Proof-of-concept clinical trial of etokimab shows a key role for IL-33 in atopic dermatitis pathogenesis

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Abstract

Targeted inhibition of cytokine pathways provides opportunities to understand fundamental biology *in vivo* in humans. The IL-33 pathway has been implicated in the pathogenesis of atopy through genetic and functional associations. We investigated the role of IL-33 inhibition in a first-in-class phase 2a study of etokimab (ANB020), an IgG1 anti-IL-33 monoclonal antibody, in patients with atopic dermatitis (AD). Twelve adult patients with moderate-to-severe AD received a single systemic administration of etokimab. Rapid and sustained clinical benefit was observed with 83% achieving Eczema Area and Severity Index (EASI)50, and 33% EASI75, with reduction in peripheral eosinophils at day 29 post-administration. We noted significant reduction in skin neutrophil infiltration after etokimab compared to placebo upon skin challenge with house dust mite, reactivity to which has been implicated in the pathogenesis of atopic dermatitis. We showed that etokimab also inhibited neutrophil migration to skin interstitial fluid *in vitro*. Besides direct effects on neutrophil migration, etokimab revealed additional unexpected CXCR1-dependent effects on IL-8-induced neutrophil migration. These human *in vivo* findings confirm an IL-33 upstream role in modulating skin inflammatory cascades and define the therapeutic potential for IL-33 inhibition in human disease, including atopic dermatitis.

Introduction

Targeted cytokine pathway intervention has been associated with enormous therapeutic advance (*1*). In addition, studying the sequelae of such approaches has shed light on basic biological and disease mechanisms. For example, TNF-inhibition in the treatment of psoriasis is highly effective, yet smoldering inflammation in some patients can require IL-17A/IL-23 pathway inhibition for greatest therapeutic efficacy. Furthermore, TNF-inhibition can occasionally associate with pustular psoriasis, or the clinical onset of multiple sclerosis (MS) and isolated demyelinating diseases, such as optic neuritis. Further studies on IL-17A inhibition have highlighted the complexities of shared and unique tissue pathotypes (*2, 3*) and more recently it has been described how IL-6 controls visceral adipose tissue mass (*4*). All these findings provide examples of unforeseen biological activities obtained from cytokine targeted therapeutic interventions in man, but also provide clues for novel therapeutic options.

Among the new wave of cytokines involved in the pathogenesis of inflammatory diseases, IL-33 has emerged a key candidate to control atopic disorders (*5*). IL-33 is an alarmin cytokine produced by a number of different cell types including epithelia, endothelia, fibroblasts, and haematopoietic cells including mast cells, neutrophils, monocytes, macrophages, and dendritic cells. IL-33 lacks a secretory signal peptide, an IL-1 family trait, and is found preformed in the nucleus under steady state conditions. IL-33 is rapidly released from damaged cells in response to stress conditions such as infection, injury, and inflammation (*5*). By binding to its heterodimeric receptor comprising ST2 and interleukin-1 receptor accessory protein (IL-1RAcP), IL-33 signals through MyD88, IRAK1, and IRAK4 with downstream activation of NF-κB or MAPK pathways (*5*). Altered gene expression, use of a decoy receptor, and post-translational modifications (for example, proteolytic processing and oxidation) all contribute to regulation of the IL-33 pathway (*5, 6*).

The IL-33:ST2 pathway contributes to inflammation associated with disorders including AD (*7*), asthma (*8*), inflammatory bowel disease (IBD) (*9*), and arthritis (*10, 11*). The most studied effects

of IL-33 relate to the initiation of innate and adaptive type 2 immune responses, characterized by the production of IL-4, IL-5, and IL-13. IL-33 administration to mice induces type 2 cytokines, serum IgE, eosinophilia, T helper 2 (Th2) and group 2 innate lymphoid cells (ILC2) accumulation and activation, epithelial cell hyperplasia, excessive mucus production, and smooth muscle contraction (*12, 13*). IL-33 over-expression in the skin leads to an AD-like phenotype with infiltration of ILC2, mast cells, and eosinophils (*14*). Rare loss-of-function mutations in the IL-33 pathway associate with reduced blood eosinophils and protection from asthma (*15*). Direct administration of IL-33 to murine skin leads to an infiltration of mast cells and neutrophils (*16*). IL-33 also drives neutrophil migration to sites of infection (*17, 18*), and contributes to their activation (*19, 20*). Furthermore, IL-33 can be cleaved into active forms by neutrophil elastase and cathepsin G (*21*) implicating an inflammatory cycle. IL-33 is known to modulate the inhibitory effects of bacterial infection on the neutrophil IL-8 receptor CXCR2 in animal models, but whether IL-33 can modulate IL-8 effects in human inflammation *in vivo* has not been studied, nor whether there is an impact of IL-33 on the other known IL-8 receptor, CXCR1 (*17, 18, 22*).

AD is a common inflammatory pruritic skin disease affecting up to 20% of children and 5-10% of adults, and is associated with atopic asthma, allergic rhino-conjunctivitis, and food allergy (*23*). Approximately 80% of affected individuals have elevated IgE specific to airborne allergens, including house dust mite, pollens and animal dander. Moderate-to-severe disease accounts for approximately 20-30% of cases and associates with a major impact on quality of life (*24*). Treatment requires optimisation of topical therapies, but lack of response to topical medications can necessitate the use of systemic approaches such as methotrexate, ciclosporin, azathioprine, and more recently, targeted inhibition of inflammatory pathways, such as IL-4Rα antagonism (*25*). The decision to use a systemic approach for treatment can be based on assessment of severity and quality of life while considering safety and efficacy, general health status, psychologic burden, and patient choice (*26*). Despite recent advances, there remains an unmet need for additional safe and efficacious treatments for AD. Here, we undertook a first-in-class phase 2a clinical trial of anti-IL-33 (etokimab) in adult patients with atopic dermatitis and investigated underlying mechanisms.

Results

We performed a phase 2a proof-of-concept study of a single intravenous 300mg dose of etokimab, a humanized IgG$_1$/kappa anti-IL-33 monoclonal antibody in twelve adult patients with moderate-to-severe AD (Fig. 1). Seven days prior to etokimab, subjects received a placebo injection. In order to understand the mechanistic role of IL-33 in atopic skin inflammation, we also sampled skin after placebo and etokimab administration. Skin suction blisters induce a split at the dermo-epidermal junction, and so provide an opportunity to investigate mechanisms of human sterile tissue inflammation, including responses to relevant antigens following skin challenge. We raised skin suction blisters, thirty minutes after saline or house dust mite (HDM) intra-epidermal skin challenge, and sampled the blister contents 24 hours later.

Safety

Etokimab was generally well-tolerated. Treatment-emergent adverse events are detailed in Table 1. Most adverse events were mild and transient, and none were thought to be etokimab-related. One participant had a transient mild neutropenia on day 8 which recovered by day 11, with no associated clinical sequelae. There were no other decreases in blood neutrophils outside the reference range throughout the study. There were no new episodes of conjunctivitis. One patient had pre-existing conjunctivitis and this did not exacerbate during the study. One patient had secondarily infected AD starting at day 76 post-etokimab which responded to oral antibiotics. The patient had previously required repeated courses of antibiotics pre-study for recurrently infected AD. One patient had an upper respiratory infection starting on day 139 post-administration which resolved without needing oral antibiotics. Two patients had a urinary tract infection starting on day 35 and day 132 post-administration which both resolved with oral antibiotics. One participant had a serious adverse event of exacerbation of pre-existing depression which started at day 140 post-etokimab and was potentially linked to concurrent immediate family serious illness. This exacerbation of depression resolved and in view of concurrent events, and timing from study drug administration, was not considered related to the study drug.

6

Efficacy

All twelve patients achieved at least EASI50 (defined as 50% improvement in EASI score from baseline) during the study following a single dose of etokimab. By 29 days after etokimab administration, we noted a marked improvement in EASI score with 83.3% of patients showing EASI50 and 33% showing EASI75, with a mean EASI improvement of 61% (P<0.0001, Fig. 2A-C). Even 15 days after etokimab, we had observed a significant improvement (mean 58% EASI decrease, P<0.01) compared to baseline. Responses were sustained with 75% showing EASI50 and 42% showing EASI75 at day 57 post-administration, with a mean EASI improvement of 62% (P<0.001). There were significant improvements in SCORAD at day 29 with 40% reduction (P<0.001, Fig. 2D). Three (25%) patients reached an IGA (Investigator Global Assessment) of 0/1 during the study (Fig. 2E). The objective clinical outcomes were associated with significant improvement in DLQI (Dermatology Life Quality Index, P<0.05, Fig. 2F) and 5D (5 Domain) Itch scores (P<0.001, Fig. 2G). Topical corticosteroid rescue therapy was negligible throughout the duration of the study. ~~We noted that patients responded irrespective of pre-study eosinophil frequency, moderate or severe disease or preceding use of systemic therapies.~~

Pharmacodynamics

We investigated the *in vivo* pharmacodynamic (PD) effects in the setting of disease using whole blood stimulation with IL-33/IL-12, and measurement of IFNγ production by ELISA. As shown in Fig. 3A, the inhibition of IFNγ production was rapid and striking, and was observed to extend beyond 57 days in all patients and to beyond 120 days in some individuals (Fig. 3A).

The EASI score pre-treatment showed a significant correlation with peripheral eosinophil percentage (r=0.623, P<0.0001, fig. S1). There was a significant reduction in peripheral eosinophil absolute counts at day 29 post-administration (mean 40% reduction, P<0.05, Fig. 3B) and eosinophil percentage count throughout the study correlated with EASI score (r=0.3419, P<0.001, Fig. 3C). It is noteworthy that in some cases, the decline in eosinophils was substantial; three patients had peripheral eosinophil percentages at 13% or above at study entry, and all three had

a decrease in eosinophils to 8% or less by day 15. There was no significant change in serum total IgE during the study (mean total serum IgE at study entry 10101±7594 kU/L; day 29 post-etokimab 9954±7394 kU/L; day 140 post-etokimab 10321±8103 kU/L).

Skin suction blisters induce neutrophilic infiltration at 24 hours

We noted infiltration of leukocytes into skin suction blisters sampled 24 hours after saline skin challenge, which was significantly enhanced after HDM challenge (P<0.05; Fig.4A). Granulocyte infiltration was reduced in response to skin suction blister formation with saline challenge after etokimab compared to that observed after placebo (mean 37% reduction, P=0.05, Fig. 4A-C). Granulocyte infiltration into the skin showed no significant difference in response to skin suction blister formation with HDM challenge after etokimab compared to that observed after placebo (mean 30% reduction, P=0.13, Fig. 4C). On further characterization of the infiltrating leukocytes 24 hours after HDM challenge as a model of antigen-specific inflammation (Fig. 4D), several immune sub-populations were detected in the blisters (fig. S2). We noted that the most profound effects of etokimab to be that of a significant inhibition of infiltration of neutrophil granulocytes in response to HDM which was reduced after etokimab compared to that observed after placebo (P<0.05, Fig. 4E).

Etokimab inhibits IL-33-induced neutrophil migration to skin interstitial fluid *ex vivo*

In order to investigate whether skin interstitial fluid can induce neutrophil migration *ex vivo*, we measured cell migration from whole blood of healthy donors using transwell IncuCyte assays, and measured neutrophil activation through shape change and elastase production. Skin interstitial fluid induced rapid neutrophil migration within 1 hour, with accompanying shape change, and neutrophil elastase production (Fig. 5A-C). Given the clinical trial data implicating a key role for IL-33 in granulocyte migration *in vivo*, we next tested whether etokimab could modulate neutrophil migration *ex vivo*. We first investigated whether peripheral blood neutrophils expressed ST2 and IL-1RAcP, and observed a mean of 40% expression of ST2 and 100% expression of IL-1RAcP (Fig. 6A). We next used the transwell assay to show that IL-33 was able to induce significant neutrophil migration (P<0.05, Fig. 6B). We observed that etokimab

disrupted the detection of IL-33 in the culture, and therefore took advantage of an enzyme linked immunosorbent assay (ELISA) to determine a concentration range of etokimab to investigate further (Fig. 6C). 5µg/ml etokimab significantly (P<0.01) inhibited detection of IL-33, and significantly reduced neutrophil migration towards IL-33 (P<0.05, Fig. 6D). These data suggested that IL-33 is a relevant skin chemoattractant for neutrophils, and that biologic therapeutic targeting is able to effectively inhibit neutrophil migration both *in vivo* and *ex vivo*.

Etokimab inhibits IL-8-induced neutrophil migration to skin interstitial fluid *ex vivo*

As part of our panel of controls from the previous experiment, we made an unexpected observation, namely that etokimab could also inhibit migration of etokimab-pre-treated neutrophils towards IL-8 (Fig. 7A). We firstly confirmed that etokimab did not bind IL-8 (fig. S3), and so proceeded to investigate the underlying mechanisms. In contrast to when neutrophils from healthy controls were pre-treated with etokimab, there was no inhibition of migration towards IL-8 if the neutrophils were not pre-treated with etokimab (Fig. 7B). Furthermore, we noted that etokimab only partially inhibited neutrophil migration towards skin interstitial fluid (Fig. 7C). In trying to reconcile these findings, we reasoned that other chemo-attractants beyond IL-33 were likely to be present in skin blister fluid *in vivo* and that etokimab had a direct effect on neutrophils during the pre-treatment phase. We were able to examine the concentrations of IL-8 in the saline interstitial fluid of 4 patients before and after etokimab administration, but we were not able to detect a significant difference (Fig. 7D). Treatment of neutrophils with etokimab did not significantly reduce IL-8 production by neutrophils in response to skin interstitial fluid (Fig. 7E). Finally, we were able to show that etokimab was able to abrogate neutrophil migration toward skin interstitial fluid if the neutrophils were pre-treated with etokimab (Fig. 7F). These data confirmed that etokimab was able to inhibit IL-8-induced migration of neutrophils through inhibiting an initial priming of IL-8 sensitivity.

Etokimab inhibits IL-33-induced neutrophil autocrine expression of CXCR1

We next investigated the underlying mechanisms of the effects of etokimab in modulating neutrophil sensitivity to IL-8. In mice, IL-33 has been shown to reduce the TLR-induced down-regulation of neutrophil CXCR2, but was not found to increase CXCR2 expression or affect CXCR1 (*17, 18, 22*). However, in contrast we found IL-33 was able to induce CXCR1 expression by human neutrophils and that this was inhibited by etokimab in all neutrophils (Fig. 8A, fig. S4A). CXCR2 was expressed by the majority of neutrophils, but there was no effect of etokimab on CXCR2 expression (Fig. 8A). Etokimab inhibited the effects of autocrine production of IL-33 on CXCR1 expression in a dose-dependent manner (Fig. 8B). When CXCR1 expression remained low, the neutrophils had reduced IL-8 sensitivity (Fig. 8C). The soluble IL-33 decoy receptor sST2 has been used in treating allergic diseases in model systems (*27*). We tested whether sST2 exhibited similar effects compared to etokimab in regulating neutrophil migration. Notably, sST2 failed to abrogate the migration of neutrophils toward IL-8, and did not reduce the CXCR1 expression on neutrophils (fig. S4B-C). This may reflect differential binding affinity or neutralisation capacity of the sST2 compared to etokimab; for example, it is of note that the affinity of etokimab for IL-33 is very high (K_d 1pM) whereas that for sST2 is 4nM (*28*). Overall, these data suggested that etokimab can inhibit an autocrine IL-33 response of neutrophils to skin interstitial fluid, which further modulates responsiveness to IL-8 via CXCR1.

Discussion

Here we capitalized on an early experimental medicine phase 2a trial of a first-in-class anti-IL-33 biologic that provided significant clinical benefit to AD patients, to understand mechanisms of disease and identify non-redundant pathways of inflammation. Suction blisters taken over saline- or HDM-challenged skin were found to provide a useful *in vivo* model inflammatory system in humans to explore efficacy and to identify previously unrecognized immune networks in skin. Furthermore, the interstitial fluid could be used *ex vivo* to define underlying mechanisms. We showed that IL-33 acts upstream in an inflammatory cascade; neutrophil migration can be directly modulated through IL-33 blockade, but also indirectly through inhibitory effects on the IL-8 pathway. Eosinophil reduction in the blood of etokimab-treated patients further supports the broad effects of IL-33 inhibition, as predicted by genetic analysis of IL-33 loss-of-function in humans (*15*). IFNγ has been implicated in the pathogenesis of chronic AD (*29, 30*). The significant and sustained inhibition of IFNγ production by whole blood in response to *ex vivo* IL-33/IL-12 suggests a prolonged *in vivo* pharmacodynamic effect in the setting of disease. Furthermore, the skin neutrophil observations associated with clinical response, confirming *in vivo* relevance of the findings. A single administration of etokimab was well-tolerated with most adverse effects being mild and transient. A single SAE occurred during the course of the current study in which there was exacerbation of pre-existing depression day 140 after etokimab administration, and was during a major family illness. The timing of the SAE (20 weeks post-dosing) and the concurrent events suggested it was unlikely to be study drug related. There were no new cases of conjunctivitis or exacerbations of previous conjunctival disease.

Specifically, we showed that skin interstitial fluid induces neutrophil migration and activation which can be inhibited by etokimab *ex vivo*. IL-33 acts independently of microbial co-factors to upregulate CXCR1 which sensitizes human neutrophils for migration to IL-8. Although excellent previous studies have demonstrated *in vitro* and in murine models that IL-33 is involved in neutrophil migration (*17, 18, 22*), the translation here to human tissue immunology shows that IL-33 plays a dominant role and effects are in part likely to be mediated instead by CXCR1.

Therefore, specific IL-33 therapeutic intervention is not targeting a redundant system, and is worthy of further investigation.

Although marked elevation of skin neutrophils is not a typical conspicuous feature of AD (*31*), there is evidence that they may nevertheless contribute to the inflammatory process (*32, 33*), particularly during acute disease if secondary infection is present (*31*). Neutrophilic infiltration to the skin may also be part of the response to HDM challenge in healthy controls, suggesting this may be a useful human *in vivo* tissue model system for wider mechanistic and therapeutic evaluation. It is likely that the clinical benefits of etokimab are related to effects beyond neutrophils and eosinophils. As well as highlighting indications amongst classical neutrophilic disease, the study therefore prompts further evaluation of the mechanistic effects of etokimab on broader pathways potentially associated with clinical efficacy, for example type 2 immune responses. Sterile neutrophilic inflammation is a feature of a wide spectrum of skin pathology including disorders such as plaque psoriasis, generalized pustular psoriasis, Sweet's syndrome, SAPHO syndrome (synovitis, acne, pustulosis, hyperostosis, and osteitis), pyoderma gangrenosum, Bechet's disease, hidradenitis suppurativa, and other auto-inflammatory diseases. We aimed to take advantage of the sub-epidermal plane of separation during skin suction blister formation to minimize the role of infection in confounding the inflammatory model. In contrast, conventional skin biopsies will include commensal organisms within the epidermis which may complicate interpretation. Furthermore, the skin suction blisters provide access to skin cells and interstitial fluid without digestion, which facilitates the development of *ex vivo* assays for investigation of underlying mechanism.

There are a number of limitations of the study, in particular that there is no separate placebo group for the long-term clinical efficacy data. However, since all treated patients showed at least fifty percent improvement in their EASI scores relative to baseline after a single dose, and changes in blood and skin biomarkers are consistent with observed efficacy, this study demonstrates an etokimab-specific drug effect. Although difficult to directly compare, it is noteworthy that placebo effects in studies with similar EASI scores at entry are typically in the

range of 10-20% EASI improvement by day 29 when topical steroids are used as rescue therapy (*25, 34*). In addition, the number of participants was small in the current experimental medicine proof-of-concept study, but we were able to explore *in vivo* skin neutrophil findings in mechanistic studies using additional human skin material *ex vivo*. Skin suction blisters take considerable participant involvement, but do offer the possibility of sampling live, unmanipulated cells from human challenged tissue.

Several targeted systemic therapies are now emerging based on the growing understanding of the involvement of type 2 immune polarization in atopic pathology (*35*). Another keratinocyte-derived alarmin, thymic stromal lymphopoietin (TSLP), which acts upstream of type 2 responses, is also considered a potential therapeutic target in the treatment of AD (*36-38*). In murine models, either IL-33 or TSLP alone is sufficient to promote the development of AD-like inflammation (*39*). Tezepelumab, an anti-TSLP humanized monoclonal antibody, has undergone a randomized phase 2a clinical trial in the treatment of patients with moderate to severe AD (*40*). ~~A positive trend of improvement on EASI score was observed after subcutaneously administered Tezepelumab plus topical corticosteroids (TCS) compared to placebo plus TCS.~~ Multiple anti-IL-33 antibodies have been developed and reported in mouse disease models (*41-44*). Peng and colleagues established the efficacy of an anti-mouse IL-33 antibody on the treatment of 2, 4-dinitrochlorobenzene (DNCB)-induced dermatitis murine model, with an improved phenotype coupled with impaired eosinophils and mast cells skin infiltration and decreased serum IgE concentrations (*41*). Although anti-mouse IL-33 antibodies show encouraging results, there have been no published studies thus far in humans. Clinicaltrials.gov lists anti-IL-33 clinical trials in patients with asthma, food allergy, chronic rhinosinusitis, chronic obstructive airways disease, and an anti-ST2 clinical trial in patients with chronic obstructive airways disease [accessed June 6th 2019]. These findings confirm significant interest in the pathway.

Experimental medicine studies provide powerful means to identify unexpected pathways and explore efficacy in inflammatory systems *in vivo*. These can be useful early signals in therapeutic development and can help identify new priority therapeutic areas. Most of the focus for IL-33

pathway inhibition has been on the type 2 cytokine axis, because of significant supporting genetic and functional data in both human and murine systems. Our findings might also provide a further dimension to explain how etokimab, by inhibiting IL-33, could provide such a rapid and persistent clinical benefit as described in this report. Furthermore, these findings open new therapeutic possible applications of etokimab to sterile neutrophilic disease as well as other inflammatory diseases with a clear neutrophilic involvement such as neutrophilic asthma. This first-in-class experimental medicine study has shown that *in vivo* in human tissue that IL-33 has key upstream effects which broadly influence different and relevant inflammatory cascades and thus widen the potential of this treatment to a larger than anticipated group of diseases.

Materials and Methods

Study design

The study was based on a phase 2a proof-of-concept study of a single unblinded intravenous 300mg dose of etokimab, a humanized IgG_1/kappa anti-IL-33 monoclonal antibody previously referred to as ANB020 (EudraCT 2016-002539-14) (*45*) ~~(US patent #10,059,764, Horlick *et al.*)~~, in twelve adult patients with moderate-to-severe AD. The clinical study was accompanied by contralateral skin suction blister analysis five days after placebo administration and then five days after etokimab administration. The protocol was written by the Sponsor (AnaptysBio, Inc.). The inclusion and exclusion criteria are provided in the Supplementary Materials, and a study summary is presented in Fig. 1. Pre-specified pharmacodynamic endpoints were changes in skin suction blister contents, and differential white blood cell counts. Main clinical endpoints were scores for Eczema Area and Severity Index (EASI), Investigator's Global Assessment (IGA), Severity scoring of atopic dermatitis (SCORAD), DLQI, and 5D Pruritus Score. Primary safety and tolerability end-points were: measure of etokimab inhibition of cytokine release (IFNγ) in an *ex vivo* test, immunogenicity to etokimab (anti-drug antibodies), potentially significant and clinically important AEs, SAEs, AEs of special interests, and AEs leading to withdrawal, physical examinations, vital signs, clinical safety laboratory tests (hematology, biochemistry, and urinalysis), electrocardiogram (ECG). Primary data are reported in data file S1.

Patients

Patients were adults diagnosed with AD based on Hanifin/Rajka criteria and recruited through a secondary/tertiary care hospital setting (Oxford University Hospitals NHS Foundation Trust). Ethical approval was given by the London-Hampstead Research Ethics Committee (16/LO/1959) and MHRA (46837/0001/001-0001) with EudraCT 2016-002539-14. Patients had an EASI>=14 at screening and a positive skin prick test to HDM. Patients were excluded if they had received systemic treatment for AD within 4 weeks of recruitment. Patients were permitted to continue using their existing emollients without change during the course of the study. Except for the skin

suction blister site, topical corticosteroids (mometasone furoate 0.1% ointment for body, hydrocortisone 2% ointment for face) were permitted in limited amounts as rescue therapy, and amounts were recorded by weight. Full inclusion and exclusion criteria and patient characteristics are presented in the Supplementary Materials.

Neutrophil isolation and culture

Peripheral blood and skin of healthy volunteers and patients with AD who were not part of the clinical trial were collected under local ethics approval [Oxford C, 09/H0606/71, National Research Ethics Service (NRES) Committee South Central]. Peripheral blood was subject to Lymphoprep (Stem Cell Technologies) isolation according to manufacturer's instructions. The lower layer was harvested and contained granulocytes and erythrocytes. The cell suspension was treated with red blood cell lysis buffer (Biolegend) for 10 minutes and neutrophils were isolated using CD16 microbeads (Miltenyi Biotec) following manufacturer's instructions and resuspended in RPMI medium. The neutrophils were immediately used in the migration assays or stained for flow cytometry analysis. For surface marker staining, neutrophils (2-5 ×10^5 cells) were treated with IL-33 (Biolegend, 50 ng/ml) for 2 hours, or with Etokimab (5 µg/ml) for 1, 2, or 3 hours.

Transwell migration assay

HTS-Transwell-96 Well Plates with 5-µm pore filters (Corning Costar) were used in the chemotaxis assay. The upper chambers were loaded with 1.5-2 ×10^5 whole blood cells or neutrophils from healthy donors in 50-100 µl of RPMI, and either treated with isotype control or Etokimab at different concentrations for 1 hour at 37°C or left untreated. The lower chambers were loaded with 150-250 µl of diluted (6x in RPMI) blister fluid or RPMI containing 10% fetal calf serum (FCS) and IL-33 (Biolegend, at 0, 12.5, 25, 50, 100, or 200 ng/ml concentration) or IL-8 (Biolegend, 50 ng/ml) with or without Etokimab (5 µg/ml) or human sST2-Fc IgG1 chimera (700 ng/ml, R&D). The IL-33 ELISA in presence of etokimab was undertaken in RPMI containing 10% FCS. Following coculture for 1 hour, the upper chambers were removed, and the plates were subjected to scanning (IncuCyte S3 Live-Cell Analysis System, Sartorius). The confluency of the cells was

determined using IncuCyte S3 Software (Sartorius) and calculated against the confluency of cells of known cell counts.

Antibodies and flow cytometry (FACS)

All stainings were performed at 4 °C in FACS staining buffer (0.5% FCS in PBS). The following anti-human antibodies were used: PerCP anti-human CD19 (H1B1, Biolegend), PerCP anti-human CD11b (M1/70, Biolegend), PerCP anti-human FcεRIα (AER-37(CRA-1), Biolegend), PerCP anti-human CD14 (HCD14, Biolegend), Alexa Fluor 700 anti-human CD45 (HI30, Biolegend), Brilliant Violet 650 anti-human CD3 (OKT3, Biolegend), PE anti-human CRTH2 (BM16, Miltenyi Biotec), Brilliant Violet 785 anti-human CD127 (A019D5, Biolegend), Brilliant Violet 605 anti-human CD56 (HCD56, Biolegend), Alexa Fluor 488 anti-human CD16 (3G8, Biolegend), PE/Cy7 anti-human CD123 (6H6, Biolegend), PE-CF594 anti-human CD15 (HI98, BD Biosciences), Brilliant Violet 421 anti-human CCR3 (5E8, Biolegend), APC/Cy7 anti-human CD11c (Bu15, Biolegend), APC anti-human CD1a (HI149, BD Biosciences), Brilliant Violet 650 anti-human CD16 (3G8, Biolegend), FITC anti-human ST2L (B4E6, MD Bioproducts), and LIVE/DEAD Fixable Aqua Dead Cell Stain (ThermoFisher). Human CXCR1 Antibody (42705, R&D system) and human CXCR2 Antibody (48311, R&D system) were conjugated in house with Alexa Fluor 488 and Alexa Fluor 647 respectively using Antibody Labeling Kits from ThermoFisher. Flow data were acquired using FACSDiva software on LSRFortessa (BD Biosciences) and further analyzed with FlowJo (Tree Star LLC) software.

ELISA

Samples were stored at -80 °C and concentrations of elastase (ThermoFisher), IL-8 (ThermoFisher), and IL-33 (R&D) were measured by sandwich ELISA according to manufacturers' instructions. According to the manufacturer, the IL-33 ELISA detects the oxidized form of IL-33, and it is not known if it detects the reduced form.

PMN cell shape analysis

Images of cell were taken using IncuCyte. Images were opened using Fiji and then inverted. Next, the 'Find Maxima' plugin was applied with a noise tolerance parameter of '160' with the output set to be as 'Segmented Particles'. From this output the "Analyze Particles" plugin was applied, and objects detected within the size range of 20-200 and with a circularity index $(4*pi(area/perimeter^2))$ of between 0.2-1.00. This filtered out any artifacts or debris which were too small, or too large to be cells. From the detected cell regions, the circularity index was measured and output in a list for subsequent analysis.

Statistical analysis

It was not possible to use power calculations in this discovery proof-of-concept study. For primary and secondary continuous endpoints, change from baseline were evaluated. Mixed effect analysis of covariance was used to assess treatment effects. All tests of treatment effects were conducted at a 2-sided alpha of 0.05 or with 2-sided 95% confidence intervals (CIs). For safety and tolerability, AEs, SAEs, vital signs, physical examinations, ECGs, and clinical laboratory assessments were evaluated. Values in *ex vivo* assays are shown as mean$\pm$ standard deviation (SD). Paired t tests, one-way or two-way ANOVA were performed using GraphPad Prism version 7.00 (GraphPad Software) to assess statistical significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; and ****$P < 0.0001$.

Condition	Patient n(%)	Condition	Patient n(%)
Nervous system disorders	4 (33.3)	Musculoskeletal and connective tissue disorders	3 (25.0)
Dizziness	1 (8.3)	Back pain	1 (8.3)
Headache	3 (25.0)	Bursitis	1 (8.3)
Migraine	1 (8.3)	Pain in extremity	1 (8.3)
Paraesthesia	1 (8.3)	Respiratory, thoracic and mediastinal disorders	2 (16.7)
Presyncope	0	Cough	1 (8.3)
Infections and infestations	4 (33.3)	Nasal congestion	1 (8.3)
Upper respiratory tract infection	2 (16.7)	Oropharyngeal pain	0
Urinary tract infection	2 (16.7)	Vascular disorders	1 (8.3)
Skin infection	1 (8.3)	Hot flush	1 (8.3)
Injury, poisoning and procedural complications	3 (25.0)	Hypertension	0
Accident	1 (8.3)	Peripheral coldness	0
Arthropod bite	1 (8.3)	Psychiatric disorders	2 (16.7)
Clavicle fracture	1 (8.3)	Depression	1 (8.3)
Contusion	0	Stress	1 (8.3)
General disorders and administration site conditions	3 (25.0)	Skin and subcutaneous tissue disorders	2 (16.7)
Peripheral swelling	2 (16.7)	Urticaria	2 (16.7)
Chest pain	1 (8.3)	Ear and labyrinth disorders	1 (8.3)
Fatigue	1 (8.3)	Tinnitus	1 (8.3)
Infusion site pain	0	Gastrointestinal disorders	1 (8.3)
Investigations	3 (25.0)	Dyspepsia	1 (8.3)
Haemoglobin decreased	1 (8.3)	Nausea	1 (8.3)
Monocyte count decreased	1 (8.3)	Vomiting	1 (8.3)
Neutrophil count decreased	1 (8.3)		

Table 1.

Supplementary Materials

Materials and Methods

Fig. S1. Peripheral eosinophil percentage and EASI score before treatment with etokimab

Fig. S2. Skin Blister Leukocytes

Fig. S3. Etokimab binding to IL-33

Fig. S4. Neutrophil migration and CXCR1

Table S1. Characteristics of the Patients

Data File S1

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Figure legends

Figure 1. Study design. (A) Schematic of study design. Saline was injected on the inside of the left upper arm and 0.05 μg HDM was injected on the inside of the right upper arm, four days after receiving either placebo or etokimab (300mg IV). After one hour, suction blister cups were applied to the sites of saline and HDM challenge with a vacuum pressure of 250 mmHg for 60 to 90 minutes to generate blisters. The blisters were protected overnight and interstitial fluid and cellular infiltrate assessed 24-hour post challenge by ELISA or FACS. (B) Schematic of skin blister analysis. The blister/interstitial fluid from all donors was collected and stored at -80°C and the cellular infiltrate was analysed by FACS.

Figure 2. Etokimab induces rapid and sustained improvements in disease severity scores and reported outcome measures. AD severity scores after administration of placebo on day -7 and 300mg IV etokimab on day 1. (A) EASI score relative to day -14(100%). (B) Percentage of patients reaching EASI50 and EASI75. (C) Changes in absolute EASI score. (D) Changes in absolute SCORAD score. (E) Changes in IGA absolute score. (F) Percentage changes in DLQI score. (G) Percentage changes in 5D itch score. Friedman test with Dunn's multiple comparison. n=12, showing mean $\pm$ SD. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 3. Pharmacodynamic effects after administration of placebo on day 1 and 300mg IV etokimab on day 8. (A) Whole blood from different timepoints was incubated at 37°C for 16 hours with IL-12 and IL-33 at 30 and 50ng/mL, respectively. IFNγ concentration in the supernatant was measured by ELISA and normalized to baseline timepoint (100%). Friedman test with Dunn's multiple comparison. n=12, showing mean +/- SD. (B) Peripheral blood eosinophil count (10^9/L), showing mean $\pm$ SD. (C) Correlation between eosinophil percentage and EASI score (Spearman correlation).

Figure 4. Skin suction blisters represent a model of sterile inflammation in humans. (A) Leukocytes from skin suction blisters of saline and HDM challenges were analyzed for their Forward Scatter (FSC) and Side Scatter (SSC) by FACS. One representative result is shown of

twelve donors, and overall analysis of the leukocyte numbers was plotted (right panel). (B) Leukocytes from skin suction blisters of saline and HDM challenges after etokimab administration were analyzed for their FSC and SSC by FACS. One representative result is shown of twelve donors. (C) 5 days after placebo or 300mg etokimab, skin suction blisters were samples 24 hours following saline or HDM skin challenge. Granulocytes were quantified using FACS and expressed as percentage total leukocytes. (D) 5 days after placebo, skin suction blisters were samples 24 hours following saline or HDM skin challenge. Neutrophils were quantified using FACS and expressed as percentage total leucocytes. (E) Overall analysis of the neutrophil percentage of leukocytes from skin suction blisters of saline and HDM challenges, 5 days after placebo or 300mg etokimab. n=12. Lines in the statistics plots represent the mean$\pm$SD. Boxes show 25/75 percentiles, whiskers show range. *P <0.05, t test.

Figure 5. Skin interstitial fluid induces neutrophil migration and activation ex vivo. (A) Whole blood cells (2×10^5 cells) from healthy donors were placed into the upper wells of a transwell chamber system and allowed to migrate through 5 μm pores toward the lower wells containing medium or non-autologous blister fluid at 37°C for 1 hour. The cell numbers from the lower wells were calculated and then the total cells (left panel) were collected for FACS analysis. One representative result is shown of three experiments (middle panel) and the overall numbers of migrated neutrophils was determined (right panel), n=5. Lines in the statistics plots represent the mean$\pm$SD. (B) Neutrophils (1×10^6 cells) were treated with medium or blister fluid for 24 hours. The concentration of elastase in the blister fluids before and after neutrophil culturing was measured by ELISA, n=5. (C) Images of neutrophils ($0.5-1\times10^5$ cells) were taken 1 hour after culturing with medium or blister fluid. One representative result is shown of fifteen experiments from 5 donors. The cell morphologies were analyzed and a circularity index for each cell was assigned. 0 is square and 1.0 is perfect circle. The average circularity index per image was calculated and overall analysis plotted. *P < 0.05; **P < 0.01; ****P < 0.0001, t test. Boxes show 25/75 percentiles, whiskers show range.

Figure 6. Etokimab inhibits IL-33-induced neutrophil migration *ex vivo*. (A) Freshly isolated neutrophils from healthy donors were analyzed for ST2 and IL-1RAcP expression by FACS, n=3-6. (B) Freshly isolated neutrophils were used for Transwell assays. Neutrophils ($2x10^5$ cells) were placed into the upper wells of a transwell chamber system and allowed to migrate through 5 μm pores toward the lower wells containing IL-33 at different concentrations at 37˚C for 1 hour. Data are shown as fold change of random migration (10% FCS in RPMI 1640), n=6. (C) The concentrations of IL-33 in the culture with or without different concentrations of Etokimab were measured by ELISA. 50 ng/ml IL-33 added shown as dotted line. (D) Neutrophils ($2x10^5$ cells) were placed into the upper wells of a transwell chamber system and allowed to migrate through 5 μm pores toward the lower wells containing IL-33 (50 ng/ml) with isotype control (5 μg/ml) or Etokimab (5 μg/ml) at 37˚C for 1 hour. Boxes show 25/75 percentiles, whiskers show range, n=8. *P < 0.05, One-way ANOVA or Nonlinear fit.

Figure 7. IL-33 has a dominant non-redundant upstream role in sterile skin neutrophilic inflammation. Freshly isolated neutrophils ($2x10^5$ cells) were used for Transwell assays. Neutrophils were (A) pre-treated (n=8) or (B) not pre-treated (n=5) with etokimab (5 μg/ml) for 1 hour and placed into the upper wells of a transwell chamber system and allowed to migrate through 5 μm pores toward the lower wells containing combinations of IL-8 (50 ng/ml), isotype control (5 μg/ml), and/or etokimab (5 μg/ml) at 37˚C for 1 hour. (C) Neutrophils were not pre-treated with etokimab and placed into the upper wells of a transwell chamber system and allowed to migrate through 5 μm pores toward the lower wells containing blister fluid, isotype control (5 μg/ml), or etokimab (5 μg/ml) at 37˚C for 1 hour, n=4. (D) The concentration of IL-8 in the blister fluids before and after etokimab administration was measured by ELISA, n=4. (E) IL-8 was measured by ELISA after neutrophils ($1x10^6$ cells) were cultured in blister fluid with isotype control (5 μg/ml) or etokimab (5 μg/ml) for 24 hours, n=5. (F) Neutrophils were pre-treated with etokimab (5 μg/ml) for an hour and placed into the upper wells of a transwell chamber system and allowed to migrate through 5 μm pores toward the lower wells containing blister fluid with isotype control (5 μg/ml) or etokimab (5 μg/ml) at 37˚C for 1 hour. Data are shown as fold change

of random migration, n=7. Boxes show 25/75 percentiles, whiskers show range. *P < 0.05; **P < 0.01; ***P < 0.005, Student's t test or two-way ANOVA.

Figure 8. Etokimab inhibits IL-33-induced neutrophil autocrine expression of CXCR1 and CXCR2.
(A) Neutrophils (2-5x10^5 cells) were cultured in medium containing isotype control (5 µg/ml) or etokimab (5 µg/ml) at 37˚C for 1, 2, and 3 hours. The expression of CXCR1 and CXCR2 was analyzed by FACS. One representative histogram is shown, n=4-5. (B-C) Neutrophils (2-5x10^5 cells) were cultured in medium containing isotype control (5 µg/ml, or different dosage of etokimab (0.625, 1.25, and 5 µg/ml) at 37˚C for 2 hours. (B) The expression of CXCR1 was analyzed by FACS, n=5. (C) Neutrophil migration toward IL-8 (50 ng/ml) was analyzed against the expression of CXCR1. Correlations between migration fold change and CXCR1 MFI expression (R^2=0.2606, P=0.0108) are shown. Each dot represents an individual paired fold-change experiment. Boxes show 25/75 percentiles, whiskers show range. *P < 0.05; **P < 0.01; ***P < 0.005, one-way or two-way ANOVA or linear regression.

Table 1. Treatment emergent adverse events