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Article in *Infection and Immunity* · January 2000

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Lymphocyte Function-Associated Antigen 1 Is a Receptor for *Pasteurella haemolytica* Leukotoxin in Bovine Leukocytes

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Received 13 August 1999/Returned for modification 4 October 1999/Accepted 14 October 1999

Pasteurella (Mannheimia) haemolytica leukotoxin (Lkt) causes cell type- and species-specific effects in ruminant leukocytes. Recent studies indicate that *P. haemolytica* Lkt binds to bovine CD18, the common subunit of all $\beta 2$ integrins. We designed experiments with the following objectives: to identify which member of the $\beta 2$ integrins is a receptor for Lkt; to determine whether Lkt binding to the receptor is target cell (bovine leukocytes) specific; to define the relationships between Lkt binding to the receptor, calcium elevation, and cytolysis; and to determine whether a correlation exists between Lkt receptor expression and the magnitude of target cell cytolysis. We compared Lkt-induced cytolysis in neutrophils from control calves and from calves with bovine leukocyte adhesion deficiency (BLAD), because neutrophils from BLAD-homozygous calves exhibit reduced $\beta 2$ integrin expression. The results demonstrate for the first time that Lkt binds to bovine CD11a and CD18 (lymphocyte function-associated antigen 1 [LFA-1]). The binding was abolished by anti-CD11a or anti-CD18 monoclonal antibody (MAb). Lkt-induced calcium elevation in bovine alveolar macrophages (BAMs) was inhibited by anti-CD11a or anti-CD18 MAb (65 to 94% and 37 to 98%, respectively, at 5 and 50 Lkt units per ml; $P < 0.05$). Lkt-induced cytolysis in neutrophils and BAMs was also inhibited by anti-CD11a or anti-CD18 MAb in a concentration-dependent manner. Lkt bound to porcine LFA-1 but did not induce calcium elevation or cytolysis. In neutrophils from BLAD calves, Lkt-induced cytolysis was decreased by 44% compared to that of neutrophils from control calves ($P < 0.05$). These results indicate that LFA-1 is a Lkt receptor, Lkt binding to LFA-1 is not target cell specific, Lkt binding to bovine LFA-1 correlates with calcium elevation and cytolysis, and bovine LFA-1 expression correlates with the magnitude of Lkt-induced target cell cytolysis.

Leukotoxin (Lkt) and lipopolysaccharide produced by *Pasteurella (Mannheimia) haemolytica* serotype 1 are considered to be the primary virulence factors contributing to lung injury in bovine pneumonic pasteurellosis (BPP) (33, 36, 38, 40), a disease of substantial economic importance to the beef and dairy cattle industries in North America (7, 28, 39). Lkt is a member of a family of gram-negative bacterial exotoxins termed RTX (for repeats in toxin) cytolysins (3). Although most RTX cytolysins interact with a variety of cell types from many different species (6), cytolysins produced by *Actinobacillus actinomycescomitans*, *Actinobacillus pleuropneumoniae* (ApxIIIa), and *P. haemolytica* are known to have cell type- and species-specific effects. The leukotoxin (LtxA) of *A. actinomycescomitans*, a human pathogen, interacts only with cells of the lymphocytic and monomyelocytic lineages of humans and some nonhuman primates (23); the Lkt of *P. haemolytica*, a ruminant pathogen, interacts only with ruminant leukocytes causing activation and cytolysis (4, 15, 25, 33, 40). A study by Lally et al. (23) has determined that two RTX cytolysins, LtxA of *A. actinomycescomitans* and alpha-hemolysin of *Escherichia coli*, bind to human myelomonocytic leukemic cell line (HL60) through a $\beta 2$ integrin lymphocyte function-associated antigen (LFA-1) and cause cytolysis. Moreover, two recent studies have identi-

fied CD18 as a receptor for *P. haemolytica* Lkt (24, 35). Since CD18 is the common subunit of all three bovine $\beta 2$ integrins CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), and CD11c/CD18 (p150/95) (1, 18), it is not clear which of the three $\beta 2$ integrins is a receptor for *P. haemolytica* Lkt.

$\beta 2$ integrins are heterodimeric cell surface glycoproteins composed of a CD11 (α) subunit and a CD18 (β) subunit and are expressed exclusively on leukocytes (5, 9). These leukocyte integrins mediate cell adhesion to endothelial cell ligands such as intracellular adhesion molecules (5, 9). The importance of $\beta 2$ integrins for host defense against microbial agents is exemplified by leukocyte adhesion deficiency, a rare genetic disease in humans that results in reduced expression of all $\beta 2$ integrins in leukocytes (21, 22), leading to life-threatening bacterial infections. A similar genetic disorder has been reported for Holstein cattle and termed bovine leukocyte adhesion deficiency (BLAD) syndrome (18, 19). Leukocytes from BLAD-homozygous calves are known to have no or reduced expression of these $\beta 2$ integrins (18, 19). However, the potential role of this reduced $\beta 2$ integrin expression in the BLAD calf model in Lkt binding and cytolysis has not been examined.

The objectives of the present study are to: (i) identify which member of the $\beta 2$ integrins is a receptor for *P. haemolytica* Lkt; (ii) determine whether Lkt binding to the receptor exhibits target cell (bovine leukocytes) specificity; (iii) define the relationship between Lkt binding to the receptor and intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) elevation and cytolysis; and (iv) determine whether a correlation exists between Lkt receptor expression

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TABLE 1. Properties and applications of the various antibodies used in this study

Specificity	Antibody	Isotype	Species reactivity	Applications ^a	Reference
CD11a	R3.1	IgG1	Cattle, dogs	FC, BE	31
CD11a	MUC76A	IgG2a	Cattle, sheep, swine	WB	8, 14
CD11b	MM12A	IgG1	Cattle, goats, sheep	FC, BE	30
CD11b	R7928	Polyclonal	Humans, cattle	WB	26
CD11c	BAQ153A	IgM	Cattle, goats, sheep	FC, BE, WB	14
CD18	R15.7	IgG1	Cattle, dogs, rabbits	FC, BE	20, 29, 31
CD18	BAQ30A	IgG1	Cattle, goats, sheep, swine, dogs, cats, rabbits	FC, BE, WB	14
CD18	BAT75A	IgG1	Cattle, goats	FC, BE, WB	35
E-selectin	EL112	IgG1	Humans	FC	34
Irrelevant	MOPC21	IgG1	None	BE	
Lkt	MAB601	IgG1	Not applicable	BE	12

^a Abbreviations: FC, flow cytometry; BE, blocking experiments include blocking of Lkt binding, calcium elevation, and cytotoxicity; WB, Western blotting.

and the magnitude of Lkt-induced target cell cytotoxicity. We used bovine neutrophils and bovine alveolar macrophages (BAMs) to study Lkt binding and functional effects, since these cells are implicated in the pathophysiology of BPP (2, 30). Porcine alveolar macrophages (PAMs) and HL60 cells are used to demonstrate whether Lkt binding is target cell specific.

MATERIALS AND METHODS

Preparation of *P. haemolytica* Lkt. Preparation of Lkt from *P. haemolytica* has been described in a previous publication (25). Briefly, crude Lkt was prepared from logarithmic-phase *P. haemolytica* D153 grown in RPMI 1640 medium supplemented with 2 mM L-glutamine. Following centrifugation, the supernatant was filter sterilized, concentrated 100-fold, and dialyzed against endotoxin-free distilled water in a spiral-wound membrane cartridge (model S1Y30; Amicon Corp., Danvers, Mass.). The retentate containing crude Lkt was lyophilized and purified to homogeneity by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified 104-kDa Lkt (monomeric, native form [40]) was lyophilized and stored at -20°C , and all studies were done with the same batch of purified Lkt. The leukotoxic activity was quantified by a colorimetric XTT (sodium,3'-(1-[phenylamino-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) assay, using the bovine lymphoid cell line (BL3) as target cells. The concentration of bioactive Lkt was expressed as Lkt units (LU) per milligram (dry weight) (41). In order to exclude the effect of postpurification lipopolysaccharide contamination in the Lkt preparations, purified Lkt fractions were incubated with 10 μg of polymyxin B per ml for 30 min on ice prior to use. Studies of lactate dehydrogenase (LDH) release were done with Lkt concentration of 50 LU/ml, since in preliminary studies this Lkt concentration resulted in $>60\%$ LDH release over a 90-min period. In $[\text{Ca}^{2+}]_i$ measurements, 5 and 50 LU/ml were used.

Preparation of leukocytes. (i) Bovine neutrophils. Peripheral blood samples were obtained from six healthy age-, breed-, and sex-matched Holstein heifers, using acid-citrate-dextrose as the anticoagulant. Blood samples from three Holstein calves homozygous for BLAD (1) containing the same anticoagulant were obtained from the U.S. Department of Agriculture, National Animal Disease Center, Ames, Iowa. Neutrophils were isolated by the method described by Olchowy et al. (27). Purified cells were $>96\%$ polymorphonuclear leukocytes and $>98\%$ viable as verified by differential counts and trypan blue exclusion, respectively.

(ii) BAMs. BAMs were isolated from six 6- to 8-week-old healthy calves as described previously (40). The cells were $>98\%$ pure and $>98\%$ viable, as determined by nonspecific esterase staining (Sigma Chemical Co., St. Louis, Mo.) and trypan blue exclusion, respectively. For $[\text{Ca}^{2+}]_i$ measurements, BAMs were plated onto round 15-mm-diameter glass coverslips at a density of 7.5×10^5 cells/ml in 12-well tissue culture plates. Cells were incubated at 37°C in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 . The medium was changed every other day, and the cells were used after 4 days of incubation.

(iii) PAMs and human promyelocytic leukemia cell line (HL60). PAMs were obtained from three 5- to 7-week-old healthy pigs as described previously (16). The HL60 cell line obtained from M. Mellancamp (University of Minnesota, St. Paul) was cultured in RPMI 1640 supplemented with 2 mM L-glutamine and 10% FBS.

Antibodies. Table 1 shows the features and applications of the various antibodies used in this study. Monoclonal antibodies (MAbs) MUC76A, MM12A, BAQ153A, BAT75A, and BAQ30A were purchased from VMRD, Inc. (Pullman, Wash.). MAbs R15.7 and R3.1 were provided by R. Rothlein (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Conn.). The Lkt-neutralizing MAb (MAB601) was provided by S. Srikumar (University of Nebraska, Lin-

coln). The R7928 polyclonal antibody was provided by C. Parkos (Emory University, Atlanta, Ga.).

Flow cytometry. The expression of $\beta 2$ integrins on neutrophils, BAMs, PAMs, and HL60 was assessed by immunofluorescence flow cytometry as described previously (34). Briefly, 10^6 cells were incubated with 1 μg of anti- $\beta 2$ integrin MAbs or control MAb for 15 min on ice. After the cells were washed, they were incubated with 1:200 diluted phycoerythrin-labeled goat anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, Pa.) in fluorescence-activated cell sorting buffer (phosphate-buffered saline [PBS] containing 2% goat serum and 5 mM NaN_3) for 15 min on ice. After the cells were washed, they were resuspended in 100 μl of fluorescence-activated cell sorting buffer and fluorescence was analyzed by a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) and expressed as mean fluorescence intensity (MFI).

Preparation of cell lysates. Lysates were prepared as described by Lally et al. (23). Briefly, 5×10^7 cells were suspended in 1 ml of lysis buffer (pH 7.5) (200 mM NaCl, 40 mM NaHCO_3 , 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 μg of leupeptin per ml, 5 μg of pepstatin per ml, 0.01% NaN_3), incubated on ice, vortexed intermittently for 30 min, and centrifuged at $100,000 \times g$ at 4°C for 1 h, and the lysates were stored at -80°C . Protein concentration of the lysates was measured with the DC-protein assay kit (Bio-Rad, Hercules, Calif.).

SDS-PAGE and Western blotting. To demonstrate the $\beta 2$ integrin expression, 30 μg of cell lysates from leukocytes of control calves and 120 μg of lysates from neutrophils of BLAD calves were loaded and proteins were separated on 4 to 15% SDS gradient gels under nonreducing conditions. Separated proteins were transferred onto a polyvinylidene difluoride membrane (Pierce Chemical Co., Rockford, Ill.), and the membrane was blocked with blocking buffer (PBS containing 0.05% Tween 20 (PBST) and 1% milk concentrate (Kirkegaard and Perry Laboratories, Gaithersburg, Md.)). The membrane was incubated with 0.1 μg of anti-CD11a (MUC76A), anti-CD11b (R7928), anti-CD11c (BAQ153A), or anti-CD18 (BAT75A and BAQ30A) antibodies for 1 h at room temperature. Membranes were washed 4 times with PBST followed by incubation with a 1:50,000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Both primary and secondary antibodies were diluted in the blocking buffer. The blots were washed with PBST and developed by using the SuperSignal ULTRA chemiluminescence detection system (Pierce Chemical Co.).

Lkt affinity chromatography. Experiments were conducted by the method of Wang et al. (35). Briefly, polystyrene beads (0.125-in. diameter) were incubated with 2 ml of a solution of 20 μg of purified Lkt per ml of PBS, pH 7.5, overnight at 4°C with gentle rocking. The beads were washed once with PBS and incubated with 1% bovine serum albumin (BSA) to block the remaining protein binding sites on the beads. Beads coated with 1% BSA served as a control. Lysates (120 μg of protein from control neutrophils and BAMs; 480 μg of protein from neutrophils of BLAD calves) were diluted 1:3 with PBS containing 1 mM CaCl_2 and 1 mM MgCl_2 and incubated with the Lkt- or BSA-coated beads at 4°C for 15 h. To demonstrate that binding is attributable to Lkt, Lkt-coated beads were incubated with MAB601 for 1 h before adding lysates. To further demonstrate the specificity of Lkt binding to the receptor, lysates from BAMs were preincubated with various anti- $\beta 2$ integrin or control (MOPC21) (final concentration of 50 $\mu\text{g}/\text{ml}$) MAbs at 37°C for 45 min before the Lkt-coated beads were added. The beads were then washed once with PBS, and the bound proteins were eluted from the beads by boiling with 50 μl of SDS-PAGE loading buffer and size fractionated on 4 to 15% SDS gradient gels under nonreducing conditions. Western blotting was performed as described earlier.

$[\text{Ca}^{2+}]_i$ measurement. $[\text{Ca}^{2+}]_i$ level was assessed by video fluorescence microscopy as described in a previous publication (17) using fura-2-acetoxymethyl ester (fura-2/AM)-loaded BAMs. Fura-2-labeled cells were alternately excited at 340 and 380 nm with a rapidly rotating filter wheel, and the fluorescence emissions were collected for each wavelength using a 510-nm-wavelength barrier

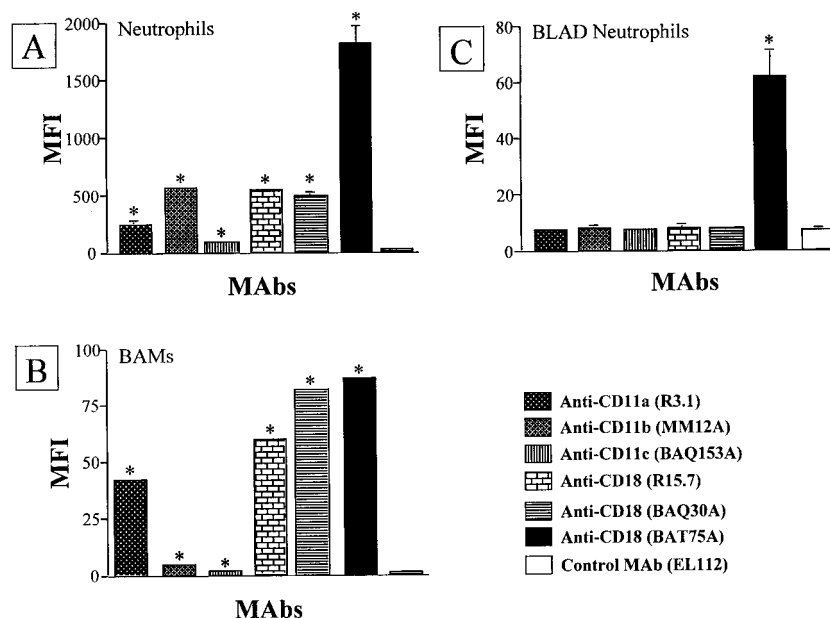


FIG. 1. Flow cytometric detection of $\beta 2$ integrin subunits. (A) Significant CD11a, CD11b, CD11c, and CD18 expression is observed in neutrophils from control calves. (B) High-level expression of CD11a and CD18 and much lower level expression of CD11b and CD11c are detected in BAMS. (C) A reduced level of CD18 expression is detected in the neutrophils of BLAD calves. The results are from four independent experiments and expressed as means \pm SEMs. Values that are significantly different from the control value ($P < 0.05$) are indicated by asterisks.

filter. Images were acquired once every second using a silicon-intensified target video camera (66 Series; DAGE-MTI Inc., Michigan City, Ind.). The integrated $[Ca^{2+}]_i$ response, a measure of total $[Ca^{2+}]_i$ elevation during the period of stimulation, was calculated (17). To examine the effects of anti- $\beta 2$ integrin MABs on Lkt-induced $[Ca^{2+}]_i$ elevation, BAMS were preincubated with anti-CD11a, anti-CD11b, anti-CD11c, anti-CD18, or control MAB for 45 min at room temperature prior to Lkt exposure. From each coverslip, ~ 30 cells were sampled and two coverslips were used for each experiment. The percent inhibition of $[Ca^{2+}]_i$ elevation was calculated as follows: percent inhibition of $[Ca^{2+}]_i$ elevation = $\{(\text{percent } [Ca^{2+}]_i \text{ elevation} - \text{percent } [Ca^{2+}]_i \text{ elevation in the presence of antibodies}) / \text{percent } [Ca^{2+}]_i \text{ elevation}\}$.

Measurement of LDH release. Lkt-induced cytotoxicity was assessed by measuring leakage of LDH activity from cells into supernatant, using a commercial kit purchased from Boehringer Mannheim (Indianapolis, Ind.). One hundred microliters of neutrophils or BAMS at a concentration of 4×10^5 cells/ml was added to each well in a 96-well U-bottom microtiter plate. Spontaneous LDH release was measured by exposing cells to assay medium (phenol red-free RPMI 1640 supplemented with 2 mM L-glutamine and 3% FBS). Total LDH release was measured by lysing the cells with 100 μ l of 2% Triton X-100 in assay medium. Experimental LDH release was measured by exposing cells to assay medium containing Lkt. Neutrophils or BAMS were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 90 min. After incubation, the cells were centrifuged at $200 \times g$ for 5 min. One hundred microliters of the supernatant from each well was transferred to each well of a 96-well flat-bottom microtiter plate. One hundred microliters of reaction reagent (prepared according to the manufacturer's recommendations) was added to each well and incubated for 30 min at room temperature in the dark. LDH activity in the supernatants was determined by measuring the optical density at 490 nm with a microplate enzyme-linked immunosorbent assay reader (Molecular Device Corp., Menlo Park, Calif.) with a reference wavelength of 620 nm. Each sample was tested in triplicate, and Lkt-induced cytotoxicity was calculated by using the following formula: percent cytotoxicity = $[(OD \text{ of C} - OD \text{ of A}) / (OD \text{ of B} - OD \text{ of A})] \times 100$, where OD is the optical density at 490 nm, A is spontaneous LDH release, B is total LDH release, and C is experimental LDH release. The effects of antibodies against the subunits of $\beta 2$ integrins on Lkt-induced cytotoxicity were also studied. Cells were incubated with different concentrations of antibodies for 45 min at 37°C in a humidified atmosphere under 5% CO_2 prior to incubation with Lkt. Inhibition of Lkt-induced cytotoxicity was calculated as follows: percent inhibition of cytotoxicity = $[(\text{percent cytotoxicity} - \text{percent cytotoxicity in the presence of antibodies}) / \text{percent cytotoxicity}] \times 100$.

Reagents. Dulbecco's modified Eagle's medium and RPMI 1640 were purchased from Celox Laboratories, Inc. (St. Paul, Minn.). Fura-2/AM was purchased from Molecular Probes (Eugene, Oreg.). Polystyrene beads were obtained from Orange Products Inc. (Allentown, Pa.). Phycoerythrin-labeled goat anti-mouse secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, Pa.). Horseradish peroxidase-conjugated goat anti-mouse and anti-

rabbit immunoglobulin G (IgG) were obtained from ICN Biomedical Research Products (Costa Mesa, Calif.). Horseradish peroxidase-conjugated goat anti-mouse IgM was obtained from Pierce Chemical Co. Other reagents were obtained from Sigma Chemical Co.

Statistical analysis. All results are expressed as means \pm standard error of means (SEMs). Comparisons are made with the unpaired Student *t* test to determine statistically significant differences. The term significant indicates a *P* value of less than 0.05.

RESULTS

Analysis of $\beta 2$ integrin expression in leukocytes. (i) Flow cytometry. Relative expression of LFA-1, Mac-1, and p150/95 in neutrophils, BAMS, PAMs, and HL60 cells was determined. Neutrophils from control calves expressed CD11a (MFI of 188), CD11b (MFI of 546), and CD11c (MFI of 80). The expression of CD18 in neutrophils from control calves was detected using three different MABs (R15.7, BAQ30A, and BAT75A) with MFIs of 550, 480, and 1,800, respectively (Fig. 1A). BAMS expressed CD11a (MFI of 41), CD11b (MFI of 4.3), CD11c (MFI of 1.3), and CD18 (MFIs of 65, 82, and 87 using R15.7, BAQ30A, and BAT75A, respectively), albeit at levels much lower than those observed for neutrophils (Fig. 1B). However, in neutrophils from BLAD calves, very low level expression of CD18 was detected by BAT75A (MFI of 60), but not by the other two anti-CD18 MABs. Neutrophils from BLAD calves also lacked expression of CD11a, CD11b, or CD11c (Fig. 1C). Expression of CD11a and CD18, comparable to levels in BAMS, was detected in PAMs and HL60 cells using anti-CD11a (R3.1) and anti-CD18 MABs (BAQ30A) (data not shown).

(ii) Western blotting. In lysates from neutrophils of control calves, a 95-kDa CD18 band was detected (Fig. 2A, lane 1). In lysates from neutrophils of BLAD calves, two lower-molecular-mass CD18 bands (90 and 85 kDa) were identified by using the BAT75A MAB (Fig. 2A, lane 3). BAMS from control calves also had two CD18 bands corresponding to 90 and 95 kDa (Fig. 2A, lane 2). A 180-kDa CD11a band was detected in both

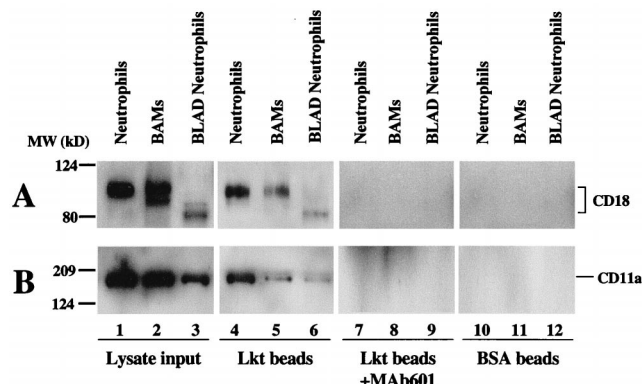


FIG. 2. Western blot analysis of CD18 (A) and CD11a (B) expression in neutrophils and BAMs using anti-CD18 (BAT75A) or anti-CD11a (MUC76A) MAb. In panel A, cell lysates from neutrophils show a 95-kDa CD18 band (lane 1), lysates from BAMs show 95- and 90-kDa CD18 bands (lane 2), and neutrophil lysates from BLAD calves show 90- and 85-kDa CD18 bands (lane 3). The lysates from these cells show only one 180-kDa CD11a band (panel B, lanes 1 to 3). Direct binding of *P. haemolytica* Lkt to bovine LFA-1 (CD11a/CD18) was also examined. Western blot analysis of eluants from Lkt-coated beads reacted with neutrophils or BAM lysates from control calves contain 95-kDa CD18 and 180-kDa CD11a bands (panels A and B, lanes 4 and 5). The eluant from Lkt-coated beads reacted with neutrophil lysates from BLAD calves contain 85-kDa CD18 and 180-kDa CD11a bands (panels A and B, lanes 6). In eluants from Lkt-coated beads preincubated with anti-Lkt MAb or from BSA-coated beads, no CD18 (panel A, lanes 7 to 12) or CD11a (panel B, lanes 7 to 12) bands are seen. Results are representative of four independent experiments. MW, molecular mass (in kilodaltons).

neutrophils and BAMs (Fig. 2B, lanes 1 and 2). However, neutrophils from BLAD calves had very low expression of CD11a (Fig. 2B, lane 3). In lysates from neutrophils and BAMs, Western blot analysis also showed two bands, one corresponding to CD11b (170 kDa) (Fig. 3A, lanes 1 and 2) and another band corresponding to CD11c (160 kDa) (Fig. 3B, lanes 1 and 2). However, no CD11b (Fig. 3A, lane 3) or CD11c

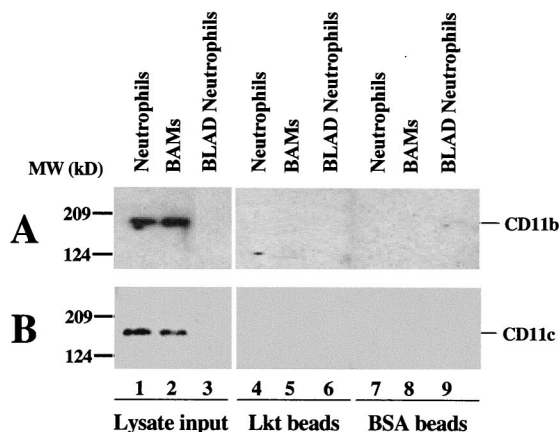


FIG. 3. Western blot analysis of CD11b (A) and CD11c (B) expression using anti-CD11b (R7928) and anti-CD11c (BAQ153A) MAbs. Lanes 1 and 2 in panels A and B show the presence of a 170-kDa CD11b band and a 160-kDa CD11c band in lysates from neutrophils and BAMs from control calves using anti-CD11b and anti-CD11c antibodies. Note that in lysates from neutrophils of BLAD calves, there are no detectable CD11b or CD11c bands (panels A and B, lanes 3). Direct binding of *P. haemolytica* Lkt to bovine CD11b or CD11c was also examined. No Lkt binding to CD11b or CD11c is observed in eluants from Lkt-coated beads reacted with lysates of neutrophils or BAMs (panels A and B, lanes 4 to 6). No CD11b or CD11c bands are observed in BSA-coated beads (panels A and B, lanes 7 to 9). Results are representative of three independent experiments. MW, molecular mass (in kilodaltons).

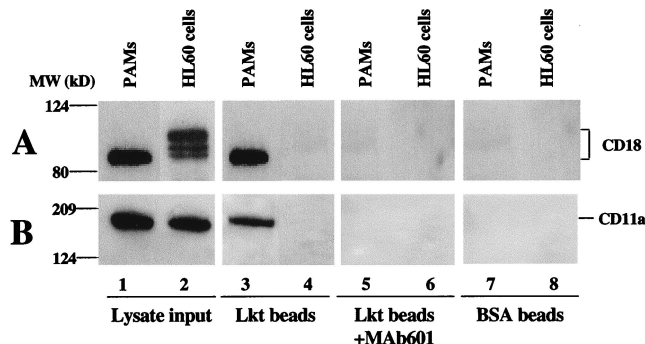


FIG. 4. Western blots showing expression of LFA-1 (probed with anti-CD18 [A] or anti-CD11a [B]). A 95-kDa CD18 band in PAM lysates and 95-, 100-, and 105-kDa CD18 bands in HL60 cell lysates (panel A, lanes 1 and 2) using anti-CD18 (BAQ30A) MAb. In both cell types, a 180-kDa band is seen (panel B, lanes 1 and 2) using anti-CD11a MAb (MUC76A). Direct binding of *P. haemolytica* Lkt to porcine and human LFA-1 was also examined. Western blots of eluants from Lkt-coated beads showing a 95-kDa CD18 band (panel A, lane 3) and a 180-kDa CD11a band in PAMs (panel B, lane 3). In HL60 cells, no CD18 or CD11a bands are detected (panels A and B, lanes 4). In eluants from Lkt-coated beads preincubated with anti-Lkt MAb or from BSA-coated beads, no CD18 (panel A, lanes 5 to 8) or CD11a bands (panel B, lanes 5 to 8) are detected. Results are representative of four independent experiments. MW, molecular mass (in kilodaltons).

(Fig. 3B, lane 3) bands were detected in lysates from neutrophils of BLAD calves. Lysates from PAMs had only a 95-kDa CD18 band (Fig. 4A, lane 1). In HL60 cell lysates, three different CD18 bands (95, 100, and 105 kDa) were detected (Fig. 4A, lane 2). A 180-kDa CD11a band was detected in lysates from PAMs and HL60 cells (Fig. 4B, lanes 1 and 2). Table 2 summarizes the molecular masses of the various $\beta 2$ integrin subunits expressed in the various cell types studied.

Detection of Lkt binding to $\beta 2$ integrins. Polystyrene beads coated with Lkt or BSA were incubated with lysates from the different cells. Bound proteins eluted from the beads were separated by SDS-PAGE under nonreducing conditions, transferred to a polyvinylidene difluoride membrane, and probed with MAbs against CD11a (MUC76A), CD11b (MM12A), CD11c (BAQ153A), and CD18 (BAQ30A and BAT75A). Bound proteins in eluants from beads coated with Lkt and incubated with lysates from neutrophils or BAMs from control calves contained a 95-kDa CD18 band (Fig. 2A, lanes 4 and 5). In contrast, bound proteins eluted from beads coated with Lkt and incubated with lysates from neutrophils from BLAD calves contained a 85-kDa CD18 band (Fig. 2A, lane 6). In addition, in the eluants from beads coated with Lkt and incubated with lysates from neutrophils of control or BLAD calves or BAMs, a 180-kDa CD11a band was detected (Fig. 2B, lanes 4 to 6). Although the eluant from Lkt-coated beads incubated with lysates from PAMs showed the 95-kDa CD18 (Fig. 4A, lane 3) and 180-kDa CD11a bands (Fig. 4B, lane 3), no such bands were detected in the eluant from beads incubated with lysates from HL60 cells (Fig. 4A and 4B, lanes 4). Table 2 summarizes Lkt binding to the various $\beta 2$ integrin subunits in the different cell types used in this study.

Several controls were included in this study to ascertain the specificity of Lkt binding to LFA-1 in bovine leukocytes and PAMs. (i) The binding was abolished by preincubating Lkt-coated beads with a neutralizing anti-Lkt MAb (MAb601) (Fig. 2A and B, lanes 7 to 9 and Fig. 4A and B, lanes 5 and 6). (ii) No evidence of binding was observed in BSA-coated beads (Fig. 2A and B, lanes 10 to 12 and Fig. 4A and B, lanes 7 and 8). (iii) Preincubating BAM lysates with anti-CD18 (Fig. 5A,

TABLE 2. Molecular masses of $\beta 2$ integrin subunits in the various cell types and Lkt binding to the various $\beta 2$ integrin subunits

Cell type	$\beta 2$ integrin subunit ^a				Lkt binding to $\beta 2$ integrin subunit ^a			
	CD11a	CD11b	CD11c	CD18	CD11a	CD11b	CD11c	CD18
Neutrophils	180	170	160	95	180	No binding	No binding	95
BAMs	180	170	160	90, 95	180	No binding	No binding	95
BLAD neutrophils	180	None	None	85, 90	180	NA	NA	85
PAMs	180	NP	NP	95	180	NP	NP	95
HL60	180	NP	NP	95, 100, 105	No binding	NP	NP	No binding

^a The values are the molecular masses (in kilodaltons) of the $\beta 2$ integrin subunits. NP, not performed; NA, not applicable.

lanes 5 and 6) or anti-CD11a (Fig. 5B, lanes 5 and 6) MAb abolished the binding.

Effects of anti- $\beta 2$ integrin antibodies on Lkt-induced $[Ca^{2+}]_i$ elevation. MABs against CD11 or CD18 subunits of $\beta 2$ integrins were used to examine the correlation between Lkt binding to the receptor and $[Ca^{2+}]_i$ elevation in BAMs. Cells were preincubated with the MABs (25 μ g/ml for 90 min at 37°C) prior to addition of Lkt (5 and 50 LU/ml). Anti-CD11a and anti-CD18 MABs significantly ($P < 0.05$) inhibited Lkt-induced $[Ca^{2+}]_i$ elevation in BAMs (65 to 94% inhibition in cells stimulated with 5 LU/ml and 37 to 98% inhibition in cells stimulated with 50 LU/ml [Fig. 6]). Anti-CD11b, anti-CD11c, or the control MAB (MOPC21) had no significant effects on Lkt-induced $[Ca^{2+}]_i$ elevation (Fig. 6). Lkt did not induce

$[Ca^{2+}]_i$ elevation in PAMs, a finding consistent with our previous study (16) (data not shown).

Effects of anti- $\beta 2$ integrin antibodies on Lkt-induced cytolysis. To examine the correlation between Lkt binding to the receptor and cytolysis, neutrophils or BAMs were preincubated with anti- $\beta 2$ integrin MABs (1 to 100 μ g/ml for 90 min at 37°C) prior to addition of Lkt (50 LU/ml). Anti-CD11a and anti-CD18 MABs inhibited Lkt-induced cytolysis in neutrophils and BAMs in a concentration-dependent manner (43% inhibition of cytolysis in neutrophils and 64% inhibition of cytolysis at 100 μ g/ml in BAMs [$P < 0.05$] [Fig. 7]). Anti-CD11b, anti-CD11c MABs, or the control MAB had no significant effects on Lkt-induced cytolysis (Fig. 7). Inhibition of Lkt-induced cytolysis by anti- $\beta 2$ integrin MABs was not studied in PAMs and HL60 cells, since Lkt did not induce cytolysis even at a concentration of 500 LU/ml (data not shown).

Comparison of Lkt-induced cytolysis in neutrophils from control and BLAD calves. To determine whether there is a correlation between Lkt receptor expression and magnitude of Lkt-induced cytolysis, cytolysis was compared in neutrophils from control and BLAD calves. Lkt caused a reduced level of

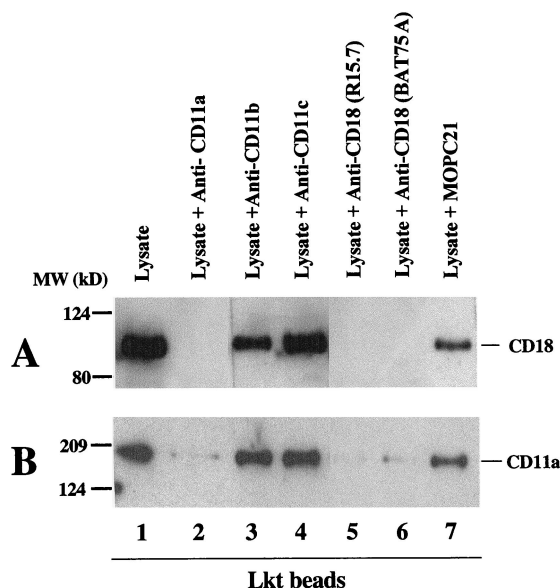


FIG. 5. Western blots probed with anti-CD18 (A) or anti-CD11a (B) showing the effects of anti- $\beta 2$ integrin antibodies on direct binding of *P. haemolytica* Lkt to bovine LFA-1. (A) Eluates from Lkt-coated beads reacted with BAM lysates and probed with anti-CD18 MAB (BAT75A; lane 1) contain a 95-kDa band; BAM lysates that had been preincubated with anti-CD11b (lane 3), anti-CD11c (lane 4), or MOPC21 (lane 7) MAB, before the Lkt-coated beads had been added and that were probed with anti-CD18 MAB contain a 95-kDa band. By contrast, for lysates preincubated with anti-CD11a (lane 2) or anti-CD18 MAB (lanes 5 and 6) before Lkt-coated beads were added, no 95-kDa band is seen. (B) Eluates from Lkt-coated beads reacted with BAM lysates and probed with anti-CD11a MAB (MUC76A; lane 1) contain a 180-kDa band; BAM lysates that had been preincubated with anti-CD11b (lane 3), anti-CD11c (lane 4), or MOPC21 (lane 7) MAB, before Lkt-coated beads had been added and that were probed with anti-CD11a MAB contain a 180-kDa band. By contrast, lysates preincubated with anti-CD11a MAB (lane 2) or anti-CD18 (lanes 5 and 6) MAB before Lkt-coated beads were added, no 180-kDa band is seen. Results are representative of three independent experiments. MW, molecular mass (in kilodaltons).

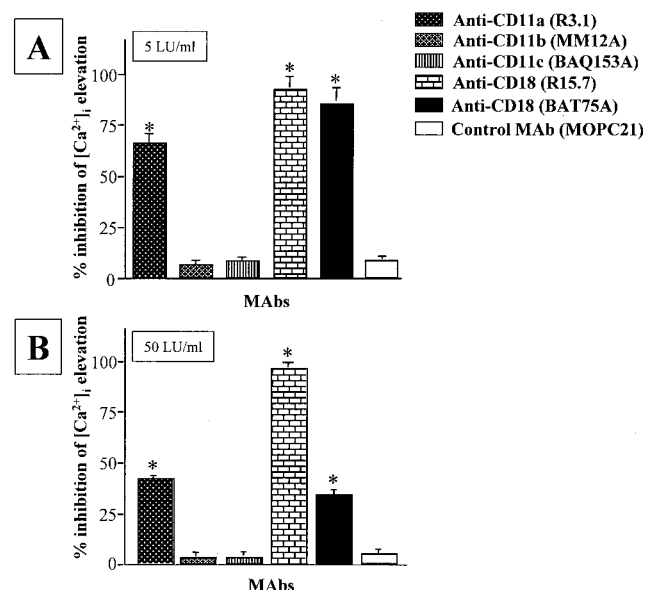


FIG. 6. Effects of anti- $\beta 2$ integrin antibodies on Lkt-induced $[Ca^{2+}]_i$ elevation. In BAMs treated with anti-CD11a or anti-CD18 MABs, there is significant inhibition of $[Ca^{2+}]_i$ response to 5 (A) and 50 (B) LU per ml. There is no inhibition of $[Ca^{2+}]_i$ response in cells treated with anti-CD11b, anti-CD11c, or the control MAB in response to 5 or 50 LU/ml. The results are from four independent experiments (~ 120 cells) and expressed as means \pm SEMs. Values that are significantly different from the control value ($P < 0.05$) are indicated by asterisks.

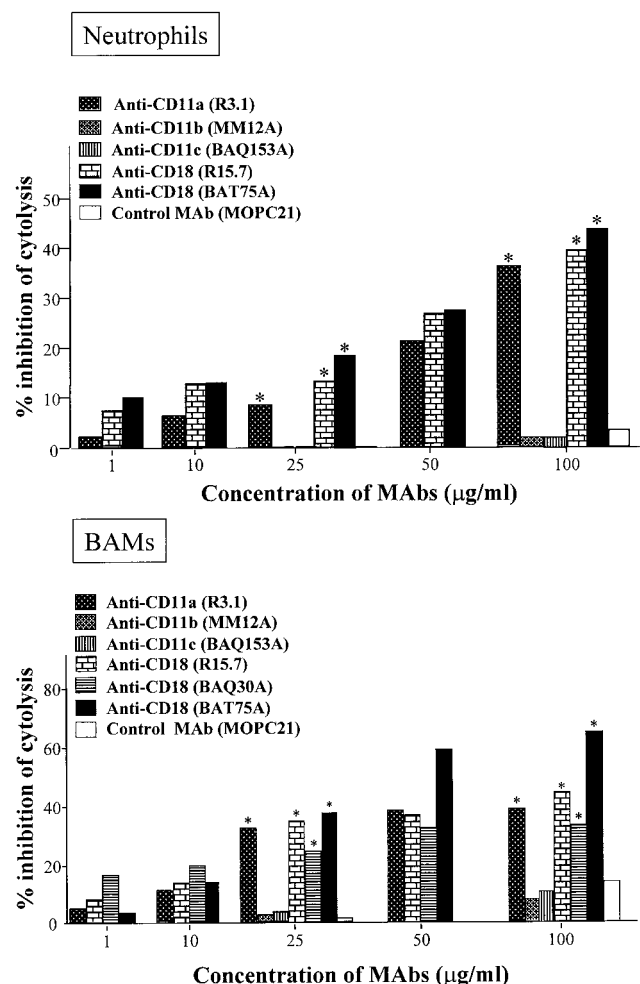


FIG. 7. Effects of anti- β 2 integrin antibodies on Lkt-induced cytolysis. Pre-incubation of neutrophils or BAMs with anti-CD11a or anti-CD18 MAb inhibits Lkt-induced cytolysis in a concentration-dependent fashion. Anti-CD11b, anti-CD11c, or control MAb at 25 or 100 μ g/ml do not inhibit Lkt-induced cytolysis. The results are from six independent experiments and expressed as means \pm SEMs. Values that are significantly different from the control value ($P < 0.05$) are indicated by asterisks.

cytolysis in neutrophils from BLAD calves (18% in BLAD neutrophils versus 62% in control neutrophils [$P < 0.05$] [Fig. 8]). Lkt-induced cytolysis was abolished by the Lkt-neutralizing MAb (Fig. 8). The isotype-matched control MAb had no effect on Lkt-induced cytolysis (Fig. 8).

DISCUSSION

In the present study, we sought to determine whether a member of the β 2 integrins is a receptor for *P. haemolytica* Lkt, whether Lkt binding is target (bovine leukocytes) cell specific, whether Lkt binding to the receptor is required for $[Ca^{2+}]_i$ elevation and cytolysis, and whether a correlation exists between Lkt receptor expression and magnitude of Lkt-induced cytolysis. The results indicate the following. (i) LFA-1 (CD11a/CD18) is a receptor for *P. haemolytica* Lkt. (ii) The Lkt binding to LFA-1 is not target cell specific, since Lkt binding is observed in PAMs. (iii) Lkt binding to bovine LFA-1 correlates with $[Ca^{2+}]_i$ elevation and cytolysis, since anti-CD11a and anti-CD18 MAb, but not anti-CD11b and anti-CD11c MAb, inhibit these responses. (iv) A reduced LFA-1 expression in the

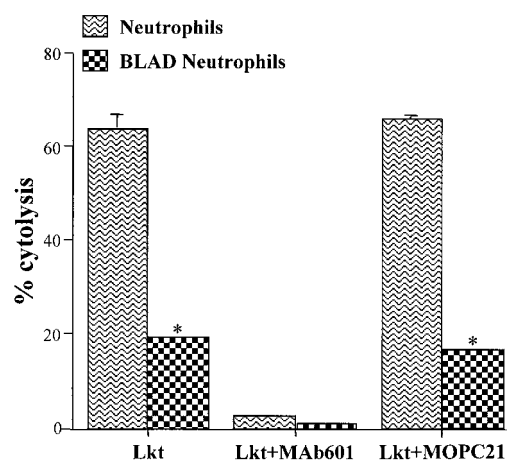


FIG. 8. Comparison of Lkt-induced cytolysis in neutrophils from control and BLAD calves. Significantly lower levels of Lkt-induced cytolysis are observed for neutrophils from BLAD calves than for those from control calves ($P < 0.05$). In cells exposed to neutralized Lkt, there is no Lkt-induced cytolysis. In cells exposed to Lkt treated with an isotype-matched control MAb (MOPC21), there is no inhibition of Lkt-induced cytolysis. The results are means \pm SEMs from four independent experiments. Values that are significantly ($P < 0.05$) reduced from Lkt-induced cytolysis values are indicated by asterisks.

neutrophils from BLAD calves correlates with reduced magnitude of Lkt-induced cytolysis. However, the presence of any additional Lkt receptor in bovine leukocytes cannot be ruled out by our studies.

Previous studies have shown that β 2 integrins are receptors for a variety of microbial virulence determinants such as fimbriae of *Porphyromonas gingivalis* (32), cryptococcal polysaccharide (10), and endotoxin (11). A study by Lally et al. (23) has shown that LFA-1 is a cell surface receptor for Lkt of *A. actinomycetemcomitans* and alpha-hemolysin of *E. coli* on human (HL60) target cells. Since *P. haemolytica* Lkt exhibits cell type- and species-specific functional effects on ruminant leukocytes (4, 13, 16, 40), it has been hypothesized that specific receptors are present on ruminant leukocytes (3, 37). In this context, two recent studies have indicated that CD18 is the receptor for *P. haemolytica* Lkt (24, 35). The evidence has been based on the fact that MAb detected a CD18 band in BL3 cell lysates reacted with Lkt. In addition, both studies showed that anti-CD18 MAb inhibited Lkt-induced apoptosis or cytolysis in BL3 cells. However, a specific β 2 integrin was not identified as the Lkt receptor by these studies. Therefore, we have extended these observations and show that LFA-1, a specific member of the β 2 integrins, is a receptor for *P. haemolytica* Lkt.

The Lkt affinity chromatography results of the present study with neutrophils and BAMs and of other investigators (24, 35) with BL3 cells demonstrate an interaction of Lkt with CD18. In addition, we provide evidence that Lkt interacts with CD11a, but not with CD11b or CD11c. Therefore, we propose that the CD18 band identified in the Western blots is the β subunit of LFA-1 but not of Mac-1 or p150/95. In this regard, a previous study by Lally et al. (23) showed that Lkt of *A. actinomycetemcomitans* binds to the LFA-1 heterodimer in HL60 cell lysates. Results from our laboratory (S. L. Hsuan, S. Jeyaseelan, M. S. Kannan, and S. K. Maheswaran, unpublished data) using immunoprecipitation of lysates from bovine leukocytes show the existence of LFA-1 as a heterodimer, rather than as dissociated CD11a and CD18 subunits. This finding, along with the finding that Lkt does not bind to CD11b or CD11c subunits, suggests

that the binding site for Lkt is the CD11a, but not CD18, subunit of the Lkt receptor LFA-1.

We have demonstrated that Lkt binding to LFA-1 in target cells (bovine leukocytes) is associated with $[Ca^{2+}]_i$ elevation and cytolysis. However, Lkt binding by itself is not sufficient for the functional effects, since a nontarget cell (PAMs) used in this study exhibits Lkt binding with no evidence of $[Ca^{2+}]_i$ elevation or cytolysis and shows NF- κ B activation (shown in a previous study [16]). Thus, the cell type- and species-specific effects of Lkt must entail both binding to LFA-1 and activation of LFA-1-associated intracellular pathways, which are present only in bovine leukocytes. Studies are in progress to further elucidate this phenomenon.

Previous investigations have provided evidence by flow cytometric analysis that neutrophils from BLAD calves have no or weak expression of all $\beta 2$ integrins (18). Our studies using flow cytometry and Western blot analysis confirm these findings. Consistent with the diminished expression of LFA-1, there is reduced Lkt binding. It is of interest to note that the CD18 bands in neutrophils from BLAD calves had molecular weights lower than those from control calves. In leukocytes from human leukocyte adhesion deficiency patients, the different size of the CD18 protein is reported to result from aberrant splicing (22). It is likely that the low-molecular-weight CD18 proteins in neutrophils from BLAD calves may also be the result of aberrant splicing. In addition, we have established a correlation between LFA-1 expression and the magnitude of Lkt-induced cytolysis, supporting the hypothesis that LFA-1 activation is required for cytolysis.

On the basis of our findings, we speculate that Lkt of *P. haemolytica* utilizes the cell adhesion molecule LFA-1 to cause activation and cytolysis, particularly in the neutrophils and macrophages in the alveolar spaces, leading to production and accumulation of a myriad of proinflammatory mediators and continuous colonization of *P. haemolytica* in the alveolar space. These events result in an uncontrollable inflammatory response leading to lung injury that is characteristic of BPP.

ACKNOWLEDGMENTS

This study was supported in part by grants from the Minnesota Agricultural Experimental Station (to M.S.K. and S.K.M.), NIH-HL057498 (to M.S.K.), NIDCR-DE09517 (to E.T.L.) and Mayo Foundation (to G.C.S.).

We thank Trevor Ames for helpful discussions, Christie Malazdrewich for help in collecting alveolar macrophages and critically reading the manuscript, and Shelia Alexander for help in flow cytometry experiments. We thank Charles Parkos of Emory University for providing R7928 polyclonal antibody and Gary Averbeck for assistance with graphics.

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Editor: J. T. Barbieri