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ORIGINAL ARTICLE

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The numbers of leukocyte subsets in lung sections differ between intercellular adhesion molecule-1$^{-/-}$, lymphocyte function-associated antigen-1$^{-/-}$ mice and intercellular adhesion molecule-1$^{-/-}$ mice after aerosol exposure to *Haemophilus influenzae* type-b

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Abstract In order to investigate the role of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen-1 (LFA-1) in pulmonary immunological processes, leukocyte populations were stained immunohistochemically on cryostat lung sections of ICAM-1$^{-/-}$ and LFA-1$^{-/-}$ mice. A further group of ICAM-1$^{-/-}$ mice was exposed to *Haemophilus influenzae* type-b (*Hib*) 24 h before being sacrificed. Comparison of the numbers of leukocytes in these groups revealed different behaviors of the leukocyte subsets: granulocytes were significantly increased in all three groups. Lymphocytes were increased in ICAM-1$^{-/-}$ mice, while there was no significant difference in LFA-1$^{-/-}$ and even a decrease in ICAM-1$^{-/-}$ mice after *Hib* exposure. Neither in ICAM-1$^{-/-}$ nor in LFA-1$^{-/-}$ mice did macrophages and dendritic cells (DCs) show significant differences to control animals. After *Hib* exposure, a significant elevation of DCs was observed. The following conclusions can be drawn: (1) all investigated leukocyte subsets can use ICAM-1- and LFA-1-independent pathways in the lungs of mice; (2) the pathways used by the leukocytes are cell-type specific; (3) ICAM-1 plays an important role in the enhanced recruitment of lymphocytes during *Hib* challenge in the lung; and (4) the alternative migratory mechanisms are able to compensate for the absence of ICAM-1 or LFA-1 or even lead to increased cell numbers. This overcompensation can be seen as a result of a balance between active alternative migratory mechanisms, which takes place in the absence of ICAM-1 or LFA-1.

Keywords Intercellular adhesion molecule-1 · Lymphocyte function-associated antigen-1 · Lung · *Haemophilus influenzae* type-b · Immunohistology

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Introduction

The current multistep model of leukocyte migration is based on investigations of vessels of the systemic circulatory system [34]. In the lung, however, a different situation exists, because leukocytes pass mainly through the pulmonary capillaries [7] rather than through high endothelial venules (HEVs). Furthermore, alveolar macrophages and pulmonary interstitial cells might influence leukocyte migration. This lung-specific leukocyte migration might explain the findings that blocking [6] or absence [12, 5] of adhesion molecules, such as the integrins or intercellular adhesion molecule (ICAM)-1 in knock-out mice, does not result in decreased leukocyte numbers in the lung as it does in organs of the systemic circulatory system. Obviously, the migratory mechanisms differ between the organs, the cell types, and even in the stimulus for emigration to the tissue [39]. Therefore, the unique situation of leukocyte migration to the lung has to be studied either in the isolated perfused lung or in specific animal models [17].

The number of leukocytes in the lung depends on the extent of immigrating and emigrating cells, local apoptosis, and proliferation [25]. The latter two aspects have a relatively small effect on the cell number in the normal lung. Apoptosis of leukocytes in the lung is regarded as a rare event, with a maximum of 2.8% of certain leuko-

cytes [18]. Krug et al. [19] did not find increased apoptosis rates in the bronchoalveolar lavage (BAL) of human lungs after allergen exposure. Furthermore, local proliferation played a minor role in healthy rat lungs, as less than 4% of lymphocytes incorporated a DNA precursor (unpublished). A similar situation was described during an immune response [30]. Therefore, the transit time or residence time and the changes in migration seem to be the most relevant parameters for the cell number found in the lung.

The purpose of this study was to determine the effect of the absence of adhesion molecules, such as ICAM-1 or lymphocyte function-associated antigen (LFA)-1, on leukocyte migration in the lung. An additional group of ICAM-1$^{-/-}$ mice was exposed to *Haemophilus influenzae* type-b *(Hib)*, which is known to induce the expression of ICAM-1 [14], in order to investigate its role during inflammation. Seven different leukocyte subsets were identified and quantified on lung sections. Because the lungs were thoroughly flushed to wash out the marginal pool before embedding, the leukocytes identified were taken as being mainly localized in the lung interstitium. In infected animals, BAL samples were also taken and investigated.

Materials and methods

Animals

The ICAM-1$^{-/-}$ animals had a mutation in exon 5 of the ICAM-1 gene produced by homologous recombination and introduction of the neomycin-resistance gene cassette [33]. The animals were divided into six groups of six animals: ICAM-1$^{-/-}$ mice with their C57BL/6 control group, LFA-1$^{-/-}$ mice, and the wild-type (WT) LFA-1$^{+/+}$ [4]. Additionally, an ICAM-1$^{-/-}$ and C57BL/6 group were exposed to *Hib*. The animals were kept under specified pathogen free conditions. All mice were 6–8 weeks old when sacrificed. All procedures in the animal experiments were performed in accordance with German animal welfare legislation.

Aerosol infection

On the day of exposure, *Hib* was suspended in a potassium–phosphate buffer at a concentration of 10^9 CFU/ml. A compressor transferred about 4 ml of this suspension to an aerosol dispersal chamber (constructed by M.L.) where up to eight mice could be exposed simultaneously for 15–30 min. Then, the mice were returned to their housing. They were killed 24 h later.

Preparation of the samples

Animals, both infected and uninfected, were killed by means of inhalation of diethyl ether for several minutes. Blood samples of infected animals were taken from the retrobulbar region. Then an intratracheal catheter was inserted, and BAL was performed with 3×0.75 ml phosphate-buffered saline (PBS, Seromed, Berlin). After opening the right atrium and the left ventricle, the lungs were perfused with PBS via the atrium until the fluid coming out of the ventricle was free of blood. The lung airways were filled with 1 ml of a solution consisting of 1:3 O.C.T.:PBS (O.C.T. Sekura Finetek, Zoeterwoude, The Netherlands). Finally, the lungs were removed en bloc, snap frozen in liquid nitrogen, and stored at –70°C.

Processing for histology and immunohistochemical staining

Cryostat sections (7 µm) were cut at –20°C. The sections contained central and peripheral lung areas providing about 30 mm^2 of each lung for each staining. After being fixed in acetone at –20°C, the sections were stained using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique. The monoclonal antibodies and their dilutions have been previously described [13]: KT3 for CD3+ lymphocytes (Serotec, Wiesbaden, Germany, [36]); L3T4 for CD4+ T cells [10], Lyt-2 for CD8+ T cells [37], and Gr-1 for neutrophils [16] (all three from Dianova, Hamburg, Germany); B220 for B lymphocytes (provided by Dr. Martin, Hannover, Germany, [3]); and NLDC-145 for dendritic cells (DCs) [26] and BM8 for macrophages [21] (both from Biomedicals AG, Augst, Switzerland).

After a 30-min incubation at room temperature with the primary antibodies and washing with Tris-buffered saline (TBS)-Tween (0.5% Tween, Serva, Heidelberg, Germany), the slices were incubated with the bridging antibody [rat anti-mouse immunoglobulin (Ig)G, Z 494, Dako, Hamburg, Germany] for another 30 min and then again, after washing, with the APAAP complex (D 488, Dako) for 25 min. In order to increase the intensity of the staining, the incubations with the bridging antibody and the APAAP complex were repeated for 15 min each. Fast Blue or Fast Red (Sigma, Munich, Germany) served as substrate for the alkaline phosphatase. Sections were counterstained with hemalaun and mounted in glycergel (Dako).

Evaluation and statistics

Stained cells were counted in ten randomly chosen fields representing 6.4 mm^2 in each section. The results were presented as cells/mm^2 (mean±SD). Differential cell counts on cytospins of infected animals using 100 µl BAL fluid were performed. The data from different groups were compared with the Mann-Whitney *U* test (SPSS Inc., Chicago, Ill.). Differences with $P<0.05$ were taken as significant.

Results

Leukocytes were scattered homogeneously in peripheral lung tissue. All leukocyte subsets were present in both ICAM-1$^{-/-}$ and LFA-1$^{-/-}$ mice, indicating alternative mechanisms of entering the lung parenchyma. Negative controls produced the expected results with no labeled cells. The lung sections of insufficient animals show higher leukocyte numbers compared with controls (Fig. 1).

ICAM-1$^{-/-}$ and LFA-1$^{-/-}$ mice

Both knock-out strains showed a significant elevation of neutrophils. Only in ICAM-1$^{-/-}$ mice were higher numbers of T (CD3+, CD4+, CD8+) and B lymphocytes found than in controls (Fig. 2A, B). There were no significant differences between the numbers of DCs and macrophages.

When both groups of deficient animals were compared, higher lymphocyte numbers in ICAM-1$^{-/-}$ but no significantly different neutrophil numbers were seen (Fig. 2C). The tendency for higher numbers of macrophages and DCs in ICAM-1$^{-/-}$ was already observed when corresponding control groups were compared (not shown).

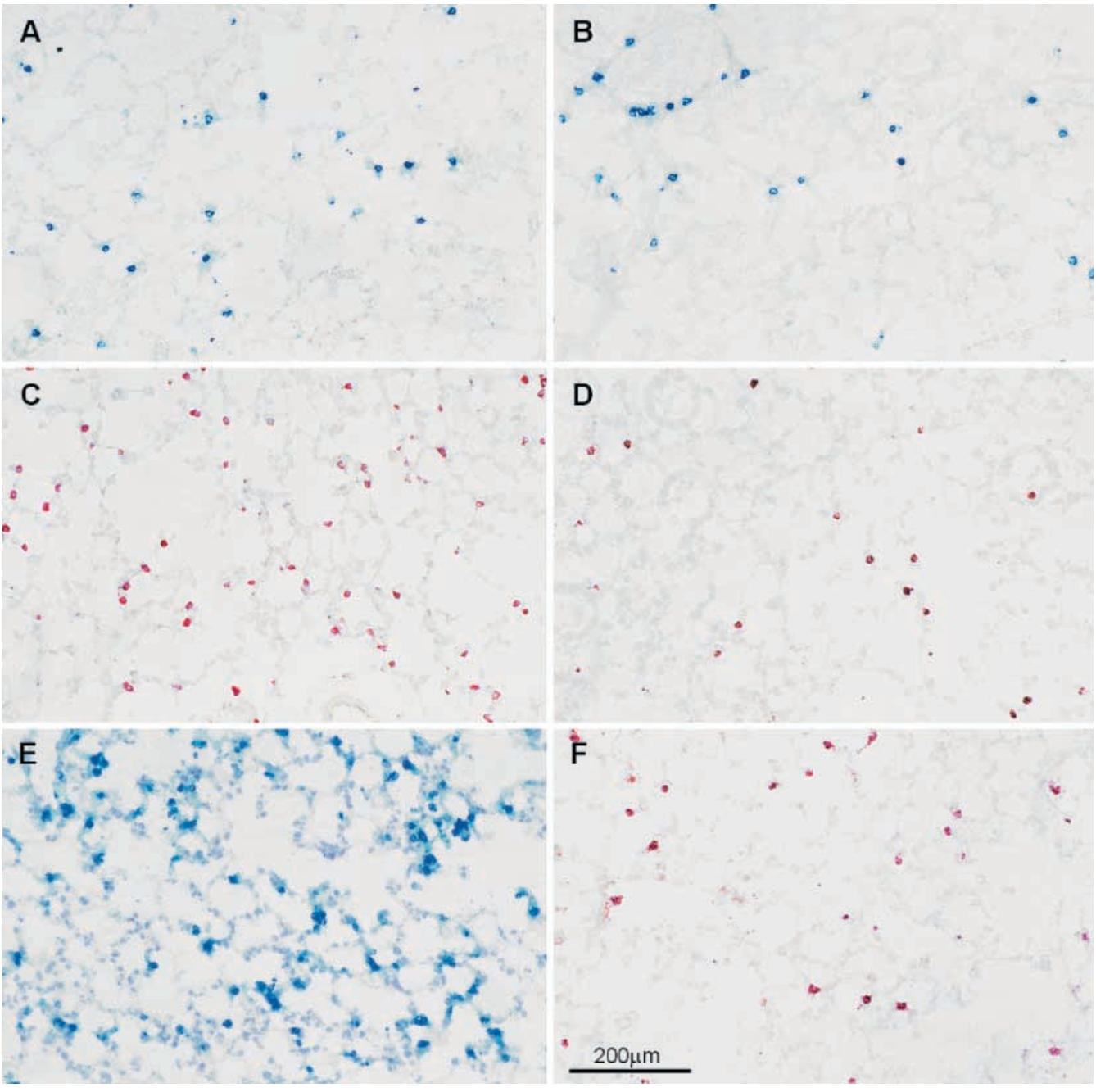

Fig. 1 Immunohistologically stained cryostat lung sections of an intercellular adhesion molecule (ICAM)-1$^{-/-}$ mouse (**A**), a lymphocyte function-associated antigen (LFA)-1$^{-/-}$ mouse (**C**), and an ICAM-1$^{-/-}$ mouse exposed to *Haemophilus influenzae* type-b (*Hib*; **E**) 24 h before facing their controls (**B**, **D**, **F**). **A**–**D** show CD3+ lymphocytes, while **E**–**F** contain Gr-1+ neutrophils. It is conspicuous that deficient animals show increased cell densities, especially in the case of the neutrophils after *Hib* exposure (**E**)

Hib-infected ICAM-1$^{-/-}$ mice

Exposure to the *Hib*-containing aerosol led to an increase of B cells and neutrophils and a decrease of DCs and macrophages in C57BL/6 control groups (Fig. 3A). This tendency was also seen in ICAM-1$^{-/-}$ mice but here, only neutrophil elevation was significant (Fig. 3B). Noteworthy is the significant increase of CD3+, CD4+, and CD8+ lymphocytes in C57BL/6 and, in contrast, the decrease of the same subsets in ICAM-1$^{-/-}$ animals. During infection, ICAM-1$^{-/-}$ mice, compared with infected controls, showed strongly increased neutrophils, which were already on a higher level without infection (Fig. 2A), and generally lower lymphocytes numbers (Fig. 3C). There was no difference in macrophage numbers. In the BAL, the most frequent subsets, the neutrophil and NLDC+ cells, the latter consisting of alveolar macrophages and DCs, were significantly increased in ICAM-1$^{-/-}$ animals (Fig. 4) as they were in the lung slices. The investigated lymphocytes from blood samples revealed no significant differences between cell numbers (not shown).

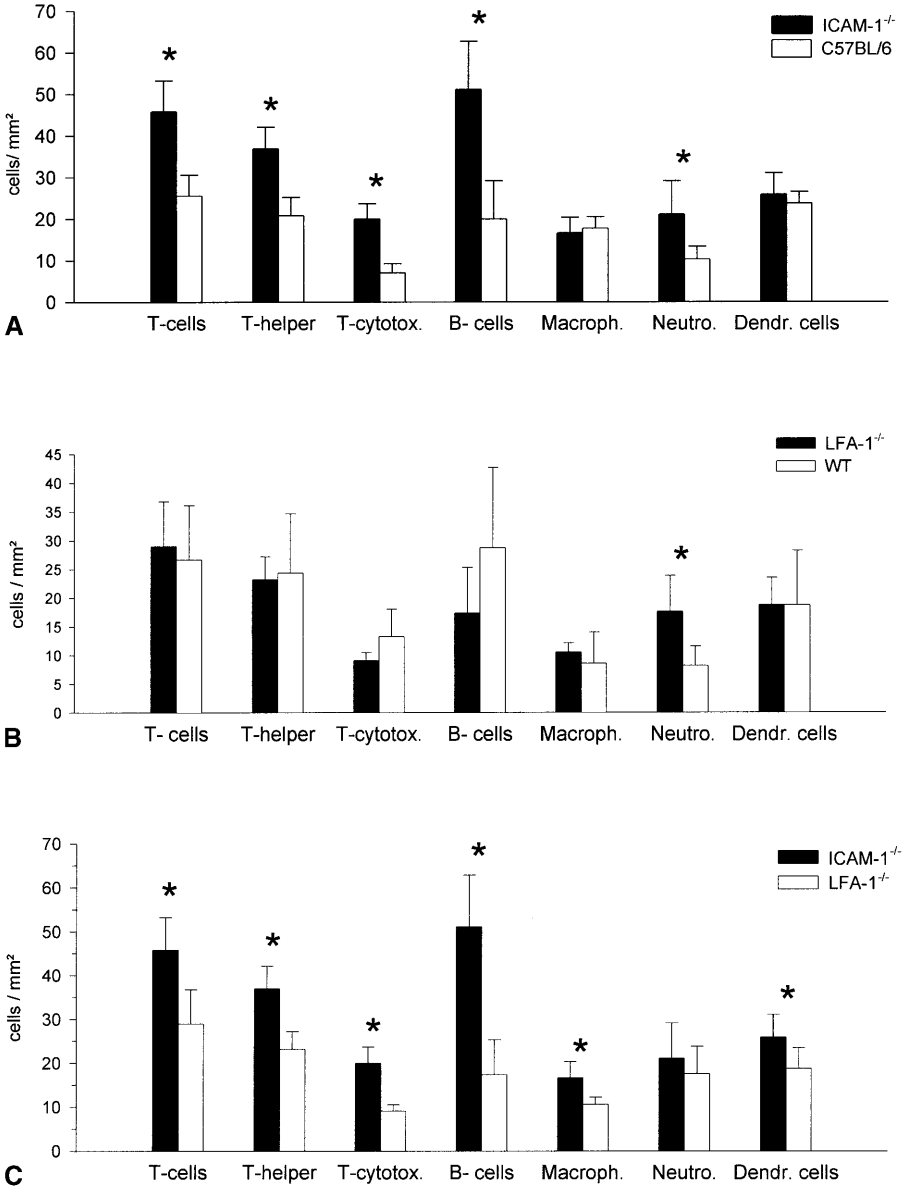

Fig. 2 Comparison between the leukocytes of lung sections of intercellular adhesion molecule (ICAM)-1$^{-/-}$ and C57BL/6 mice (**A**), lymphocyte function-associated antigen (LFA)-1$^{-/-}$ and wild-type LFA-1$^{+/+}$ mice (**B**), and ICAM-1$^{-/-}$ and LFA-1$^{-/-}$ mice (**C**). Mean±SD; *P<0.05

Discussion

The role of adhesion molecules in the immigration of leukocytes from the blood into the parenchyma of an organ can be studied using 'knock-out' animals. In using antibodies against adhesion molecules, there is the risk of an incomplete blockade of function or the activity of endothelial cells or leukocytes being influenced by the antibody. However, animals deficient in a molecule might still produce isoforms, which could have intact ligand binding sites, as reported for ICAM-1$^{-/-}$ C57BL/6 mice [15]. The role of these alternatively spliced ICAM-1 molecules is unknown. Qin et al. [27] reported that the monoclonal antibody (mAb) YN1/1, which binds to both ICAM-1 and its isoforms, does not inhibit the immigration of neutrophils in mice deficient in ICAM-1. Therefore, it can be assumed that isoforms do not contribute to the immigration of neutrophils.

In this study, cells were mainly counted in the lung tissue, so it was not possible to observe the migration across the different barriers within the lung directly. The cell density was thus taken as an indicator for cell migration, i.e., as the result of leukocyte traffic in the lung. The role of other factors that could contribute to the cell density, e.g., the local proliferation, apoptosis, or changes of blood cell counts, can be seen as being of minor relevance. However, even minor differences in migration could possibly result in obvious effects over time [24].

It is not easy to compare results from experiments carried out with mAbs with those from experiments using animals genetically deficient in the same molecule. The effective blockade with mAbs and increased cell counts in deficient animals show that in deficiency, migratory mechanisms may be employed which, in the case of acute blockade, are not available. Elevated neutrophils have been reported in CD18$^{-/-}$ calves after inoculation

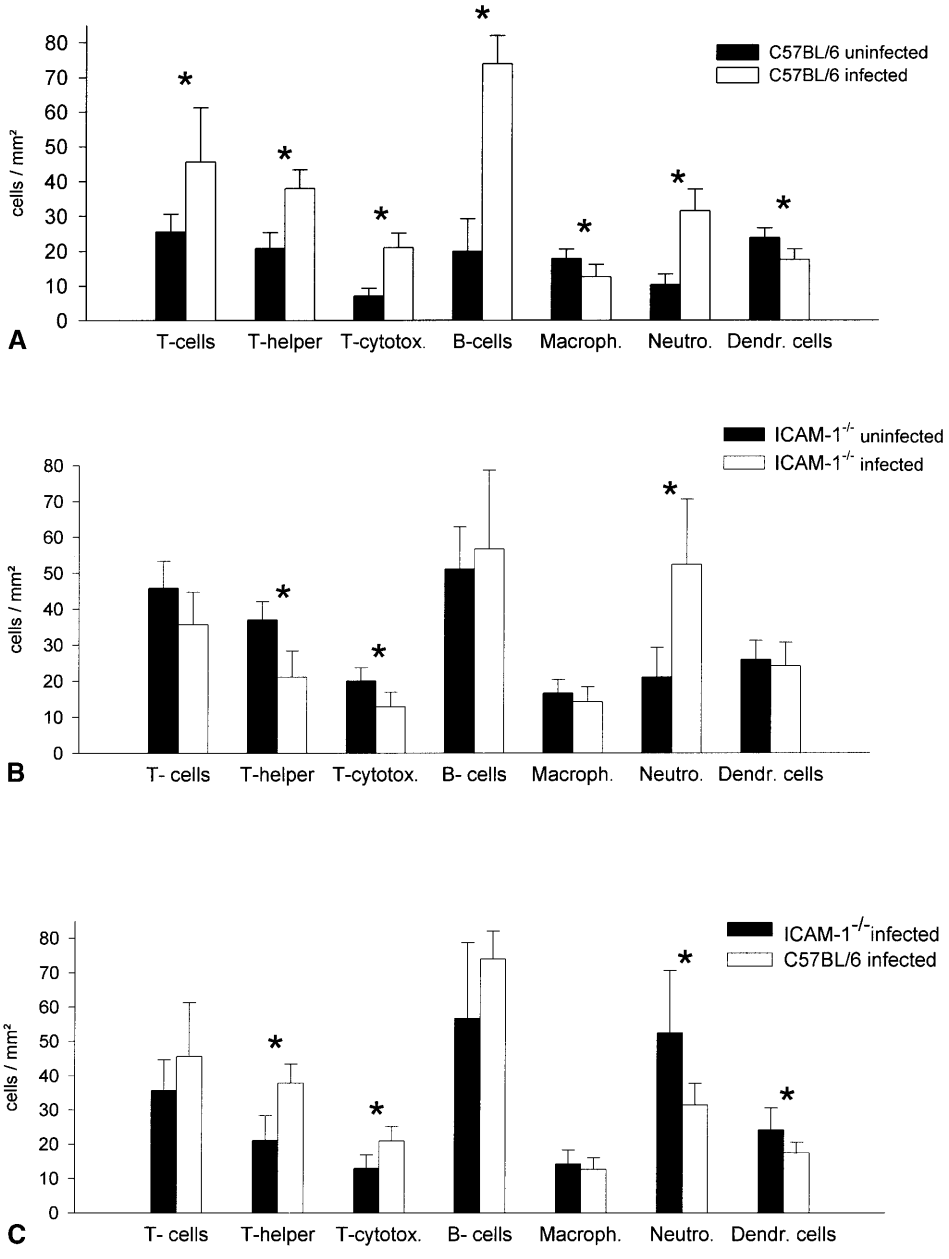

Fig. 3 Comparison between the leukocytes of lung sections of uninfected and *Haemophilus influenzae* type-b-infected C57BL/6 mice (**A**) and intercellular adhesion molecule (ICAM)-1$^{-/-}$ mice (**B**) and of C57BL/6 and ICAM-1$^{-/-}$ mice, both infected (**C**). Mean±SD; **P*<0.05

with sterile solution [1]. Furthermore, increased neutrophil numbers in the lungs of ICAM-1$^{-/-}$ mice [28] suggest pathways independent of these adhesion molecules that cause an increase of cell numbers.

Macrophages and DCs that enter the lung as monocyte precursors [2] show similar behavior in our experiment. There is no elevation in ICAM-1$^{-/-}$ mice (Fig. 2A). This correlates with a stronger expression of ICAM-1 on alveolar macrophages than on blood monocytes [35]. According to Shang et al. [31, 32], monocytes can use both β_1- and β_2-integrins for transendothelial migration. In LFA-1$^{-/-}$ mice, Mac-1 (CD11b/CD18) can be the ligand for ICAM-1. β_1-Dependent pathways or pathways that are Mac-1-dependent and ICAM-1-independent could compensate for the absence of ICAM-1 in the lung.

The different effects of the absence of ICAM-1 and LFA-1 are most obvious in the case of lymphocytes. Greenwood et al. [11] distinguished between adhesion to and transmigration through the endothelium of retina, where adhesion depended on LFA-1 and diapedesis on the pair ICAM-1/LFA-1. In an experiment with umbilical endothelial cells by Oppenheimer-Marks et al. [23], ICAM-1/LFA-1 and vascular cell adhesion molecule (VCAM)-1/very late activation antigen (VLA)-4 mediated adhesion to resting and interleukin (IL)-1-activated endothelium, respectively, while in all cases, diapedesis was ICAM-1 dependent. This ICAM-1 dependency was not observed in the lungs of our experiment, and there was, in fact, an elevation of lymphocytes in the absence of ICAM-1. Even after blockade of all four adhesion molecules, i.e., ICAM-1, LFA-1, VCAM-1, and VLA-4,

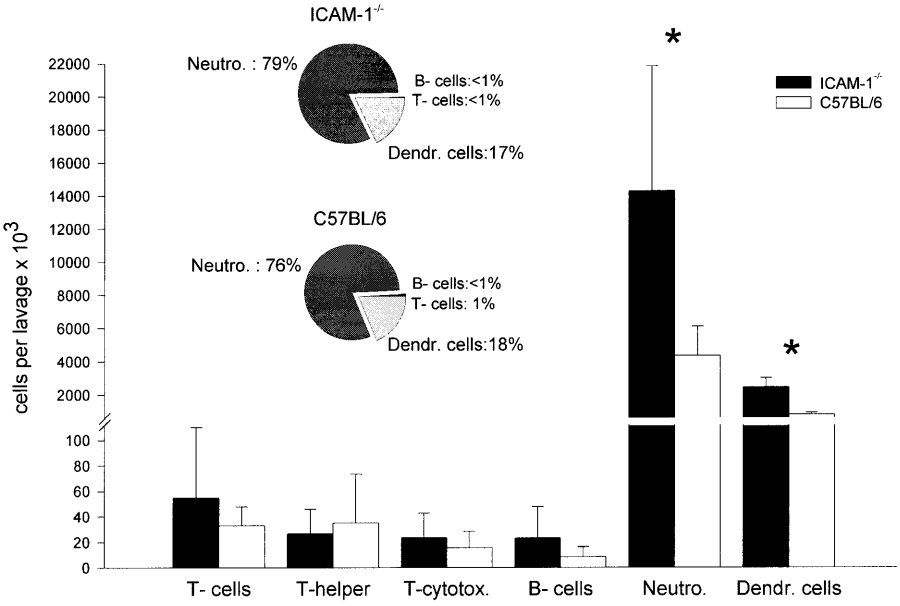

Fig. 4 Comparison of leukocytes of the bronchoalveolar lavage of *Haemophilus influenzae* type-b-infected intercellular adhesion molecule-1$^{-/-}$ and C57BL/6 mice. The break in the Y-axis comprises the area from 112,000 to 600,000. The relative composition of the subsets and absolute numbers are shown. Mean±SD; *P<0.05

there was no absolute blockade of cell adhesion [23], so that additional mechanisms must be assumed to be active. Further indications of the causes of cell number elevation can be found during infection.

Hib aerosol exposure

Exposure to *H. influenzae* caused an increase of lymphocytes and neutrophils in control mice. In ICAM-1$^{-/-}$ mice, the elevation of neutrophils was even stronger, while there was no increase in lymphocyte numbers, indicating dependency during infection, which was not observed in uninfected mice. Because there were no significant differences in lymphocyte numbers of the blood samples, systemic lymphocytosis and lymphocytopenia can be excluded as possible reasons for changes within the lung tissue. Like neutrophils, DC numbers increased in the lung tissue and in the BAL.

ICAM-1-independent immigration during infection was already mentioned by Kumasaka et al. [20] when comparing different methods of interfering with ICAM-1. In concordance with our results, Mizgerd et al. [22] reported increased neutrophil numbers in the lung after infection in CD18$^{-/-}$ mice. The reason was assumed to be an existing neutrophilia, an explanation we could not confirm. During an infection, leukocyte "stiffness" increases, so they can get stuck in the narrow capillary bed of the lung [8]. The diapedesis can occur in a manner which does [1, 9] or does not damage [38] the capillary. The increased cell counts could be due to pathways that are already present but in the absence of some adhesion molecules are more active or to molecules induced by insufficiency. Presumably, an important role is played by chemokines, which can induce the production of adhesion molecules. They seem to have a relatively low redundancy and great specificity [40]. Therefore, they could be a promising target for interference. Some chemokines are believed to perform integrin-independent adhesion [29].

The alternative pathways still remain undetermined. If they are compensating for the missing adhesion molecules, there is an overcompensation of lymphocytes and neutrophils in ICAM-1$^{-/-}$ mice and of neutrophils in LFA-1$^{-/-}$ mice. There are no indications that ICAM-1 or LFA-1 have inhibiting effects on cell migration or on other adhesion molecules. Therefore, the increased cell numbers could be seen as a consequence of the adjusted balance between the active migratory mechanisms.

Conclusion

All leukocyte subsets studied can enter the lungs of mice using specific ICAM-1- or LFA-1-independent pathways. Therefore, the cell density changed relative to the WT. ICAM-1 plays an important role in lymphocyte recruitment during an *Hib* infection. One cell type has a variety of migration mechanisms at its disposal. A simple addition of the capacity of these different pathways is not enough to explain all observed changes in cell numbers, such as an elevation in deficient animals, which might result from the balance of active migratory mechanisms. It remains an interesting question whether the redundancy of migration pathways might prevent precise intervention or whether the blockade of a group of adhesion molecules could open the door to a new form of therapy.

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