

**Plasma IL-33 in atopic patients correlates with pro-inflammatory cytokines and changes
cholesterol transport protein expression: a surprising neutral overall impact on
atherogenicity**

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Abstract

Objective. Interleukin (IL)-33 has been associated with atopic and inflammatory conditions. IL-33 may be atheroprotective inducing a Th1-to-Th2 immunologic switch. However, the role of IL-33 in cardiovascular disease remains unclear. This study examines the effect of physiological

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and elevated IL-33 levels in plasma from atopic patients (AP) on cholesterol metabolism in human macrophages as compared to plasma from healthy controls (HC).

Methods. 25 AP and 25 HC were enrolled in this study. Plasma samples were analyzed for levels of IL-33, IFN- γ , TNF- α , IL-17 α , IL-5 and soluble ST2. THP-1 differentiated macrophages were exposed to HC and AP plasma. Expression of proteins involved in reverse cholesterol transport (ABCA1, ABCG1 and 27-hydroxylase) and scavenger receptors, responsible for uptake of modified lipids (CD36, ScR-A1, CXCL16 and LOX-1) were measured using QRT-PCR and immunoblotting techniques.

Results. IL-33 was significantly higher in AP plasma: 106.7 $\pm$ 95 pg/ml versus HC plasma (53.4 $\pm$ 23pg/ml). IL-33 concentration strongly correlated with levels of IFN- γ ($r=0.85$), TNF α ($r=0.9$) and IL-17 α ($r=0.94$). No significant difference was found in soluble ST2 levels. An important contrast was observed for 27-hydroxylase: normal IL-33 in AP plasma amplified 27-hydroxylase while increased IL-33 suppressed it. Expression of CD36 and SR-A1 was greater in macrophages exposed to plasma with high IL-33, while CXCL16 was higher in cells grown in the presence of plasma with normal IL-33.

Conclusions. Here we demonstrate that high levels of IL-33 and a high IL-33/soluble ST2 ratio correlates with elevated levels of IFN- γ , TNF- α and IL-17 α as well as IL-5, demonstrating that IL-33 has pleiotropic effects. However, elevated IL-33 did not significantly impact lipid accumulation in macrophages overall. Given the wide variety of cellular responses regulated by IL-33, further investigation with a larger sample size will allow us to clarify the threshold concentration of IL-33 that leads to optimal cholesterol balance.

Keywords: IL-33, sST2, IFN γ , TNF α , Reverse Cholesterol Transport, Scavenger Receptors

Abbreviations: ABC transporters, ATP-binding cassette transporters; IFN γ , interferon- γ ; TNF α , tumor necrosis factor- α , IL, interleukin; RCT, reverse cholesterol transport; LDL, low-density lipoprotein; ScR-AI - scavenger receptor class A type I; PMA, phorbol 12-myristate 13-acetate; CD36 - cluster of differentiation 36; LOX-1- lectin-like oxidized low-density lipoprotein receptor; sST2, soluble suppression of tumorigenicity 2

1. Introduction

Interleukin (IL)-33 (also known as DVS27 and NF-HEV) is the most recently discovered member of the IL-1 cytokine family [1-3]. Over the last decade, various research approaches have presented evidence for its dual role in cellular immune response and inflammation [3, 4]. Thus, IL-33 activates different cells of the innate immune system: mast cells [5, 6], neutrophils and dendritic cells [7, 8], inciting multiple inflammatory responses. The potent activating

abilities of IL-33 led to its association with multiple allergic and immunologic conditions: asthma [9, 10]; atopic dermatitis [4, 11]; chronic allergic conjunctivitis [12]; anaphylactic shock [13]; in patients with Japanese cedar pollinosis [14] and allergic rhinitis [15].

In alveolar macrophages [16] and in a murine atherosclerotic model (apolipoprotein E deficient mice) [17] IL-33 stimulates polarization of M2 macrophages and induces a switch from a pro-atherogenic TH1- to a protective TH2-type of immune responses. However, in natural killer cells and in bone marrow derived human macrophages, IL-33 enhances production of both TH2-type cytokines and TH1-type cytokines [18, 19].

In patients with allergic diseases levels of IL-33 have been reported 3-5 times that of physiologic. Therefore, it remains unclear whether this cytokine will affect the progression of cardiovascular disease in this cohort of patients. Genetic studies [20] and a few documented cases of ischemic heart disease or stroke accompanying allergic reactions suggest an existing link [21-24].

Macrophages play a central role in regulation of lipid trafficking and atherosclerosis progression. Lipid overload occurs when the balance between cholesterol flow into and out of the cell is disturbed. McLaren et al [17], demonstrated that IL-33 possesses anti-atherogenic properties, reducing macrophage foam cell formation. Our group has developed an *in vitro* assay to estimate pro-atherogenic properties of plasma from patients with immune dysfunction of varying etiology [25, 26]. This assay consists of exposing naïve cultured cells representing those found in the arterial wall (monocytes/macrophages, endothelium, smooth muscle cells) to plasma isolated from human subjects, followed by gene and protein expression analysis directed at

analyzing cholesterol transport capacity. We have identified a set of proteins that provide an assessment of cholesterol transport in and out of the cell. This set includes: (for influx) scavenger receptors CD36, scavenger receptor (ScR)-A1, lectin-like oxidized LDL receptor (LOX-1), CXCL16 [27-29] and (for efflux) cholesterol 27-hydroxylase, ATP-binding cassette transporter (ABC) A1 and ABCG1 [30-32]. This profile has already proven to be a useful tool in prediction of pro-atherogenic changes in autoimmune rheumatic diseases.

In this study we utilized THP-1 human macrophages exposed to plasma of atopic patients in order to uncover the link between level of IL-33 and macrophage lipid accumulation and processing. Here we report that despite some pro-atherogenic changes in expression of cholesterol transport proteins instigated by atopic patient (AP) plasma with elevated IL-33, overall lipid accumulation in THP-1 macrophages in our model system was not affected.

2. Materials and Methods

Cell Culture

THP-1 monocytes (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine and 50 µg per ml of penicillin-streptomycin at 37°C in a 5% CO₂ atmosphere to a density of 10⁶ cells per ml. Cell culture media and supplementary reagents were obtained from Invitrogen (Grand Island, NY). Differentiation of the monocytic THP-1 cells into adherent macrophages was stimulated by 48h exposure to 100nM phorbol 12-myristate 13-acetate (PMA), obtained from Sigma-Aldrich (St. Louis, MO). When differentiated phenotype was achieved, the PMA-containing medium was removed, and replaced with complete RPMI 1640 supplemented with 10% FCS. The macrophages were cultured for another 24h prior to treatment.

Subject Inclusion and Exclusion Criteria

Human subject studies were performed under protocol #251894-7 approved by the Institutional Review Board of Winthrop University Hospital. Written informed consent was obtained from all enrolled subjects. 25 patients with atopic disease (AP) age 18-55, 25% male, 75% female (100% allergic rhinitis, 50% asthma) and 25 healthy controls (HC) were enrolled.

Inclusion criteria for the study were as follows: (1) patients ages 18 years or older, seen in the Winthrop Allergy and Immunology Practice (2) diagnosed with an atopic disorder, defined as asthma, allergic rhinoconjunctivitis, atopic dermatitis or/and food allergy. Exclusion criteria include the following: (1) patients currently taking or recently taking (within the past 3 months) medications prescribed for hypercholesterolemia, such as HMG-CoA reductase inhibitors (statins).

Healthy control are healthy volunteers 18 years or older with no history of atopic disorder, not on statins or medications for hypercholesterolemia.

Measurement of cytokine levels in patient plasma

Plasma samples from HC and AP were analyzed for levels of IL-33, IFN- γ , TNF- α , IL-1 β , IL-17 α , IL-10, IL-5 with MILLIPLEX map Kit (Millipore, Billerica, MA) according to the manufacturer's instructions. Soluble ST2 (suppression of tumorigenicity 2) levels were measured with Presage® ST2 Assay (Critical Diagnostics). Briefly, cell supernatants were incubated with magnetic beads coated with antibodies for the previously stated proteins in a 96 well plate. After incubating with detection antibodies and streptavidin - phycoerythrin protein concentrations were

quantified using the Bio-Plex 200 system (BioRad, Hercules, CA). The minimum detectable level of each ELISA was as IL-33 < 3.9 pg/ml, 2.0 pg/ml for IFN- γ and TNF- α , 3.0 pg/ml for IL-10, and < 4 pg/ml for IL-5. All samples were measured in duplicate, and the mean of the two values of optical density were reported.

Experimental Conditions

Upon PMA stimulation, the THP-1 differentiated macrophages were cultured for an additional 24h prior to treatment. THP-1 macrophages were incubated in 12-well plates for 18h in RPMI medium containing each of the following: 10% FCS; 10% pooled normal human plasma (Innovative Research, Novi, MI); 10% plasma from each HC; 10% plasma from each AP. No FCS was added to cells incubated in human plasma.

Human IL-33 antibody (R&D systems, Minneapolis, MN) were used at 5 μ g/ml concentration (0.75 - 3 μ g/ml is enough for neutralization of 1 ng/ml IL-33) to neutralize IL-33 present in AP plasma upon incubation of macrophages.

RNA Isolation and QRT-PCR

Immediately after the incubation period, total RNA was isolated with the Trizol reagent and dissolved in nuclease-free water. The quantity of total RNA from each condition was measured by absorption at 260 and 280 nanometer wavelengths by ultraviolet spectrophotometry (Hitachi U2010 spectrophotometer).

QRT-PCR analysis was performed using the FastStart SYBR Green Reagents Kit according to the manufacturers' instructions on the Roche Light Cycler 480 (Roche Applied Science, Indianapolis, IN). cDNA was copied from 1 µg of total RNA using Murine Leukemia Virus reverse transcriptase primed with oligo dT and random hexamers. Equal amounts of cDNA were taken from each reverse transcription reaction mixture for real-time PCR amplification using gene specific primers for ABCA1, ABCG1, 27-hydroxylase, ScR-A1, CD36, LOX-1 and CXCL16 as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Table 1).

QRT-PCR was performed using techniques standardized in our laboratory. Each reaction was done in triplicate. To correct for differences in cDNA load among samples, the target PCRs were normalized to a reference PCR involving the endogenous housekeeping gene GAPDH. Non-template controls were included for each primer pair to check for significant levels of any contaminants. A melting-curve analysis was performed to assess the specificity of the amplified PCR products.

Protein Isolation and Western Blot Analysis

Cellular extracts were prepared with Lysis Kit - RIPA Buffer (ProteinSimple, Santa Clara, CA). The immunoreactive proteins were detected using Wes Assay kit (ProteinSimple, Santa Clara, CA).

Rabbit anti-human ABCA1 (sc-20794) (Santa Cruz, CA) and rabbit anti-human ABCG1 (ab-36969) (Abcam Inc., Cambridge, MA) were used as primary antibodies for detection of ABCA1 and ABCG1, respectively. Rabbit anti-human LOX-1 (ab60178), CD36 (ab64014),

ScR-A1 (ab36625), CXCL16 (ab101404) were purchased from Abcam Inc. (Cambridge, MA). As a loading control, GAPDH was detected using rabbit anti-human GAPDH antibody (ab9485).

Quantization of detected proteins and image preparation were performed with Compass Software (ProteinSimple, Santa Clara, CA).

Evaluation of Foam Cell formation by DiI-oxLDL Internalization Assay

THP-1 differentiated macrophages were cultured and exposed to 10% plasma from HC (n=8), AP with elevated level of IL-33 (n=8), AP with normal level of IL-33 (n=8) as described in the Experimental Conditions section. After treatment cells were incubated with 5 µg/ml 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI)-oxLDL (Intracel, Frederick, MD) for 4h. Slides were prepared using Vectashield mounting medium containing DAPI stain (Vector Laboratories, Inc., Burlingame, CA). After incubation, accumulation of DiI-oxLDL in cells was determined by fluorescent intensity with a Nikon A1 microscopy unit with 40X magnification and photographed with a DS-Ri1 digital camera. Fluorescent intensity was quantified from at least 3 random fields (1024x1024 pixels) per slide, from 3 slides per experimental condition and graphed.

Statistical Analysis of Experimental Data

Statistical analysis was performed using Graphpad Prism, version 5.01 (GraphPad Software, San Diego, CA). All data were analyzed by one-way analysis of variance, and pairwise multiple comparisons were made between control and treatment conditions using Bonferroni correction. Probability values less than 0.05 were regarded as significant.

3. Results

Clinical characteristics

25 AP and 25 healthy controls age 18-55 were enrolled in this study (Table 2). 75% of patients were female. All atopic patients had allergic rhinitis, and 50% of them had asthma. Of these patients, 42% were polysensitized to more than one allergen.

Level of IL-33 and Other Cytokines in Plasma from Atopic Patients

Plasma samples from HC and AP were analyzed for levels of IL-33, IFN γ , TNF α , IL-1 β , IL-17 α , IL-10, IL-5. We found that the IL-33 level was significantly higher in AP plasma - 106.7 $\pm$ 95pg/ml as compared to HC plasma (53.4 $\pm$ 23pg/ml) (P<0.001, n=2) (Fig. 1A).

No significant difference was found in the sST2 levels between HC and AP (22 $\pm$ 3 vs.19 $\pm$ 5ng/ml) (Fig 1B). However, the IL-33/sST2 ratio was 2.5 times higher for AP versus HC (Fig 1C).

In our cohort of AP, plasma concentration of IL-33 strongly correlated with levels of IL-17 α (r=0.67), IFN γ (r=0.85), TNF α (r=0.9) and IL-5 (r=0.66) (Fig. 2). The highest concentration of IL-33 was detected in AP with the highest levels of Th1 and Th17 mediated cytokines: IFN γ , TNF α and IL-17 α as well as the Th2 mediated cytokine IL-5.

Effect of Plasma from Atopic Patients on the Expression of Cholesterol Efflux Proteins in THP-1

Human Macrophages

Gene expression analysis was performed comparing 25 HC and 25 AP. Then, both gene and protein expression assays were completed using a subset of plasmas: 8 random HC, 8 AP with

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elevated level of IL-33 (higher than 80pg/ml), 8 random AP with normal level of IL-33, comparable to that observed in HC plasma (53.4 ± 23 pg/ml).

For presentation purposes, the average mRNA and protein levels in THP-1 macrophages exposed to HC plasma were set at 100% and all results are relative to this standard.

First, we compared changes in THP-1 macrophages exposed to plasma from AP vs. plasma from HC, irrespective of IL-33 level. Thus, incubation of THP-1 human macrophages with plasma from AP significantly decreased cholesterol efflux via downregulation of ABCA1 and ABCG1 to $72.1 \pm 26.7\%$ ($P < 0.05$, $n=3$) and $24 \pm 11\%$, respectively ($P < 0.001$, $n=3$) (Fig. 3A) compared to expression in cells exposed to HC plasma. The level of 27-hydroxylase was not significantly affected by AP plasma.

Next, we analyzed whether the effect of plasma from patients with an elevated level of IL-33 differed from the effect of plasma with normal IL-33. Consequently, upon exposure of macrophages to plasma from AP patients with elevated IL-33 we did not observed significant changes in the expression of ABCA1 compared to cells incubated with HC plasma (Fig. 3B). In contrast, plasma from AP with normal IL-33 downregulated expression of ABCA1 to $71.3 \pm 21\%$ ($P < 0.05$, $n=3$). Difference in plasma concentration of IL-33 did not affect expression of ABCG1: it was decreased in cells exposed to AP plasma with both – normal ($23 \pm 7.5\%$) and elevated IL-33 ($40.5 \pm 11\%$) ($P < 0.001$, $n=3$) (Fig. 3B). A substantial contrast was observed in the expression of 27-hydroxylase dependent upon plasma IL-33 level (Fig. 3B). Thus, plasma with an elevated level of IL-33 caused a reduction in the expression of 27-hydroxylase to $50.2 \pm 25\%$ versus cells

exposed to HC plasma ($P<0.001$, $n=3$). Exposure of THP-1 macrophages to plasma from AP patients with normal IL-33 led to amplified expression of 27-hydroxylase to $142.3\pm23\%$ ($P<0.001$, $n=3$).

Results of protein expression analysis on a subset of plasmas confirmed the pattern of changes described for gene expression, but revealed significant changes only for 27-hydroxylase (Fig. 3C). Thus, protein expression of 27-hydroxylase in THP-1 macrophages exposed to AP plasma with an elevated level of IL-33 was significantly downregulated to $43.1\pm8.3\%$ ($P<0.05$, $n=3$). Conversely, cells exposed to AP plasma with a normal level of IL-33 expressed a high level of 27-hydroxylase $167.7\pm27.7\%$ ($P<0.05$, $n=3$).

Effect of Plasma from Atopic Patients on the Expression of Cholesterol Influx Proteins in THP-1 Human Macrophages

On average, plasma from AP did not significantly change expression of the major receptors responsible for oxLDL uptake: CD36, LOX-1 and CXCL16 compared to cells exposed to HC plasma (set at 100%), (Fig. 4A). To our surprise, we observed a decrease in the expression of ScR-A1 ($66\pm12\%$) in macrophages exposed to plasma from AP ($P<0.05$, $n=3$).

Next, we evaluated the effect of plasma from AP with different levels of IL-33 on scavenger receptor expression in THP-1 macrophages. We observed that elevated level of IL-33 caused an increase in the expression of CD36, ScR-A1 and LOX-1 (Fig. 4B). Thus, mRNA for CD36 reached $158.4\pm58\%$ ($P<0.001$, $n=3$), ScR-A1 increased to $132.4\pm28\%$ ($P<0.001$, $n=3$) and LOX-1 to $144.7\pm49\%$ ($P<0.001$, $n=3$) compared to cells exposed to HC plasma. In cells exposed to

plasma with a normal level of IL-33, the level of ScR-A1 remained low: $64.6 \pm 12\%$ (Fig. 4B). mRNA level of CXCL16 in THP-1 macrophages was elevated in the presence of plasma from AP with normal IL-33 level $145.6 \pm 89\%$ ($P < 0.05$, $n=3$).

For our subset of 24 plasmas (8 HC, 8 AP with elevated IL-33, 8 AP with normal IL-33, all chosen randomly from within each group), results of gene expression analysis for CD36, CXCL16 and SRA1 were confirmed by Western blot (Fig. 4C). Protein expression of LOX-1 did not differ significantly among all compared groups. Protein expression of CD36 and SR-A1 was significantly higher on THP-1 macrophages exposed to plasma from AP with high level of IL-33 reaching $206 \pm 64\%$ ($P < 0.001$, $n=3$) and $157.6 \pm 16.5\%$ ($P < 0.05$, $n=3$), respectively. In contrast protein expression of CXCL16 was higher in cells grown in the presence of plasma from AP with normal level of IL-33 $191.5 \pm 42\%$ ($P < 0.05$, $n=3$).

Effect of IL-33 Neutralization on the Expression of Cholesterol Efflux and Influx Proteins in THP-1 Human Macrophages

We evaluated the expression of ABCA1, 27-hydroxylase and scavenger receptors in THP-1 human macrophages exposed to AP plasma with different levels of IL-33 with and without IL-33 neutralization.

Incubation of macrophages with plasma from AP with elevated IL-33 after exposure to IL-33 neutralizing antibody rescues expression of ABCA1 and 27-hydroxylase (Fig. 5). Thus, message and protein levels of ABCA1 and 27-hydroxylase doubled upon neutralization of IL-33 in THP-1 macrophages. We detected similar upregulation of ABCA1 and 27-hydroxylase upon

neutralization of IL-33 in the presence of plasma with an IL-33 level comparable to that of healthy controls only at the level of mRNA. No significant changes were detected for the ABCA1 or 27-hydroxylase protein (Fig. 5B, C).

Neutralization of the IL-33 in AP plasma with different levels of IL-33 demonstrated doubled gene expression of CXCL16 (Fig. 6A). The CXCL16 protein level was increased upon IL-33 neutralization in AP with high IL-33 and not affected in AP plasma with normal IL-33 (Fig. 6B). mRNA and protein expression of the rest of the scavenger receptors were not affected by neutralization of IL-33 (Fig. 6).

DiI-oxLDL Uptake and Macrophage Foam Cell Transformation

Next we tested how different levels of IL-33 impact DiI-oxLDL uptake by THP-1 macrophages. Again, we used the same subset of 24 plasmas (8 HC, 8 AP with high IL-33, 8 AP with normal IL-33). On average, THP-1 macrophages incubated in the presence of plasma from HC and AP with different levels of IL-33 internalized a similar amount of DiI-oxLDL and did not differ significantly from each other (Fig. 7). Thus, DiI-oxLDL uptake by THP-1 macrophages exposed to AP plasma with elevated level of IL-33 uptake was $107 \pm 23\%$ vs. cells exposed to HC plasma. Cells grown in the presence of AP with normal IL-33 level internalized DiI-oxLDL at $110 \pm 34\%$ compared to cells with HC plasma, set at 100%.

4. Discussion

IL-33 has been associated with atopic and inflammatory conditions [33-36]. Overall in allergic disease, levels of IL-33 reported 3-5 times higher than physiologic, though numbers vary per study. Specifically, Matsuba-Kitamura et al [37] demonstrated that IL-33 might play a

crucial role in the induction and augmentation of allergic conjunctivitis. Additionally, Asaka et al [38] found elevated levels of IL-33 in the nasal secretions of patients experiencing allergic rhinitis exacerbations compared to healthy controls. IL-33 levels in anaphylactic shock have been shown to be 30-40 times that of physiologic levels [13, 39]. In our cohort of patients with atopic diseases (100% allergic rhinitis, 50% asthma) the level of IL-33 was significantly higher - 106.7 ± 95 pg/ml as compared to plasma of healthy volunteers (53.4 ± 23 pg/ml) (Fig. 1A). No significant difference was found in the sST2 levels between HC and AP (22 ± 3 vs. 19 ± 5 ng/ml) (Fig 1B). In this study, we observed that elevated levels of IL-33 and a low IL-33/sST2 ratio correlates with elevated level of Th1 and Th17 mediated cytokines: IFN- γ , TNF- α and IL-17 α as well as Th2 mediated IL-5. This might indicate and stay in accord with previous findings [17, 18] of a dual role for IL-33 in patients with atopic disease. The strong correlation of IL-33 and IL-17 α in AP might indicate a possible link for IL-33 involvement in IL-17 mediated activation of the adaptive T-cell response and inflammatory cytokine induction.

Our group has published multiple reports on the pro-atherogenic nature of the cytokines IFN- γ and TNF- α [40, 41]. Correlation of IL-33 with Th1 and Th17 mediated cytokines led us to investigate possible abnormalities in lipid handling in macrophages exposed to plasma from AP. However, a detailed examination of the effect of physiological and elevated levels of IL-33 in plasma from AP on cholesterol metabolism in human macrophages revealed an overall neutral effect.

Therefore, plasma from patients with elevated IL-33 did not change ABCA1-mediated cholesterol efflux to apoA1 [42] compared to cells exposed to HC plasma (Fig. 3B). Surprisingly, downregulation of ABCA1 mRNA to $71\pm 21\%$ was observed in THP-1 macrophages exposed to plasma from AP with normal IL-33 ($P<0.05$, $n=3$). Expression of another transporter - ABCG1, mediating efflux to HDL [42], was decreased in macrophages exposed to both – normal ($23\pm 7.5\%$) and elevated levels of IL-33 ($40.5\pm 11\%$) (Fig. 3B). Even though we detected changes in gene expression of ABCA1 and ABCG1, no significant alterations were observed on the protein level. Neutralization of IL-33 increased gene expression of ABCA1, but did not alter protein expression significantly in THP-1 macrophages (Fig. 5).

For the first time, we demonstrate that IL-33 is crucial for the expression of one of the mediators of intracellular catabolism of cholesterol to polar sterols, cytochrome P450 27-hydroxylase [43, 44] (Fig. 3B). AP plasma with elevated IL-33 caused a reduction in the expression of 27-hydroxylase to $50.2\pm 25\%$ versus cells exposed to HC plasma. Plasma from AP patients with normal IL-33 amplified 27-hydroxylase expression. Since normal IL-33 in AP plasma is associated with low ABCA1 and high 27-hydroxylase while high IL-33 in AP plasma is associated with higher ABCA1 and low 27-hydroxylase, it is possible that this leads to an overall neutral effect on efflux. Thus, message and protein levels of 27-hydroxylase were doubled upon neutralization of IL-33 (Fig. 5).

AP plasma from those with an elevated level of IL-33 caused an increase in the protein expression of scavenger receptors CD36 and ScR-A1 reaching $206\pm 64\%$ ($P<0.001$, $n=3$) and $157.6\pm 16.5\%$ ($P<0.05$, $n=3$), respectively in THP-1 macrophages. Neither mRNA nor protein

expression for the rest of scavenger receptors were affected by neutralization of IL-33 (Fig. 6). In contrast protein expression of CXCL16 was higher in cells grown in the presence of plasma from AP with normal level of IL-33 $191.5 \pm 42\%$ ($P < 0.05$, $n=3$). Neutralization of the IL-33 in AP plasma with either high or normal IL-33 level doubled gene expression of CXCL16 (Fig. 6A). CXCL16 protein level was increased upon IL-33 neutralization in AP with high IL-33 and not affected in AP plasma with normal IL-33.

Observed changes in cholesterol efflux and influx upon exposure of THP-1 macrophages to plasma from AP did not produce notable changes in lipid accumulation and foam cell transformation.

IL-33 has known anti-atherogenic properties [29, 45]. However, it remains uncertain whether this cytokine affects the progression or prevents atherosclerotic cardiovascular disease in patients with allergic conditions. Interestingly, results of these study contrast with our previous findings using the same approach with plasma from rheumatoid arthritis and systemic lupus erythematosus patients [46-48]. In subjects with these autoimmune rheumatic diseases, changes in cholesterol transport resulted in augmented foam cell formation and lipid accumulation. The lupus and rheumatoid arthritis patient populations are known to have exceedingly high risk of developing cardiovascular sequelae including myocardial infarction and stroke. Their plasma shows consistent atheroma-promoting properties. Therefore, the alterations described in AP might suggest better compensatory mechanisms or less pro-atherogenic properties of plasma from patients with allergic diseases.

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Taken together, the findings of this study indicate that an elevated level of IL-33 in patients with atopic diseases causes moderate alteration in the expression of cholesterol efflux and influx proteins. However, this might be a result of synergistic effects of multiple pro-inflammatory cytokines elevated in plasma from AP. This effect is not strong enough to produce pro-atherogenic changes in human macrophages in a cell culture system. Furthermore, our findings might add support to previous reports that IL-33 functions as an alarmin released upon cellular stress and injury [49, 50]. The work presented here using plasma from AP sets the stage for further research on the role of IL-33 in atopic and inflammatory conditions.

Nevertheless, our study indicates that patients with atopy might have an elevated risk of atherosclerosis progression when the presence of pro-inflammatory cytokines is chronic. Just as in atherosclerosis, oxidative stress may play a role in atopic diseases such as atopic dermatitis [51]. The inflammatory background in atherosclerosis and atopy may converge although not as resoundingly as they do in lupus or rheumatoid arthritis. Larger studies may be needed to detect risk.

Mild agents with anti-atherogenic properties might be explored as treatments for AP that could mitigate oxidative stress and improve lipid transport. For example, the dietary supplement resveratrol has the potential to alleviate the central causative factor in atherosclerosis – namely the accumulation of lipids [52]. Since resveratrol also reduces oxidative stress and has anti-inflammatory properties, it may be of interest in allergy as well as the cardiovascular disciplines.

Our study limitations included small sample size, and although patients had atopic disease, the disease activity state was not quantified. Therefore our IL-33 measurements represented a snapshot level in our patient population. Additionally, although a history of hypercholesterolemia was an exclusion criterion, future studies can include cholesterol level measurement at the time of IL-33 measurement in order to further assess alterations in lipid metabolism in this cohort of patients.

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Acknowledgements

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Fig. 1. Level of IL-33(A), sST2 (B) and IL-33/sST2 ratio in the plasma of healthy control volunteers (HC) and atopic patients (AP) (n=25). *-P<0.001**

Fig. 2 Correlation between level of IL-33 and TH1/TH2 cytokines in plasma from AP. ELISA measurements done in duplicates.*-P<0.05, *-P<0.001.**

Fig. 3. Effect of AP plasma on the expression of cholesterol efflux proteins in THP-1 human macrophages. (A) THP-1 macrophages were incubated in the presence of 10% plasma from each of 25 AP or each of 25 HC, as indicated in methods. Following incubation, total RNA isolated from cells of each condition was reverse transcribed and amplified by QRT-PCR with GAPDH message as an internal standard. Gene expression levels were graphed as relative mRNA expression. The data represent the mean $\pm$ SEM of three independent experiments. (B) Gene expression analysis in THP-1 macrophages exposed to plasma from: 8 random HC, 8 AP with elevated level of IL-33 ($\uparrow$ IL-33), 8 random AP with level of IL-33, observed in HC plasma (normal IL-33). The data represent the mean $\pm$ SEM of three independent experiments. (C) Protein expression analysis in macrophages exposed to 8 random HC, 8 AP with elevated level of IL-33 ($\uparrow$ IL-33), 8 random AP with normal IL-33 level. * - P<0.05; ** - P<0.01; *** - P<0.001 vs. THP-1 macrophages exposed to HC plasma.

Fig. 4. Effect of AP plasma on the expression of scavenger receptors in THP-1 human macrophages. (A) THP-1 macrophages were incubated in the presence of 10% plasma from each of 19 AP or each of 20 HC, as indicated in methods. Following incubation, total RNA isolated from cells of each condition was reverse transcribed and amplified by QRT-PCR with GAPDH message as an internal standard. Gene expression levels were graphed as relative mRNA expression. The data represent the mean $\pm$ SEM of three independent experiments. (B) Gene expression analysis in THP-1 macrophages exposed to plasma from: 8 random HC, 8 AP with elevated level of IL-33 ($\uparrow$ IL-33), 8 random AP with level of IL-33, observed in HC plasma

(normal IL-33). The data represent the mean $\pm$ SEM of three independent experiments. (C) Protein expression analysis in macrophages exposed to 8 random HC, 8 AP with elevated level of IL-33 ($\uparrow$ IL-33), 8 random AP with normal IL-33 level. * - $P<0.05$; ** - $P<0.01$; *** - $P<0.001$ vs. THP-1 macrophages exposed to HC plasma.

Fig. 5. Effect of IL-33 neutralization in AP plasma on the expression of ABCA1 and 27OH in THP-1 human macrophages. THP-1 macrophages were incubated in the presence of 10% plasma from: 8 AP with elevated level of IL-33 ($\uparrow$ IL-33) and 8 random AP with normal IL-33 in the presence/absence of IL-33 neutralizing antibodies, as indicated in methods. (A) Total RNA isolated from cells of each condition was reverse transcribed and amplified by QRT-PCR with GAPDH message as an internal standard. Gene expression levels were graphed as relative mRNA expression with mean of HC set at 100%. Protein expression analysis was done using Western blot technique. Protein levels were normalized to GAPDH and graphed (B). Western blot images (C) presented for 4 random AP $\uparrow$ IL-33 and 8 random APNIL-33. * - $P<0.05$ vs. THP-1 macrophages exposed to AP $\uparrow$ IL-33 plasma; # - $P<0.05$; ## - $P<0.01$; ### - $P<0.001$ vs. cells without IL-33 neutralization.

Fig. 6. Effect of IL-33 neutralization in AP plasma on the expression of scavenger receptors in THP-1 human macrophages. THP-1 macrophages were incubated in the presence of 10% plasma from: 8 AP with elevated level of IL-33 ($\uparrow$ IL-33) and 8 random AP with normal IL-33 in the presence/absence of IL33 neutrilized antibodies, as indicated in methods. (A) Total RNA isolated from cells of each condition was reverse transcribed and amplified by QRT-PCR with GAPDH message as an internal standard. Gene expression levels were graphed as relative mRNA expression with mean of HC set at 100%. Protein expression analysis was done using western blot technique. Protein levels are normalized to GAPDH and graphed (B). Western blot images (C) presented for 4 random AP $\uparrow$ IL-33 and 8 random APNIL33. # - $P<0.05$; ## - $P<0.01$ vs. cells without IL33 neutralization.

Fig. 7. Impact of IL-33 level in plasma from AP patients on oxidized low-density lipoprotein (ox-LDL) uptake. Cultured THP-1 macrophages were exposed for 24h to plasma from 8 random HC, 8 AP with elevated level of IL-33 ($\uparrow$ IL-33), or 8 AP with level of IL-33, observed in HC plasma (normal IL-33). DiI-oxLDL internalization was measured as fluorescent intensity of integrated DiI-oxLDL and presented as a percent of cells included DiI-labeled oxLDL (A). Cells exposed to HC plasma were set at 100%. All results are expressed as mean $\pm$ SEM of three independent experiments. Representative images of DiI-oxLDL uptake shown in green (B) and counterstained with DAPI (blue). HC-healthy controls, APH – AP with high level of IL33, APN - AP with normal level of IL33. Scale bar – 10 μ m.

Table 1. The list of specific primers used for QRT-PCR.

Gene	Primer
ABCA1	F 5'-GAAGTACATCAGAACATGGGC-3' R 5'-GATCAAAGCCATGGCTGTAG-3'
ABCG1	F 5'-CAGGAAGATTAGACACTGTGG-3' R 5'-GAAAGGGGAATGGAGAGAAG-3'
27-hydroxylase	F 5'-AAGCGATACCTGGATGGTTG-3' R 5'-TGTTGGATGTCGTGTCCACT-3'
CD36	F 5'-GAGAACTGTTATGGGGCTAT-3' R 5'-TTCAACTGGAGAG-GCAAAGG-3'
LOX-1	F 5'-TACTCTCCATGGTGGTGCC-3' R 5'-AGCTTCTTCTGCTTGTTGCC-3'
ScR-A1	F 5'-CTCGTGTTTGCAGTTCTCA-3' R 5'-CCATGTTGCTCATGTGTTCC-3'
CXCL16	F 5'-ACTACACGACGTTCCAGCTCC-3' R 5'-CTTTGTCCGAGGACAGTGATC-3'
GAPDH	F 5'- ACCATCATCCCTGCCTCTAC-3' R 5'-CCTGTTGCTGTAGCCAAAT-3'

Patient Characteristics	Atopic	Normal Healthy Controls
Number of Subjects	25	25
Sex	75% Female	75% Female
Age Range (yrs)	18-55	18-50
Allergic Rhinitis	100%	
Polysensitized (skin test positive to > one aeroallergen)	42%	
Asthma	50%	













