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Mac-1 deficiency induces respiratory failure by affecting type I alveolar epithelial cells

J. Wang[1], Y.B. Ci[2], C.L. Liu[1] and H.M. Sun[3]

[1]Department of Respiratory Medicine, People's Hospital of Laizhou City, Laizhou, Shandong, China
[2]Department of Severe Medical Science, People's Hospital of Laizhou City, Laizhou, Shandong, China
[3]Department of Nursing, People's Hospital of Laizhou City, Laizhou, Shandong, China

Corresponding author: J. Wang
E-mail: wnagjunsv@sina.com

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ABSTRACT. As a b2 integrin family member, Mac-1 plays an important role in the inflammatory response. Inflammation and lung injury are closely associated, but the involvement of Mac-1 in the occurrence and development of such pathologies remains poorly understood. We aimed to investigate the relationship between Mac-1 deficiency and respiratory failure in Mac-1 knockout {Mac-$1^{-/-}$} mice, using C57BL/6J mice as a control. The newborn survival rate of Mac-$1^{-/-}$ mice was calculated, and mouse lung tissue was treated with hematoxylin and eosin and subjected to immunofluorescent staining. Moreover, western blotting and immunohistochemistry were used to detect the expression of molecules specific to type I and type II alveolar epithelial cells, as well as alveolar surfactant proteins secreted by the latter. Survival of Mac-$1^{-/-}$ pups was significantly lower than that of newborn C57BL/6J mice. In a float test, lung tissues from C57BL/6J mice were buoyant, whereas those of Mac-$1^{-/-}$ mice were

not. Compared with C57BL/6J mice, expression of proSP-C {specific to type II alveolar epithelial cells} and alveolar surfactant proteins in Mac-$1^{-/-}$ mice was not significantly different, implying that type II cell function was unaltered. However, western blotting revealed expression of T1α, Aqp5, and Snx5 {type I alveolar epithelial cell markers} in Mac-$1^{-/-}$ mice to be significantly decreased {$P < 0.05$}. In conclusion, Mac-1 may play an important role in respiratory failure. Its absence leads to this condition not by influencing type II alveolar epithelial cells or their secreted surfactant proteins, but rather by reducing type I alveolar cell numbers.

Key words: Mac-1; Type I alveolar epithelial cells; Respiratory failure; Type II alveolar epithelial cells

INTRODUCTION

Respiratory failure is a common medical emergency, and severe cases that do not receive timely and effective treatment can lead to a series of serious complications, including multiple organ dysfunction syndrome, and eventually death (Morrisey and Hogan, 2010; Pei et al., 2011). Alveolar epithelial cells are divided into type I and type II subsets. The latter are synthesized by original stem cells and have the potential to give rise to the former. Moreover, type II cells can synthesize and secrete alveolar surfactant, and are of great significance to the maintenance of alveolar stability (McGough and Cullen, 2011; Weinmaster and Fischer, 2011). Many detailed investigations have been conducted concerning type II alveolar cells in the pathogenesis of lung injury. The large and flat type I alveolar epithelial cells cover more than 95% of the alveolar surface, and are the cells most affected by lung damage of various kinds. Type I cells demonstrate a variety of biological functions besides their involvement in the formation of air-blood barriers. For instance, they are involved in water and ion transport and play a role in immune regulation (Lee et al., 2010; Geng et al., 2011). However, few studies have examined the function of type I alveolar cells in the pathogenesis of lung injury.

Mac-1 is a member of the b2 integrin family. As an important adhesion molecule, it is involved in body defense and the immune response, and is expressed in a wide range of tissues by certain white blood cells, including neutrophils, monocytes, eosinophils, and natural killer cells. It is also expressed to a lesser extent by T cells, macrophages, and B cells (Ho and Springer, 1982; Gao et al., 2011; Simmons et al., 2011). Signal transduction following the binding of ligands by Mac-1 leads to inflammatory cell activation, inducing the expression of genes encoding inflammatory cytokines such as IL-1beta and TNF-alpha (Fan and Edgington, 1993; Rezzonico et al., 2000). Mac-1 deficiency affects white blood cell migration and tissue infiltration, components of the inflammatory response. Inflammation is closely related to the occurrence and development of lung injury (Matsumoto et al., 2011; Heald-Sargent and Gallagher, 2012); however, the involvement of Mac-1 in these processes remains poorly understood. The aim of the present study was to investigate the role of Mac-1 in respiratory failure and identify the mechanism responsible using Mac-1 knockout mice.

MATERIAL AND METHODS

Materials

Experimental animals and breeding

Homozygous Mac-1 knockout mice (B6.129S6-*Itgam*^{tm1Myd}/J; Mac-1$^{-/-}$; stock No. 003991) were purchased from the Jackson Laboratory (Bar Harbor, ME, US). C57BL/6J mice (hereinafter referred to as C57), purchased from the Medical Laboratory Animal Center of Shandong Province (Jinan, China; production license SCXK 2008-0002), were used as control animals. Mice were raised in individual ventilated cages (VMC64S7; Suhang Experimental Animals Equipment Factory, Jinan, China) in a specific pathogen-free environment at 22°-28°C, with a relative humidity level of 50-70%, and automatic light control (12/12-h light/dark cycle). Feed was purchased from Shandong Animal Center, and underwent ^{60}Co irradiation sterilization. Drinking water consisted of sterilized municipal water.

Mice were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the People's Hospital of Laizhou City.

Main instruments and reagents

The following were used in our experiments: polymerase chain reaction (PCR) thermal cycler (Biometra, Göttingen, Germany); stereoscopic microscope (Motic, Xiamen, China); proteinase K, deoxynucleotides (Shanghai Univ-bio Company, Shanghai, China); PCR primers (Invitrogen Trading Shanghai Co., Ltd., Shanghai, China); *Taq* polymerase, Tris-saturated phenol, chloroform (Sigma Co., Ltd., St Louis, MO, USA); rabbit polyclonal anti-SP-C and anti-Aqp5 antibodies (Santa Cruz Biotechnology, Dallas, TX, USA); rabbit polyclonal anti-β-actin, anti-Snx5, and anti-T1α antibodies (Abcam, Cambridge, MA, USA).

Experimental methods

Detection of Mac-1 knockout

Identification of Mac-1 knockout mice was achieved with the following primers, which amplified a band of 700 bp: 5'-TAG GCT ATC CAG AGG TAG AC-3' and 5'-ATC GCC TTC TTG ACG AGT TCA-3'. The wild-type sequence was amplified using the following primer pair, resulting in a 325-bp product: 5'-TAG GCT ATC CAG AGG TAG AC-3' and 5'-CAT ACC TGT GAC CAG AAG AGC-3'. PCR cycling conditions were as follows: denaturation at 94°C for 3 min, then 35 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min.

Treatment and control groups

Male and female full siblings (at a ratio of 1:2) were mated, and pregnant mice were placed in a separate cage. C57 and Mac-1$^{-/-}$ mice, as control and treatment groups, respectively, were placed in different cages. The feeding regime was identical for both groups. All procedures were conducted according to ethics guidelines.

Lung float test

After euthanizing Mac-1$^{-/-}$ (N = 5) and C57 mice (N = 5), lung tissues were immediately removed and placed in a microcentrifuge tube containing 0.5 mL phosphate-buffered saline (PBS).

Hematoxylin and eosin (HE) staining

Lung tissues were fixed in formalin overnight, then dehydrated, cleared, and embedded as follows: 70% ethanol for 3 h, 80% ethanol for 3 h, 95% ethanol for 2 h, 100% ethanol for 1.5 h (twice), xylene for 0.5 h (twice), paraffin for 1 h, and paraffin for 2 h (at 60°C). Tissues were then sliced into 3-mm sections, and dewaxed by 10-min incubations in xylene, anhydrous ethanol, and 95, 90, 85, and 80% ethanol. Sections were stained with hematoxylin for 1 min, and washed in water until blue. Eosin staining was then carried out for 10 s, before sections were washed with water, dried, and mounted. The center of each tissue section was then examined under a microscope by three different pathology technicians at a magnification of x 20.

Immunofluorescence staining

After having been fixed, tissues were dehydrated using a sucrose gradient, frozen, and sliced into 6-mm sections. Antigen repair was performed at a high temperature for 5 min. Once the sections had cooled down, they were given three 5-min PBS washes, and blocked with 10% bovine serum albumin for 50 min. Primary antibody staining was conducted overnight, before rewarming sections for 40 min, subjecting them to three 5-min PBS washes, and exposing them to the fluorescent secondary antibody. The primary antibody was rabbit anti-mouse proSP-C (diluted 1:100), and the secondary antibody was anti-rabbit immunoglobulin G (488 nm; 1:100; Santa Cruz Biotechnology). Sections were dried and mounted, and the positive rate was calculated using ImageJ software (Schneider et al., 2012) as the ratio of the number of positive cells to the area of the selected field of view.

Western blotting

Tissue proteins were extracted using a kit (T-PER™ Tissue Protein Extraction Reagent, ThermoFisher Scientific), and a bicinchoninic acid (BCA) assay was used for quantitative analysis (Pierce™ BCA Protein Assay Kit, ThermoFisher Scientific). BCA assay working solution was prepared by mixing reagents A and B at a ratio of 50:1. Cell lysate supernatant (2 μL) was mixed with 18 μL PBS and 200 μL working solution. All protein samples were adjusted to the same concentration before being mixed with 1X bromophenol blue, which accounted for 1/5 of the total sample volume. The samples were then transferred to a polyacrylamide gel, along with 6 μL protein marker. Proteins were electrophoresed at 80 V on the stacking gel, then 120 V on the resolving gel. Electrophoresis was terminated when the target protein reached 1 cm from the edge of the gel. Proteins were transferred at a constant current of 250 mA for 90 min to a polyvinylidene fluoride membrane, which had been previously immersed in methyl for 5 min. The membrane was then removed from the transfer cell, lightly rinsed with Tris-buffered saline-Tween 20 (TBST), and rocked slowly in blocking solution (5% skim milk) for 1 h. After further light rinsing with TBST, the membrane was incubated with rabbit polyclonal anti-SP-C (1:100), anti-Aqp5 (1:100), anti-Snx5 (1:100),

anti-T1α (1:200), or anti-β-actin (1:200) primary antibody overnight. After being warmed at room temperature for 40 min, the membrane was given three 5-min washes with TBST. The horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000), chosen according to the primary antibody, was then applied, and the membrane rocked gently at room temperature for 1 h. The membrane was subsequently washed with TBST three times, each wash lasting 5 min, and developed using enhanced chemiluminescence reagents.

Statistical analysis

Western blotting results were processed by ImageJ software and are reported as means ± standard deviations of at least three experiments. SPSS 11.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The *t*-test was employed, with $P < 0.05$ indicating a statistically significant difference.

RESULTS

Survival rates of newborn C57 and Mac-1$^{-/-}$ mice

The survival rate of newborn C57 mice was 100% 10 h after birth (Figure 1A), whereas that of newborn Mac-1$^{-/-}$ mice was 60% 1 h after birth, although this latter figure decreased as pup age increased. By 70 h after birth, Mac-1$^{-/-}$ newborn survival was less than 40%. The difference in survival rate between the two groups was statistically significant ($P < 0.05$). Compared with newborn C57 mice, the bodies of Mac-1$^{-/-}$ pups showed obvious cyanosis. The images in Figure 1B show mice 24h after birth.

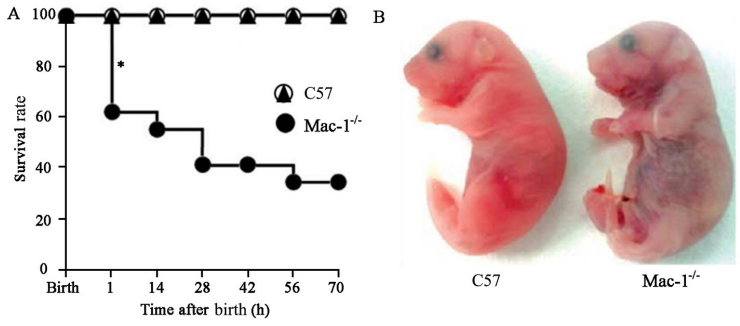

Figure 1. Survival rates and cyanosis of newborn C57 and Mac-1$^{-/-}$ mice. **A.** Survival rates of newborn C57 and Mac-1$^{-/-}$ mice; *$P < 0.05$; **B.** Obvious cyanosis in the body of a newborn Mac-1$^{-/-}$ mouse, compared with a C57 pup.

Alveolar thickening in Mac-1$^{-/-}$ mice

Lung tissue taken from C57 mice floated when placed in PBS. Concerning Mac-1$^{-/-}$ mice, some lung tissue floated, but most sank to the bottom of the tube due to increased density caused by insufficient lung expansion (Figure 2A). These observations indicated that Mac-1$^{-/-}$ mice may suffer respiratory failure at birth. HE staining of lung tissue showed thickening of alveoli in Mac-1$^{-/-}$ mice, with alveolar cell monolayers appearing double- or even multi-layered. Lung tissue was removed from mice 24 h after birth (Figure 2B).

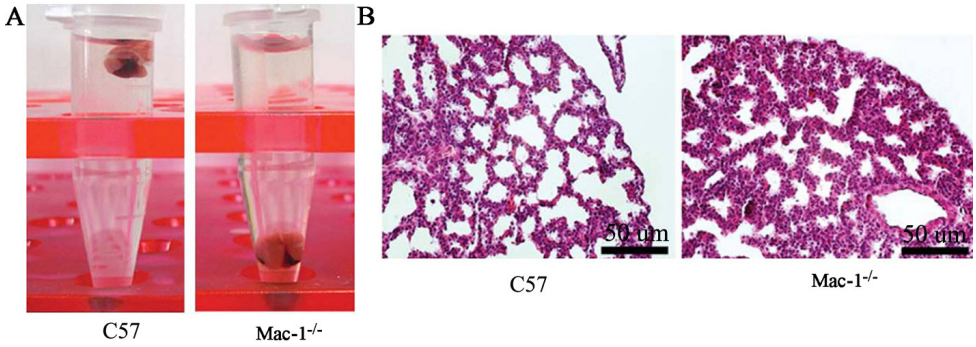

Figure 2. Alveolar thickening in Mac-1$^{-/-}$ mice. **A.** Lung float test. In contrast to C57 mice, Mac-1$^{-/-}$ mice may suffer respiratory failure at birth. **B.** Alveoli of Mac-1$^{-/-}$ mice were thickened compared to those of C57 mice.

Structure and function of type II alveolar cells were relatively unaltered in Mac-1$^{-/-}$ mice

To investigate the mechanism causing respiratory failure in newborn Mac-1$^{-/-}$ mice, proSP-C, a protein specific to type II alveolar epithelial cells, was detected by immunofluorescence. Figure 3A and B depict proSP-C expression in C57 and Mac-1$^{-/-}$ mice, respectively. No significant difference was noted in this respect. In addition, western blotting revealed that expression of the secreted protein SP-C did not significantly differ between C57 and Mac-1$^{-/-}$ mice (Figure 3C). Therefore, the structure and function of the alveolar surface were comparable in these two groups, which did not significantly differ in terms of alveolar surfactant protein expression.

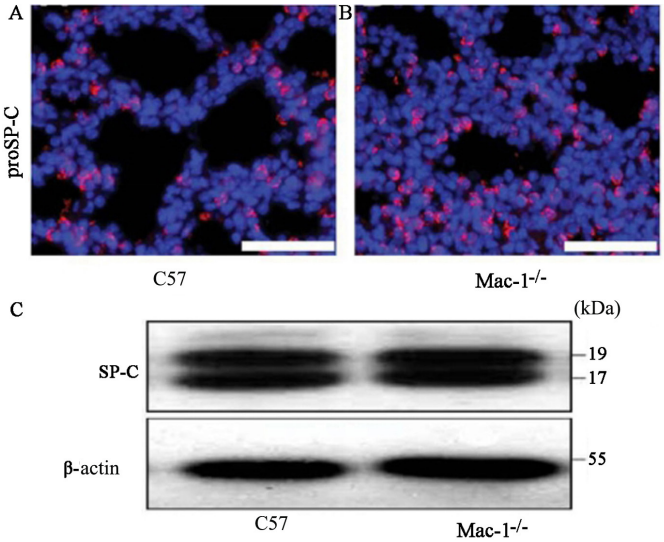

Figure 3. Expression of alveolar surfactant proteins specific to type II alveolar epithelial cells did not significantly differ between C57 and Mac-1$^{-/-}$ mice. **A.** and **B.** Immunofluorescence assay of proSP-C expression. **C.** Western blotting of SP-C protein.

Expression of proteins specific to type I alveolar cells was diminished in Mac-1$^{-/-}$ mice

Levels of proteins specific to type I alveolar cells (T1α, Aqp5, and Snx5) were also measured. Western blotting revealed that expression of these three proteins in Mac-1$^{-/-}$ mice was significantly lower than that in C57 mice ($P < 0.001$, Figure 4), indicating that the occurrence of respiratory failure may be related to a decrease of the numbers of type I alveolar epithelial cells.

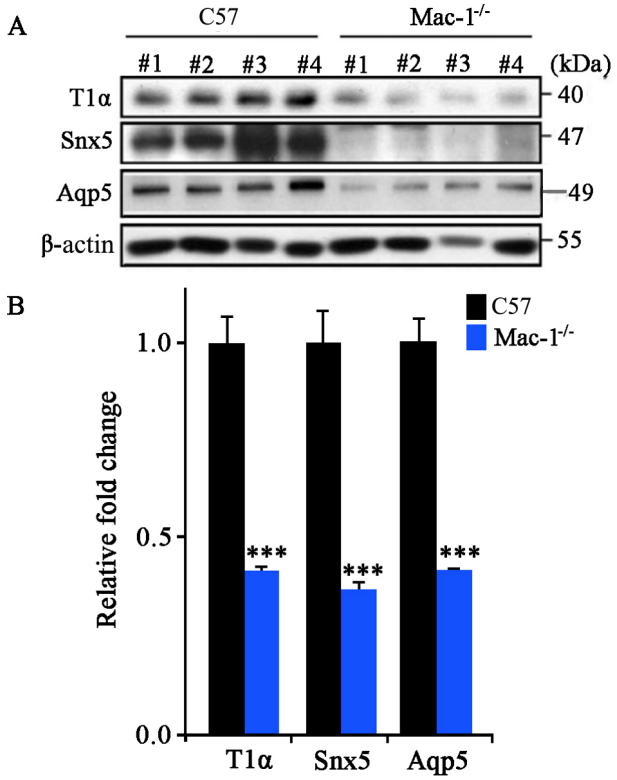

Figure 4. Expression of T1α, Aqp5, and Snx5 proteins, specific to type I alveolar cells, was significantly reduced in Mac-1$^{-/-}$ mice. **A.** Western blotting. **B.** Quantitative analysis. ***$P < 0.001$.

DISCUSSION

Mac-1 is a member of the b2 integrin family. As an important adhesion molecule, it is involved in physical defense against pathogens and the immune response, and is a significant participant in inflammation, a key factor in lung injury (Ye et al., 2007). It has been reported that Mac-1 plays a role in the occurrence of lung disease (Qian et al., 2013); however, studies of this protein *in vivo* are scarce. The current work investigated the effect of Mac-1 on respiratory failure. Compared with C57 mice, the neonatal mortality of Mac-1$^{-/-}$ mice was significantly increased, and the bodies of dead Mac-1$^{-/-}$ pups showed obvious cyanosis, indicating that respiratory failure may have occurred before death. In float tests, the lung tissues of Mac-1$^{-/-}$ mice sank, indicating excessive pulmonary hyperplasia leading to an increase in tissue

density. It was evident from pathological examination that the cause of death was very likely to be respiratory failure. Some studies have suggested that respiratory failure is caused by incomplete differentiation of type I or II alveolar epithelial cells; therefore, we examined the presence of these cells in affected lung tissue.

To explore the mechanism responsible for the lung injury observed, specific markers of each alveolar epithelial cell type were measured. Based on this, we determined that Mac-1 deficiency caused poor differentiation of type I alveolar epithelial cells, the number of which was consequently reduced, eventually leading to respiratory failure. Other studies have shown that Mac-1 plays an important function in the maturation and differentiation of these cells, and our study sheds light on the mechanism underlying this relationship (Pyrc et al., 2010; Wang et al., 2011; Weinheimer et al., 2012; Dijkman et al., 2013).

Type I alveolar epithelial cells cover more than 95% of the alveolar surface, and are vulnerable to various injuries. In addition to participating in the formation of the air-blood barrier, they also transport water and ions, and exhibit certain immune regulatory functions (Mossel et al., 2008; Yu et al., 2011). These cells are distinct from type II alveolar cells, the involvement of which in lung injury has been comprehensively studied (Jamaluddin et al., 2009). Our results imply that Mac-1 does not directly affect type II alveolar epithelial cells, as the expression and localization of SP-C were unaffected by Mac-1 deletion. However, the main limitation of the current study is that we cannot exclude the possibility that Mac-1 affects these cells in a non-autonomous manner. In the future, we plan to investigate the role of Mac-1 in alveolar cells using a conditional knockout model specific to each cell type. In summary, loss of Mac-1 affects the differentiation of type I alveolar cells, resulting in compromised lung structure and function, eventually leading to respiratory failure. The mechanism responsible needs to be further studied.

Conflicts of interest

The authors declare no conflict of interest.

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