Mice lacking uteroglobin are highly susceptible to developing pulmonary fibrosis

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Abstract Uteroglobin (UG) is an anti-inflammatory protein secreted by the airway epithelia of all mammals. UG-knockout (UG-KO) mice sporadically develop focal pulmonary fibrosis (PF), a group of complex interstitial disorders of the lung that has high mortality and morbidity; however, the molecular mechanism(s) remains unclear. We report here that UG-KO mice are extraordinarily sensitive to bleomycin, an anti-cancer agent known to induce PF and readily develop PF when treated with an extremely low dose of bleomycin that has virtually no effect on the wild type littermates. We further demonstrate that UG prevents PF suppressing bleomycin-induced production of pro-inflammatory T-helper 2 cytokines and TGF-β, which are also pro-fibrotic. Our results define a critical role of UG in preventing the development of PF and provide the proof of principle that recombinant UG may have therapeutic potential.

1. Introduction

Uteroglobin (UG) or Clara cell 10 kDa (CC10) protein, the founding member of the newly recognized *Secretoglobin* superfamily [1], is a steroid-inducible, multifunctional protein secreted by the airway epithelia of all mammals, including humans (for review see Ref. [2]). Non-steroidal hormones, such as prolactin, has been reported to augment the effects of steroid hormones [3]. In numerous experiments, it has been demonstrated that this protein has potent anti-inflammatory and anti-chemotactic properties. We previously noted that UG-knockout (UG-KO) mice [4], generated by targeted disruption of the UG gene in embryonic stem (ES) cells, with age sporadically develop focal pulmonary fibrosis (PF). PF is a complex disease with high mortality and morbidity [5,6]. However, the molecular mechanism(s) of PF in UG-KO mice is not clearly understood. As stated above, UG is secreted by the mucosal epithelial cells that line the airways of all mammals and these cells have emerged as the key site of initial injury that precedes the development of PF [7]. Thus, UG may play a protective role against the development of PF.

Bleomycin, an anti-cancer drug, is limited in its clinical use in humans because it causes widespread alveolar epithelial cell injury and mediates the development of PF [7]. The later property of this drug has been widely used to generate animal models of PF [6]. Due to the lack of an effective treatment, PF is a disease with high mortality [5,6]. Understanding the molecular mechanism(s) of pathogenesis of this disease may facilitate the development of rational therapeutic approaches. The generation of UG-KO mice, which sporadically develop focal PF, provided an opportunity to understand the molecular mechanism(s) by which UG may play a protective role against PF.

The pathologic features of PF include progressive alveolar inflammation, deposition of extracellular matrix proteins such as collagen and proliferation of fibroblasts. Although the molecular mechanisms of this group of diseases remain unclear, it appears that a breakdown of homeostatic mechanisms to prevent pulmonary inflammation plays critical pathogenic roles. However, it has been reported that injury to the mucosal epithelial layer that lines the mammalian airways is one of the primary events that leads to PF [7]. We previously reported that UG-KO mice manifest exaggerated pulmonary inflammation when challenged with allergens such as ovalbumin (OVA; Ref. [8,9]). Most importantly, recombinant UG treatment abrogates allergen-induced inflammation in the lungs of these mice. Since airway inflammation precedes PF and since UG is a potent anti-inflammatory protein present in the airways, we sought to determine whether UG may have protective roles against PF.

Here we report that mice lacking UG manifest extreme sensitivity to bleomycin and are highly predisposed to developing PF even when these mice are treated with the lowest dose of this drug that does not appear to affect the WT littermates. The results of our study suggest that UG plays a pivotal role in maintaining homeostasis in the airways by preventing T_H2–mediated inflammation and the development of PF.

2. Materials and methods

2.1. Animals

UG-knockout (UG-KO) mice were generated as described previously [4]. Animals were housed and maintained under germ-free condition, and all experiments were performed according to a protocol approved by the NICHD Animal Care and Use Committee.

2.2. Bleomycin-induced pulmonary fibrosis

In all experiments sex-matched UG-KO and WT littermates were used. Four to six weeks old mice were anesthetized with ketamine and varying doses (0.075 U, 0.0375 U or 0.0075 U/mouse) of bleomycin

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(Calbiochem, La Jolla, CA) in 50 μl sterile saline were intratracheally administered. Control mice were treated with saline alone in the same manner.

2.3. Histological analysis

The animals were killed 28 days after bleomycin or saline treatment, the lungs were fixed by intratracheal administration of 3.7% neutral buffered formalin. After fixation, the tissues were embedded in paraffin and sectioned. Lung sections were stained with hematoxilin and eosin or Masson's trichrome stain (American Histolab, Inc., Gaithersburg, MD). The stained tittue sections were examined using an Axiophot Microscope (Carl Zeiss Microimaging, Inc.) and digital photomicrographs were recorded.

2.4. Determination of hydroxylproline content in the lungs

The hydroxyproline content of mouse lung was determined colorimetrically as described previously [10], with minor modifications. Briefly, the lungs were excised and weighted, trimmed free of surrounding conducting airways and hydrolyzed in 2 ml of 6 N HCl by autoclaving at 120 °C for 20 min. The resultant hydrolysate was neutralized with 2 ml of 6 N NaOH. Five microlitre of each sample was mixed with 5 μl of citrate–acetate buffer (5% citric acid, 1.2% glacial acetic acid, 7.25% sodium acetate, and 3.4% sodium hydroxide). One hundred microlitres of chloramine-T solution (1.4% chloramines-T, 10% *N*-propanol, and 80% citrate–acetate buffer) was added, and the mixture was incubated for 20 min at room temperature. Ehrlich's solution was added and the samples were incubated at 65 °C for 18 min. The colored product was measured at 560 nm and the amount of hydroxyproline was calculated from a standard curve constructed using hydroxyl-L-proline (Sigma) at concentrations of 0.25–40 μg/ml. The data were expressed as micrograms of hydroxyproline per gram of fresh lung tissue.

2.5. Analysis of bronchoalveolar lavage fluid (BALF)

Seven days after bleomycin or saline treatment, bronchoalveolar lavage (BAL) was performed as previously described [8]. For differential counts, cells were spun onto glass slides by cytocentrifugation and were stained with HEMA3 (Fisher Scientific Co., Kalamazoo, MI). A total of 500 cells per sample were analyzed.

2.6. RNA isolation and quantitative real-time PCR

Total RNA was isolated using TriZol (Invitrogen) following the manufacturer's protocol and further purified by QIAGEN RNeasy Mini Kit and treated with DNAse (DNAse I, 30 U/μg total RNA) (QIAGEN). Total RNA (2 μg) was reverse transcribed using Super-Script III First-Strand Synthesis System (Invitrogen). Real time, quantitative RT-PCR analysis was performed by using the SYBR Green PCR Master Mix and an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The cDNA equivalent of 10 ng total RNA was used as the template. The PCR primers used are: IL-4: forward primer: GGA GAT GGA TGT GCC AAA CG and reverse primer: CGA GCT CAC TCT CTG TGG TGT T; IL-5: forward primer: TTG ACA AGC AAT GAG ACG ATG AG and reverse primer: TCC AAT GCA TAG CTG GTG ATT T; IL-13: forward primer: CGC AAG GCC CCC ACT AC and reverse primer: AAA GTG GGC TAC TTC GAT TTT GG; TGF-beta: forward primer: CGG AGA GCC CTG GAT ACC A and reverse primer: TGC CGC ACA CAG CAG TTC; pro-collagen Iα1: forward primer: GCT TCA CCT ACA GCA CCC TTG T and reverse primer: GAT GAC TGT CTT GCC CCA AGT T and β-actin: forward primer: ACG GCC AGG TCA TCA CTA TTG and reverse primer: TGG AAA AGA GCC TCA GGG C. The data, normalized to β-actin, were analyzed using ABI Prism Software version 1.01 (Applied Biosystems) and presented as fold increase compared with normal controls. Each experiment was performed in triplicate for each group.

2.7. Western blot analysis

Total protein (15 μg) from each serum sample was resolved by SDS–polyacrylamide gel electrophoresis, eletrotransferred to PVDF membrane (Immobilon P, Millipore Corporation) and immunodetected. The primary antibodies used are: anti-mouse IL-13 (1:1000; R&D) and anti-bovine serum albumin (BSA) (1:2000; upstate). The second antibodies used are: donkey anti-goat IgG and goat anti-rabbit IgG (Santa Cruz Biotechnology).

2.8. Statistical analysis

Comparisons of data among each experimental group were evaluated with Student's *t* test of variance. Data are expressed as the means (*n*=3) ± S.D. Data were considered significant at $P < 0.05$.

3. Results and discussion

3.1. High mortality of UG-KO mice treated with bleomycin

To delineate whether the UG-KO mice are more susceptible than their WT littermates to develop bleomycin-induced PF, we first studied the dose–response of bleomycin on UG-KO mice and their wild type (WT) littermates by intratracheal administration of varying doses of this drug. For each dose, we treated 6–13 mice that are 4–6 weeks old. At this age, the UG-KO mice do not develop any sign of pulmonary fibrosis. We used a dose of bleomycin (0.075 U/mouse) that is commonly used to generate pulmonary fibrosis in WT mice. We found that 28 days after intratracheal instillation of bleomycin the mortality in UG-KO mice reached 50%, compared with only 7% in WT mice (Fig. 1). Thus, we lowered the bleomycin dose to 0.0375 U/ mouse and even then, 25% of the UG-KO mice died whereas all of their WT littermates remained viable. We then used even a smaller dose of bleomycin (0.0075 U/mouse) and at this dose, none of these mice died throughout 28 days duration of the experimental period (Fig. 1). Therefore, we used this lowest dose for all of our experiments. Taken together, these results show that lack of UG in these mice confers extreme sensitivity to bleomycin and causes high mortality.

3.2. UG-KO mice are highly susceptible to developing bleomycin-induced PF

The results described above prompted us to determine whether the lowest dose of bleomycin (0.0075 U/mouse) even though it does cause death, induces PF in UG-KO mice. Accordingly, this dose of bleomycin was intratracheally administered in the UG-KO mice and their WT littermates. The mice were killed 28 days after bleomycin treatment and the lungs were analyzed histologically for the presence of PF. The results show that compared with WT littermates (Fig. 2A) UG-KO mice develop extensive PF at this lowest dose of bleomycin (Fig. 2B). To confirm the development of PF, we stained the lung sections with Mason's trichrome stain, which readily identifies the presence of collagen, characteristically found in PF, because of its blue color. The results show that compared with the lungs of WT littermates (Fig. 2C) those of the UG-KO mice manifest extensive deposition of collagen (Fig. 2D) confirming the presence of fibrosis.

3.3. Low dose bleomycin stimulates the expression of additional PF markers in the lungs of UG-KO mice

To determine the level of fibrosis, we further analyzed additional molecular markers, which are associated with PF. First, we measured the lung hydroxyproline content by colorimetric method and analyzed the levels of collagen by quantitating pro-collagen $1\alpha_1$-mRNA expression by real time PCR using total RNA from the lungs. The results show that compared with bleomycin-treated WT mice, the levels of both hydroxyproline (Fig. 2E), and pro-collagen-$1\alpha_1$-mRNA (Fig. 2F) expressions are significantly elevated in the lungs of bleomycin-treated UG-KO mice. Since transforming growth factor-β (TGF-β) is a pro-fibrotic cytokine that stimulates the migration
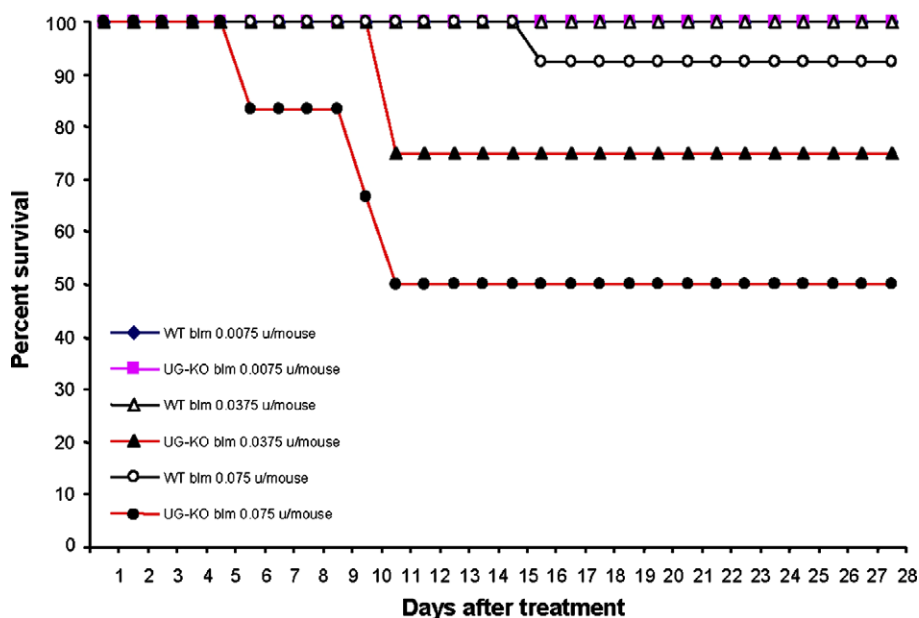
Fig. 1. Uteroglobin-knockout mice are highly susceptible to developing bleomycin-induced pulmonary fibrosis. Survival of UG-KO and WT mice treated with varying doses of bleomycin. UG-KO or WT mice ($n = 6–13$) were treated with indicated doses of bleomycin and percent survival was determined for each group throughout 28 days of experimental period. The data are representative of at least two separate experiments. Note the strikingly lower percentage of survival of the UG-KO mice, which were treated with bleomycin dose 0.075 U/mouse and 0.0375 U/mouse, respectively. Also note that 0.0075 U of bleomycin/mouse was tolerated by both UG-KO and WT littermates. Bleomycin-induced pulmonary fibrosis in WT and UG-KO mice.

as well as proliferation of fibroblasts and are characteristically associated with PF[11], we also analyzed the level of TGF β-mRNA expression by quantitative real time PCR. Our results show that TGF β-mRNA expression levels are significantly elevated ($P < 0.005$) in the lungs of UG-KO mice treated with bleomycin compared with those of their bleomycin-treated WT littermates (Fig. 2G). These results confirmed that all three critical molecular indicators of PF are markedly elevated in the lungs of UG-KO mice treated with the lowest dose of bleomycin that does not cause PF in WT littermates. These data strongly indicate that lack of UG predisposes mice to developing PF even at an extremely low dose of bleomycin suggesting a protective role of UG in the respiratory system.

3.4. Lowest dose of bleomycin induces markedly higher levels of airway inflammation in UG-KO mice

It is well known that inflammation precedes fibrosis. Thus, we sought to determine whether the UG-KO mice are more prone to developing exaggerated inflammatory response in the lungs when treated with bleomycin. To explore this possibility, we histologically analyzed the lungs of UG-KO mice and their WT littermates 28 days after bleomycin treatment. The results show that compared with the lungs of WT mice those of the UG-KO mice contained markedly higher levels of inflammatory cells in the airways, and in the lung parenchyma (Supplemental Figure 1). In related experiments, we further analyzed the number and type of inflammatory cell populations in the bronchoalveolar lavage fluid (BALF) at seven days after bleomycin administration, when the inflammatory response is reported to be maximal. The results show that in the UG-KO lungs the basal levels of all four categories of inflammatory cells, eosinophils (Fig. 3A), neutrophils (Fig. 3B), lymphocytes (Fig. 3C) and monocytes/macrophages (Fig. 3D), are higher than in those of the WT littermates. Most interestingly, while

bleomycin treatment caused a moderate elevation in the number of these cells in WT lungs they were significantly higher ($P < 0.05$) in the bleomycin-treated UG-KO mouse lungs (Fig. 3A–D). Taken together, these results suggest that the extreme susceptibility of UG-KO mice to bleomycin-induced inflammation may predispose them to develop PF.

3.5. Bleomycin induces higher levels of T_H2 cytokine expression in UG-KO lungs

Fibrosis is a particular concern in numerous persistent inflammatory states driven by T helper type 2 (T_H2) responses [12]. It has been reported that the transcription of several genes that are known to be involved in wound healing and fibrosis is upregulated by T_H2 cytokines [5]. Cytokines have also been reported to play critical roles in orchtrating allergen-mediated airway inflammation [13]. Further, we previously reported that UG-KO mice manifest exaggerated airway inflammation induced by a model allergen, ovalbumin [8]. Thus, we analyzed the transcription levels of T_H2 cytokines, IL-4, IL-5 and IL-13 by real-time PCR. The results show that the baseline levels of T_H2 cytokines, especially IL-4 and IL-13, are already higher in the lungs of UG-KO mice (Fig. 4A and B). Interestingly, while there were no significant difference in the basal levels of IL5-mRNA expression in the lungs of the WT and UG-KO mice bleomycin treatment appears to significantly ($P < 0.05$) augment these basal levels (Fig. 4C). Moreover, while low dose bleomycin treatment did not markedly elevate the T_H2 cytokine levels in WT lungs, there were significant ($P < 0.05$) augmentation of the already elevated baseline levels of these cytokines in the bleomycin-treated UG-KO mouse lungs (Fig. 4A–C). These results indicate that bleomycin induces pulmonary inflammation by stimulating the expression of pro-inflammatory T_H2 cytokines. Moreover, these results also indicate the likelihood that
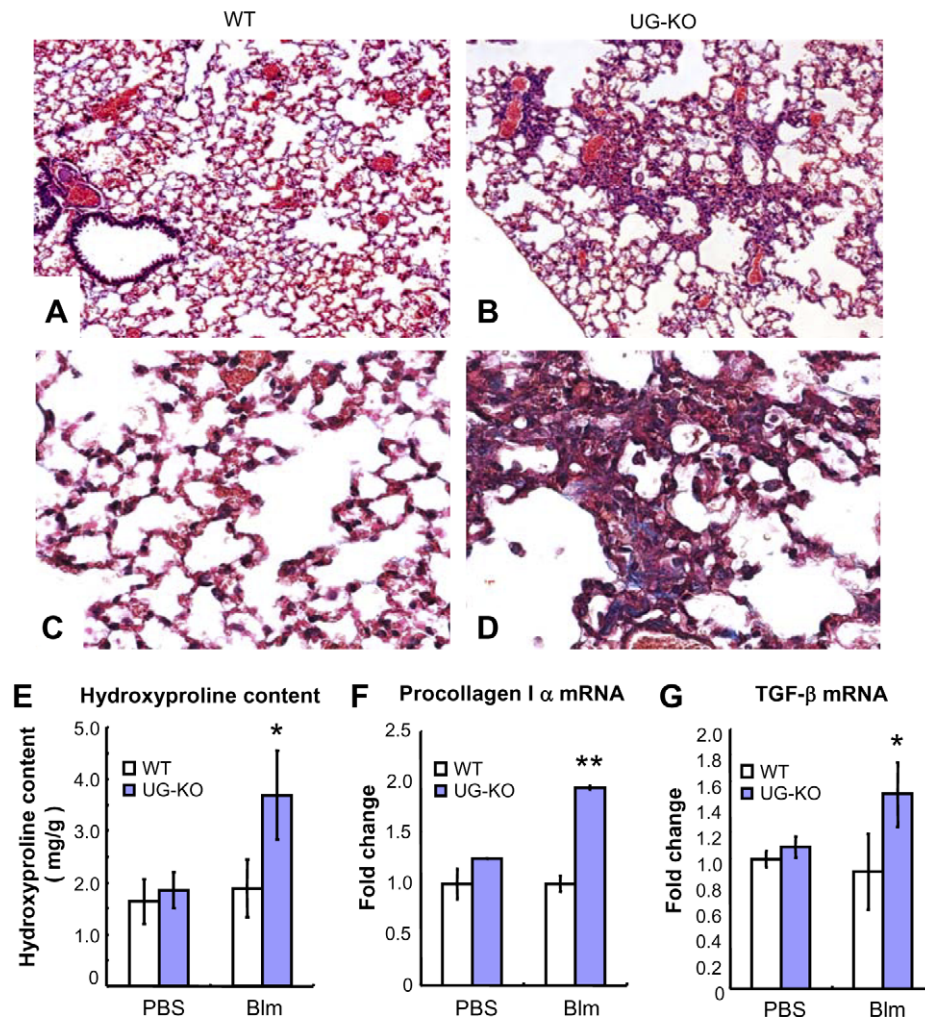
Fig. 2. Histological detection fibrosis. Lung tissues from UG-KO mice and their WT littermates 28 days after bleomycin-treatment were stained with hematoxylin–eosin (panels A and B) and Masson's Trichrome (panels C and D) stains: WT (panels A and C) and UG-KO (panels B and D). Compared with the lungs of WT littermates (panel C), Masson's Trichrome staining show deposition of collagen (blue staining) in those of the UG-KO mice (panel D). Magnification: 200× (panels A and B) and 630× (panels C and D). Hydroxyproline content (panel E) and pro-collagen 1α_1 mRNA levels (panel F) in the lungs of bleomycin-treated mice and the profibrotic cytokine TGF-β-mRNA expression in UG-KO mice and in their WT littermates (panel G). Bars represent means ± S.E. with $n = 3$. [*], $P < 0.05$; [**], $P < 0.01$ compared with bleomycin-treated wild type mice group.

UG suppresses this stimulation in WT mouse lungs. Among the T_H2 cytokines, IL-13 has been reported to play the most critical role in inducting fibrosis. Therefore, we analyzed the level of IL-13 protein in the serum of bleomycin treated and untreated UG-KO as well as WT mice by Western blot analysis. The tissue expression of IL-13 in the lungs were also evaluated by immunohistochemical analysis. The results show that compared with bleomycin-treated WT littermates the IL-13 protein levels in the serum of UG-KO mice are markedly elevated (Fig. 4D). We also found that compared with the lungs of WT littermates (Supplemental Figure 2A) a markedly higher basal level of IL-13-specific staining was readily detectable in the airways of the UG-KO mice (Supplemental Figure 2B). More interestingly, while bleomycin treatment moderately increased IL-13 staining in the airways of WT littermates (Supplemental Figure 2C), it dramatically augmented that in the airways of UG-KO mice (Supplemental Figure 2D). These results strongly suggest that the bleomycin-induced exaggerated inflammatory response in the lungs of UG-KO mice is mediated by the infiltration of the lungs with T_H2 cells that secrete T_H2 cytokines such as IL-13.

In this study, we sought to understand the molecular mechanism(s) of PF phenotype in UG-KO mice. We found that UG, and indirectly the airway epithelial cells that secrete UG, play critical roles in protecting the mammals from developing PF. It has been reported that the expression pattern of epithelial cell-specific markers (e.g. surfactant protein C and Clara cell specific protein, CC10) in the lungs of mice treated with bleomycin is strain-specific [14–17]. It has also been demonstrated that mice with the C57 genetic background are most sensitive to bleomycin treatment [18]. Our UG-KO mice and their WT littermates are of the C57 background. This is significant, as we have used the lowest dose of bleomycin that does not induce PF in the WT mice of this sensitive C57 strain although this low dose readily induces inflammation and extensive PF in UG-KO littermates. These results strongly suggest that UG confers resistance against the development of PF.

How might UG protect against PF? Our results show that the anti-fibrotic effects of UG stems from its ability to prevent pulmonary infiltration of T_H2 cells that secrete pro-fibrotic T_H2 cytokines such as IL-4, IL-5 and IL-13. The anti-chemotactic effects of UG have been reported previously [19] and this property
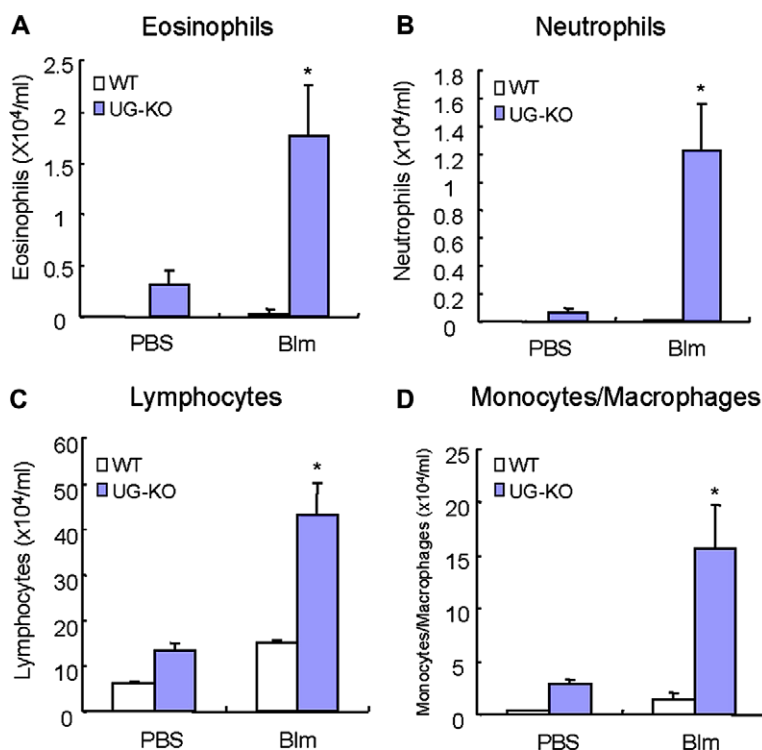
Fig. 3. Extreme susceptibility of mice lacking uteroglobin to bleomycin-induced airway inflammation. Eosinophil (panel A), neutrophil (panel B), lymphocyte (panel C) and monocyte/macrophage (panel D) cells in bronchoalveolar lavage fluid (BALF) samples from WT and UG-KO mice at seven days after administration of 0.0075 U/mouse of bleomycin (Blm) or PBS control (PBS). Bars represent means ($n = 4$–7) ± S.E. *, $P < 0.01$ compared with bleomycin-treated wild type mice group.
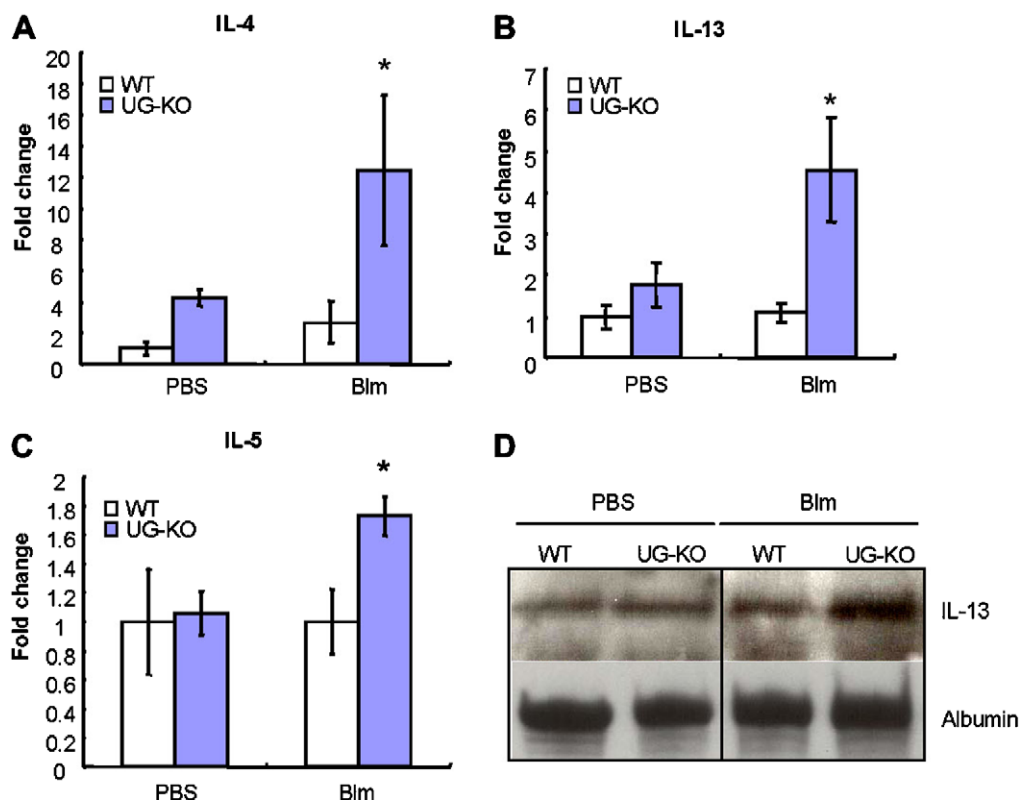

Fig. 4. Bleomycin induces high levels of T_H2 cytokine expression in uteroglobin-knockout mouse lungs. The mRNA levels of T_H2 cytokines were determined by real-time PCR using total RNA from the lungs of WT and UG-KO mice at 28 days after treatment with bleomycin (Blm) or PBS (control). IL-4 (panel A), IL-13 (panel B), IL-5 (panel C). Bars represent means ($n = 3$) ± S.E. *, $P < 0.05$. The Western blot analysis of IL-13 in the serum of untreated and bleomycin-treated WT and UG-KO mice (panel D).

of UG may be instrumental in preventing T$_H$2 cell infiltration of the lungs. It has been reported that TGF-β is one of the cytokines that is consistently associated with PF[20,21]. Our results show that bleomycin treatment markedly increases the level of TGF-β-mRNA in the lungs of UG-KO mice. In addition, the monocytes/macrophages, the cell type significantly increased in the lungs of bleomycin-treated UG-KO mice, are known to express TGF-β [5]. Therefore, we sought to determine whether UG downregulates the expression of TGF-β-mRNA in a murine monocyte/macrophage cell line, RAW 264.7. The results show that UG treatment indeed suppresses the expression of TGF-β-mRNA in these cells (Supplemental Figure 3). The mechanism(s) by which UG downregulates TGF-β-mRNA expression is not yet clear. However, it is possible that UG exerts this effect via its cell surface receptor(s) [22–24].

Although the molecular mechanism(s) of PF is yet to be clearly defined, it appears that a breakdown of homeostatic mechanisms to prevent pulmonary inflammation plays critical pathogenic roles. Pulmonary fibrosis affects a large population worldwide and currently, there is no effective treatment for this disease except for lung transplantation and supportive oxygen therapy [5,6,10]. Current therapies are directed to suppress lung inflammation that often precedes fibrosis. However, these treatments are effective only in a small proportion of the affected population. Further, while these treatments may inhibit inflammation, they do not appear to prevent fibrosis. Clearly, understanding the homeostatic mechanism(s) in the mammalian respiratory system that keeps inflammation at bay may provide insight into the molecular mechanism(s) of this disease and the development of novel therapeutic approaches. In this regard, UG appears to maintain homeostasis in the mammalian respiratory system to keep this vital organ free of inflammation consequently, protects it from the development of fibrosis. Thus, the findings of this study, at least in part, provide a proof of principle that recombinant UG may have therapeutic potential for PF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.07.031.

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