative intracellular pathogen. We present data demonstrating that freshly explanted alveolar Mϕ do not efficiently bind *M. tuberculosis* in a serum-free system, although a small subpopulation of these Mϕ (10–15%) can bind mycobacteria. In contrast, almost 100% of a peritoneal Mϕ population bind mycobacteria under the same conditions. The poor binding of mycobacteria by alveolar Mϕ does not reflect a general inability to associate with particles; binding and ingestion of latex beads and zymosan particles were comparable with that seen with peritoneal Mϕ. Resident alveolar Mϕ did not efficiently bind mycobacteria in the presence of serum and expressed poorly several Mϕ surface receptors, including CR3. Furthermore, we demonstrate that bovine surfactant protein A does not enhance the association of *M. tuberculosis* with alveolar Mϕ. Differentiation of alveolar Mϕ in vitro resulted in increased expression of Mϕ surface receptors and an increased capacity to bind mycobacteria in the presence and absence of serum. Evidence is presented that opsonic binding of *M. tuberculosis* by differentiated alveolar Mϕ is mediated by complement and CR3, and that the poor binding by resident alveolar Mϕ is due to their poor expression of CR3. The receptor mediating nonopsonic binding of *M. tuberculosis* to differentiated alveolar Mϕ was not unequivocally identified in this study, but could also be CR3. ***The Journal of Immunology*****, 1998, 160: 5514–5521.**

Mycobacterium tuberculosis is a facultative intracellular pathogen found almost exclusively in Mϕ[3] and located primarily in the apical regions of the lung. To better understand the interaction of *M. tuberculosis* with its host cell, investigators have developed models that utilize Mϕ obtained from humans and mice. Presumably, the best model would be the human alveolar Mϕ. Unfortunately, due to the constant exposure of human lungs to inhaled particles and microbes, the normal healthy human lung will undoubtedly have a mixed population of cells in the pulmonary lavage, including resident alveolar Mϕ, elicited (monocyte-derived) Mϕ, and possibly immune activated Mϕ. We have shown previously that the phenotype of a Mϕ greatly affects its interaction with mycobacteria (1). Thus, to fully understand the pathogenesis of mycobacteria in the different lung Mϕ populations, it will be necessary to study the interaction of mycobacteria with pure populations of Mϕ. Defined, pure, or enriched Mϕ populations can be obtained readily from animal models following experimental manipulations (e.g., the introduction of phlogistic agents into the lung will induce a population of predominantly elicited Mϕ). As the association of mycobacteria with human and murine Mϕ is known to be mediated by similar mechanisms (1, 2), we consider the mouse alveolar Mϕ to be a representative model for the human alveolar Mϕ. Moreover, the mouse model facilitates the acquisition of populations of Mϕ of a desired phenotype (resident, elicited, immune activated) and also favors reproducibility between samples. However, it will still be of importance to study the human pulmonary lavage Mϕ population as a representation of the in situ situation probably encountered by an inhaled mycobacterium.

Whereas opsonic phagocytosis of mycobacteria appears to be mediated by complement receptors binding to complement components fixed to the mycobacteria (3–6), the process of nonopsonic binding of mycobacteria is understood poorly. It has been suggested that mycobacteria can bind to Mϕ nonopsonically via the vitronectin receptor, CR1, CR3, FcγR, transferrin receptor, mannose receptor, or a glucan receptor (4–8). We have shown previously that the nonopsonic binding of *M. tuberculosis* to mouse peritoneal Mϕ is mediated predominantly by an epitope within CR3 distinct from that which binds iC3b (1), possibly the glucan binding site of CR3 recently described (9). These results have been confirmed using CHO cells transfected with human CD11b/CD18 (2). In contrast, little is known about the interaction of mycobacteria and alveolar Mϕ (8, 10, 11), the cell with which the bacteria initially associate.

We have investigated the ability of murine alveolar Mϕ to bind *M. tuberculosis* and have related those binding characteristics to the expression of Mϕ surface receptors. We have compared the alveolar Mϕ to the murine peritoneal Mϕ, as we have previously characterized the latter cell's ability to bind *M. tuberculosis* (1). We describe a number of observations concerning the interaction of alveolar Mϕ and *M. tuberculosis*: 1) nonopsonic binding of *M. tuberculosis* and other mycobacteria by resident alveolar Mϕ is extremely poor; 2) resident alveolar Mϕ do not efficiently bind mycobacteria (or other particles) in the presence of normal serum,

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[3] Abbreviations used in this paper: Mϕ, macrophage; BCG, bacille bilié de Calmette-Guérin; bSP-A, bovine surfactant protein A; EIgG, sheep red blood cells coated with immunoglobulin G; EIgMC′, sheep red blood cells coated with immunoglobulin M and inactivated complement component C3b; iC3b, inactivated complement component C3b; MDM, monocyte-derived macrophage; MOI, multiplicity of infection; SP-A, surfactant protein A.

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presumably because they do not express receptors for serum opsonins (12–17 and this study) or because there are minimal amounts of serum in the lung (18), consequently creating a redundancy for receptors on alveolar Mϕ that recognize serum opsonins; 3) SP-A does not enhance binding of *M. tuberculosis* to resident alveolar Mϕ; and 4) differentiation of resident alveolar Mϕ results in an increase in their ability to bind mycobacteria nonopsonically and opsonically, correlating with the increased expression of a number of Mϕ surface receptors.

Materials and Methods

Mycobacteria

M. tuberculosis, strain Erdman (Trudeau Mycobacterial Collection (TMC) No. 107; American Type Culture Collection (ATCC) No. 35801, Rockville, MD); *M. tuberculosis*, strain H37Rv (TMC 102, ATCC 27294); *M. tuberculosis*, strain H37Ra (TMC 201, ATCC 25177); and *Mycobacterium bovis* BCG, strain Pasteur (TMC 1011, ATCC 35734) were grown and stored as previously described (1).

Macrophages

Resident and thioglycollate-elicited murine peritoneal Mϕ from 6- to 8-wk-old female BALB/c mice were isolated and maintained as previously described (1). To obtain murine alveolar Mϕ, 6- to 8-wk-old female BALB/c mice were injected with a lethal dose of pentabarbitol, and the heart and lung were dissected out into cold PBS and washed free of blood. A 22G catheter (Critikon, Tampa, FL) was inserted into the trachea and tied off. While supporting the lungs in a jig, they were lavaged with 10 ml of PBS, containing 0.1% EDTA, in 1- to 2-ml aliquots (preliminary studies showed that the inclusion of EDTA increased the yield of Mϕ, but did not affect subsequent Mϕ functions). Pooled washings were pelleted and washed with supplemented RPMI (RPMI 1640 medium (Life Technologies, Grand Island, NY) plus 10% v/v FCS (Life Technologies), 10 mM L-glutamine, and 10 mM sodium pyruvate). The final pellet was resuspended in supplemented RPMI at 5×10^5/ml, and 100-μl aliquots were placed onto 13-mm coverslips. The cells were allowed to adhere for 1 h at 37°C in 5% CO_2, washed, and used immediately in experiments or, if being maintained in vitro, transferred to 24-well plates containing 1 ml supplemented RPMI and reincubated at 37°C in 5% CO_2 (if kept past day 4, the media were replenished on that day by removing 500 μl supernatant and adding 500 μl supplemented RPMI).

Particles for probing Mϕ receptors

The function of FcγR was investigated using EIgG, and complement receptors were identified using EIgMC′, whereas zymosan particles (prepared from bakers yeast, Sigma, St. Louis, MO) were used to probe for lectin-like phagocytic receptors, all as described previously (1).

In vitro assay for binding of particles to Mϕ

Adherent peritoneal and alveolar Mϕ were washed twice using binding medium (19), 138 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.6 mM $CaCl_2$, 1 mM $MgCl_2$, and 5.5 mM D-glucose. A 500-μl aliquot of binding medium was added to each well, and the cells were acclimatized for 10 min at 37°C, 5% CO_2. In some experiments, the Mϕ were incubated for an additional 15 min in the presence of mAb, bSPA (a gift from Dr. F. Possmeyer and K. Inchely, University of Western Ontario, London, Ontario), or PMA (Sigma) in binding medium. The overlay was then removed and replaced with 250 μl of binding medium containing any material to be tested in the system and 250 μl of binding medium containing particles, as previously described (1). Particles and Mϕ were then gently rocked for 1 h at 37°C, 5% CO_2 (Nutator; Becton Dickinson, Mountain View, CA), followed by an additional 2 h stationary at 37°C, 5% CO_2. The monolayers were then processed as previously described (1). In experiments comparing the binding characteristics of Mϕ maintained in vitro for different times (e.g., freshly explanted alveolar Mϕ and alveolar Mϕ maintained in vitro for 4 days), the Mϕ were set up on different days so that their binding characteristics could be compared on the same day using the same preparation of particles.

The binding of zymosan, EIgG, and EIgMC′ was assessed by counting the total number of particles associated with 100 Mϕ (Association Index), whereas the association of mycobacteria with Mϕ was estimated by counting the percentage of Mϕ with 0, 1 to 5, 6 to 10, or >10 associated bacteria, as previously described (1). The purpose of these studies was to identify the binding mechanisms involved in Mϕ/mycobacteria interactions, so no attempt was made to differentiate attachment and ingestion.

In some experiments, the association of *M. tuberculosis* with Mϕ in the presence of serum was assessed. Serum from BALB/c mice, prepared as described previously (20), was added to Mϕ and mycobacteria to give a final concentration of 1% serum. Heat-inactivated serum was prepared by heating at 56°C for 30 min.

FACS analysis

The expression of surface Ags on alveolar Mϕ was investigated using FACS analysis. Cells from five mice were pooled and incubated in supplemented RPMI in a Teflon container at 37°C, 5% CO_2 for 4 days before processing. On day 4, the cells were gently resuspended and pelleted by centrifugation. At the same time, cells from three mice were pooled and processed immediately after lavage. Both freshly isolated and day 4 cells were washed in wash buffer (RPMI with 2% v/v FCS, 20 mM HEPES, and 20 mM azide), resuspended in wash buffer, processed for FACS, as previously described (1), and read on a Becton Dickinson FACScan.

The mAbs used in this study were, unless stated otherwise, prepared from hybridomas obtained from ATCC and were as follows: M1/9.3.4.HL.2 (rat IgG2a, α CD45 (21)); F4/80 (rat IgG2b, α murine Mϕ marker (22)); 2.4G2 (rat IgG2b, α mouse FcγRII (23)); M1/70.15.11.5.HL (rat IgG2b, α mouse CD11b, recognizing an epitope that binds iC3b (21)); 5C6 (rat IgG2b, α mouse CD11b, recognizing an epitope that is involved in Mϕ attachment and spreading, but is distinct from that which binds iC3b (24, 25)); M17/4.4.11.9 (rat IgG2a, α mouse CD11a (26), was obtained from Developmental Studies Hybridoma Bank); M18/2.a.12.7 (rat IgG2a, α mouse CD18 (26), was obtained from Developmental Studies Hybridoma Bank); 8C12 (rat IgG from nude mouse ascites, α mouse CR1 (27), a generous gift from Dr. T. Kinoshita, Osaka University, Osaka, Japan); 2F8 (rat IgG2b, α murine scavenger receptor (28), a generous gift from Dr. S. Gordon, University of Oxford, Oxford, England); and N418 (hamster IgG, α mouse CD11c (29)). Controls for the mAb were as follows: SFR8-B6 (30) for the rat IgG and 1% normal hamster sera in wash buffer for N418. Following incubation with the primary Ab, cells were incubated with donkey anti-rat IgG FITC, or goat anti-hamster IgG FITC (Jackson ImmunoResearch, West Grove, PA), as appropriate, and processed as previously described (1).

Statistical analysis

Data are expressed as mean ± SEM. Student's *t* test for independent means was used to evaluate Mϕ binding; $p < 0.05$ was considered significant.

Results

Binding of M. tuberculosis *by freshly explanted alveolar Mϕ: a comparison with peritoneal Mϕ*

Confirming earlier studies (1), we demonstrated that freshly explanted murine, resident peritoneal Mϕ readily bind *M. tuberculosis* (Fig. 1). In contrast, freshly explanted, resident alveolar Mϕ did not bind *M. tuberculosis* efficiently (Fig. 1); the percentage of the cell population binding mycobacteria was significantly less ($p < 0.001$) than that seen with peritoneal Mϕ. Assessment of the distribution of mycobacteria within the Mϕ populations demonstrated that, in addition to very few freshly explanted, resident alveolar Mϕ binding *M. tuberculosis*, those Mϕ that did bind mycobacteria bound only one to five bacteria. In contrast, the majority of freshly explanted resident peritoneal Mϕ bound mycobacteria and, in addition, bound more bacteria (approximately 40% of the population bound more than five bacteria). Furthermore, the binding seen with alveolar Mϕ was obtained using an MOI 10 times higher than that used for peritoneal Mϕ. When mycobacteria were added to resident peritoneal Mϕ at an equal MOI as had been added to resident alveolar Mϕ (approximately 500:1), almost 100% of the population bound >10 bacteria (data not shown).

No significant difference ($p > 0.05$) was seen between the two Mϕ populations in their ability to bind latex particles (Fig. 1). This demonstrated that the poor binding of mycobacteria by alveolar Mϕ was not the result of a global inability to bind particles, and also demonstrated that the procedure for obtaining the Mϕ had not damaged them.

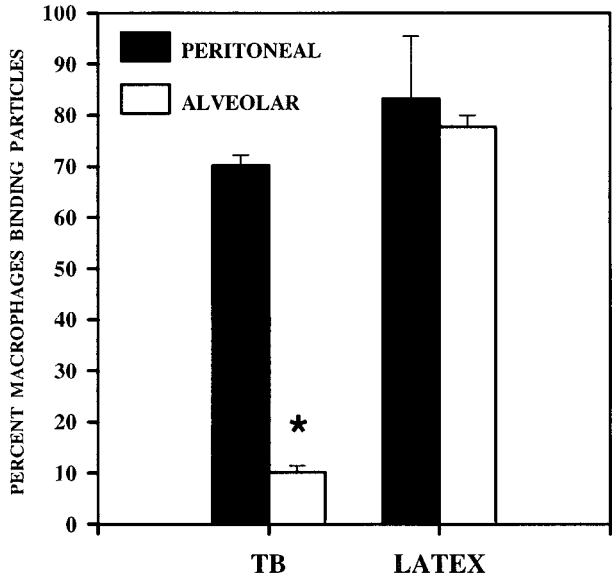

FIGURE 1. Resident peritoneal and alveolar Mϕ were obtained from BALB/c mice and adhered to coverslips in serum-supplemented medium for 2 h. The ability of these Mϕ to bind *M. tuberculosis* (TB) and 0.8-μm polyvinyl latex beads (LATEX) in a serum-free system was then assessed. The percentage of the Mϕ population binding ≥1 mycobacterium or ≥1 latex bead is shown. The mean ± SEM is shown for 14 (TB) or 3 (LATEX) experiments, each with triplicate coverslips. *$p < 0.001$ when comparing alveolar and peritoneal Mϕ.

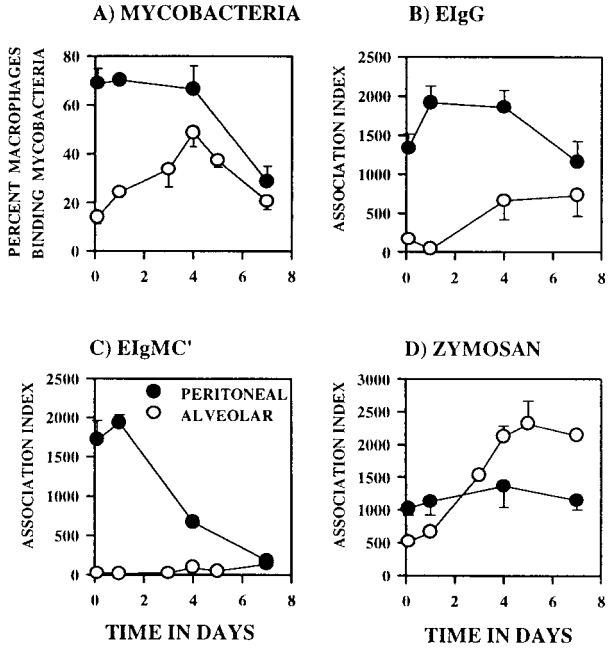

FIGURE 2. Resident alveolar (○) and peritoneal (●) Mϕ were obtained from BALB/c mice, adhered to coverslips, and maintained in vitro in serum-supplemented medium for up to 7 days. After each maintenance period, the adherent Mϕ were then tested for their ability to bind mycobacteria (*A*), EIgG (*B*), EIgMC′ (*C*), or zymosan (*D*) in a serum-free system for 3 h. Binding was assessed as percentage of Mϕ binding ≥1 mycobacterium or (for all other particles) as the number of particles associated with 100 Mϕ (Association Index). Each point represents the mean ± SEM of three experiments with duplicate coverslips.

The poor binding of *M. tuberculosis*, strain Erdman, by resident alveolar Mϕ was not limited to that strain; binding of *M. tuberculosis*, strains H37Rv and H37Ra, and of *M. bovis* BCG to alveolar Mϕ was equally poor (data not shown).

Binding of M. tuberculosis, *EIgG, EIgMC′, and zymosan by pulmonary and peritoneal Mϕ maintained in vitro for up to 7 days*

During the in vitro maintenance of resident alveolar Mϕ, the ability to bind *M. tuberculosis* was enhanced transiently, peaking at about day 4 and then declining (Fig. 2*A*). Over the same time period, resident peritoneal Mϕ were observed to efficiently bind *M. tuberculosis* until day 4, whereupon this ability began to decline (Fig. 2*A*).

Binding of control particles was used to probe for the expression of functional receptors for IgG (EIgG), complement component iC3b (EIgMC′), and glycans (zymosan) by resident alveolar and peritoneal Mϕ during their maintenance over 7 days. Freshly explanted resident peritoneal Mϕ bound all of the particles efficiently, with more than 90% of the population binding each particle. Over a 7-day period, peritoneal Mϕ lost their ability to bind EIgMC′ (Fig. 2*C*), while their ability to bind EIgG (Fig. 2*B*) and zymosan (Fig. 2*D*) increased initially and then returned to base level. Fresh alveolar Mϕ did not bind EIgG (Fig. 2*B*) or EIgMC′ (Fig. 2*C*) as efficiently as they did zymosan (Fig. 2*D*) with, respectively, approximately 40, 10, and 90% of the population binding each of these particles. Their ability to bind zymosan and EIgG increased with time in culture, but their ability to bind EIgMC′ remained negligible over the 7 days. Over the 7-day period, no correlation was seen between the binding of *M. tuberculosis* by alveolar Mϕ and their ability to bind control particles.

Table I. *Effect of PMA on the binding of* M. tuberculosis *and EIgMC′ by macrophages*[a]

Particle	PMA	Macrophages: Thioglycollate elicited	Macrophages: Day 0 alveolar	Macrophages: Day 4 alveolar
M. tuberculosis	−	31.5 ± 4.3[b]	17.5 ± 2.3[b]	39.0 ± 6.3[b]
M. tuberculosis	+	37.0 ± 4.8[b]	13.2 ± 2.6[b]	38.0 ± 11.5[b]
EIgMC′	−	0[c]	10 ± 5[c]	0[c]
EIgMC′	+	242 ± 56[c]	1 ± 1[c]	0[c]

[a] Binding of *M. tuberculosis* and EIgMC′ by thioglycollate elicited peritoneal Mϕ (thioglycollate elicited), freshly explanted resident alveolar macrophages (day 0 alveolar) and alveolar macrophages maintained in vitro for 4 days (day 4 alveolar) in the presence (+) or absence (−) of PMA. The mean ± SEM of two experiments, each with three coverslips, is shown.

[b] Binding of *M. tuberculosis* was measured as the percentage of the Mϕ population that bound ≥1 mycobacterium.

[c] Binding of EIgMC′ was measured as the number of particles per 100 Mϕ.

Binding and ingestion of zymosan by alveolar Mϕ were particularly efficient. More than 90% of freshly explanted alveolar Mϕ ingested zymosan, and after 4 days maintenance in vitro, this had increased to 100%. The number of zymosan particles ingested by each alveolar Mϕ at day 4 was between 20 and 30, resulting in the Mϕ appearing to be completely full of zymosan. The binding and ingestion of zymosan by alveolar Mϕ further demonstrated that the poor binding of mycobacteria by alveolar Mϕ was not the result of a general inability to bind particles.

Induction of particle binding by treatment of Mϕ with PMA

Elicited peritoneal Mϕ express CR3 in a nonfunctional state that can be activated to become functional by treatment with PMA (1, 31). We investigated the possibility that alveolar Mϕ could be stimulated with PMA to induce functional binding of particles by

Table II. *Effect of bSP-A on the binding of* M. tuberculosis *by macrophages*[a]

			Percent Macrophages Binding[b]			Association Index[c]		
Mϕ	bSP-A	*n*	>1 mycobacterium	>10 mycobacteria	*n*	Zymosan	*n*	EIgMC′
ALV	−	5	5.0 ± 2.1	0.2 ± 0.2	4	751 ± 68	4	2 ± 1
ALV	+	5	3.0 ± 1.5	0	4	1007 ± 16	4	1 ± 1
PER	−	5	74.8 ± 4.3	38.0 ± 6.4	4	1565 ± 21	4	1488 ± 46
PER	+	5	85.0 ± 5.8	55.6 ± 9.9	4	1960 ± 32	4	851 ± 123

[a] Binding of *M. tuberculosis*, zymosan, and EIgMC′ by freshly explanted resident alveolar (ALV) or peritoneal (PER) Mϕ, in the presence (+) or absence (−) of bSP-A. Mean ± SEM of two experiments, each with two or three coverslips (*n* = total number of coverslips assessed).

[b] Binding of *M. tuberculosis* is expressed as the percentage of the Mϕ population that bound ≥1 mycobacterium and the percentage that bound ≥10 mycobacteria.

[c] Binding of EIgMC′ and zymosan was measured as the number of particles per 100 Mϕ (Association Index).

CR3. In these experiments, we used thioglycollate-elicited peritoneal Mϕ as a positive control for the action of PMA on Mϕ. As previously reported (1), treatment of thioglycollate-elicited peritoneal Mϕ with PMA induced a significant ($p < 0.05$) increase in EIgMC′ binding, but not ($p > 0.05$) *M. tuberculosis* binding (Table I). PMA did not induce an increase in the binding of EIgMC′ or of mycobacteria by freshly explanted alveolar Mϕ (Table I). Day 4 alveolar Mϕ were similarly unaffected by PMA (Table I). This experiment shows that alveolar Mϕ do not express CR3 in a nonfunctional state.

Induction of particle binding by treatment of Mϕ with bSP-A

There are reports describing how the binding of some bacteria by alveolar Mϕ can be increased by treatment with SP-A (32–34). We tested whether bSP-A could enhance the binding of *M. tuberculosis* to murine, resident alveolar Mϕ (Table II). No increase in binding of *M. tuberculosis* by alveolar Mϕ was observed after treatment with bSP-A. Identical treatment of resident peritoneal Mϕ appeared to induce an observable, albeit statistically insignificant, increase in binding of mycobacteria (Table II). Binding of zymosan by both resident alveolar and peritoneal Mϕ appeared to be increased by bSP-A treatment (Table II), although this increase was not statistically significant. Binding of EIgMC′ by alveolar Mϕ was unaffected by bSP-A, whereas peritoneal Mϕ demonstrated a decrease in their ability to bind EIgMC′ following bSP-A treatment, although this decrease was not statistically significant.

Reactivity of mAb recognizing Mϕ surface ligands with alveolar Mϕ

FACS analysis of alveolar Mϕ (Table III) revealed that freshly explanted, resident cells expressed high levels of CD45 (>20 times that of the control background fluorescence) and intermediate levels of CD18, CD11a, CD11c, and FcγR (2–5 times that of the control fluorescence). Expression of CD35, CD11b, the Mϕ marker F4/80, and the scavenger receptor 2F8 by resident alveolar Mϕ was very low (less than twice that of the control fluorescence). Interestingly, the expression of the epitope within CD11b, which is recognized by 5C6, was expressed strongly by a small subpopulation (approximately 10%) of freshly explanted alveolar Mϕ (data not shown), whereas the epitope recognized by M1/70 was not expressed at all.

FACS analysis of alveolar Mϕ maintained in vitro for 4 days (Table III) showed that mAb-reactive receptor expression had increased during the maintenance of the phagocytes. High levels of CD45 were still expressed. Surprisingly, the expression of CD11a had declined. The expression of CD11b (both epitopes) and CD11c had increased to more than 5 times that of the control values. While all of the other ligands investigated were expressed at higher levels than at day 0, expression still remained relatively low (less than 5 times that of control levels). More than 90% of the alveolar Mϕ expressed the 5C6-reactive epitope after 4 days in culture (data not shown). It should be noted, however, that at no time did

Table III. *Expression of surface Ag on alveolar macrophages*[a]

		Fluorescence Intensity			
Ab	Epitope	Day 0 alveolar	*n*	Day 4 alveolar	*n*
SFR8-B6	Rat IgG control	7.8 ± 3.2	4	8.1 ± 0.9	4
F4/80	Macrophage	8.7 ± 0.8	3	26.6 ± 4.2	4
M1/9	CD45 (LCA)	159.1 ± 10.1	3	200.3 ± 41.6	4
2.4G2	FcR	19.7 ± 6.5	3	35.6 ± 4.3	4
2F8	Scavenger receptor	5.0 ± 0	2	21.3 ± 4.0	3
M18	CD18 (β_2 integrin)	23.9 ± 10.5	3	30.3 ± 6.4	4
M17	CD11a (LFA)	32.2 ± 5.4	3	16.5 ± 0.6	3
M1/70	CD11b (CR3)-iC3B	4.1 ± 0.2	3	56.0 ± 19.8	4
5C6	CD11b(CR3)-NOT iC3b	13.6 ± 6.8	4	51.3 ± 18.4	4
8C12	CD35(CR1)	11.7 ± 6.5	3	38.9 ± 28.8	4
Hamster sera	Control	6.5 ± 0.0	2	14.1 ± 4.2	2
N418	CD11c (CR4)	31.2 ± 8.9	2	56.7 ± 4.8	2

[a] Alveolar washings were processed immediately (day 0 alveolar) or maintained in vitro for 4 days before processing (day 4 alveolar). The mean ± SEM fluorescence intensity from 2 to 4 (*n*) separate measurements is given for each mAb. For comparison, the fluorescence intensity of some of the mAb reacting with resident peritoneal Mϕ was: IgG control, 16.7; F4/80, 187.7; FcR, 63.9; CD11b (CR3)-iC3b, 433.2; CD11b (CR3)-NOT iC3b, 177.8 (from Ref. 1).

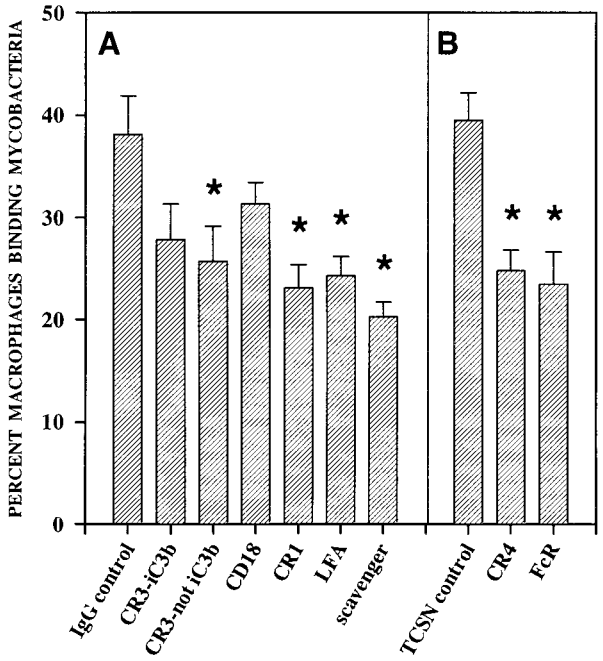

FIGURE 3. Resident alveolar Mϕ were maintained in vitro in serum-supplemented medium for 4 days, and then assessed for their ability to bind *M. tuberculosis* in a serum-free system ± mAb recognizing Mϕ receptors. An isotype control (IgG control) was used for purified mAb (*A*) and a tissue culture supernatant containing control IgG (TCSN control) for mAb in tissue culture supernatant (*B*). The epitope recognized by each mAb is shown; for details of the mAb used, see *Materials and Methods*. The mean ± SEM percentage of the Mϕ population binding ≥1 mycobacterium is shown for four (*A*) or two (*B*) experiments, each with two or three coverslips. *$p < 0.05$ when compared with the appropriate control group.

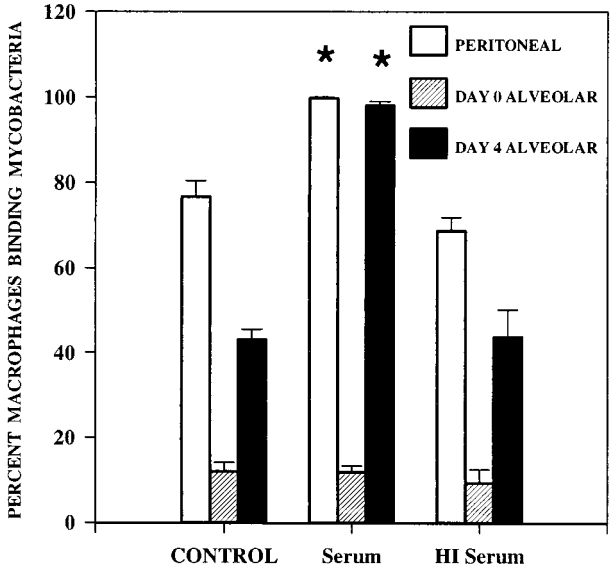

FIGURE 4. Resident alveolar and peritoneal Mϕ were obtained from BALB/c mice, adhered to coverslips, and maintained in vitro in serum-supplemented medium. After 2 h of adherence (peritoneal and day 0 alveolar) or 4 days in vitro (day 4 alveolar), the binding of *M. tuberculosis* to these cells was assessed in the absence (control) or presence of 1% normal mouse serum, either whole (serum) or heat-inactivated (HI serum). The mean ± SEM percentage of the Mϕ population binding ≥1 mycobacterium is shown for two experiments, each with triplicate coverslips. *$p < 0.001$ when compared with the appropriate control group.

the expression of surface receptors on alveolar Mϕ approach the levels seen for resident peritoneal Mϕ (Table III) (1).

Inhibition of binding of M. tuberculosis *to day 4 alveolar Mϕ by mAb recognizing Mϕ receptors*

The demonstration that day 4 alveolar Mϕ were better able to bind mycobacteria (Fig. 2) than were freshly explanted alveolar Mϕ and that this ability coincided with an increase in surface receptor expression (Table III) suggested that the increased binding was mediated by these receptors. To test this, mAb recognizing Mϕ receptors were investigated for their ability to inhibit the enhanced binding of mycobacteria by day 4 alveolar Mϕ. Binding was significantly ($p < 0.05$) inhibited by six of the eight mAb tested (recognizing CR3 (not the iC3b epitope), CR1, LFA, CR4, FcγR, and the scavenger receptor) and appeared to be slightly suppressed by the remaining two mAb (recognizing CD18 and the iC3b epitope of CR3), albeit at a statistically insignificant ($p > 0.05$) level (Fig. 3). In contrast to these results, no significant inhibition ($p > 0.05$) of mycobacteria binding to freshly explanted alveolar Mϕ was observed in the presence of the same mAb (data not shown).

Binding of M. tuberculosis *by murine peritoneal and alveolar Mϕ in the presence of serum*

Binding of *M. tuberculosis* to freshly explanted resident peritoneal Mϕ was significantly ($p < 0.001$) enhanced in the presence of 1% normal murine serum. This increase was dependent on a heat-labile component of serum, as heat-inactivated serum did not significantly ($p > 0.05$) enhance binding (Fig. 4). Normal serum (1%) had no significant effect ($p > 0.05$) on the binding of *M. tuberculosis* to freshly explanted, resident alveolar Mϕ (Fig. 4), whereas the binding of *M. tuberculosis* by day 4 alveolar Mϕ was significantly ($p < 0.001$) increased in the presence of 1% whole, but not ($p > 0.05$) heat-inactivated, serum (Fig. 4). Serum-mediated increases in binding were manifested as an increase in the percentage of the Mϕ population binding mycobacteria (Fig. 4) and as an increase in the number of mycobacteria bound; the percentage of peritoneal Mϕ binding >10 mycobacteria increased from 39 ± 6.8 in serum-free controls to 94.8 ± 1.4 in the presence of serum, and for day 4 alveolar Mϕ increased from 4 ± 0.6 to 83.8 ± 3.5, each significant ($p < 0.001$) increases. The increased binding of *M. tuberculosis* by day 4 alveolar Mϕ in the presence of 1% serum was inhibited significantly ($p < 0.001$) by mAb recognizing CR3, but not those recognizing other receptors (CR1, CR4, FcγR) for serum opsonins (Fig. 5).

Discussion

It is commonly accepted that inhaled mycobacteria are ingested readily by the host's resident alveolar Mϕ and that this event initiates an infection, the outcome of which is then regulated by such factors as the virulence of the mycobacteria and the inherent microbicidal power of the alveolar Mϕ (35). Our data demonstrate that the supposition that mycobacteria are ingested readily by the alveolar Mϕ should be modified. We have shown previously that elicited and activated peritoneal Mϕ are less able to bind and ingest mycobacteria nonopsonically than are resident peritoneal Mϕ (1). In this study, we extend those observations to show that resident alveolar Mϕ are considerably less able to bind *M. tuberculosis* than are resident peritoneal Mϕ. This inferior binding was evident for both nonopsonic and opsonic binding of mycobacteria, but was not the result of a generalized inability of alveolar Mϕ to bind and ingest particles: their ability to ingest latex beads and zymosan was comparable with that of peritoneal Mϕ.

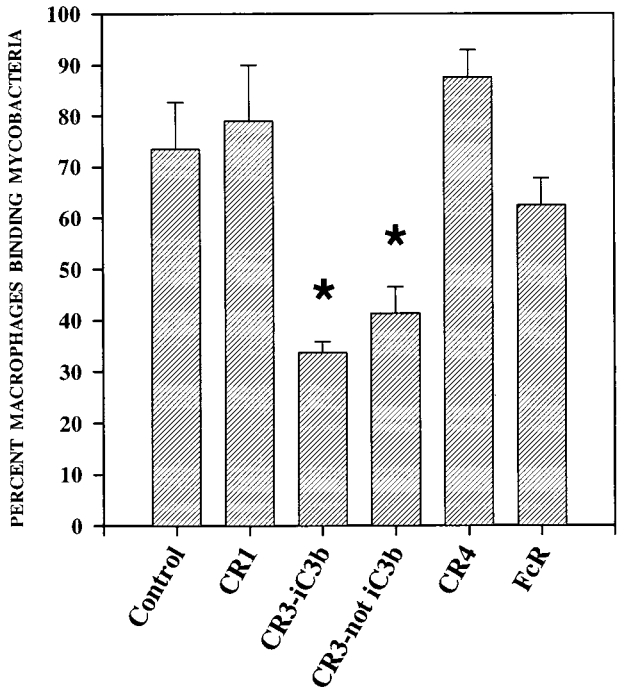

FIGURE 5. Resident alveolar Mϕ were maintained in vitro for 4 days, and then assessed for their ability to bind *M. tuberculosis* in the presence of 1% whole mouse serum ± mAb recognizing Mϕ receptors. The epitope recognized by each mAb is shown; for details of the mAb used, see *Materials and Methods*. The mean ± SEM percentage of the Mϕ population binding ≥1 mycobacterium is shown for two experiments, each with three coverslips. *$p < 0.001$ when compared with the control group.

Freshly explanted alveolar Mϕ were also poorly able to bind particles coated with IgG or iC3b. This is in agreement with earlier reports (12–17) and suggests a lack of functional receptors for serum opsonins. This failing appeared to be due to a lack of expression of receptors rather than a lack of receptor function, as FACS analysis of freshly explanted alveolar Mϕ showed minimal expression of receptors for IgG (FcγR) or C3b/iC3b (CR1/CR3/CR4). Furthermore, treatment of alveolar Mϕ with PMA did not induce an increase in the binding of EIgMC′; PMA has been shown to increase binding of EIgMC′ by some Mϕ populations due to the induction of functional binding of iC3b by receptors expressed on the Mϕ surface in a previously nonfunctional state (1, 31, and Table I). The nonopsonic binding of bacteria by murine alveolar Mϕ has not been studied extensively, but appears to be uniformly poor; they do not bind *Listeria monocytogenes* (13), *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* (32), *M. bovis* BCG (36), or *M. tuberculosis* (this study) very efficiently. In contrast to their poor binding of bacteria, alveolar Mϕ avidly bind fungal pathogens (37, 38), presumably by the mechanism with which they bind zymosan.

Analysis of surface receptor expression of resident alveolar Mϕ from humans and other primates shows low levels of CD11b and CD35 with relatively higher expression of CD11a, CD11c, and CD18 (39–44), just as we demonstrate in this work for resident murine alveolar Mϕ. Human alveolar Mϕ, like their murine counterpart, bind bacteria poorly, both in the absence and presence of normal serum. Binding of *P. aeruginosa* by human alveolar Mϕ was minimal and was not enhanced by normal serum (40). Similarly, the binding of *M. tuberculosis* to human alveolar Mϕ has been shown to be very poor; using an infection ratio of 100 bacteria to 1 Mϕ in the presence of 2% normal serum, only 20% of a Mϕ population ingested an average of 3 mycobacteria each (10). In contrast, the association of *M. tuberculosis* with human MDMs under similar conditions (MOI of 100 to 1 in the presence of 1% serum) results in 95% of the Mϕ binding in excess of 10 bacteria each (R. W. Stokes, unpublished observations).

The maintenance of alveolar Mϕ in vitro resulted in an increase in surface expression of a number of receptors (F4/80, FcγR, CR3, CR4, and scavenger receptor), as detected by FACS, which corresponded with the increased binding of *M. tuberculosis* by these Mϕ. It is possible that the day 4 alveolar Mϕ are representative of an in vivo Mϕ population that has differentiated after recent ingress into the lung. However, in the same way that there is uncertainty as to whether the in vitro differentiated human MDM represents any population of Mϕ found in vivo, we cannot be sure that the in vitro differentiated alveolar Mϕ truly represent a possible Mϕ phenotype found in vivo. This question could be addressed by characterizing the phenotype of Mϕ obtained from the lungs of mice infected with mycobacteria or treated with phlogistic agents and is currently under investigation.

M. tuberculosis binds nonopsonically to resident peritoneal Mϕ predominantly via an epitope within CR3 distinct from that which binds iC3b (1, 2). If resident alveolar Mϕ use the same mechanism, their poor binding of *M. tuberculosis* could be explained by their low level of CR3 expression, and consequently, the increased binding of *M. tuberculosis* by alveolar Mϕ maintained in vitro for 4 days could be due to their increased expression of CR3. However, inhibition of nonopsonic binding of mycobacteria to alveolar Mϕ by mAb recognizing Mϕ receptors did not support the above contention. Binding by freshly explanted alveolar Mϕ was not inhibited significantly by any mAb, but as initial binding levels of mycobacteria by resident alveolar Mϕ were so low, it is likely that inhibition studies were unresolvable. With day 4 alveolar Mϕ, all of the mAb tested appeared to inhibit binding to some degree, ranging from 25 to 50% of the control value. This may reflect a diverse array of receptors mediating the nonopsonic binding of *M. tuberculosis* to alveolar Mϕ, as has been previously suggested (8). Alternatively, it may indicate nonspecific effects of the mAb interacting with Mϕ (45), thus demonstrating the problem with relying on mAb inhibition as the only indication of receptor involvement in the binding of particles to Mϕ. We consider the latter explanation to be the most likely, as inhibition of binding appeared to be equally effective with mAb that recognized receptors that had been up-regulated (e.g., CR3) or down-regulated (e.g., LFA) during in vitro maintenance. It is hard to reconcile the importance of a receptor in binding mycobacteria (as shown by the mAb inhibition) with the observation that the receptor is down-regulated over the same period that binding of mycobacteria is up-regulated. Thus, we consider that the receptor that mediates the nonopsonic binding of mycobacteria to alveolar Mϕ has yet to be determined.

Evidence that binding of *M. tuberculosis* to day 4 alveolar Mϕ in the presence of serum is mediated by heat-labile complement components that bind to CR3 was obtained. These results suggested that complement fixes to the mycobacterial surface and mediates binding to differentiated alveolar Mϕ CR3, as has been reported in other models of serum-mediated binding of mycobacteria by Mϕ (3–6). Other receptors for complement components (CR1 and CR4) and the receptor for IgG (FcγR) did not appear to be involved in the serum opsonin-mediated binding of *M. tuberculosis* to day 4 alveolar Mϕ. This is in contrast to a study on serum opsonin-mediated binding of mycobacteria to human alveolar Mϕ in which CR4 was shown to predominate, with CR1 and CR3 playing a minor role (10). However, in another study (46), it was found that human alveolar Mϕ bind complement-coated particles

predominantly via CR3 with little involvement of CR4. These contrasting results may reflect variation in the constituent populations of Mϕ within the human alveolar lavages of these two studies.

The observation that day 4 alveolar Mϕ bind only complement-coated mycobacteria and not complement-coated SRBC, whereas peritoneal Mϕ bind both (Figs. 4 and 2*C*) was a surprising observation that we currently cannot explain. Our favored explanation is that resident alveolar and peritoneal Mϕ express different densities of CR3 on their surface (1 and Table III), which, coupled with the difference in size of the SRBC and the mycobacteria, results in the variation in binding. This possibility and other possibilities are currently being investigated further. However, it must be emphasized that the freshly explanted alveolar Mϕ binds neither SRBC nor mycobacteria in the presence of serum.

It is possible that other, nonserum opsonins may mediate increased binding of mycobacteria to resident alveolar Mϕ. One candidate, the lung surfactant SP-A, has been suggested to increase the binding of mycobacteria to human MDM and alveolar Mϕ (47) and to murine alveolar Mϕ (34). SP-A is also reported to enhance the association of other microorganisms with Mϕ in a species-dependent manner (32, 33, 38). We found that bSP-A did not affect the binding of *M. tuberculosis* to resident murine alveolar Mϕ, whereas binding to peritoneal Mϕ was slightly increased (Table II). The difference between our result and those of others may be due to the different origins of the SP-A that was used. Alternatively, it may merely reflect the way the data are presented; in one report, the binding of *M. tuberculosis* by normal alveolar Mϕ was said to increase in the presence of SP-A and was defined as an increase of 2% (from 10 to 12) of the Mϕ-binding mycobacteria (34), whereas in another report, binding of *M. tuberculosis* by alveolar Mϕ increased in the presence of SP-A, but it was not clear how substantial was this increase, as only percentage changes in binding were reported and not the actual levels of binding (47). However, bSP-A was not without effect on the binding characteristics of murine Mϕ. In agreement with earlier studies using rat SP-A (16), we found that bSP-A inhibited binding of iC3b-coated particles to peritoneal Mϕ. In addition, the binding of zymosan by resident alveolar Mϕ (and peritoneal Mϕ) was increased in the presence of bSP-A. We conclude from these experiments that bSP-A has no affect on the binding of *M. tuberculosis* to resident, murine alveolar Mϕ, but can increase binding of the bacteria to other Mϕ populations. Thus, within the human alveolar Mϕ population of mixed phenotypes, there may be a subpopulation of Mϕ (comparable with MDM) that can bind mycobacteria opsonized with SP-A.

Our observations have major implications for the pathogenesis of *M. tuberculosis* in the lung. On deposition in the lung, an inoculum of *M. tuberculosis* has been considered previously to be avidly ingested by alveolar Mϕ (35). We now present evidence that this is not so; a very small subpopulation of resident Mϕ will ingest the mycobacteria nonopsonically. If the mycobacteria are able to survive and replicate, they could induce the production of cytokines from cells in the vicinity of the infection, which could result in the differentiation of resident Mϕ and also in the deposition of elicited Mϕ, along with accompanying serum at the site of infection. Differentiated alveolar Mϕ (as represented by day 4 alveolar Mϕ in this study) are better able to bind mycobacteria nonopsonically and opsonically. Elicited Mϕ bind mycobacteria poorly in the absence of serum opsonins (1), but strongly in the presence of normal serum (R. W. Stokes, unpublished observations). Thus, in this study, we provide further evidence for our contention (1) that the interaction of mycobacteria with Mϕ is dependent on the phenotype of the Mϕ. This interaction is impacted further by the presence or absence of opsonins. Mycobacteria will therefore enter their host cell via a number of possible receptor/ligand interactions, and the nature of this receptor/ligand interaction will preferentially direct the bacteria to certain Mϕ phenotypes. For example, if the bacterium is coated with iC3b, it will direct it away from resident alveolar Mϕ, but toward differentiated alveolar Mϕ and elicited Mϕ. We would suggest that the survival and subsequent pathogenesis of a mycobacterium are affected by the precise nature of the interaction of a bacterium with its host cell and by the phenotype of the Mϕ that ingests that bacterium.

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