IL-6 amplifier activation in epithelial regions of bronchi after allogeneic lung transplantation

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Abstract

The IL-6 amplifier, a positive feedback loop for NFκB signaling, which was originally found to be activated by IL-17A and IL-6 stimulation in non-immune cells, is molecularly a simultaneous activator of NFκB and signal transducer and activator of transcription 3 (STAT3), functionally a local chemokine inducer and pathologically a machinery for inflammation development. It has been shown that IL-6 amplifier activation in epithelial cells contributes to rejection responses in a mouse chronic rejection model that develops a bronchiolitis obliterans (BO)-like disease. We investigated whether the IL-6 amplifier is activated in BO regions of a human lung graft after allogeneic transplantation. NFκB and STAT3 molecules were phosphorylated in the epithelial regions of bronchi that localized in the BO regions. Additionally, chemokine ligand 2 (CCL2), and CD4+ T cells and macrophages increased in these regions. Furthermore, human lung epithelial cells expressed CCL2 after stimulation by IFNγ in the presence of IL-6 and epidermal growth factor via enhanced STAT3 signaling, which parallels behavior seen in the mouse model. Thus, our results suggest that the IL-6 amplifier in the epithelial cells of grafts is involved in chronic rejection after lung transplantation, suggesting that the amplifier may be a valuable therapeutic target to prevent chronic rejection after lung transplantation.

Keywords: cytokines, inflammation, NFκB, STAT3, IL-6, transplantation

Introduction

Together with the signal transducer and activator of transcription 3 (STAT3) stimulator IL-6, NFκB stimulators including IL-17A induce a positive feedback of IL-6 signaling in type 1 collagen+ non-immune cells. We named this feedback loop IL-6-triggered positive feedback for NFκB signaling, or ‘IL-6 amplifier’ for short, and investigated its role in the development of autoimmune disease models (1). Subsequent results showed that the IL-6 amplifier is molecularly a simultaneous activator of NFκB and STAT3, functionally a local chemokine inducer and pathologically a machinery of inflammation development via a NFκB loop (1–3).

Along with disease outcomes, acute and chronic inflamations are often seen in graft rejection after allogeneic transplantations. One initial factor for such inflammations is T cells, which directly and/or indirectly recognize MHC differences between the donor and recipient (4,5). Therefore, many therapeutic drugs for allogeneic transplantations suppress T-cell activation pathways. As such, clinicians have successfully controlled acute T-cell-triggered inflammation after transplantation (6). However, chronic inflammation has proven to be a greater challenge. To control chronic inflammation, a delicate balance is required: too much immune suppression increases the risk of pathogenic invasions in the recipient and donor graft, whereas too little of the same increases the risk of allogeneic responses against the donor graft by the recipient’s immune system. Therefore, it is possible that a low, but
certain level of allogeneic T-cell responses might be involved in the chronic rejection response.

Such a situation is especially true in lung transplantation, which is a common treatment in various end-stage lung and cardiac diseases (7). Most chronic rejection responses after lung transplantation cause bronchiolitis obliterans (BO) (8).

From a pathological point of view, BO features an intra-luminal polyoid plug that consists of the granulation tissue between terminal and respiratory bronchioles that have already lost epithelial cells. From a clinical pulmonologist's perspective, BO is a chronic scarring process that affects the small airways of the lung and results in progressive obliteration of the small airways. In either case, epithelial damage, which impairs the lung's function for oxygen exchange, is a serious risk in patients suffering from chronic rejection responses after lung transplantation.

Because we recently showed IL-6 amplifier activation in a BO-like animal disease of allogeneic airway transplantation (9), we hypothesized that the IL-6 amplifier might also be a factor for chronic rejection after human lung transplantation. Here, we investigated the sites of IL-6 amplifier activation in a lung graft after allogeneic transplantation, finding that IL-6 amplifier activation is evident in the epithelial cells of graft regions showing BO. Therefore, we suggest that attenuating IL-6 amplifier activation may offer a new therapeutic target to reduce inflammation-related transplantation rejection including chronic inflammation.

Methods

Antibodies and reagents

Anti-CD4 (Sigma-Aldrich, Tokyo, Japan), anti-CD11b (Sigma-Aldrich), anti-chemokine ligand 2 (CCL2) (Biolegend, Tokyo), anti-IL-6 (Sigma-Aldrich), anti-phospho-STAT3 (Cell Signaling), anti-phospho-NFκB p65 (Cell Signaling), human epidermal growth factor [epidermal growth factor (EGF); R&D Systems, Minneapolis, MN, USA] and anti-phospho-ErbB1 antibody (Sigma-Aldrich) were all purchased. Rabbit IgG (Sigma-Aldrich), rat IgG (Sigma-Aldrich), mouse IgG (Biolegend) and rabbit mAb (Cell Signaling) were used for the control staining.

Human BO sample preparation

Human lung tissue samples were obtained from a 54-year-old woman who received a left hemi-lateral lung transplant from a cadaveric donor in July 2001 and died from BO in 2010 at Osaka University Hospital. The use of these samples was approved by the Ethics Committees of Osaka University Hospital.

Immunohistochemistry

Immunohistochemistry was performed as described previously with slight modifications (7).

Cells, stimulation conditions and ELISA/qPCR

Human primary small airway epithelial cells (CC-2547, Sanko, Tokyo) were cultured in small airway cell basal medium (Sanko) with supplement reagents (SAGM SingleQuots, Lonza, Tokyo). Human primary lung fibroblasts (CAF9) isolated at Osaka University Hospital were cultured in fibroblast basal medium (Sanko) with supplement reagents (FGM-2 SingleQuots, Lonza). Cells passaged <5 times were used for the assay. The use of these cells was approved by the Ethics Committees of Osaka University Hospital. Cells were stimulated with cytokines and/or growth factors, followed by ELISA and real-time polymerase chain reaction (qPCR) experiments as described in the following paragraphs.

Human primary lung fibroblasts or small airway lung epithelial cells were starved for 1 h, followed by stimulation with the indicated cytokines (100 ng ml−1 each) for 24 h (ELISA) or 3 h (qPCR). Human CCL2 concentrations in the supernatant were determined by an ELISA kit (Biolegend). Quantitative PCR of human IL-6 or CCL2 was performed with a SYBR green system. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference. For inhibition experiments, human primary lung fibroblasts or small airway lung epithelial cells were starved for 1 h, followed by stimulation with IL-6, EGF and IFNγ for 3 h. STAT3 inhibitor [5,15-diphenylporphyrin (5,15-DPP) at 10 or 30 μM, Calbiochem], inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKK2) inhibitor IV (at 1 or 3 μM, Calbiochem) or anti-IL-6 neutralizing antibody (200 μg ml−1, Abcam) was added to the culture 30 min before cytokine stimulation. Human IL-6 or CCL2 qPCR was performed with a SYBR green system. Human GAPDH was used as a reference.

A 7300 Fast Real-Time PCR system (Applied Biosystems, Tokyo) and SYBR green PCR Master Mix (Sigma-Aldrich and KAPA, Woburn, MA, USA) were used to quantify the levels of CCL2, EGF, epidermal growth factor receptor (ErbB1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1 (HPRT) mRNA. Total RNA was prepared from cells and graft tissues using a GenElute Mammalian Total RNA kit and DNase I (Sigma-Aldrich). The PCR primer pairs were as follows: human type 1 collagen primers, 5′-GTCAGGGGCAAGACGAAG-3′ and 5′-CAGATACGTCATCGCAACAAC-3′; human CCL2 primers, 5′-CAGCGGATGCAATCATGCACC-3′ and 5′-TGGATCTCCTGAACCCACTTCT-3′; and human GAPDH primers, 5′-GAGTTTGCTGCT-3′ and 5′-CGCTCCTGGAAGATGTTG-3′. The conditions for qPCR were 40 cycles at 94°C for 15 s, followed by 40 cycles at 60°C for 30 s and 72°C for 30 s. The relative mRNA expression levels were normalized to HPRT or GAPDH mRNA levels.

Western blotting

Human epithelial cells were lysed with NP40 buffer and subjected to SDS–PAGE and western blotting with anti-STAT3, anti-phospho-STAT3, anti-NFκB p65 and anti-phospho-NFκB p65.

Confocal microscopy

Cells were adhered to poly-l-lysine-coated glass base dishes (Asahi glass), starved for 1 h and stimulated with IL-6, EGF and/or IFNγ (100 ng ml−1 each) for 30 min. Intracellular staining of phospho-NFκB p65 (5276) was performed using the Cytofix/Cytoperm kit (BD Bioscience). Alexa Flour 488-conjugated anti-rabbit IgG (Invitrogen) was used as the secondary antibody. Hoechst33342 (eBioscience) was used for nuclear staining. Confocal microscopy was performed with the LSM5 Pascal system (Carl Zeiss).

Statistical analysis

Student’s t-tests (two-tailed) were used for the statistical analyses of differences among groups.
Results

NFκB and STAT3 are activated in epithelial basal cells of the bronchi in BO regions after lung transplantation.

We investigated whether the IL-6 amplifier is activated during the rejection of human lung transplantations. We investigated the activation of NFκB and STAT3 in patient lung samples, because the IL-6 amplifier simultaneously activates these two transcriptional factors in non-immune cells. Employing an anti-phospho-STAT3 (Tyr705) antibody treatment followed by immunohistochemistry caused clear phospho-STAT3 staining in the epithelial basement cells of tracheal ducts in BO regions (Fig. 1A, upper panel) but not in normal regions (Fig. 1A, lower panel). The epithelial cells, including basement and columnar cells, were confirmed to be type 1 collagen+ cells (Supplementary Figure 1, available at International Immunology Online). In the BO regions, 73.0% of epithelial basement cells were nuclear...
phospho-STAT3+, whereas only 1.4% of epithelial columnar cells in the tracheal ducts were so. On the other hand, only 3.7% of epithelial basement cells were phospho-STAT3+ in normal regions. Additionally, phospho-STAT3 staining was easily seen in epithelial basement cells located beneath well-regulated epithelial columnar cells but not in those located under stacked epithelial columnar cells, which were found only in the tracheal ducts of the BO samples (Fig. 1A, white arrow). Moreover, small numbers of phospho-STAT3-stained cells were seen in small tracheal ducts that showed the typical BO phenotype of granulation minus epithelial layers (Fig. 1C).

NFκB activation, monitored by p65 phosphorylation via an anti-phospho-NFκB p65 (Ser276) antibody, was observed in epithelial columnar cells and their corresponding basement cells (Fig. 1B). In BO regions, 48.5% of epithelial basement cells were nuclear phospho-NFκB+, but only 26.2% of epithelial columnar cells from the tracheal ducts were so. On the other hand, we found only 15.2% of epithelial basement cells were phospho-NFκB+ in normal samples. In contrast, the phospho-NFκB staining was weak in epithelial basement cells located beneath well-regulated epithelial columnar cells, but clearly seen in those located beneath stacked columnar epithelial cells (Fig. 1B, white arrow). Moreover, we did not observe obvious phospho-NFκB staining in the intra-luminal polypoid plug of granulation tissue within terminal and respiratory bronchioles that showed epithelial loss (Fig. 1D). Thus, the IL-6 amplifier appears activated in the epithelial basal cells of bronchi in BO-affected regions.

ErbB1 was activated in epithelial regions
We previously identified EGF as an NFκB activator and IL-6 as a STAT3 activator for IL-6 amplifier activation in our murine heterotopic airway transplantation model (9). We therefore investigated here whether these two activators are also expressed in human transplants. Consistent with the mouse model, highly phosphorylated ErbB1 and high IL-6 expressions were present in epithelial columnar cells and epithelial basement cells of BO regions (Fig. 2A and B). In fact, 93.4% of epithelial basement cells and 95.7% of epithelial columnar cells were phospho-ErbB1+ in the tracheal ducts of BO regions. This number was only 49.3% for epithelial basement cells in normal regions. The results of the serial section experiments were consistent with Figs 1 and 2: phosphorylation of both ErbB1 and NFκB occurred in epithelial basement cells, but only ErbB1 phosphorylation was much higher in epithelial columnar cells in the BO case (Supplementary Figure 2, available at International Immunology Online). On the other hand, phospho-STAT3 was observed only in epithelial basement cells, not in epithelial columnar cells (Supplementary Figure 3, available at International Immunology Online).

Therefore, we concluded that activation of the IL-6 amplifier occurs in the epithelial basement cells, but not in the epithelial columnar cells, because the IL-6 amplifier is induced after simultaneous activation of NFκB and STAT3. Thus, we hypothesized that the activation of the IL-6 amplifier might augment phosphorylation of NFκB to trigger positive feedback of NFκB in epithelial basement cells.

Moreover, a certain level of phospho-ErbB1 staining was seen in the intra-luminal polypoid plug of the granulation
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(A) Phospho-ErbB1

(B) IL-6

Control Ab
tissue found within terminal and respiratory bronchioles that showed epithelial loss, although phospho-ErbB1 staining was also seen outside the bronchioles (Fig. 2C, arrows). Additionally, there was an observable level of IL-6 staining within the terminal and respiratory bronchioles (Fig. 2D, arrows). These results suggest that the ErbB1 ligand EGF might be an NFκB activator and that IL-6 might be a STAT3 activator in the epithelial region of human lung and therefore potentially cause IL-6 amplifier activation.

A chemotactic factor for Th1 and macrophages, CCL2, is increased in epithelial regions

We next sought to identify the target of the IL-6 amplifier. CCL2, a chemotactic factor for Th1 and macrophages, was considered a candidate, because our mouse model results indicated that it is a target in lung epithelial cells and lung fibroblasts (9). In the mouse model, we also found that CCL2 plays a role in the rejection response. We therefore investigated CCL2 expression in human samples.

Epithelial regions in BO lung samples expressed excess CCL2, whereas those not showing BO did not express the same (Fig. 3A). Consistent with this enhanced expression of CCL2, CD4+ T cells and CD11b+ cells, both of which may express various cytokines including IFNγ, were evident in epithelial regions that showed IL-6 amplifier activation in the tracheal ducts of BO human lung samples (Fig. 3B and C). Additionally, we observed a certain level of CCL2 staining in the intra-luminal polypoid plug of the granulation tissue found within terminal and respiratory bronchioles that had epithelial loss (Fig. 3D). CD4+ T cells and CD11b+ cells were scattered outside tracheal ducts that had granulation and no epithelial layers (Fig. 3E and F, arrows). Consistent with the accumulation of CD4+ T cells and CD11b+ cells, phospho-STAT1, which is a downstream event of IFNγ stimulation, was present, particularly in epithelial basement cells (Fig. 3G, upper panel, arrows). In the BO regions, 14.0% of epithelial basement cells and 5.9% of epithelial columnar cells in the tracheal ducts were nuclear phospho-STAT1+. On the other hand, only 1.2% of epithelial basement cells were phospho-STAT1+ in normal regions. The results of the serial section experiments confirmed that phospho-STAT1, phospho-STAT3 and phospho-NFκB are present in epithelial basement cells (Supplementary Figure 3, available at International Immunology Online).

Moreover, human type 1 collagen+ cells, including primary lung epithelial cells and lung fibroblasts, expressed excess CCL2 mRNA and protein after the IL-6 amplifier was activated by IFNγ, particularly in the presence of IL-6 and EGF (Fig. 4A–D). IL-6 expression is also enhanced by IL-6 and IFNγ stimulation, but not by EGF stimulation in both cells (Fig. 4E and F). Unexpected, however, was the observation that EGF stimulation could suppress IL-6 expression (Fig. 4E and F), although further studies are needed to understand the molecular mechanism of this EGF-mediated inhibition. IFNγ-mediated IL-6 amplifier activation in human type 1 collagen+ cells was dependent on STAT3, NFκB and IL-6 as confirmed by using inhibitors for STAT3/NFκB and anti-IL-6 neutralizing antibody in each cell (Fig. 5A–H).

Furthermore, IFNγ stimulation mainly induced STAT3 activation, whereas it had a negligible effect on NFκB activation in human type 1 collagen+ cells (Fig. 6A). The nuclear localization of NFκB was unaltered with or without IFNγ treatment in the presence of IL-6 and EGF stimulation in primary human epithelial cells (Fig. 6B). These results suggest that CCL2
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expression is enhanced by IL-6 amplifier activation in allogeneic grafts via the IFNγ–STAT3 axis after CD4^+ T-cell and activated macrophage accumulation.

**Discussion**

We have recently shown a molecular mechanism for allogeneic rejections using a tracheal heterotopic transplantation mouse model that incorporates BO-like lesions (9). In the previous study, we proposed that IL-6 amplifier activation in a graft’s type 1 collagen+ cells in tracheal epithelial regions may cause the observed pathology. The primary signals responsible for IL-6 amplifier activation were IL-6 and IFNγ for STAT3 stimulation and EGF for NFκB stimulation in the model system. CCL2, a T,1/macrophage chemokine, was found to be a critical target of the IL-6 amplifier, as it was hyper-induced by IFNγ via enhanced STAT3 signaling. We here show similar phenomena in the epithelial regions of human lung transplant samples that show BO. Activation of STAT3 and NFκB in these epithelial regions suggests that the IL-6 amplifier is activated there. We also found excess expression of IL-6 and CCL2 and evident phosphorylation of ErbB1 in the epithelial regions of BO samples. Therefore, we argue that IL-6 amplifier activation may regulate chronic lung rejection responses after allogeneic lung transplantation. Thus, the IL-6 amplifier could be an important determinant for transplantation rejection, including chronic rejection responses, not only in mouse but also in humans.
Based on the activation of STAT3 and NFκB, here we show IL-6 amplifier activation in epithelial regions including epithelial basement cells in the tracheal ducts of BO human lung samples. This activation was not seen in small tracheal ducts that had granulation-absent epithelial layers despite typical BO phenotypes. These results suggest that IL-6 amplifier might be a triggering mechanism for chronic rejection responses, but not a maintenance mechanism. This conclusion is strengthened by the observation that fibroblastic cells in the granulation of BO trachea did not show evident NFκB and STAT3 activation. Therefore, we propose that chronic rejection is divided into at least two steps: (i) destruction of the epithelial region in its early phase, a process that depends on IL-6 amplifier activation in epithelial cells and their corresponding basement cells and (ii) granulation in bronchioles at a relatively late phase that corresponds to minimal IL-6 amplifier activation.

High NFκB and STAT3 activation was seen in epithelial layers, particularly in basement cells, near BO-affected regions. On the other hand, this was not the case for stable regions more distant from the BO-affected ones. We hypothesized that there exist specific factors that enhance IL-6 amplifier activation in specific lung site(s) that show the BO phenotype and that their activation might depend on a positive feedback mechanism. For example, once the IL-6 amplifier is locally activated, the amplifier itself could induce NFκB and STAT3 activators directly and/or indirectly. Because the main pathway of the IL-6 amplifier is NFκB signaling (unpublished results), we suspect a feedback loop involving NFκB stimulators, possibly including an EGF pathway.
Fig. 4. IFNγ enhances IL-6 amplifier activation in the presence of IL-6 and EGF in human type 1 collagen+ cells. (A–F) Two types of type 1 collagen+ human primary cells—human small airway epithelial cells (A, C and E) and human lung fibroblasts (B, D and F)—were stimulated with a combination of IL-6, EGF and IFNγ (100 ng ml−1 each) for 24 and 3h, respectively, followed by CCL2 or IL-6 expression analysis using ELISA (24h; A and B) and real-time PCR (3h; C–F), respectively. Error bars represent the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. Experiments were performed at least three times; representative data are shown.
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A. Human small airway epithelial cells

B. Human lung fibroblasts

C. IL-6/EGF/IFNγ

D. IL-6/EGF/IFNγ

E. IL-6/EGF/IFNγ

F. IL-6/EGF/IFNγ

G. IL-6/EGF/IFNγ

H. IL-6/EGF/IFNγ
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Fig. 5. IFN-γ-mediated IL-6 amplifier activation in human type 1 collagen+ cells is dependent on STAT3, NFκB and IL-6. (A–H) Two types of type 1 collagen+ human primary cells—human small airway epithelial cells (A–D and I–L) and human lung fibroblasts (E–H and M–P)—were stimulated with a combination of IL-6, EGF and IFNγ (100 ng ml⁻¹ each) in the presence of inhibitors for STAT3 or IKK2 (A, B, E, F, I, J, M, and N) or with anti-hIL-6 antibody C, D, G, H, K, L, O, and P for 3 h, followed by CCL2 or IL-6 expression analysis using real-time PCR. Error bars represent the mean ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Experiments were performed at least three times; representative data are shown.
What is the role of accumulated CD4+ T cells and macrophages in the BO lesions? Because we showed that IFNγ enhances the activation of the IL-6 amplifier at least in vitro via STAT3 activation (Fig. 6A), it would appear that these cell populations might be a source of IFNγ. However, we cannot dismiss the possibility that the IFNγ–STAT1 pathway in epithelial cells directly and/or indirectly functions as a NFκB stimulator during IL-6 amplifier activation. Although the number of accumulated macrophages in the grafts of our mouse model was almost an order of magnitude less than that of CD4+ T cells, we found in our human specimen that the increase between the two populations was comparable (see Fig. 3B and C). Because we did not apply immune-suppressive drugs, which particularly suppress T-cell activation, in our previous mouse model experiments, it is likely those results were affected by T cells, particularly allo-reactive ones. In human cases including this one, however, immune-suppressive drugs were applied. Therefore, the fact that we can dismiss the influence of T cells in our results suggested that macrophages had a critical role in the chronic rejection of lung transplantation and produced various cytokines including IFNγ, a strong IL-6 amplifier stimulator.

In summary, we here show that the IL-6 amplifier, a key regulator for inflammation in disease models, is activated in epithelial cells and their basement cells in the BO lesions of lung samples following transplantation. These regions are marked by activation of NFκB, STAT3 and ErbB1 and the production of IL-6 and CCL2 in epithelial layers, particularly epithelial basement cells, near BO lesions. Thus, we suggest that IL-6 amplifier activation in grafts plays a critical role for allogenecic chronic rejection responses in humans after lung transplantation and that the IL-6 amplifier is a possible therapeutic target for transplantation rejection.

Supplementary data
Supplementary data are available at International Immunology Online.

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Conflict of Interest: The authors declare no conflicting financial interests.

References

Fig. 6. IFNγ enhances IL-6 amplifier activation in the presence of IL-6 and EGF via STAT3 activation in human type 1 collagen+ cells. (A) Human lung fibroblast cells were stimulated with a combination of IL-6 (100 ng ml⁻¹), EGF (100 ng ml⁻¹) and IFNγ (100 ng ml⁻¹) for 10 min, followed by analysis of phosphorylation of STAT3 or p65 NFκB by western blotting. Experiments were performed at least three times; representative data are shown. (B) Human small airway epithelial cells were starved for 1 h and then stimulated with the indicated cytokines (100 ng ml⁻¹ each) for 30 min. Nuclear localization of p65 was calculated. **P < 0.001; ns, not significant. Experiments were performed at least three times; representative data are shown.
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