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Influence of β_2-Integrin Adhesion Molecule Expression and Pulmonary Infection with *Pasteurella haemolytica* on Cytokine Gene Expression in Cattle

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β_2-Integrins are leukocyte adhesion molecules composed of alpha (CD11a, -b, -c, or -d) and beta (CD18) subunit heterodimers. Genetic CD18 deficiency results in impaired neutrophil egress into tissues that varies between conducting airways and alveoli of the lung. In this study, we investigated whether CD18 deficiency in cattle affects proinflammatory cytokine (PIC) expression in pulmonary tissue after respiratory infection with *Pasteurella haemolytica*. Cattle were infected with *P. haemolytica* via fiberoptic deposition of organisms into the posterior part of the right cranial lung lobe. Animals were euthanized at 2 or 4 h postinoculation (p.i.), and tissues were collected to assess PIC gene expression using antisense RNA probes specific for bovine interleukin-1α (IL-1α), IL-1β, IL-6, gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) along with the β-actin (β-Act) housekeeping gene. Expression of PIC was induced at 2 h p.i. in *P. haemolytica*-infected cattle and continued to 4 h p.i. At 2 h p.i., induction of gene expression and increase of cells that expressed PIC were observed both in $CD18^+$ and $CD18^-$ cattle after inoculation of *P. haemolytica*. The induction of gene expression with *P. haemolytica* inoculation was more prominent in $CD18^-$ cattle than in $CD18^+$ cattle by comparison to pyrogen-free saline (PFS)-inoculated control animals. At 4 h p.i., however, the induction of PIC, especially IL-1α, IL-6, and IFN-γ, in the lungs of $CD18^+$ cattle inoculated with *P. haemolytica* was greater than that in lungs of the $CD18^-$ cattle. IFN-γ and TNF-α genes were not increased in *P. haemolytica*-inoculated $CD18^-$ cattle lungs compared to the PFS-inoculated control lungs at 4 h p.i. In PFS-inoculated lungs, we generally observed a higher percentage of cells and higher level of gene expression in the lungs of $CD18^-$ cattle than in the lungs of $CD18^+$ cattle, especially at 4 h p.i. The rate of neutrophil infiltration into the lungs of $CD18^-$ cattle at 2 h p.i. was significantly higher than that of $CD18^+$ cattle; at 4 h p.i., there was no difference between the two groups. These data suggest that β_2-integrins may contribute to the induction of expression of some PIC genes, as a consequence of *P. haemolytica* infection.

Biotype A serotype 1 *Pasteurella haemolytica* is the primary bacterium responsible for shipping fever, or bovine pneumonic pasteurellosis (34), a disease characterized by acute lobar fibrinonecrotizing pneumonia (34, 49, 50). Several virulence factors of *P. haemolytica* have been identified (15). Lipopolysaccharide (LPS) and leukotoxin are the best-known stimulators of inflammation in bovine pneumonic pasteurellosis (11, 13, 15, 42, 46, 47, 49, 50). These virulence factors stimulate a variety of respiratory tract cells such as alveolar and intravascular macrophages, mast cells, and endothelial cells, and these cells express and produce inflammatory mediators. The inflammatory mediators secreted by respiratory tract cells are proinflammatory cytokines (PICs) such as interleukin-1 (IL-1), IL-6, tumor necrosis factor alpha (TNF-α), complement components, hydrolytic enzymes, and chemokines (20, 28, 33, 38, 40, 41, 48). Once secreted, these inflammatory mediators trigger an inflammatory cascade. Neutrophils are known to be the primary infiltrating inflammatory cells involved in clearing infections in the lungs; however, prolonged activation of neutrophils may induce severe tissue damage by the production of oxygen-derived free radicals and enzymes such as elastase (9, 10, 17, 27, 45). Furthermore, these inflammatory mediators induce expression of adhesion molecules on leukocytes and endothelial cells that facilitate the infiltration of additional leukocytes into inflamed tissues (6, 9, 10, 27, 45).

Bovine leukocyte adhesion deficiency (BLAD) is an autosomal recessive genetic disease resulting from one amino acid substitution (D128G) in the β subunit (CD18) of the β_2-integrin (CD11/CD18) superfamily (LFA-1, Mac-1, p150, 95) (23–25, 32, 35, 39). This β_2-integrin mediates tight adhesion of leukocytes onto endothelial membrane of inflamed tissue (3). Leukocytes from BLAD-affected animals express no functional CD18 (3). The affected animals (referred to as BLAD or $CD18^-$) suffer recurrent bacterial, viral, or fungal infections (5, 23–25, 30, 31). $CD18^-$ cattle often die because of respiratory or enteric infections despite antibiotic therapy (4). Another prominent characteristic of BLAD is progressive neutrophilia due to impaired transmigration of neutrophils across the vascular endothelium into sites of infection (2, 4, 19). In pneumonic lungs of $CD18^-$ cattle, however, remarkable levels of neutrophil infiltration were observed in the alveolar lumina (2,

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TABLE 1. Sequences of oligomers used for PCR amplification of bovine cytokine-specific cDNA fragments used for synthesis of riboprobes[a]

Specificity	Orientation	Sequence (5′-3′)
IL-1α	Forward	GCGAATTCACAGCAGTTGGAATAAGCCGTG
	Backward	CCAAGCTTGCACAGAGTTGGACATGACTGAAG
IL-1β	Forward	GCGAATTCAAGGCTCTCCACCTCCTCTC
	Backward	CCAAGCTTGTCTACTTCCTCCAGATGCA
IL-6	Forward	GCGAATTCTGGGTTCAATCAGGCGATTTG
	Backward	CCAAGCTTAGGTCAGTGTTTGTGGCTGGAGTG
IFN-γ	Forward	GCGAATTCTTACTGCTCTGTGGGCTTTTGG
	Backward	CCAAGCTTTGCTCCTTTGAATGACCTGGTTATC
TNF-α	Forward	GCGAATTCAAGCCCTGGTACGAACCCATCTAC
	Backward	CCAAGCTTTAGACCTGCCCAGACTCAGCATAG
β-Act	Forward	GCGAATTCCTGTCCACCTTCCAGCAGATGT
	Backward	CCAAGCTTTTCGAAAACGCCACCTGTTACC

[a] The amplified DNA fragments of 156, 180, 201, 250, 119, and 80 bp for IL-1α, IL-1β, IL-6, IFN-γ, TNF-α, and β-Act, respectively, were cloned into a pGEM4 vector (Promega) and transformed into *Escherichia coli* JM109, and their sequences were confirmed by dideoxy sequencing. For riboprobe synthesis, plasmid DNA was isolated and linearized with either *Eco*RI or *Hin*dIII for antisense- or sense-strand probe, respectively.

4). Binding of β_2-integrin with LPS or the counter receptor, intercellular adhesion molecule, activates resting leukocytes (8, 19, 22, 29). In this study, we compared PIC gene expression in lungs of $CD18^+$ and $CD18^-$ cattle at 2 and 4 h after inoculation with *P. haemolytica*. In situ hybridization was used to measure PIC gene expression.

MATERIALS AND METHODS

Animals and *P. haemolytica* inoculation. Twelve Holstein cattle were used for this experiment. Half of them were confirmed $CD18^-$ homozygous, and the other half were age-matched normal $CD18^+$ cattle (23–25, 35, 39). Both $CD18^-$ (BLAD) and $CD18^+$ (normal) cattle were inoculated with 8 ml of 10^7 CFU of *P. haemolytica* per ml in pyrogen-free saline (PFS) followed by 10 ml of sterile PFS by fiberoptic bronchoscopy into the right cranial lung lobe as described previously (12). The left lobe of the lung, inoculated with 18 ml of sterile PFS, served as saline-treated control. Animals were euthanized at 2 and 4 h after inoculation with *P. haemolytica*. Lung tissues were collected at necropsy and fixed in neutral buffered 10% zinc formalin for histological preparation.

Probe synthesis for in situ hybridization. Bovine sequence-specific RNA probes for five different PICs, IL-1α, IL-1β, IL-6, gamma interferon-γ (IFN-γ), and TNF-α, and the β-actin (β-Act) housekeeping gene were synthesized. Briefly, cDNA was reverse transcribed from bovine leukocyte total RNA using random hexameric primers and subjected to PCR amplification using probe-specific primer pairs (Table 1).

The linear plasmid DNA was transcribed in vitro using either T7 or SP6 RNA polymerase with ATP, CTP, GTP, and digoxigenin-labeled UTP (Boehringer Mannheim, Indianapolis, Ind.) as a labeling agent for antisense- or sense-strand probe, respectively. The antisense-strand probe was used for detection of gene expression because it hybridizes with mRNA by forming a complementary double strand. The sense strand probe was used as a negative control to assess nonspecific binding.

In situ hybridization. Paraffin-embedded tissue sections were deparaffinized and treated with proteinase K (10 μg/ml; Boehringer Mannheim) for 30 min at 37°C. Slides were washed with diethyl pyrocarbonate (Sigma, St. Louis, Mo.)-treated phosphate-buffered saline and then dried. Fifty microliters of RNA probe (0.5 mg of RNA/ml) in hybridization solution (50% formamide, 25% diethyl pyrocarbonate-treated H_2O, 3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1× Denhardt's solution, 0.2 mg of yeast tRNA per ml, 50 mM sodium phosphate [pH 7.4], 10% dextran sulfate) was applied onto a sample slide and covered with a coverslip. Slides were heated at 90°C for 10 min and were hybridized 16 h in a humidified chamber at 60°C. To remove unbound probe, slides were incubated for 30 min at 37°C with RNase A (20 mg/ml; Boehringer

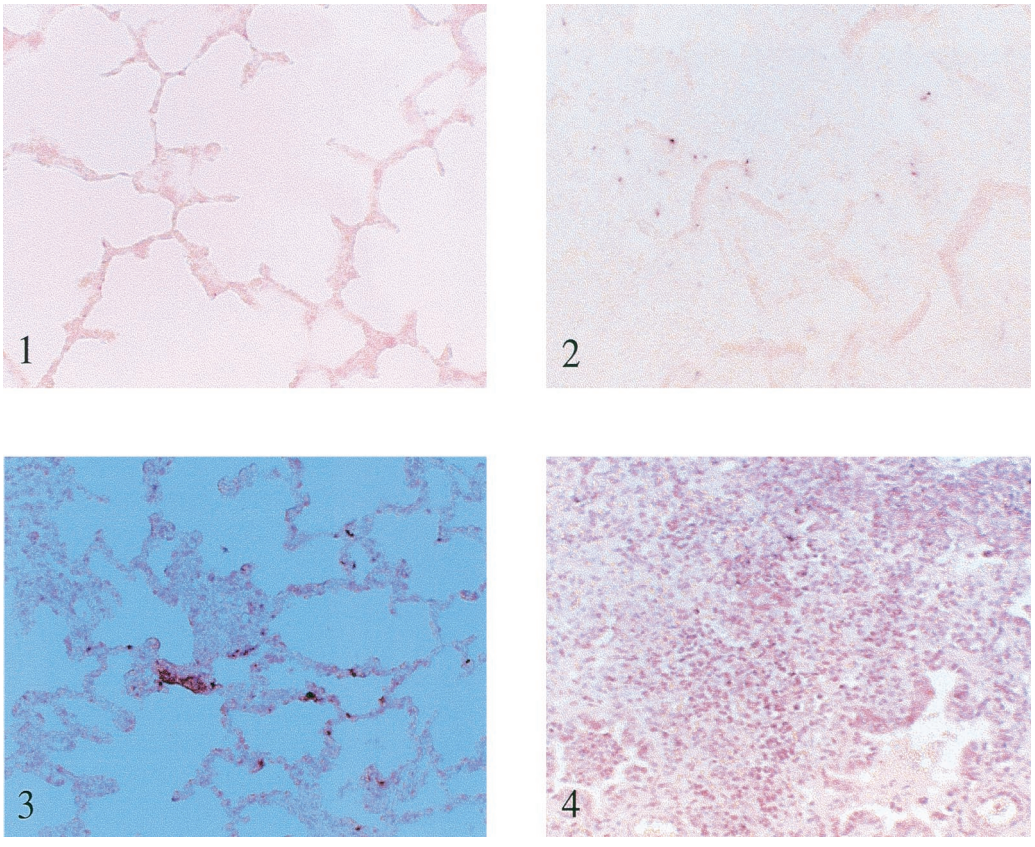

FIG. 1. Photomicrographs of bovine lung tissues in which IL-1α or IFN-γ mRNA is detected by in situ hybridization. Hybridized cells were stained with alkaline phosphatase and NBT-BCIP substrate (magnification, ×100). 1, $CD18^+$ animal inoculated with PFS and examined for IFN-γ; 2, $CD18^-$ animal inoculated with *P. haemolytica* and examined for IFN-γ; 3, $CD18^+$ animal inoculated with PFS and examined for IL-1α; 4, $CD18^-$ animal inoculated with *P. haemolytica* and examined for IL-1α.

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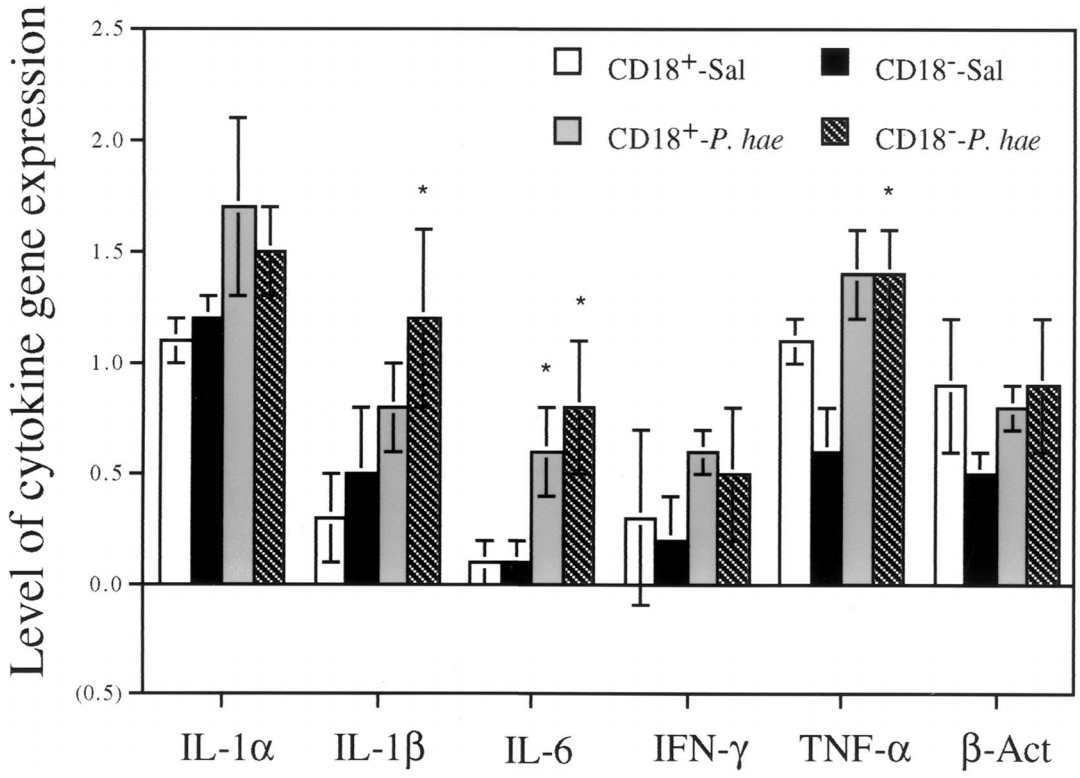

FIG. 2. Differences of proinflammatory cytokine gene expression in pulmonary tissues from *P. haemolytica*-infected (*P. hae*) and noninfected (Sal) lobes in $CD18^+$ and $CD18^-$ cattle 2 h p.i. Level of cytokine gene expression means the intensity of staining per cell. Subjective scoring was applied according to the color reaction: 0, no signal; 1, low expression; 2, moderate expression; 3, high expression. The scores represent the average of an individual tissue within a group with standard error of mean ($n = 3$). The data were examined using the Kruskal-Wallis one-way analysis of variance by ranks to evaluate the effects of CD18 and *P. haemolytica* inoculation on expression of the PIC genes. The asterisk indicates a statistically significant difference ($P < 0.05$) between *P. haemolytica*- and PFS-inoculated lungs within a genetic group for expression of a given cytokine.

Mannheim), washed sequentially in 2× SSC for 5 min, 1× SSC for 5 min, and 0.5× SSC for 1 h at 60°C, and finally washed with 0.5× SSC for 5 min at room temperature. Slides were then incubated with sheep antidigoxigenin antibody labeled with alkaline phosphatase and developed in 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (NBT-BCIP; Boehringer Mannheim) solution in the dark for 18 h. After chromogen development, slides were counterstained with nuclear fast red for 3 min and coverslipped with aqueous mounting medium (Accurate Chemical & Scientific Corp., Westbury, N.Y.) (7).

Macrophage staining. Monoclonal antibody EBM11 (anti-CD68; DAKO, Carpenteria, Calif.) was used for detection of macrophages in lung tissues (1). Briefly, sections of lung tissues were deparaffinized and treated with 0.25% bacterial protease (type XIV; Sigma) in Tris buffer (pH 7.6) for 30 min at 37°C. The primary antibody, monoclonal mouse anti-CD68, was diluted 1:25 and applied and incubated overnight at 4°C. Peroxidase-labeled goat anti-mouse immunoglobulin G (IgG) was used as the secondary antibody with 3′,3-diaminobenzidine substrate (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif.). Slides were counterstained with hematoxylin.

Neutrophil staining. For neutrophil staining, lead thiocyanate [Pb(II) SCN; Aldrich, Milwaukee, Wis.] was used for antigen retrieval (unpublished data). Deparaffinized tissues were treated in boiling Pb(II) SCN (1%) for 1.5 min and then remained in the same solution additional 10 min with the heat turned off (beaker still on heating block). Slides were allowed to cool for 15 min at room temperature in the saturated Pb(II) SCN solution under a hood. Endogenous peroxidase blocking was performed for 20 min at room temperature. The sections were treated with 10% normal goat serum for 15 min and labeled with a 1:150 dilution of secondary antibody (anti-mouse IgG [Fab-specific] biotin conjugated; Sigma) for 1 h at room temperature. The sections were incubated with streptavidin-horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) for 30 min and subjected to a color reaction with diaminobenzidine (Kirkegaard & Perry Laboratories) for 15 min. After 15 min of incubation, the sections were counterstained with hematoxylin for 1 min and coverslipped.

Data analysis. Two scoring system were applied to five independent fields on each slide. The first assessment was to evaluate the level of gene expression on a per-cell basis. Subjective scoring of signal intensity was scaled according to the color reaction: 0, no chromogen staining; 1, pale blue; 2, blue to purple; 3, dark purple. Another criterion for the scoring system was the percentage of positive cells identified in the first evaluation. Subjective scoring of positive cells was as follows: 0, no positive cells; 1, 1 to 30% of cells positive; 2, 30 to 70% of cells positive; 3, >70% of cells positive. The mean of each slide was calculated from five independent observations in a given sample. The mean of each group was an average of individual tissue means, with standard error of mean.

The data were examined using the Kruskal-Wallis one-way analysis of variance by ranks to evaluate the effects of CD18 and *P. haemolytica* inoculation for the expression of the PIC genes.

RESULTS

Various levels of cytokine gene expression were detected by cytokine-specific RNA probes (Fig. 1). Inoculation of *P. haemolytica* induced PIC gene expression and increased numbers of cells expressing PIC genes in lungs of both $CD18^+$ and $CD18^-$ cattle at both 2 and 4 h postinoculation (p.i.) compared to PFS-inoculated lungs (Fig. 2 to 5). The induction of IL-1β, IL-6, and TNF-α gene expression at 2 h after inoculation with *P. haemolytica* was statistically significant in $CD18^-$ cattle ($P < 0.05$), and only IL-6 was induced significantly in $CD18^+$ cattle compared to PFS-inoculated animals (Fig. 2). The induced level of PIC gene expression between *P. haemolytica*-inoculated $CD18^+$ and $CD18^-$ cattle, however, was not statistically different (Fig. 2). The number of cells that expressed PIC except IFN-γ also increased substantially at 2 h after inoculation with *P. haemolytica* in lungs of both $CD18^+$ and $CD18^-$ animals (Fig. 3). From 2 to 4 h p.i., expression of some cytokines especially IL-1α and IFN-γ, increased progressively in $CD18^+$ cattle treated with *P. haemolytica*, but no significant increase was observed in $CD18^-$ animals (Fig. 2 and 4). Proin-

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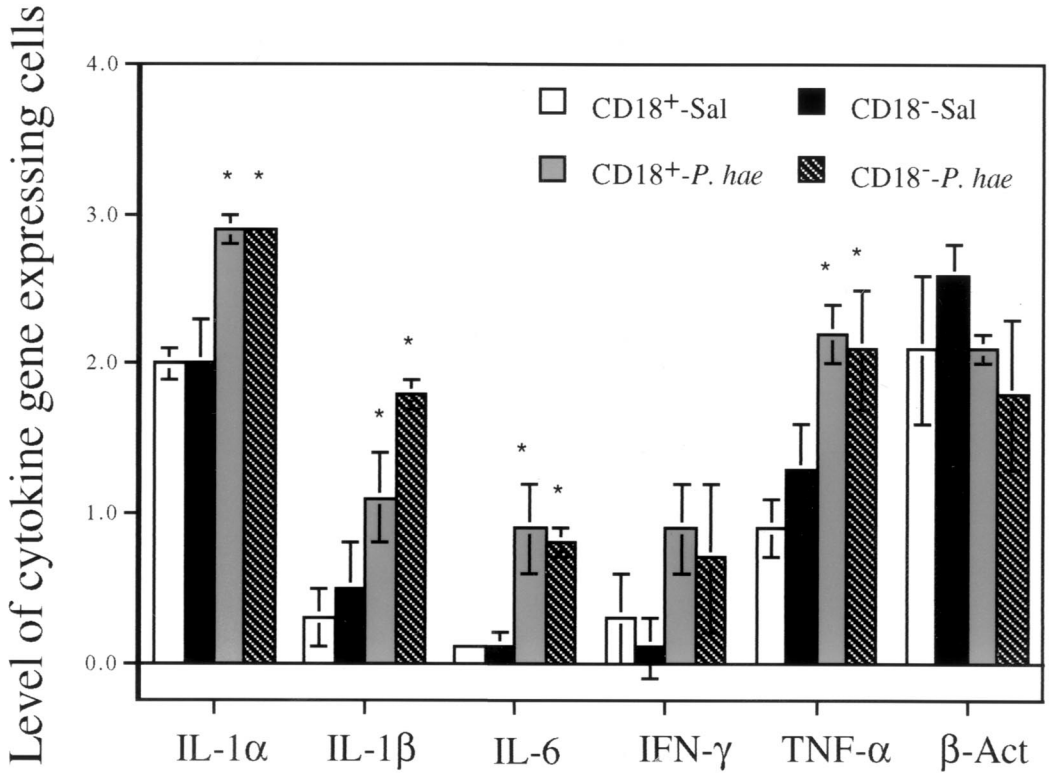

FIG. 3. Amount of cells that expressed proinflammatory cytokine gene in pulmonary tissues from *P. haemolytica*-infected (*P. hae*) and noninfected (Sal) lobes in $CD18^+$ and $CD18^-$ cattle 2 h p.i. Values for cytokine expression-positive cells are estimates of the percentage of cells that hybridized with a given cytokine RNA probe. Most stained cells were macrophages and neutrophils. Subjective scoring was used for estimation of positive cells: 0, no positive cells; 1, <30% of positive cells among total cells; 2, 30 to 70% positive cells among total cells; 3, >70% positive cells among total cells. Scores represent the average for each tissue within a group, with standard error of the mean ($n = 3$). The data were examined using the Kruskal-Wallis one-way analysis of variance by ranks to evaluate the effects of CD18 and *P. haemolytica* inoculation on expression of the PIC genes. The asterisk indicates a statistically significant difference ($P < 0.05$) between *P. haemolytica*- and PFS-inoculated lungs within a genetic group for expression of a given cytokine.

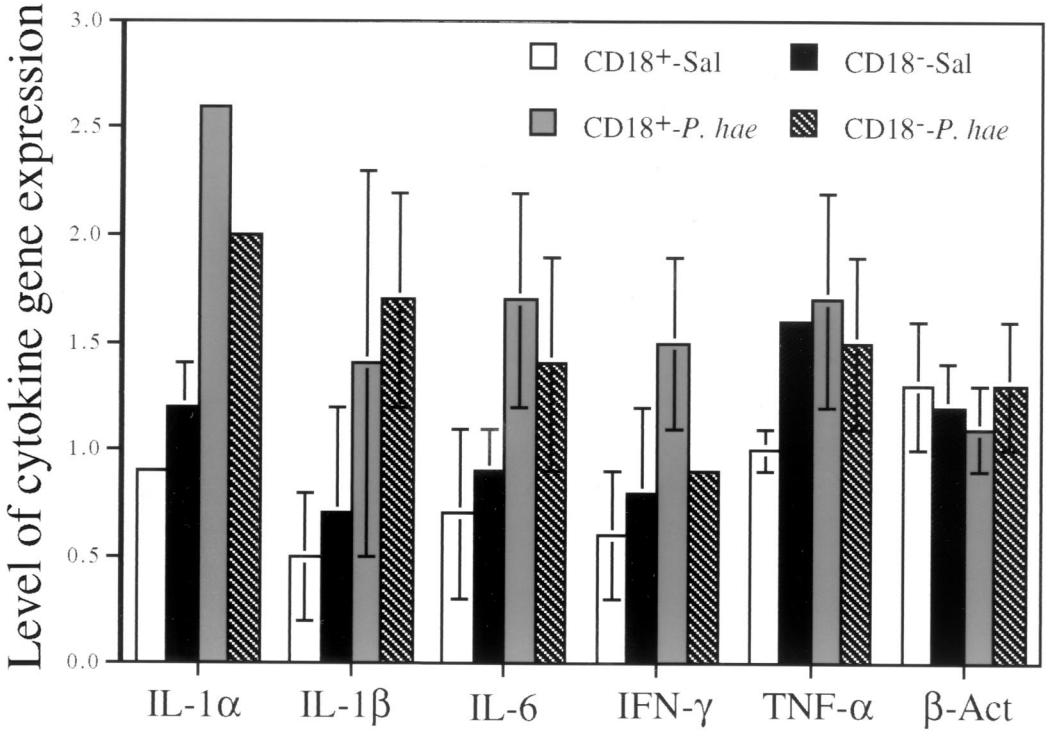

FIG. 4. Differences of proinflammatory cytokine gene expression in pulmonary tissues from *P. haemolytica*-infected (*P. hae*) and noninfected (Sal) lobes in $CD18^+$ and $CD18^-$ cattle 4 h p.i. For details, see the legend to Fig. 2.

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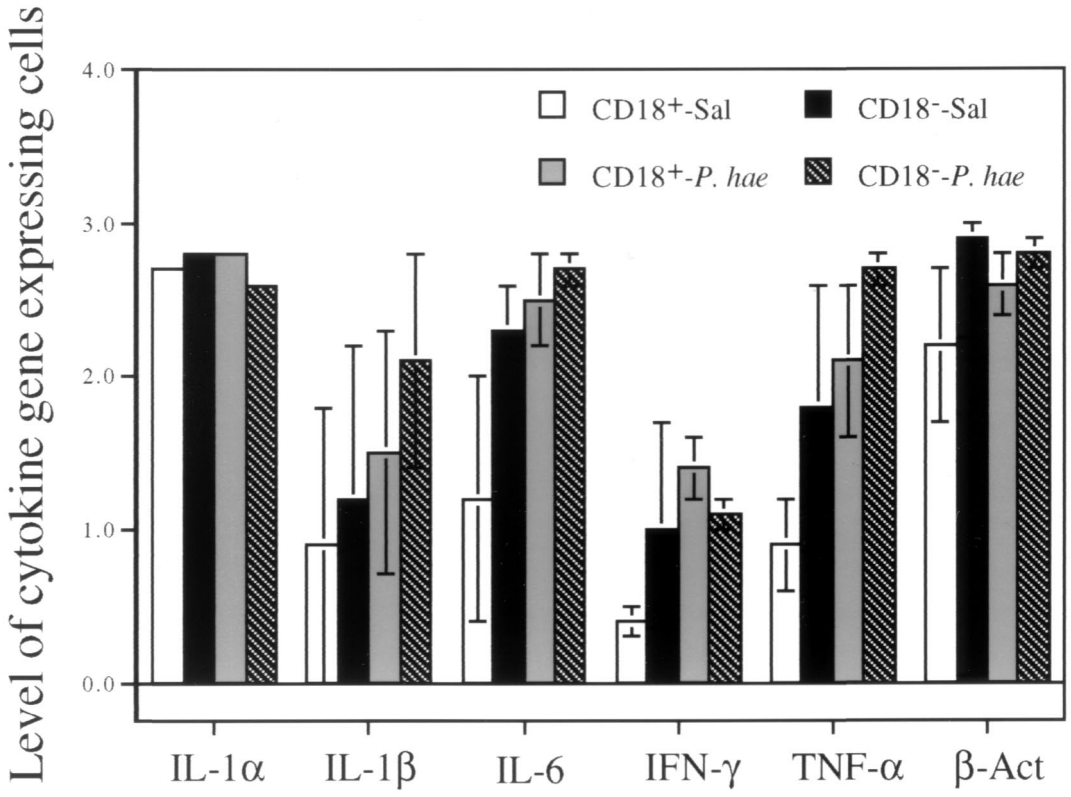

FIG. 5. Amount of cells that expressed proinflammatory cytokine genes in pulmonary tissues from *P. haemolytica*-infected (*P. hae*) and noninfected (Sal) lobes in $CD18^+$ and $CD18^-$ cattle 4 h p.i. For details, see the legend to Fig. 3.

flammatory cytokine expression by cells in both $CD18^+$ and $CD18^-$ animals also increased 4 h after inoculation with *P. haemolytica*, but the increase was not statistically significant compared to PFS-inoculated animals (Fig. 4). The same result was observed in the increase of cell numbers that expressed PIC genes at 4 h after inoculation with *P. haemolytica* (Fig. 5). In the PFS-inoculated lungs, $CD18^-$ cattle had a tendency (that was not statistically significant) to express higher levels of PIC genes and had more cells expressing PIC genes than $CD18^+$ cattle, especially in 4-h-p.i. groups (Fig. 4 and 5).

Inoculation with *P. haemolytica* increased infiltration of circulating leukocytes. Increased numbers of neutrophils and macrophages in *P. haemolytica*-inoculated lungs were observed (Fig. 6 and Table 2). Neutrophils were more prominent than macrophages in *P. haemolytica*-inoculated bovine lungs at both 2 and 4 h p.i. (Table 2). The increase of neutrophils in lungs of $CD18^-$ animals was statistically significant both 2 and 4 h after inoculation with *P. haemolytica* but at only 4 h p.i. in $CD18^+$ animals; the increase in macrophages was not statistically significant (Table 2).

DISCUSSION

Basal PIC gene expression in PFS-inoculated lung tissues of $CD18^-$ cattle is generally higher than in healthy $CD18^+$ cattle, possibly the result of chronic activation of $CD18^-$ animals' immune system due to recurrent and/or chronic infection (23–25, 30, 31). Kehrli et al. reported selected functional abnormalities of neutrophils in $CD18^-$ cattle (23, 24, 31). They observed diminished levels of phagocytosis, ingestion and associated reactive oxygen generation, myeloperoxidase-dependent iodination, and extracellular release of elastase by neutrophils from $CD18^-$ animals (23, 24, 31). All of these dysfunctions of $CD18^-$ neutrophils are related to the lack of functional activities of complement receptors (CR3 and CR4) that are part of β_2-integrin functions.

The function of integrin is not restricted to mediating cell-to-cell adherence and migration of immune cells but also involves signal transduction (17, 18, 36, 37, 43). Expression of PIC genes increased in tissues of both $CD18^+$ and $CD18^-$ cattle following inoculation with *P. haemolytica*. At 2 h p.i., the augmentations of PIC gene expression and of percentages of PIC gene-expressing cells with *P. haemolytica* inoculation were almost the same between $CD18^+$ and $CD18^-$ cattle. At 4 h p.i., however, the lung tissue of $CD18^+$ cattle expressed higher levels of IL-1α and IFN-γ genes than the lung tissue of $CD18^-$ cattle. In the $CD18^-$ cattle, expression of the IFN-γ and TNF-α genes was no greater in *P. haemolytica*-inoculated lungs than in PFS-inoculated lungs at 4 h p.i. The main difference between the two groups of calves, $CD18^+$ and $CD18^-$, was suspected to be the ability to sustain gene expression. The molecular mechanism of the signal transduction of β_2-integrins is not fully understood (37). It is clear, however, that these proteins play an important role in immune responses by coordinating various immunological and mechanical stimulations via cytoskeletal proteins, resulting in a stronger immune response by the host (36, 37). Flaherty et al. observed that β_2-integrin mediates signal transduction in response to LPS in a β_2-integrin gene-transfected Chinese hamster ovary fibroblast cell line (18). These data suggest that β_2-integrins contribute in vivo to induce PIC (especially IFN-γ and TNF-α) gene expression, as a consequence of *P. haemolytica* infection. Therefore, $CD18^-$ cattle could not respond properly to the *P. haemolytica* infection. Since RTX toxins bind to β_2-integrins and induce

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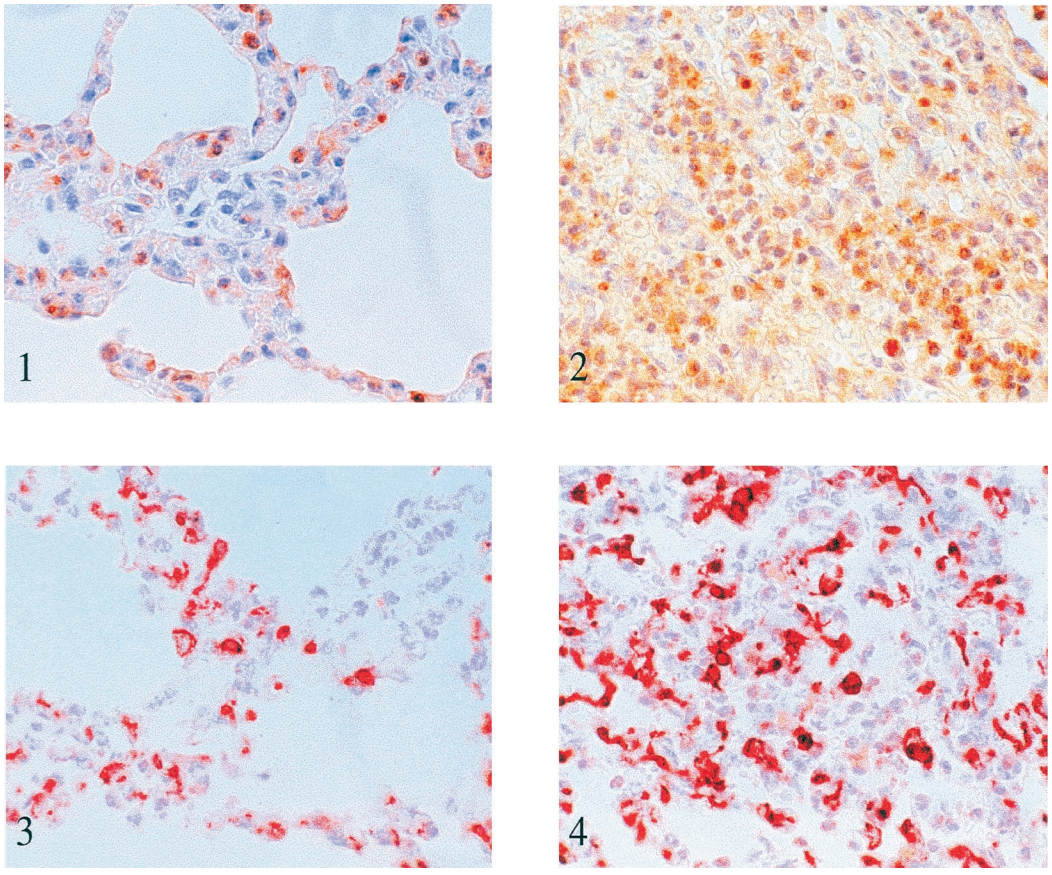

FIG. 6. Photomicrographs of bovine lung tissue stained with either monoclonal mouse anti-CD68 for macrophages or goat anti-mouse IgG (Fab specific) for neutrophils (magnification, ×250). 1, CD18$^-$ animal inoculated with PFS and examined for neutrophils; 2, CD18$^-$ animal inoculated with *P. haemolytica* and examined for neutrophils; 3, CD18$^-$ animal inoculated with PFS and examined for macrophages; 4, CD18$^-$ animal inoculated with *P. haemolytica* and examined for macrophages.

apoptosis of target cells, we considered leukotoxin of *P. haemolytica* a potentially important mediator (26, 44). However, we could not determine whether *P. haemolytica* leukotoxin had a significant role in this experiment.

Ackermann et al. reported that the number of resident inflammatory cells in the alveolar septum of CD18$^-$ animals is substantially greater than the number in CD18$^+$ animals (2). They proposed three possible mechanisms for neutrophil infiltration into the lung alveoli of CD18$^-$ cattle: migration through porelike fenestrae, CD18-independent adherence, and septal degradative processes (2). At 2 h p.i., the increase of neutrophils in the lung of *P. haemolytica*-inoculated CD18$^-$ cattle, but not CD18$^+$ cattle, was statistically significant ($P < 0.05$). At 4 h p.i., both CD18$^+$ and CD18$^-$ animals had significant neutrophil infiltration ($P < 0.05$). Neutrophils have been shown to be the primary inflammatory cells that migrate to the infection site (17). The higher percentage and faster infiltration of inflammatory cells seen in the CD18$^-$ animals at 2 h after inoculation with *P. haemolytica* may be a mechanism to compensate for the immune malfunction of the host. One of the characteristics of CD18$^-$ animals is elevated leukocyte counts in the blood due to a striking neutrophilia (19, 23, 25). The leukocytosis may contribute to the high number of leukocytes in the lung tissues of CD18$^-$ animals after *P. haemolytica* inoculation by simply increasing the chance of random migration. In contrast to neutrophils, the percentages of macrophages in the lungs after challenge with *P. haemolytica* were not significantly different at the two time points. Doerschuk et al. also observed CD18-independent migration of neutrophils into the lungs of rabbits challenged with *Streptococcus pneumoniae* (14, 16). Recently, it was demonstrated that neutrophils may transmigrate the endothelium by activation of C5a and adhesion ligands such as E-selectin, independent from CD18 (2, 21). Furthermore, neutrophils within the alveolar septum may release many tissue-damaging materials such as proteases, fatty acid metabolites, and radical oxygen metabo-

TABLE 2. Percentage of labeled neutrophils and macrophages in lungs of CD18$^+$ and CD18$^-$ cattle 2 or 4 h after inoculation with PFS or *P. haemolytica*

Group	2 h p.i.		4 h p.i.	
	Neutrophils (%)	Macrophages (%)	Neutrophils (%)	Macrophages (%)
CD18$^+$				
PFS	13.3 ± 4.1	13.3 ± 4.1	13.3 ± 4.1	13.3 ± 5.4
P. haemolytica	23.3 ± 4.1	20.0 ± 7.1	43.3 ± 4.1[b]	23.3 ± 4.1
CD18$^-$				
PFS	16.7 ± 2.0	16.7 ± 4.1	13.3 ± 4.1	21.7 ± 5.4
P. haemolytica	36.7 ± 4.1[b]	16.7 ± 8.2	50.0 ± 12.2[b]	30.0 ± 7.1

[a] Percentages were calculated by dividing the number of specifically stained cells from all cells counted in five randomly chosen sites.

[b] Statistically significant difference ($P < 0.05$) between *P. haemolytica*- and PFS-inoculated lungs within a genetic group for expression of the given cytokine.

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lites, thus facilitating leukocyte infiltration into parenchymal tissue regardless of CD18 expression (2).

This study suggests that even though leukocytes of infected CD18$^-$ animals can transmigrate into the lungs, the cells are unable to clear invading pathogens efficiently because of the impairment of selective immune functions. Therefore, the CD18$^-$ animals have more episodes of infections and more persisting infections.

ACKNOWLEDGMENTS

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