Nasal administration of IL-33 induces airways angiogenesis and expression of multiple angiogenic factors in a murine asthma surrogate

Running Title: IL-33 and angiogenesis in asthma

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Summary

Th2-promoting cytokine IL-33 has been implicated in asthma pathogenesis. Angiogenesis is a feature of airways remodelling in asthma. We hypothesised that IL-33 induces airways angiogenesis and expression of angiogenetic factors in an established murine surrogate of asthma. In the present study, BALB/c mice were subjected to serial intranasal challenge with IL-33 alone for up to 70 days. In parallel, OVA-sensitized mice were subjected to serial intranasal challenge with OVA or normal saline to serve as positive and negative controls, respectively. Immunohistochemical analysis of expression of Von Willebrand factor (vWF) and erythroblast transformation-specific (ETS) related gene (ERG), both blood vessel markers, and angiogenetic factors angiogenin, insulin-like growth factor (IGF-1), endothelin-1, epidermal growth factor (EGF) and amphiregulin was performed in lung sections *ex vivo*. An established in house assay was used to test whether IL-33 is able to induce microvessel formation by human vascular endothelial cells. Results showed that serial intranasal challenge of mice with IL-33 or OVA resulted in proliferation of peribronchial vWF^{+} blood vessels to a degree closely related to the total expression of the angiogenic factors amphiregulin, angiogenin, endothelin-1, EGF and IGF-1. IL-33 also induced microvessel formation by human endothelial cells in a concentration-dependent fashion *in vitro*. Our data are consistent with the hypothesis that IL-33 has the capacity to induce angiogenesis at least partly by increasing local expression of multiple angiogenetic factors in an allergen-independent murine asthma surrogate, and consequently that IL-33 or its receptor are potential novel molecular

targets for asthma therapy.

Keywords: IL-33, asthma, angiogenesis, murine model

Abbreviations:

AHR: Airways hyperresponsiveness

EGF: Epidermal growth factor

ERG: ETS-related gene

ETS: erythroblast transformation-specific

HUVEC: Human umbilical vein endothelial cells

IGF-1: Insulin-like growth factor

IL-33: interleukin 33

OVA: ovalbumin

vWF: Von Willebrand factor;

Introduction

Asthma is a chronic disease affecting more than 300 million people worldwide and its prevalence is still rising.[1] It is characterised pathophysiologically by bronchial mucosal inflammation with Th2-type immune bias and episodic airways obstruction, reflecting airways hyperresponsiveness (AHR).[2] Neo-angiogenesis in the airways mucosa is a distinct feature of asthma pathophysiology which is generally considered to be an important component of airways remodelling, which may contribute to irreversible airways obstruction. Neo-angiogenesis may be defined as the emergence of new blood vessels from pre-existing vasculature and is regulated by a balance of promoting (angiogenic) and counteracting (angiostatic) factors.[3,4] In the course of chronic inflammation, neo-angiogenic processes are initiated as a result of the dominance of pro-angiogenic effects likely involving many growth factors.[5] Given that neo- angiogenesis is a typical feature of remodelling in asthmatic airways, asthma is not only an airways disease, but also a vascular disease.[6] Nevertheless there are relatively few reports addressing its pathogenesis in asthma.

IL-33 is a member of the IL-1 cytokine family and is a ligand of the receptor ST2.[7] Recent studies in human subjects and murine asthma surrogates suggest a central role for IL-33 in driving Th2-mediated inflammation.[8.9] Direct administration of IL-33 alone into the airways of naive mice via the intranasal route resulted in elevated expression of Th2 cytokines, eosinophilic inflammation and AHR.[10-12] Deletion or blockade of the IL-33 receptor (T1/ST2) has been shown to attenuate allergic airways inflammation in animal asthma surrogates.[9,11] Some studies suggest

that IL-33 also promotes angiogenesis and increases vascular permeability, possibly at least partly by promoting endothelial cellular production of nitric oxide and urokinase in human endothelial cells.[13,14]

To examine the potential role of IL-33 to promote angiogenesis in asthma and clarify its mechanisms of action we utilized a murine surrogate of asthma initiated by direct IL-33 challenge which we developed as previously described[15] to compare, in parallel, the effects of direct IL-33 challenge of the airways with those of "classical" ovalbumin (OVA) challenge necessitating prior IgE anti-OVA sensitisation of the animals as a positive control. In addition, using an *in vitro* model we investigate whether IL-33 exerts direct effects on human angiogenesis i*n vitro*.

Materials and methods

Animals

Female BALB/c mice (8-10 weeks old) were obtained from Vital River Laboratories (Beijing, China) and maintained in a pathogen-free mouse facility located in the Department of Laboratory Animal Sciences, Capital Medical University, Beijing, China. All of the experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

IL-33- and ovalbumin (OVA)-challenge

The protocols employed for sensitization and inhalational challenge have previously been described.[15] Mice were randomly distributed into 3 groups including a group serially challenged with 50μg/dose of OVA as a positive control;[16,17] a group administered saline as a negative control and a group serially challenged with IL-33.[15,18] The OVA-challenged mice were first sensitised by intraperitoneal injection of 100μg/dose OVA (Sigma-Aldrich, Beijing, China, emulsified in $Al[OH]_3$) on days 0 and 12. These mice were then further intranasally challenged daily on days 18 to 23 with 50μg/dose of OVA (OVA50) in 50μL saline. For IL-33-challenge, naïve mice were challenged intranasally daily from days 18 to 23 with recombinant mouse IL-33 (mIL-33, R&D Systems, 100 ng in 50μL saline = 1.1 x 10-7M).Then the mice in each group were further challenged intranasally with OVA or IL-33 every 2 days for a further 30 days. Some of them were observed for a further 17 days after cessation of the challenges. Control mice were intraperitoneally injected with same amount of

$Al[OH]_3$ then nasally challenged with normal saline at corresponding time-points. Lung tissues were collected on days 20, 24, 36, 48, 54 and 70 (each time point n=5 for per group), respectively, 24 hrs after the most recent intranasal challenge e animals.[15]

Lung immunohistochemistry

Resected left lung tissue was fixed in 10% neutral-buffered formalin for 24 hr, then dissected and embedded in paraffin. Immunohistochemistry was used to detect major angiogenic biomarkers in 5- μ m lung sections. Primary monoclonal antibodies against mouse erythroblast transformation-specific (ETS) related gene (ERG, 1:50), angiogenin (1:50), insulin-like growth factor(IGF-1, 1:400), endothelin-1 (1:600), and epidermal growth factor (EGF, 1:100) were purchased from Abcam (Hong Kong, China). Primary monoclonal antibody against mouse Von Willebrand factor (vWF, 1:50) and amphiregulin (1:100) were purchased from Merck Millipore (Shanghai, China) and Santa Cruz Biotechnology, Inc (CA, USA) respectively. The PAP (peroxidase anti-peroxidase) technique was used for detection as previously described.[15,16] Sections were anonymised by coding then independently analysed by 2 observers using a Leica DM6000B microscope (Leica, Wetzlar, Germany) connected with a Leica Application Suite Version 3.6. Image-Pro Plus software was used to delineate and measure the total areas of each section showing positive staining in an objective fashion. For each biomarker three independent experiments were performed. The data were expressed as percentages of the entire areas of the lung sections showing positive staining.

In vitro angiogenesis assay

We used a well established *in vitro* angiogenesis assay (AngioKit; TCS CellWorks, Buckingham, UK)[19,20] which is based on co-culture of HUVEC over a monolayer of irradiated fibroblasts. Cultures were incubated at 37°C in a humidified atmosphere with 5% CO2. Culture medium with recombinant human IL-33 (0.1, 1.0 and 10 ng/mL, R&D Systems, Abingdon, UK) was replenished on days 4, 7, and 9. On day 11 cultured cells were fixed and vascular structures visualised by labelling with mouse anti-CD31 according to the manufacturer's instructions. Culture medium alone and recombinant human VEGF-A (R&D Systems, 10 ng/mL) served as negative and positive assay controls. The analysis software (AngioSys; TCS CellWorks) segmented the images using a grey level threshold tool to select CD31-labelled cells. The overall numbers of vascular junctions, tubules, and tubule length were determined as described previously.[19,20]

Statistical analysis

All data are reported as the mean ± SEM and analysed using InStat 2.01 software (GraphPad, San Diego, CA, USA). Between-group comparisons at specific time-points were performed using 2-way parametric ANOVA followed by a Bonferroni post test or the Student's unpaired t test. Data generated from the angiogenesis assay were analysed using Mann Whitney test. A p value <0.05 was considered significant.

Results

IL-33 increased airways vascularity

Compared with saline challenge, IL-33 induced marked airways angiogenesis as indicated by significantly elevated numbers of peribronchial, vWF^+ immunoreactive blood vessels, which appeared as early as day 20, peaked at day 48 and persisted until the end of the experiment (day70) (Figure 1). OVA challenge was associated with similar, statistically equivalent changes in vWF^+ immunoreactive vessels.

ERG is constitutively expressed by endothelial cells and is considered as another biomarker of endothelial cell numbers and angiogenesis. Immunohistochemical analysis of ERG immunoreactivity similarly confirmed that, compared with saline challenge, both OVA- and IL-33-challenge induced significant expression of ERG, which was first apparent at day 36, peaked at day 48 and persisted until the end of the experiment (day 70) (Figure 2).

IL-33 induced airways angiogenin and EGF expression

Immunohistochemical analysis showed that compared with control, saline challenge, both OVA- and IL-33-challenge of airways significantly increased the expression of immunoreactivity for lung and parenchymal and peribronchial angiogenin and EGF. Elevated immunoreactivity for angiogenin and EGF was apparent from day 24, peaked at day 48 and persisted until day 70, the end of the experiments (Figures 3 and 4).

IL-33 induced elevated airways IGF-1, endothelin-1 and amphiregulin expression

Immunoanalysis showed that IL-33 as compared with saline challenge of the airways significantly increased expression of lung parenchymal and peribronchial IGF-1, endothelin-1 and amphiregulin immunoreactivity from day 20 (endothelin-1) or day 24 (IGF-1 and amphiregulin) which persisted until the end of the experiment(Figures 5, 6 and 7). OVA challenge again induced elevated expression of these growth factors with temporal courses statistically equivalent to those induced by IL-33 challenge.

IL-33 induced human angiogenesis *in vitro*

In an in vitro angiogenesis assay, IL-33 induced statistically significant increases in the numbers, lengthens and branching of microvessels produced by HUVEC in a concentration-dependent fashion (Figure 8). The effects of IL-33 at concentration of 10 ng/mL were equivalent to those of VEGF at the same concentration, employed as a positive control.

DISCUSSION

Angiogenesis, a feature of airways remodelling in asthma, can be defined as the emergence of new blood vessels from pre-existing vasculature. Although pathophysiological significance of this change still needs to be further explored, increased blood flow and permeability in asthmatic lung might contribute to oedema and local inflammation because neo-angiogenesis provides prerequisite for extravasation of inflammatory cells into local tissue. Some studies have shown that vascularity is associated with asthma severity.[1,3,4-6] Previous investigations have also suggested that angiogenesis in asthmatics is closely related to the local expression of angiogenic factors. [6,19, 21] IL-33 and its ST2 receptor are expressed in the endothelial cells of human veins and arteries.[22] In human endothelial cells, IL-33 induced inflammatory activation as evidenced by increased vascular permeability, increased production of inflammatory cytokines and the stimulation of angiogenesis.[13.23] The data from the present study confirm that IL-33 has the capacity to induce angiogenesis *in vivo* and to increase local expression of multiple angiogenic growth factors in the epithelium and submucosal tissues of the airways in an established IL-33-induced allergen- independent murine surrogate of asthma. These changes were comparable to those induced by conventional OVA challenge of IgE-sensitised animals, a surrogate of allergic asthma, and significantly more marked with some analytes, particularly at the peak time point of expression at day 48. Furthermore, using an *in vitro* model of human angiogenesis, we have showed that IL-33 effects concentration-dependent microvessel formation, by endothelial cells in a manner comparable to that of VEGF,

a well established angiogenic agent.

Angiogenesis is a complex process, likely regulated by a variety of both discrete and interacting local mechanisms. ERG, expressed throughout the life of the endothelium, appears to contribute to angiogenesis by regulating vascular homeostasis, vascular integrity and endothelial cell growth.[24, 25] Inhibition of ERG in human umbilical vein endothelial cells causes loss of cell-cell contact and attenuation of microvessel formation.[26] Angiogenin also appears to play a fundamental, if ill-defined role in vascular growth.[26,27] Amphiregulin is a potent mitogen for several types of cell, including epithelial cells, vascular smooth muscle cells and endothelial cells acting via the epidermal growth factor receptor (EGFR).[28] In addition, both innate and adaptive immune cells are able to express this molecule, suggesting that amphiregulin might play a role in orchestrating immunity, inflammation and tissue repair.[29] Interestingly, IL-33 can induce type 2 innate lymphoid cells to produce amphiregulin and Th2-type cytokines *in vitro*.[30] Endothelin-1 is a potent vasoconstrictor which may also cause vascular remodelling, cellular proliferation, extracellular matrix production and capillary permeability.[31,32] As a multifunctional vascular protein vWF has been implicated in the regulation of angiogenesis in many diseases from cancer to atherosclerosis.[33] EGF promotes cellular proliferation and is involved in embryogenesis, angiogenesis and cellular differentiation.[34] IGF-1 plays an important role in the physiology of endothelial cell structure and function by promoting migration, microvessel formation and production of VEGF and the vasodilator nitric oxide.[35]

Because angiogenesis is such a complex process, the question of how IL-33 regulates angiogenesis directly or indirectly through effects on expression of these angiogenic factors in asthma and other diseases remain to be clarified. Recent studies indicate that IL-33 might contribute to the pathogenesis of angiogenesis-dependent and inflammatory vascular diseases by promoting production of endothelial NO and a range of additional mediators including IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesionmolecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and endothelial E-selectin.[13, 23,36] IL-33 is constitutively expressed in the nuclei of vascular endothelial cells in various human tissues, with the apparent exception of tumor vessels.[37] In line with these studies, we here show that IL-33 directly promotes angiogenesis *in vitro*, and *in vivo* in the setting of asthma-like inflammation of the airways, directly and/or indirectly by up-regulating a range of pro-angiogenic factors. Whatever the case, IL-33 may be a key molecular target for the inhibition of neo-angiogenesis and its consequences in the setting of asthma.

Acknowledgements

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Conflicts of interest

The authors declare no conflicts of interest.

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Figure legends

Figure 1: IL-33 increased airways vWF immunoreactivity.

A: Representative photomicrographs of vWF immunoreactive blood vessels in lung sections from saline (NS)-, OVA- and IL-33-challenged mice at various time points as indicated (light microscopy, ×200). **B:** Quantitative analysis of numbers of vWF^{+} blood vessels per unit area of lung sections. Data are expressed as mean ± SEM (n = 5 mice in each group at each time-point). *P < 0.05.

Figure 2: IL-33 increased airways ERG expression.

A: Representative photomicrographs of ERG immunostaining in lung sections from saline (NS)-, OVA- and IL-33-challenged mice at various time points as indicated (light microscopy, ×400). **B:** Data are expressed as mean ± SEM (n = 5 mice in each group at each time-point). *P < 0.05.

Figure 3: IL-33 increased airways angiogenin expression.

A: Representative photomicrographs of angiogenin immunoreactivity in lung sections from saline (NS)-, OVA- and IL-33-challenged mice at various time points as indicated (light microscopy, ×200). **B:** Data are expressed as mean ± SEM (n = 5 mice in each group at each time-point). *P < 0.05.

Figure 4: IL-33 increased airways EGF expression.

A: Representative photomicrographs of EGF immunoreactivity in lung sections from

saline (NS)-, OVA- and IL-33-challenged mice at various time points as indicated (light microscopy, ×200). **B:** Data are expressed as mean ± SEM (n = 5 mice in each group at each time-point). *P < 0.05.

Figure 5: IL-33 increased airways IGF-1 expression.

A: Representative photomicrographs of IGF-1 immunoreactivity in lung sections from saline (NS)-, OVA- and IL-33-challenged mice at various time points as indicated (light microscopy, ×200). **B:** Data are expressed as mean ± SEM (n = 5 mice in each group at each time-point). *P < 0.05.

Figure 6: IL-33 increased airways endothelin-1 expression.

A: Representative photomicrographs of endothelin-1 immunoreactivity in lung sections from saline (NS)-, OVA- and IL-33-challenged mice at various time points as indicated (light microscopy, ×200). **B:** Data are expressed as mean ± SEM (n = 5 mice in each group at each time-point). *P < 0.05.

Figure 7: IL-33 increased airways amphiregulin expression.

A: Representative photomicrographs of amphiregulin immunoreactivity in lung sections from saline (NS)-, OVA- and IL-33-challenged mice at various time points as indicated (light microscopy, ×200). **B:** Data are expressed as mean ± SEM (n = 5 mice in each group at each time-point). *P < 0.05.

Figure 8: IL-33 induced angiogenesis *in vitro*.

Top panel: representative light photomicrographs (4x original magnification) show formation of primitive vascular tubule structures by human vascular endothelial cells after 11 days of culture with medium control, VEGF (10 ng/mL) and IL-33 (0.1, 1.0 and 10 ng/mL). Bottom panel: computer-assisted quantification of mean total numbers of tubules, mean total tubule lengths and mean numbers of branch points. Bars show the mean ± SEM of three separate experiments performed in duplicate. *P < 0.05.