A Macrophage Invasion Mechanism for Mycobacteria Implicating the Extracellular Domain of CD43

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Abstract

We studied the role of CD43 (leukosialin/sialophorin), the negatively charged sialoglycoprotein of leukocytes, in the binding of mycobacteria to host cells. CD43-transfected HeLa cells bound *Mycobacterium avium*, but not *Salmonella typhimurium* or *Shigella flexneri*. Quantitative bacteriology showed that macrophages (Mϕ) from wild-type mice ($CD43^{+/+}$) bound *M. avium*, *Mycobacterium bovis* (bacillus Calmette-Guérin), and *Mycobacterium tuberculosis* (strain H37Rv), whereas Mϕ from CD43 knockout mice ($CD43^{-/-}$) did not. Fluorescence microscopy demonstrated that the associated *M. avium* had been ingested by the $CD43^{+/+}$ Mϕ. The inability of $CD43^{-/-}$ Mϕ to bind *M. avium* could be restored by addition of galactoglycoprotein (Galgp), the extracellular mucin portion of CD43. The effect of Galgp is not due to opsonization of the bacteria, but required its interaction with the Mϕ; other mucins had no effect. CD43 expression by the Mϕ was also required for optimal induction by *M. avium* of tumor necrosis factor (TNF)-α production, which likewise could be reconstituted by Galgp. In contrast, interleukin (IL)-10 production by *M. avium*–infected Mϕ was CD43 independent, demonstrating discordant regulation of TNF-α and IL-10. These findings describe a novel role of CD43 in promoting stable interaction of mycobacteria with receptors on the Mϕ enabling the cells to respond specifically with TNF-α production.

Key words: mycobacteria • CD43 • macrophages • tumor necrosis factor α • interleukin 10

Introduction

Mycobacteria are among the most important infectious agents in the world. *Mycobacterium tuberculosis*, which is spread by airborne transmission, causes more deaths globally than any other infectious agent. Estimates indicate 90 million people infected with *M. tuberculosis* during the current decade, 30 million of whom will die as a result of infection (1). In the United States, *Mycobacterium avium* complex has emerged as the most prevalent opportunistic infection among patients with advanced HIV-1 infection. *M. avium* infections in patients with AIDS and other immunodeficiencies typically manifest with widespread visceral organ involvement and high-grade bacteremia; the patients suffer considerable morbidity and shortened life span (2–5).

Macrophages (Mϕ),[1] the predominant host cells, are the first line defense against spread of mycobacterial infection. In the successful process, Mϕ eliminate mycobacterial in-

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[1]*Abbreviations used in this paper:* AFGP, antifreeze fish glycoprotein; BCG, bacillus Calmette-Guérin; Galgp, galactoglycoprotein; CFDA, carboxyfluorescein diacetate; Mϕ, macrophage(s); PSM, porcine submaxillary gland mucin; SP-A, surfactant protein A.

fection by a complex network of events involving TNF-α production (6) and leading to apoptosis and elimination of the host Mϕ containing endocytosed microorganisms (7, 8). Pathogenic mycobacteria have evolved mechanisms to survive and replicate within endosomes of Mϕ (9) after entering the cells by one of several Mϕ receptor pathways. These include complement receptors CR1, CR3, and CR4, mannose receptor, CD14, Fc receptors, scavenger receptors, and one or more receptors for pulmonary surfactant protein A (SP-A) (10, 11). To some extent, the outcome of mycobacterial infection is influenced by the receptor, in that uptake via Fc receptors, but not complement receptors, induces reactive oxygen metabolites, a process believed to decrease survival of endocytosed mycobacteria (12).

This study aims to understand the role of nonreceptor surface molecules in regulating binding and uptake of mycobacteria by Mϕ. CD43, a cell surface mucin on macrophages, was considered as a candidate regulatory molecule because of its known ability to interfere with adhesion processes. For example, CD43-transfected HeLa cells, unlike wild-type HeLa cells, display decreased intracellular adhesion molecule (ICAM)-mediated adhesion (13), and targeted disruption of the CD43 gene in mice increased adhesiveness of splenic T cells (14). Therefore, we used CD43-transfected and wild-type HeLa cells as well as Mϕ from CD43 knockout and wild-type mice to determine whether the presence of CD43 diminishes binding of mycobacteria to Mϕ and, by doing so, diminishes or abrogates important downstream events such as cytokine production.

Materials and Methods

Animals. CD43 knockout mice were generated by homologous recombination via embryonic stem cell chimeras (14). The $CD43^{-/-}$ mice and control wild-type mice (in C57 × SVJ 129 background) were maintained under specific pathogen-free conditions at the Tufts University School of Medicine. For all experiments, 6–8-wk-old mice matched for sex were used. In select experiments, gene status was verified by flow cytometric analysis of cells stained with PE-conjugated S7 rat anti–mouse CD43 mAb (BD PharMingen).

Microorganisms. *Shigella flexneri* and *Salmonella typhimurium* were provided by Dr. J. Cohen, Imperial College of Science, Technology and Medicine (London, UK). BCG (*M. bovis* strain bacillus Calmette-Guérin) was obtained from the Trudeau Institute, Saranac Lake, NY. The *M. tuberculosis* strain H37Rv was a gift of Dr. Hardy Kornfeld, Boston University School of Medicine. *M. avium* serovar 4, isolated from an AIDS patient and typed by the Mycobacterial Culture Collection (National Jewish Hospital, Denver, CO), was grown to log phase in Middlebrook 7H9 broth with 5 mg/ml albumin, 1 mg/ml dextrose, and 3 μg/ml catalase (OADC; Difco). The mycobacteria were harvested by centrifugation (2,000 *g*), washed twice in HBSS, suspended in Middlebrook 7H9 broth, sonicated for 10 s at 500 W, and stored in aliquots at −70°C. *M. avium*, BCG, and *M. tuberculosis* were thawed as needed, sonicated for 20 s to disperse the bacteria, and quantified by plate counting. 10-fold dilutions (5 replicates per dilution) were plated on Middlebrook 7H11 agar with albumin-dextrose-catalase enrichment. The plates were incubated at 37°C in 90% humidity for 10 d to 4 wk, and colonies were counted. *M. avium* colonies were exclusively of the smooth, transparent morphotype (15) and appeared homogeneously dispersed.

HeLa Cell Lines. The generation of HeLa cell CD43 transfectants was described previously (13). Wild-type HeLa cells and HeLa cells transfected with vector (pSV2Neo) were used as controls. The cells in RPMI 1640 with 10% heat-treated (56°C for 30 min) FCS and 25 mM Hepes were propagated by plating 2×10^6 cells/ml in 24-well culture plates (Costar) and passaging by EDTA treatment. Immediately before infection, confluent HeLa cells were washed three times with HBSS, and fresh medium was added.

Live *M. avium*, *S. flexneri*, and *S. typhimurium* were stained with carboxyfluorescein diacetate (CFDA; Molecular Probes). The bacteria at 10^7/ml were incubated with 4 μM CFDA in DMEM at 37°C for 30 min. After extensive washing, the CFDA-stained microorganisms were used to challenge confluent cultures of CD43-transfected, wild-type, and vector-transfected HeLa cells by coculture at 1, 5, or 10 microorganisms/cell for 2–4 h. The cells were washed three times in HBSS and analyzed by fluorescence microscopy.

Murine Spleen Mϕ. Murine spleens were removed sterilely and teased, and the released cells were washed three times in HBSS and suspended in RPMI 1640 with 10% heat-treated (56°C for 30 min) FCS, 25 mM Hepes, and 2×10^{-5} M 2-ME (Sigma-Aldrich). All media tested negative for LPS (<0.05 ng/ml) in the Limulus amebocyte lysate assay (BioWhittaker). Spleen cells were plated at 2×10^6 cells/well in 24-well plates (Costar) for 4–6 d in 5% CO_2 at 37°C. Immediately before infection, nonadherent cells were discarded, and the Mϕ populations, which were 97–99% pure by esterase stain, were washed four times in HBSS, and fresh medium was added.

Triplicate wells of adherent Mϕ (~1.0×10^5 cells/well) were inoculated with *M. avium*, BCG, or *M. tuberculosis* strain H37Rv at 2, 10, or 20 microorganisms/cell and cultured for 1–4 h for mycobacteria binding assays or 4–48 h for cytokine measurements. To quantify bound mycobacteria, the adherent Mϕ were washed four times with HBSS, lysed by addition of SDS (500 μl of 0.2%), and the effect of SDS was terminated with FCS (500 μl 50%). Cell lysates (100 μl) were cultured at 37°C in 5-ml vials containing culture broth and [^{14}C]palmitic acid (Bactec vials; Becton Dickinson). Accumulated $^{14}CO_2$ was quantified at 24-h intervals using the Bactec model 460TB system (Becton Dickinson). Plots of accumulated $^{14}CO_2$ versus time of culture yielded T-100 values, defined as time needed to reach $^{14}CO_2$ accumulation value of 100 on a scale of 0–999 (16). Linear correlation was found between T-100 and the log of bacterial number over the range 10^1 to 10^7 organisms, and assay results, expressed as number of mycobacteria associated with Mϕ, were determined from a calibration plot. Calibration was provided by periodic parallel assay of mycobacteria that had been quantified by plate counting.

Light Microscopy. Monolayers of adherent Mϕ were prepared by plating 2×10^6 murine spleen cells on 13-mm-diameter plastic coverslips (Thermanox™, no. 174950; Nunc); these were incubated with *M. avium*, harvested and washed as described above, and dried. The coverslips were stained with TB carbolfuchsein KF and counterstained with TB brilliant green K following the manufacturer's instructions (Kinyoun acid-fast staining procedure for mycobacteria; Difco). Mϕ containing >10 detectable bacteria were scored as positive. Quantitation is based on visual scoring of 3–5 fields with 30–80 cells each.

Fluorescence Microscopy. Monolayers of adherent Mϕ on plastic coverslips as described above were incubated with *M. avium*, harvested, washed, and fixed with 4% paraformaldehyde in PBS.

The mycobacteria in the fixed monolayers were stained with the rhodamine-auramine TB Fluorescent stain kit (no. 4312521; Becton Dickinson) according to the manufacturer's directions. The stained cells were examined by phase–contrast and fluorescent microscopy with a Nikon microscope.

Galactoglycoprotein and Other Mucins. Galactoglycoprotein (Galgp; provided by Dr. Karl Schmid, Department of Biochemistry, Boston University School of Medicine, and Dr. H. Gerhard Schwick, Behringwerke AG, Marburg/Lahn, Germany), was purified from pooled normal human plasma by anion exchange chromatography of the supernatant of Cohn fraction V followed by gel filtration (17) with the additional step of solid-phase immunoabsorption using antiserum raised in a rabbit that had been immunized with Galgp but that produced antibodies only against the trace contaminants (18). Galgp appeared as a single ~120-kD band on SDS gels stained with Alcian blue, a glycoprotein stain (19), and no component was detected when overloaded gels (50 μg samples) were stained with the protein stain Coomassie blue (data not shown).

Antifreeze fish glycoprotein (AFGP) (20) isolated from Northern cod (mol wt of ~3,000) was provided by A/F Protein, Inc. (Waltham, MA). Purified porcine submaxillary gland mucin (PSM) was reduced, carboxymethylated, and treated with trypsin to generate a monomeric mucin species (PSM-RT) (21). The mucins were added to Mϕ cultures at the same time as *M. avium*, unless otherwise noted.

Measurement of TNF-α and IL-10. Murine spleen Mϕ were isolated, cultured in 24-well plates, and inoculated with *M. avium* as described above. At 4–48 h after infection, supernatants were harvested and the concentrations of TNF-α and IL-10 were determined by ELISA using matched antibody pairs and cytokine standards (Endogen).

Phagocytosis. Monolayers of murine spleen Mϕ were prepared by plating 2×10^6 murine spleen cells on plastic coverslips as described for microscopy experiments. 4–6 d later, the adherent monolayers were washed and polystyrene (latex) fluorescent microspheres of 1-μm diameter (Fluoresbrite YG Microspheres; Polysciences, Inc.) were added to triplicate wells at a ratio of 200 beads/Mϕ and incubated for 30 min. The coverslips were washed five times in HBSS at 37°C and air-dried. The Mϕ were examined in a Nikon microscope; cells with at least two phagocytized beads were scored as positive.

Statistical Analysis. The results are expressed as the mean ± SEM. Statistical differences were determined using SigmaStat Statistical Software (Jandel Scientific), using the *t* test for normally distributed data with equal variances and the Mann-Whitney rank sum test for data populations with nonnormal distributions and/or unequal variances.

Results

CD43 Is Required for Optimal Binding of Mycobacteria to Mϕ. As our initial approach, we incubated CD43-transfected HeLa cells and control HeLa cells with *M. avium*, *S. typhimurium*, and *S. flexneri*. After 4 h exposure to the pathogen, we quantified the cells that had bound bacteria. Contrary to expectations, *M. avium* were found to be stably associated with the CD43-transfected HeLa cells (Fig. 1, left) and were not associated with control HeLa cells, both wild-type (data not shown) and vector-transfected cells (Fig. 1, right). The binding of *M. avium* to CD43-expressing HeLa cells appeared to be specific, since neither of the other tested bacterial species, *S. typhimurium* and *S. flexneri*, bound to CD43-transfected (or control) HeLa cells (Fig. 1).

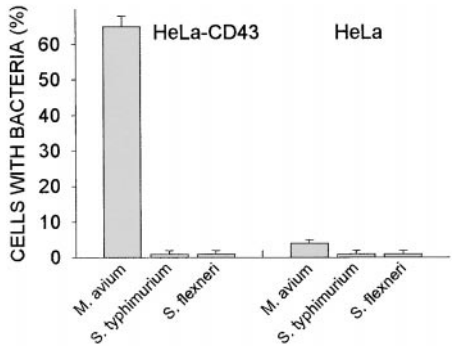

Figure 1. Specific association of *M. avium* with CD43-transfected HeLa cells. HeLa cells transfected with CD43 (HeLa-CD43) and vector-transfected cells (HeLa) were incubated as indicated with stained live *M. avium*, *S. typhimurium*, and *S. flexneri* at 10 organisms/cell for 4 h. Cells were washed, and the infected cells were counted using fluorescence microscopy (see Materials and Methods). Shown is a representative experiment. The number of CD43-transfected HeLa cells that scored positive for *M. avium* binding was significantly different than the number of vector-transfected cells ($n = 3$, $P < 0.0001$).

The surprising finding that *M. avium* bind to $CD43^+$ HeLa cells prompted us to examine the role of CD43 in

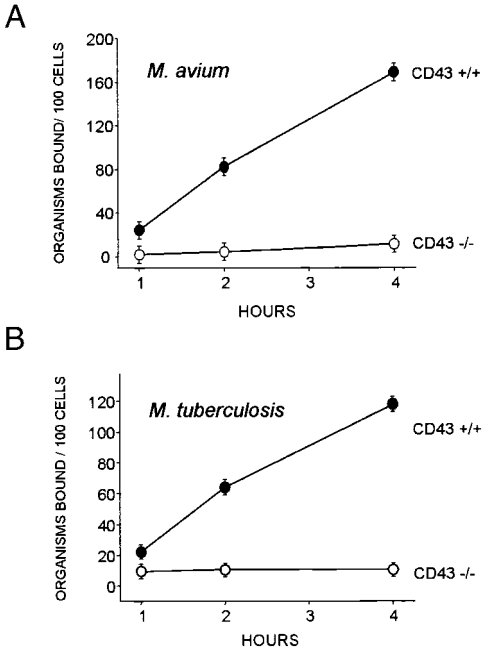

Figure 2. Binding of *M. avium* and *M. tuberculosis* to murine $CD43^{+/+}$ and $CD43^{-/-}$ Mϕ examined by quantitative bacteriology. (A) *M. avium* serovar 4 were incubated with adherent splenic Mϕ ($\sim 1 \times 10^5$) at a bacteria/cell ratio of 2:1 for the indicated time; the Mϕ were extensively washed, and the adherent bacteria were quantified. (B) *M. tuberculosis* strain H37Rv were similarly analyzed. Shown are mean values ± SEM for three mice of each group. The number *of M. avium* and *M. tuberculosis* associated with $CD43^{-/-}$ and $CD43^{+/+}$ Mϕ are significantly different at all time points ($P < 0.0001$). Although the absolute numbers of bound mycobacteria varied, similar differences between $CD43^{+/+}$ and $CD43^{-/-}$ Mϕ were observed in two additional independent experiments.

binding of mycobacteria by Mϕ, their normal host cell. Monolayers of splenic Mϕ were prepared from wild-type ($CD43^{+/+}$) and CD43 gene–deleted ($CD43^{-/-}$) mice. When these were challenged with *M. avium*, quantitative bacteriology showed that the $CD43^{+/+}$ Mϕ bound significant numbers of *M. avium* (Fig. 2 A). In contrast, $CD43^{-/-}$ Mϕ failed to bind *M. avium* or bound minimal numbers of the bacteria. The binding of *M. avium* to $CD43^{+/+}$ Mϕ was dependent on bacterial dose (shown below) and time of coincubation (Fig. 2 A). An exposure time of 4 h was chosen for further experiments because, at this time, binding of the *M. avium* to the Mϕ was substantial and bacterial growth was not yet observed.

To examine whether other mycobacterial species are also dependent on CD43 for uptake, splenic mouse Mϕ were challenged with *M. tuberculosis* H37Rv, a virulent strain. Whereas substantial binding was seen for $CD43^{+/+}$ Mϕ, $CD43^{-/-}$ Mϕ failed to bind *M. tuberculosis* (Fig. 2 B). Similar results, i.e., binding to $CD43^{+/+}$ Mϕ and minimal or absent binding to $CD43^{-/-}$ Mϕ, were obtained also for BCG (data not shown). Collectively, these findings strongly indicate a positive role for CD43 in mycobacterial binding.

In an independent approach, Mϕ incubated with *M. avium* were evaluated by light microscopy after acid-fast staining of the mycobacteria. On infection with *M. avium* at a 20:1 ratio, 57 ± 6% of $CD43^{+/+}$ Mϕ had >10 bacteria detectable by staining compared with 8 ± 3% of $CD43^{-/-}$ Mϕ ($n = 4$).

In other experiments, Mϕ incubated with *M. avium* were examined by fluorescence microscopy after staining of the mycobacteria with rhodamine-auramine. When incubated at a 20:1 ratio, fluorescent micrographs showed multiple mycobacteria associated with all or most of the $CD43^{+/+}$ Mϕ (Fig. 3 A, left) and negligible association of mycobacteria with $CD43^{-/-}$ Mϕ (right). Higher magnification micrographs showed that the mycobacteria associated with $CD43^{+/+}$ Mϕ are localized predominantly

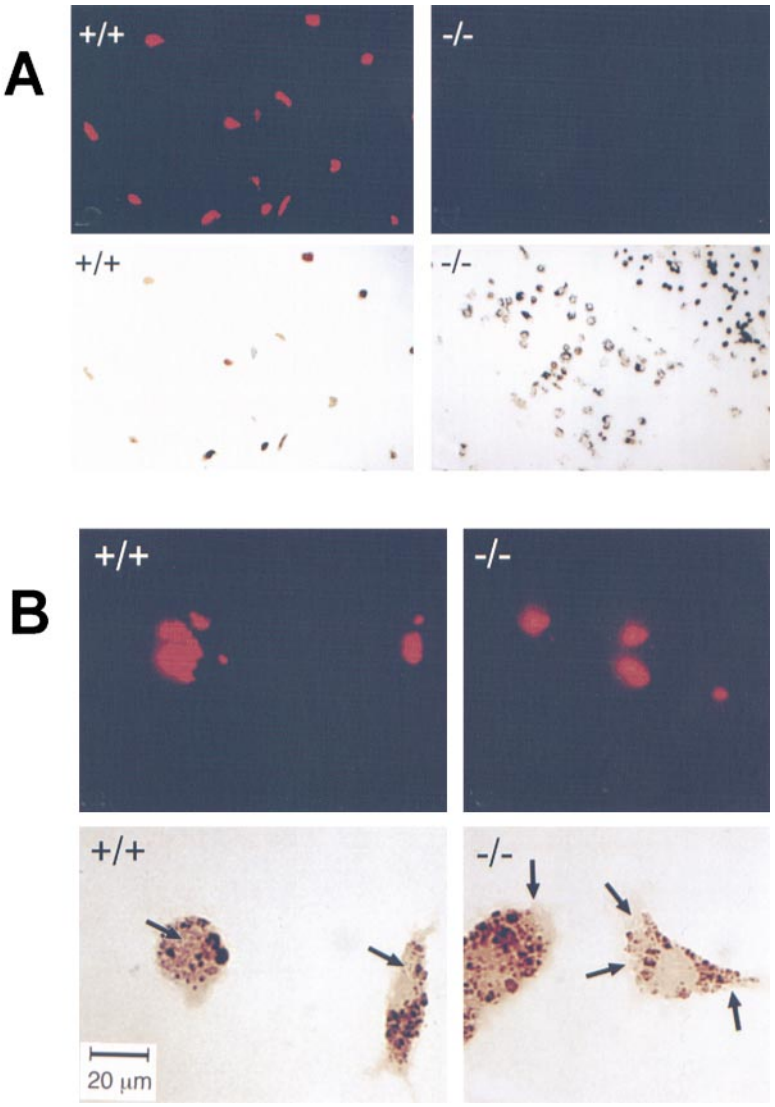

Figure 3. Association of *M. avium* with $CD43^{+/+}$ and $CD43^{-/-}$ Mϕ examined by fluorescence microscopy. *M. avium* were incubated with Mϕ at a ratio of 20:1 for 4 h. After washing and fixing, mycobacteria associated with the cell monolayers were stained with auramine-rhodamine (TB staining reagents), and the preparations were examined by microscopy. (A) Shown are fluorescent micrographs (top) and phase–contrast images of the same frames (bottom) of $CD43^{+/+}$ (left) and $CD43^{-/-}$ (right) Mϕ. Note that almost all of the $CD43^{+/+}$ Mϕ have multiple associated mycobacteria; in contrast, negligible numbers of mycobacteria are associated with $CD43^{-/-}$ Mϕ. (B) Higher magnification images. Description as in A. The arrows on the phase–contrast micrographs indicate the regions of the cells with positive signals in the corresponding fluorescent micrographs. Note that mycobacteria associated with $CD43^{+/+}$ Mϕ are localized within the cells. In contrast, in the very rare $CD43^{-/-}$ Mϕ with associated mycobacteria, the organisms are localized to the periphery of the cell. Bar, (B) 20 μm.

within the cells (Fig. 3 B, left). In contrast, in the rare cases where mycobacteria were found associated with CD43$^{-/-}$ Mϕ, the microorganisms were localized to the periphery of the cells (Fig. 3 B, right).

To test whether the diminished association of mycobacteria with CD43$^{-/-}$ Mϕ is due to a global defect in phagocytosis, monolayers of wild-type and CD43$^{-/-}$ Mϕ were incubated with nonopsonized inert latex fluorospheres (200 spheres/cell) for 30 min (22). Uptake of the latex beads examined by fluorescence microscopy showed that 76 ± 5% of CD43$^{+/+}$ and 73 ± 5% of CD43$^{-/-}$ Mϕ contained fluorescent beads. The finding that CD43$^{-/-}$ Mϕ do not take up less beads than CD43$^{+/+}$ Mϕ ($n = 3$, $P < 0.6$) suggests that the cells are not globally defective in phagocytosis.

Galgp Restores M. avium Binding to CD43$^{-/-}$ Mϕ. The extracellular mucin region of CD43 was identified as a normal component of human plasma, given the name Galgp, and isolated (17). We reasoned that if CD43 functions as a receptor for mycobacteria, addition of soluble extracellular CD43 to wild-type Mϕ might abrogate mycobacterial binding by competing with cell surface CD43. However, addition of Galgp to CD43$^{+/+}$ Mϕ resulted in enhanced mycobacterial binding (not shown). More importantly, addition of 100 μg/ml Galgp to CD43$^{-/-}$ Mϕ at the time of infection restored the time-dependent (Fig. 4 A) and bacterial dose–dependent (Fig. 4 B) association of *M. avium* with CD43$^{-/-}$ Mϕ. Restoration of *M. avium* binding to CD43$^{-/-}$ Mϕ by Galgp (100 μg/ml) was also seen when the cells were evaluated by acid-fast staining (data not shown). These findings suggest that Mϕ CD43, rather than serving as a receptor for mycobacteria, functions by promoting or stabilizing binding or uptake of mycobacteria.

The proposed collaborative role of CD43 in enabling mycobacterial association with Mϕ was further tested by preincubating *M. avium* with Galgp for 4 h, washing the bacteria extensively, and adding them to CD43$^{-/-}$ Mϕ for an additional 4-h incubation. Whereas coincubation of Galgp, *M. avium*, and Mϕ enhanced the binding of mycobacteria to Mϕ, no enhancement was observed when mycobacteria were preincubated with Galgp, washed, and then added to the cells (Fig. 5), indicating that Galgp does not opsonize the mycobacteria.

We next determined whether other mucins are also able to restore the association of *M. avium* with CD43$^{-/-}$ Mϕ. *M. avium* were added to CD43$^{-/-}$ Mϕ together with 100 μg/ml of Galgp or AFGP (20) or PSM that had been reduced, carboxymethylated, and trypsin-treated to generate linear monomeric mucin molecules (PSM-RT) (21). After 4 h, association of bacteria with Mϕ was assessed. *M. avium* binding to CD43$^{-/-}$ Mϕ was enhanced only by the presence of Galgp. AFGP and PSM-RT failed to enhance association of *M. avium* with Mϕ (Fig. 6).

In contrast, addition of Galgp did not enhance association of *M. avium* with wild-type (CD43^{-}) HeLa cells. On infection with a 1:1 ratio, the number of bacteria associated per 100 cells was 5, 7, and 40, respectively, for vector-transfected HeLa, vector-transfected HeLa with Galgp (100 μg/ml), and CD43-transfected HeLa. At an infection ratio of 5:1, the corresponding results were 10, 10, and 70 bacteria bound per 100 cells. Similar results were obtained when *M. avium*–challenged HeLa cells (infection ratio of 15:1)

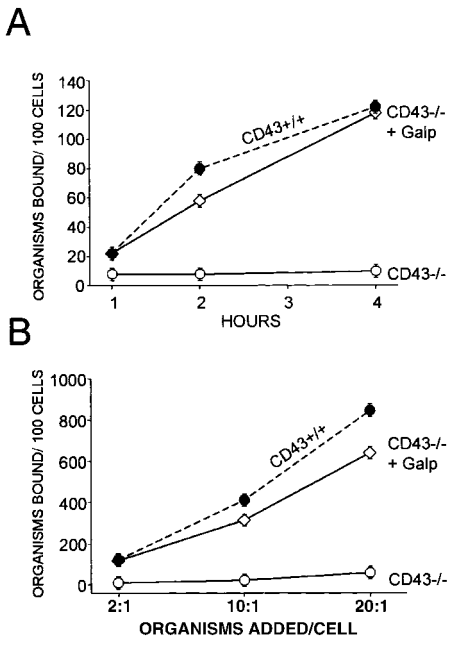

Figure 4. Addition of Galgp restores binding of *M. avium* by CD43$^{-/-}$ Mϕ. (A) *M. avium* were incubated for varying times with CD43$^{-/-}$ Mϕ in the absence (○) and presence (◇) of 100 μg/ml Galgp. Bacteria/cell ratio was 2:1. The Mϕ were harvested and extensively washed, and adherent bacteria were quantified. The binding of *M. avium* to CD43$^{+/+}$ Mϕ in the absence of Galgp is shown for comparison (●, dashed lines). (B) *M. avium* at varying bacteria/cell ratios as indicated were incubated with CD43$^{-/-}$ Mϕ for 4 h in the absence (○) and presence (◇) of 100 μg/ml Galgp. Shown are mean values ± SEM for three mice of each group. The number of mycobacteria associated with CD43$^{-/-}$ Mϕ in the presence and absence of 100 μg/ml Galgp was significantly different in all conditions ($P < 0.0001$). Comparable effects of Galgp addition were observed in two additional experiments. Galgp at 200 μg/ml produced similar results (data not shown).

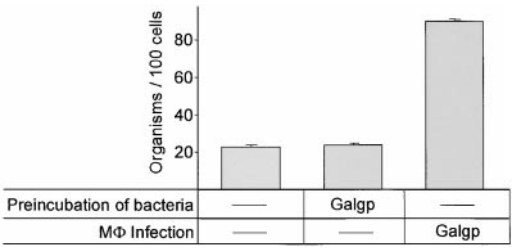

Figure 5. Preincubation of *M. avium* with Galgp does not enhance their binding to CD43$^{-/-}$ Mϕ. *M. avium* were preincubated for 4 h with 100 μg/ml Galgp or without additive, as indicated, and then extensively washed. The bacteria were used to infect CD43$^{-/-}$ Mϕ (infection ratio of two organisms per cell). Galgp (100 μg/ml) or saline was added, as indicated at the time of infection. After extensive washing, *M. avium* associated with Mϕ were quantified. Shown are mean values ± SEM for cells from three mice. The number of *M. avium* associated with Mϕ were significantly different for mycobacteria preincubated with Galgp compared with *M. avium* infected in the presence of Galgp ($P < 0.0001$). Similar effects of Galgp treatment were seen in one additional experiment.

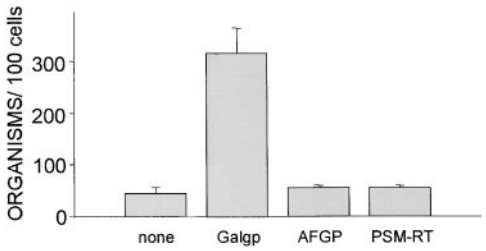

Figure 6. Galgp, but not other mucins, enables CD43$^{-/-}$ Mϕ to bind *M. avium*. *M. avium* at a bacteria/cell ratio of 2:1 were incubated with CD43$^{-/-}$ Mϕ in the absence (none) or presence of Galgp, AFGP, or PSM reduced and trypsin-treated (PSM-RT) at 100 μg/ml. After 4 h, the cells were washed and the mycobacteria associated with cells were quantified. Shown are mean values ± SEM for cells from three mice. The number of *M. avium* associated with the CD43$^{-/-}$ Mϕ incubated with the mycobacteria in the presence of Galgp, AFGP, or PSM were significantly different ($P < 0.0001$). Similar effects of mucin treatments were seen in one additional experiment.

were evaluated by acid-fast staining and light microscopy. 70% of CD43-transfected HeLa cells, but <10% of vector-transfected HeLa and vector-transfected HeLa with Galgp, scored positive for bacterial association (≥5 bacteria/cell). In the case of CD43-transfected HeLa cells, the associated mycobacteria were clearly located outside the cell perimeter as anticipated for these nonphagocytic cells (23).

In Contrast to CD43$^{+/+}$ Mϕ, CD43$^{-/-}$ Mϕ Fail to Produce TNF-α in Response to M. avium and This Deficiency Is Corrected by Galgp. Early after inoculation with mycobacteria, wild-type Mϕ respond by producing the proinflammatory cytokine TNF-α (24), which plays a central role in defense against microbial organisms including mycobacteria (25, 26). To investigate whether *M. avium*–dependent TNF-α production is altered in CD43$^{-/-}$ Mϕ, we measured TNF-α levels produced by CD43$^{-/-}$ and CD43 $^{+/+}$ Mϕ over the 4–48-h period after inoculation. *M. avium* inoculation of CD43$^{+/+}$ Mϕ resulted in the production of 1,150 ± 200 ng/ml TNF-α at 8 h. In contrast, CD43$^{-/-}$ Mϕ inoculated with the same *M. avium*/cell ratio produced <200 ng/ml TNF-α (Fig. 7 A). The possibility of a TNF-α expression defect in CD43$^{-/-}$ Mϕ was ruled out by finding that CD43$^{-/-}$ and wild-type Mϕ produced comparable levels of TNF-α when stimulated for 8 h with LPS (100 ng/ml; data not shown), suggesting that the defective induction of TNF-α production is *M. avium* specific. Importantly, the inability of CD43$^{-/-}$ Mϕ to produce TNF-α in response to *M. avium* was overcome by the addition of Galgp during inoculation (Fig. 7 B), and the extent of the restorative effect was dependent on Galgp concentration.

Incubation of wild-type Mϕ with mycobacteria also induces production of the immunosuppressive cytokine IL-10, which counteracts the effects of TNF-α (27). Incubation with *M. avium* of CD43$^{-/-}$ and CD43$^{+/+}$ Mϕ induced comparable levels of IL-10 (Fig. 7 C), indicating that the lesser interaction of mycobacteria with CD43$^{-/-}$ Mϕ is sufficient to induce IL-10 production. The discordant regulation of TNF-α and IL-10 demonstrates that the failure

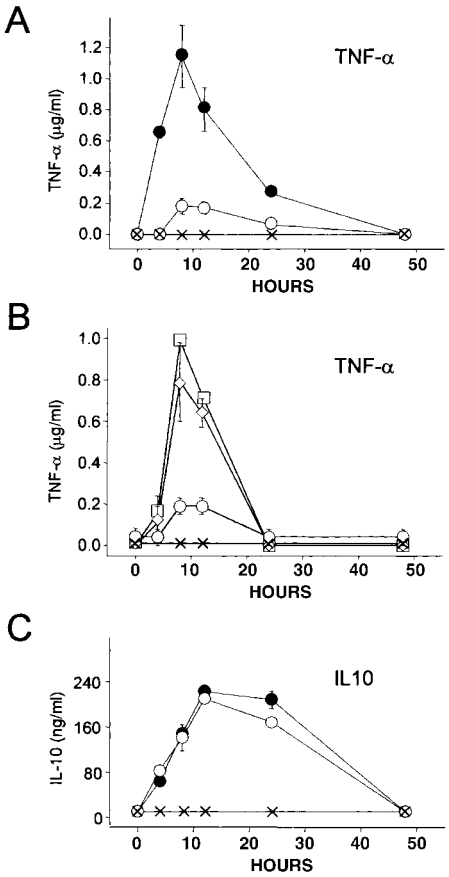

Figure 7. TNF-α and IL-10 production examined in CD43$^{+/+}$ and CD43$^{-/-}$ Mϕ challenged with *M. avium*. (A) *M. avium*–induced TNF-α production is significantly impaired in CD43$^{-/-}$ Mϕ. Mϕ from CD43$^{+/+}$ (●) and CD43$^{-/-}$ (○) mice were inoculated with *M. avium* (two bacteria per Mϕ), and the supernatants harvested at the indicated times were assayed by ELISA. The levels of TNF-α produced by *M. avium*–challenged CD43$^{+/+}$ and CD43$^{-/-}$ Mϕ are significantly different at 4, 8, and 12 h ($P < 0.001$, 0.007, and 0.01, respectively). In the absence of mycobacterial challenge, neither CD43$^{+/+}$ (×) nor CD43$^{-/-}$ Mϕ (not shown) produced TNF-α. (B) Impaired TNF-α production by *M. avium*–challenged CD43$^{-/-}$ Mϕ is restored by Galgp. CD43$^{-/-}$ Mϕ were inoculated with *M. avium* (two bacteria per Mϕ) in the absence (○) and presence of 100 (◇) and 200 (□) μg/ml Galgp. Galgp was tested also at 50 μg/ml, and the resulting TNF-α levels were indistinguishable from levels produced in the absence of added Galgp (○). Note that 200 μg Galgp increases TNF-α to levels of CD43$^{+/+}$ cells at 8 and 12 h (compare with A). The levels of TNF-α are significantly different for *M. avium*–challenged CD43$^{-/-}$ Mϕ in the absence and presence of 100 and 200 μg/ml Galgp at 8 and 12 h ($P < 0.02$). Note also that Mϕ cultured with Galgp (200 μg/ml) in the absence of mycobacterial challenge (×) did not produce TNF-α. (C) *M. avium*–dependent IL-10 production is not impaired in CD43$^{-/-}$ Mϕ. The Mϕ supernatants from A were assayed for IL-10 by ELISA. The levels of IL-10 produced by CD43$^{+/+}$ (●) and CD43$^{-/-}$ (○) Mϕ after inoculation with *M. avium* are not significantly different at any time point (*P* values range from 0.06 to 0.85). Neither CD43$^{+/+}$ (×) nor CD43$^{-/-}$ Mϕ (not shown) produced IL-10 in the absence of mycobacterial challenge. Values are mean ± SEM of replicate assays of fractions from three mice of each group.

to produce TNF-α in response to mycobacterial challenge is a specific defect of $CD43^{-/-}$ macrophages.

Discussion

Mycobacterial diseases, some of which are spread by airborne transmission, are a serious global public health problem (3). In this communication, we provide the first evidence identifying a new component of apparent importance for the uptake of mycobacteria by their primary host cell, the Mϕ. This component, CD43, is the predominant sialoglycoprotein on leukocytes. We show that the presence of CD43 is obligatory for firm association of mycobacteria with Mϕ. Mϕ from wild-type mice and CD43-transfected HeLa cells bound high numbers of *M. avium*. In contrast, negligible numbers of *M. avium* associated with untransfected HeLa cells and with Mϕ from CD43 gene–deleted mice. The role of CD43 in the binding of mycobacteria is specific because other bacteria, *S. typhimurium* and *S. flexneri*, did not bind to $CD43^{+}$ HeLa cells. Also, Mϕ from $CD43^{+/+}$ mice bound two other species of mycobacteria, *M. tuberculosis* and *M. bovis*, and these also failed to bind to $CD43^{-/-}$ Mϕ. Fluorescence microscopy of Mϕ incubated with *M. avium* showed that the associated mycobacteria had been ingested by the $CD43^{+/+}$ Mϕ. CD43 was also required for the production of TNF-α, but not of IL-10, by Mϕ challenged with *M. avium*, strongly suggesting a functional link between the action of CD43 in mycobacterial binding and/or uptake and induction of TNF-α production.

CD43, also called sialophorin or leukosialin, is a prevalent sialoglycoprotein on monocytes, neutrophils, and T lymphocytes (28, 29). On T cells, CD43 extends 45 nm from the phospholipid bilayer, making it the largest glycoprotein on the cell surface (30). One function of CD43 is that of a repulsive or barrier molecule restricting cell to cell contact (13, 14, 31) due to its negative charge, prevalence, size, and rigidity. In addition, in vitro binding of CD43 mAb transduces intracellular signals that lead to T lymphocyte proliferation (e.g., 32) and activation of macrophages, increasing homotypic adhesion and hydrogen peroxide production (32, 33). At the mechanistic level, evidence suggests that ligation of CD43 induces tyrosine phosphorylation reactions (34, 35), and ligated CD43 itself binds the actin filament linker proteins, moesin and ezrin, which function in cytoskeletal reorganization (36).

The inability of Mϕ that lack CD43 to bind mycobacteria and produce TNF-α was corrected by the addition of Galgp, a purified mucin glycoprotein equivalent to the extracellular region of CD43. Galgp, which is 76% carbohydrate, was originally identified as a plasma component. Its high content of galactose and *N*-acetylgalactosamine suggested its identity with the extracellular region of CD43, and this relationship was confirmed by amino acid sequencing (18). Isolated plasma Galgp is heterogeneous; all molecules have the NH_2-terminal CD43 sequence, but they have varying COOH termini. The longest species encompasses 226 of the 235 extracellular CD43 amino acids. It is believed that Galgp originates as leukocyte surface CD43 primarily because the single copy (human) CD43 gene encodes a single polypeptide that includes a transmembrane and a cytoplasmic region, missing from Galgp (discussed in reference 18). Relative to other plasma glycoproteins that originate as leukocyte surface molecules, the levels of Galgp are extremely high, >10 μg/ml of normal plasma (37). It is not known whether Galgp is released by proteolysis from intact cells or arises by another mechanism, e.g., as a stable residue remaining after leukocyte death.

Because Galgp was obtained by chromatography (from a human plasma Cohn fraction), we cannot eliminate the possibility that the active component that enhances mycobacterial adherence is a contaminating species. This scenario is unlikely because contaminants were not seen on overloaded SDS gels stained for glycoprotein and protein. Also, trace proteinaceous materials remaining after conventional chromatographic purification were removed by a final immunoabsorption step. Importantly, addition of Galgp to uninfected $CD43^{-/-}$ Mϕ did not induce TNF-α or IL-10 (Fig. 7, A–C), indicating that the action of Galgp is not due to induction of cytokines by trace contaminants.

Two other monomeric mucins, AFGP, which consists of tandem repeats of an *O*-glycosylated tripeptide, and PSM-RT, a derivative of PSM, which consists of tandem repeats of 81 residues, failed to mediate adherence of *M. avium* with Mϕ (Fig. 6), indicating that the action of Galgp is specific. Whereas AFGP lacks sialic acid, PSM-RT, like Galgp, is heavily sialylated, bearing *N*-glycolylneuraminic acid on 40–50% of its *O*-glycans (21). These findings indicate that the effect of Galgp is not simply due to the presence of sialylated *O*-glycans.

The finding that Galgp, a soluble species, enables $CD43^{-/-}$ Mϕ to bind mycobacteria indicates that CD43 does not by itself function as a surface receptor for mycobacteria. Clues to the mode of action of CD43/Galgp might be found by reference to other functionally interchangeable membrane protein/soluble protein pairs. These include the IL-6 receptor (IL-6R), which exists in an integral membrane and a solubilized form elaborated by cleavage from the cell surface (38). Soluble IL-6R is able to bind IL-6 and associate with the IL-6R coreceptor gp130, thereby mediating signaling in IL-6R–negative cells (39). Similar behavior is observed for the membrane and soluble forms of CD14. On binding LPS, soluble CD14, like cell surface CD14, can trigger intracellular signaling by interacting with its coreceptor toll-like receptor 2 (TLR-2) (40, 41).

Previous studies have shown that binding and phagocytosis of mycobacteria by Mϕ can be mediated by several surface receptors, including complement receptors CR1, CR3, and CR4, Fc receptors, CD14, mannose receptor, receptors for SP-A, and scavenger receptors (for a review, see reference 11). In light of this plethora of receptors, the near absence of mycobacterial uptake in $CD43^{-/-}$ Mϕ suggests that CD43 functions as a coreceptor or facilitator and/or cofactor of the mycobacterial uptake process. The finding that soluble Galgp restores *M. avium* binding to $CD43^{-/-}$ Mϕ but not to $CD43^{-}$ HeLa cells also attests to the contribution of Mϕ components other than CD43/Galgp in the interaction with mycobacteria.

The action of Galgp to enhance mycobacteria binding and/or uptake resembles the action of SP-A. Addition of SP-A, a member of the collectin (collagen-like lectin) family of multimeric innate defense glycoproteins, increases the adherence and phagocytosis of *M. tuberculosis* by Mϕ (42). Like Galgp, SP-A must be present during the interaction of mycobacteria with Mϕ. Indeed, reference to earlier studies suggests a candidate Mϕ molecule that may functionally interact with both CD43/Galgp and SP-A. This molecule, a surface glycoprotein called C1qRp, binds SP-A (43), and the binding of SP-A to C1qRp enhances the Mϕ capacity for phagocytosis of opsonized targets (44). Since it was also shown that CD43 and C1qRp copurify and coimmune precipitate (45), Galgp/CD43 and C1qRp may be components of a multimeric Mϕ complex that enhances mycobacteria binding and uptake.

Inoculation of wild-type Mϕ with mycobacteria induces the proinflammatory cytokine TNF-α (46), which is known to decrease the survival of *M. avium* (6) by promoting apoptosis of the infected host Mϕ (47), an apparent innate defense mechanism that prevents systemic spread of infection (8). *M. avium*–induced TNF-α production failed to occur in $CD43^{-/-}$ Mϕ and was restored by addition of Galgp, strongly suggesting that the action of CD43 in mycobacterial binding and/or uptake and TNF-α induction are functionally linked.

On the other hand, *M. avium*–dependent induction of IL-10, an antiinflammatory cytokine and TNF-α antagonist (27), was not impeded in $CD43^{-/-}$ Mϕ, strengthening existing evidence (48) that mycobacteria induce separate or divergent pathways for TNF-α and IL-10 production.

In summary, this study identifies the surface mucin CD43 as a component essential for robust binding and/or uptake of mycobacteria by Mϕ and for mycobacteria-induced TNF-α production, but not for production of IL-10. Further studies are warranted to identify the Mϕ molecules that cooperate with CD43 and the mechanism that enhances mycobacterial binding.

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