Haptoglobin reduces lung injury associated with exposure to blood

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Departments of 1Cellular and Structural Biology and 2Medicine, University of Texas Health Science Center, San Antonio, Texas 78229; 3Department of Biological Sciences, University of South Carolina, Columbia, South Carolina 29208; and 4National Health and Environmental Effects Research Laboratory, Environmental Protection Agency, Research Triangle Park, North Carolina 27711

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Yang, Funmei, David J. Haile, Franklin G. Berger, Damon C. Herbert, Emily Van Beveren, and Andrew J. Ghio. Haptoglobin reduces lung injury associated with exposure to blood. Am J Physiol Lung Cell Mol Physiol 284: L402–L409, 2003. First published October 18, 2002; 10.1152/ajplung.00115.2002.—The biological functions of the acute-phase protein haptoglobin (Hp) may be related to its ability to bind hemoglobin (Hb) or to modulate immune response. Hp is expressed at a high level in lung cells, yet its protective role(s) in the lung is not known. With the use of transgenic mice overexpressing Hp in alveolar macrophages, we demonstrated that Hp diminished Hb-induced lung injury when the lung was exposed to whole blood. In transgenic mouse lungs, Hb was more efficiently removed, and the induction of stress-responsive heme oxygenase-1 gene was significantly lower when compared with wild-type mice. At 24 h after blood treatment, the ferritin level that serves as an index for intracellular iron content was also lower in alveolar macrophages in transgenic mice than in wild-type mice. We propose that an Hp-mediated Hb catabolism process exists in alveolar macrophages. This process is likely coupled to an iron mobilization pathway and may be an efficient mechanism to reduce oxidative damage associated with hemolysis.

extravasation of erythrocytes; lung diseases; hemorrhage; metal transporter protein-1; heme oxygenase-1

EXTRAVASATION OF ERYTHROCYTES into the lower respiratory tract occurs in numerous lung injuries, including chronic bronchitis, bronchiectasis, cystic fibrosis, lung cancer, pulmonary embolism, pneumonia, tuberculosis, traumatic injury, and diffuse alveolar hemorrhage. After hemolysis, the catalytic iron present in hemoglobin (Hb) can induce the formation of reactive oxygen species and lead to oxidative damage in lung tissues. Both free Hb and erythrocytes have been shown to induce lung injury in experimental animal models (3, 14).

Haptoglobin (Hp), an α2-acid glycoprotein, has long been known as the major Hb-binding protein associated with Hb catabolism. It is produced mainly by the liver and secreted into the circulation. As a major positive acute-phase reactant, Hp increases in plasma during inflammation, infection, trauma, tissue damage, and malignancy. In patients with severe hemolysis, on the other hand, Hp decreases in plasma to a nondetectable level with the formation of Hp-Hb complexes. The Hb scavenger receptor CD163 has recently been identified to mediate endocytosis of Hp-Hb complexes by monocytes/macrophages (17).

In our previous studies, we identified the lung as a major site of extrahepatic synthesis of Hp. Hp gene expression can be detected in airway epithelial cells in mice and baboons and in alveolar macrophages and eosinophils in humans (22, 23). As in the liver, expression of Hp gene in the lung increases severalfold upon exposure to inflammatory stimuli and during some diseased states, suggesting protective roles of Hp in lung tissues. To understand the functions of Hp in the lung, we produced transgenic mice that express the human Hp gene at high levels in several tissues, including lung. The cell type-specific expression of human Hp gene is maintained in the transgenic mice. Although the endogenous mouse Hp gene is expressed in airway epithelial cells, the human Hp transgene is expressed in alveolar macrophages in these mice (23). In this paper, we have used these transgenic mice to investigate the protective effects of Hp against blood-induced lung injury. We also measured the expression of other proteins likely involved in the Hp-mediated Hb catabolism and iron mobilization in alveolar macrophages.

MATERIALS AND METHODS

Production of transgenic mice. Transgenic mice carrying a 9-kb human Hp genomic DNA, which contains the entire Hp2 gene plus 1 kb of the 5′- and 1.5 kb of the 3′-flanking regions, were produced in a background of CB6F1, as described previously (23). The transgene was introduced in an inbred background C57BL/6.

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Tracheal instillation of blood. Blood was collected from normal healthy adult mice by intracardiac puncture into heparinized syringes after the animals were anesthetized with halothane. Animals were then euthanized by further exsanguination through the abdominal aorta. To induce lung injury, transgenic and wild-type mice (3–4 mo old) were anesthetized with Metofane (Pitman-Moore, Mundelein, IL) and intratracheally instilled with 50 μl of blood. The dose was determined according to the previous study in rats (14). Control animals from each group received the same volume of normal (0.9%) saline. Previous investigation using this method of exposure has demonstrated a uniform distribution of instilled material in the lung. After the instillation, all mice were allowed to recover from the anesthesia and return to animal care facilities.

Collection of lavage fluids and lavage cells from mice. Mice were anesthetized, euthanized, and tracheally lavaged with 1.0 ml of normal saline. The lavage procedure was repeated twice. The combined lavage fluid was centrifuged at 600 g for 10 min at 4°C to separate cells from supernatant. The cells were resuspended in HBSS, and, with a cytospin, 2 x 10^5 cells were then pelleted onto slides. With the use of modified Wright’s stain (Diff-Quick stain; American Scientific Products, McGaw Park, IL), neutrophils were enumerated, and values were expressed as the percentage of total cells recovered.

Biochemical analysis. Lavage protein levels were determined using the Pierce Coomassie Plus protein assay reagent (Pierce, Rockford, IL). This assay was modified for use in the Cobas Fara II centrifugal spectrophotometer (Hoffman-LaRoche). Bovine serum albumin served as the standard. The lactate dehydrogenase concentration in the lavage fluid was measured using a commercially prepared kit (Sigma Diagnostics) modified for automated measurement. Concentrations of macrophage inflammatory protein-2 (MIP-2) in the lavage fluids were measured by the method of ELISA using Quantikine kits purchased from R&D Systems (Minneapolis, MN). Total Hb in bronchoalveolar lavage supernatant was quantified by the cyanomethemoglobin method (Sigma Chemical, St. Louis, MO).

In situ hybridization and immunohistochemistry. Surgical specimens of human lung and normal mouse lung tissues were obtained, quick-frozen in Tissue-Tek optimum cutting temperature compound, and stored at −80°C until used for the preparation of cryosections. Human alveolar macrophages were obtained from volunteers (13) and pelleted onto coated glass slides. In situ hybridization of lung sections was conducted as previously described (23) using antisense mouse metal transport protein-1 (MTP-1) cRNA probes. The MTP-1 cDNA template used for the preparation of riboprobe was described in a previous publication (1). Immunohistochemistry for CD163 was performed using the Vectastain Elite ABC peroxidase kit and the VIP peroxidase substrate kit (Vector Laboratories, Burlingame, CA). Anti-CD163 antibody was a gift from Dr. Sören K. Moestrup (University of Aarhus, Aarhus, Denmark). Immunohistochemical study for heme oxygenase-1 (HO-1) and ferritin was performed as described previously (7, 14).

Statistics. Data are expressed as means ± SE. Differences between multiple groups were analyzed using analysis of variance. Two-tailed tests of significance were employed. Significance was assumed at P < 0.05.
RESULTS

The human Hp transgene is expressed in several tissues, including liver, lung, adrenal glands, uterus, ovary, and submaxillary glands. A high level of Hp2 protein, which can be distinguished from the mouse Hp protein, is detected in the sera of transgenic mice (23). In most of the tissues in which Hp is produced, the human Hp2 transgene and the mouse endogenous Hp gene are expressed in the same type of cells. In the lung, however, the human Hp transgene is expressed in alveolar macrophages as it is in the human lung, whereas the endogenous mouse Hp gene is expressed in airway epithelial cells. The level of Hp protein in the lavage fluid was barely detectable in the wild-type animals because of extensive dilution of the lung fluids. However, an average of 15 μg/ml of Hp was found in the lavage fluids of transgenic mice.

To determine whether an elevated level of Hp in the lung can attenuate lung injury associated with exposure to the blood, mice were intratracheally instilled with a small volume of whole blood (50 μl) derived from normal healthy mice. Twenty-four hours after treatment, lung tissues and lavage fluids were collected and analyzed. The transgenic mice consistently displayed a higher degree of tolerance to blood-induced lung injury compared with wild-type animals. As shown in Fig. 1, the commonly used indexes for lung tissue injury, i.e., levels of total protein and lactate dehydrogenase in the lavage fluids, were elevated in both transgenic and wild-type mice after the animals were exposed to blood. However, the change was significantly less in the transgenic mice than in the wild-type mice.

Additionally, a lower degree of lung inflammation was found in transgenic mice compared with wild-type mice after blood treatment. The level of the major inflammatory cytokine MIP-2 was lower in the transgenic mouse lungs than in the wild-type mouse lungs (Fig. 2). We also studied the profiles of lavage cells to determine whether there is a difference in the degree of inflammatory cell infiltration between wild-type and transgenic mice. After blood treatment, the percent of neutrophils in wild-type mice is twice as high as in transgenic mice (Table 1). The total cell number was also higher in wild-type than in transgenic mice. There was no significant difference in the numbers of total cells or neutrophils between wild-type and transgenic mice in response to saline treatment.

As predicted, the amount of Hb retained in the lung after blood treatment was much lower in transgenic mice than in wild-type mice (Fig. 3). To identify the lung cells that are involved in the clearance of Hb in the lung, we studied the expression of the Hb scavenging protein CD163 in the lung. This protein, a monocyte/macrophage cell surface receptor, was recently shown to bind Hp-Hb complexes. An immunohistochemical experiment was performed on human lung sections, freshly isolated human alveolar macrophages, and baboon lung sections using an antibody against human CD163 that cross-reacts with baboon, but not mouse, antigen. A strong immunostain for CD163 was localized in alveolar macrophages but not in any other lung cell (Fig. 4). These results indicated that alveolar macrophages are the major cells responsible for Hb catabolism in the lung via CD163-mediated endocytosis.

Because the safe sequestration of iron is crucial for lung defense, we studied the molecular mechanisms involved in iron recycling after hemolysis in the lung. After endocytosis, iron can be released from Hb catalyzed by heme oxygenase. The inducible form of heme oxygenase, HO-1, is known to be activated by...
its substrate heme and numerous stress stimuli (reviewed in Ref. 16). To investigate the response of the HO-1 gene in lung cells in vivo to blood exposure, we performed immunohistochemical analysis of HO-1 on lung specimens collected 4, 8, 16, and 24 h after intratracheal instillation of blood. HO-1 expression was increased in alveolar macrophages, endothelial cells, and alveolar epithelial cells, but not in airway epithelial cells, in both wild-type and transgenic mice at 4 h after blood exposure (Table 2). HO-1 levels peaked at 4–8 h after exposure and declined after that. At all times, the level of HO-1 was much higher in wild-type than in transgenic mice. This was particularly noticeable in alveolar macrophages (Table 2). By 24 h after blood treatment, the HO-1 expression in transgenic mouse lung had decreased to the same level as in the untreated wild-type or transgenic mouse lung. However, it remained high in the alveolar macrophages in wild-type mice (Table 2 and Fig. 5). There was also a more obvious increase in the number of alveolar macrophages in wild-type (Fig. 5B) than in transgenic mouse lungs after blood exposure. This is in agreement with our observations for the profiles of lavage cells in these lungs (see Table 1).

After Hb is catabolized in alveolar macrophages, free iron released from heme can be sequestered in ferritin or mobilized to extracellular space. Previous studies have shown that macrophages are capable of releasing iron in the form of ferritin and transferrin (4, 9, 20), especially during iron overload (20, 21). To investigate the molecular mechanism underlining this process in alveolar macrophages, we studied the expression of an iron export protein, MTP-1 (also known as ferroportin 1, Ireg1), that has recently been shown to be responsible for iron export in zebrafish yolk sac, *Xenopus* oocytes (11), and cultured

**Table 2. Levels of HO-1 in different lung cells after blood exposure**

<table>
<thead>
<tr>
<th>Hours Posttreatment</th>
<th>Lung Cell Type</th>
<th>Saline Treated*</th>
<th>Transgenic Blood Treated</th>
<th>Wild-type Blood Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Alveolar macrophages</td>
<td>++</td>
<td>++</td>
<td>+++</td>
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<tr>
<td></td>
<td>Endothelium</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
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<tr>
<td></td>
<td>Airway epithelium</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td></td>
<td>Alveolar epithelium</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>Alveolar macrophages</td>
<td>nd</td>
<td>+</td>
<td>+++</td>
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<tr>
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<td>Endothelium</td>
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<tr>
<td>8</td>
<td>Airway epithelium</td>
<td>nd</td>
<td>−</td>
<td>−</td>
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<td></td>
<td>Alveolar epithelium</td>
<td>+</td>
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<td>−</td>
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<td></td>
<td>Alveolar macrophages</td>
<td>nd</td>
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<td>+</td>
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<tr>
<td></td>
<td>Endothelium</td>
<td>nd</td>
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<td>+</td>
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<tr>
<td>16</td>
<td>Airway epithelium</td>
<td>nd</td>
<td>−</td>
<td>−</td>
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<td></td>
<td>Alveolar epithelium</td>
<td>+</td>
<td>+</td>
<td>++</td>
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<td></td>
<td>Alveolar macrophages</td>
<td>+</td>
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<tr>
<td></td>
<td>Alveolar macrophages</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Semiquantitative analysis of the heme oxygenase-1 (HO-1) levels was conducted by comparing the intensities of the immunostain for HO-1 in different mouse lung specimens after the immunohistochemical reaction without prior knowledge of the genotypes of the mice. *There is no difference between wild-type and transgenic mice after saline treatment. nd, = not done; −, not detectable; +, low level; ++, medium level; ++++, high level; +++++, very high level.
cells (1). We found that MTP-1 is expressed in alveolar macrophages and airway epithelial cells in human lungs (unpublished observations). By using the technique of in situ hybridization, we demonstrated that a high level of MTP-1 mRNA was present mainly in the alveolar macrophage in the mouse lung (Fig. 6). This result suggests MTP-1 plays a role in iron metabolism in alveolar macrophages after Hb catabolism.

To investigate the efficiency of iron mobilization in alveolar macrophages, we conducted immunohistochemical analysis of ferritin, which reflects the level of intracellular iron, in wild-type and transgenic mouse lungs 24 h after blood exposure. The results are summarized in Table 3. In the endothelium, airway epithelium, and alveolar epithelium, the ferritin level was not significantly different between transgenic and wild-type mice whether the animals were treated with saline or blood. In alveolar macrophages, the ferritin level was the same between transgenic and wild-type mice treated with saline. There was a marked increase in both transgenic and wild-type mice after blood exposure. However, the level of ferritin in alveolar macrophages was much greater in wild-type mice than in transgenic mice (Fig. 7), suggesting a higher efficiency of Hb catabolism and iron mobilization in transgenic mice than in wild-type mice.

DISCUSSION

In numerous acute and chronic lung injuries, frequently, red blood cells can be present in the lower respiratory tract. Extravasated erythrocytes can be removed by phagocytosis or destroyed by proteases, resulting in the release of Hb and reactive iron. These byproducts potentially participate in oxidant generation and contribute to injury (15, 24). Ghio et al. (14) has shown that intratracheal instillation of whole blood in the rat can induce a neutrophilic lung injury with a marked increase in both TNF-α and MIP-2. This injury is also associated with a disruption of iron equilibrium, which was made evident by quantifying iron and staining for Hb and ferritin. Nevertheless, the clearance processes for both erythrocytes and Hb in the lung are considered to be very efficient, and after a few days, little free Hb remains after exposure to whole blood (14).

As a major acute-phase protein, Hp has been shown to have several biological functions, including a role in Hb catabolism (10). In previous studies, we
have found that the airway epithelium is a major site of Hp gene expression in baboons and mice. In humans, the Hp gene is not expressed in normal healthy lung but is greatly activated in alveolar macrophages in diseased lung tissues. In mouse lungs, alveolar macrophages can also be induced to synthesize Hp during inflammation (unpublished observations). The roles of Hp in lung health and lung defense have not been elucidated. Results from this study indicate that a major pathway for the removal of Hb in the lung involves the formation of Hp-Hb complexes. Using a transgenic mouse system, we have shown that overexpression of Hp in the lung promotes Hb removal after intratracheal instillation of whole blood. This was accompanied by a lower degree of neutrophilic lung injury and inflammation in transgenic compared with wild-type mice. After blood exposure, the increase of both TNF-α (data not shown) and MIP-2 was significantly less in transgenic than in wild-type mice. We have conducted a similar study using wild-type mice and Hp knockout mice generated by Lim et al. (18). In this experiment, the wild-type mice were much more tolerant to blood-induced lung injury than the Hp knockout mice (unpublished observations), providing further support for the protective effects of Hp during lung injury.

The fate of the Hp-Hb complexes in the lung is not clear. In the circulation, the Hp-Hb complexes are delivered to the parenchymal cells of the liver where Hb is broken down to bilirubin. A mechanism, which could be responsible for the transport of high-molecular-weight Hp-Hb complexes from respiratory tract to circulation, is yet to be established. In this study, we have shown that CD163, which was recently identified as an Hp-Hb scavenging receptor (17), is present in alveolar macrophages but not other lung cells. CD163 has a high affinity for Hp-Hb complexes but does not bind to Hp or Hb. CD163-mediated endocytosis of Hp-Hb complexes by alveolar macrophages could be the major pathway responsible for the removal of Hb in the lung. Increased production of Hp by alveolar macrophages and airway epithelial cells at the site of inflammation could contribute significantly to the clearance of Hb and thus protect the lower respiratory tract against Hb-mediated oxidative damage. In humans, there are two different genetic alleles for Hp, the Hp1 and Hp2. Interestingly, complexes of Hb and multimeric Hp (Hp 2-2 phenotype) exhibit a 10-fold higher functional affinity for CD163 than do complexes of Hb and dimeric Hp (Hp 1-1 phenotype) (17). It is not known whether individuals with the Hp1-1 type are more susceptible to Hb-induced lung injury and inflammation.

Table 3. Levels of ferritin in different lung cells 24 h after blood exposure

<table>
<thead>
<tr>
<th>Lung Cell Type</th>
<th>Transgenic Saline Treated</th>
<th>Transgenic Blood Treated</th>
<th>Wild-type Saline Treated</th>
<th>Wild-type Blood Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alveolar macrophage</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>Endothelium</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Airway epithelium*</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Alveolar epithelium</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Semiquantitative analysis of ferritin levels was conducted as described in Table 2. *Level of ferritin in the airway epithelium was very high and was difficult to compare among different groups of animals.

Fig. 7. Immunohistochemical analysis of ferritin in mouse lungs after blood exposure. Immunostaining of ferritin was performed on paraffin sections derived from mouse lungs exposed to blood for 24 h. There is diffuse uptake of the antibody for ferritin by all cell types in the lower respiratory tract. However, large, intra-alveolar macrophages (arrows) that stain strongly for the antibody are evident in the wild-type animals (A) but not in the transgenics (B). Original magnification was ×400.
In alveolar macrophages, Hb catabolism involves the key enzyme heme oxygenase. Expression of the inducible form of this protein, HO-1, is known to be elevated in the lung during injury, inflammation, hyperoxia, and other stressful conditions. In this study, we have shown that HO-1 was induced at the early time points in alveolar macrophages in both transgenic and wild-type mouse lungs but diminished at later time points in transgenic mice. Because HO-1 gene expression is induced by its substrate heme in Hb, these results support our hypothesis that Hb is removed and catabolized more efficiently in transgenic mice than in wild-type mice. It is likely that the elevated level of Hp in the transgenic mouse lung, especially in the vicinity of alveolar macrophages, promotes a speedy clearance of Hb from the alveolar space. Shortly after blood exposure, this protective effect allowed the transgenic mice to activate only the needed level of HO-1, which was much lower than that required in the wild-type mice. The structure of the heme molecule and the mechanism involved in the induction of HO-1 by heme are not clear. Alternative explanations for a lower degree of HO-1 induction in transgenic mice could include the compartmentalization of Hp-Hb complexes in alveolar macrophages in these mice. Disregarding the mechanism involved, the level of HO-1 induction appeared to serve as an excellent index for Hb-associated oxidative stress. The anti-inflammatory effect of Hp in transgenic mouse lungs was also evident by a lower level of inflammatory cytokines, such as MIP-2 and TNF-α.

In alveolar macrophages, iron released from Hb may either enhance the production of ferritin and intracellular iron storage or be excreted as a metal bound to ferritin and/or transferrin. Iron efflux in alveolar macrophages has been shown to increase during iron overload (21) and may reflect a cellular protective mechanism against cell death as a result of oxidative stress (12). The molecular mechanism(s) involved in intracellular iron trafficking and iron transport across the cell membrane is not completely understood. Our finding that the iron exporter MTP-1 is expressed at a high level in alveolar macrophages suggests that MTP-1 may be another key protein that participates in an iron detoxification (recycling) process in alveolar macrophages. Our proposed model for this process is illustrated in Fig. 8. In this model, Hb released during hemolysis is first captured by Hp synthesized locally at a high level by alveolar macrophages (in human) or airway epithelial cells (in mice and baboon). The Hp-Hb complexes are taken up by alveolar macrophages through CD163-mediated endocytosis. After Hb is degraded, iron can be sequestered in the cells or released in less reactive forms as transferrin and/or ferritin. Some of these iron-containing proteins may be captured within mucus and may subsequently be expelled from the lung. A high concentration of iron-containing protein has been found in sputum from patients with lung diseases (2). Interestingly, all the key proteins participating in this Hb catabolism and iron detoxification process are elevated during inflammation (5, 6, 8, 19, unpublished observations), further suggesting their importance in lung defense.

Using transgenic mice overexpressing Hp, we have shown that an increased level of Hp can promote Hb clearance and attenuate blood-induced lung injury and inflammation. Our results suggest that Hb catabolism in alveolar macrophages is linked to iron mobilization to ensure the safe sequestration of iron derived from Hb. Alveolar macrophages appear to produce all the key proteins and enzymes needed in this catabolic pathway. Hb and other proteins involved in iron mobilization may play important roles in lung defense.

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