Interleukin-33 Induces Expression of Adhesion Molecules and Inflammatory Activation in Human Endothelial Cells and in Human Atherosclerotic Plaques

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Objective—Interleukin (IL)-33 is the most recently described member of the IL-1 family of cytokines and it is a ligand of the ST2 receptor. While the effects of IL-33 on the immune system have been extensively studied, the properties of this cytokine in the cardiovascular system are much less investigated.

Methods/Results—We show here that IL-33 promoted the adhesion of human leukocytes to monolayers of human endothelial cells and robustly increased vascular cell adhesion molecule-1, intercellular adhesion molecule-1, endothelial selectin, and monocyte chemoattractant protein-1 protein production and mRNA expression in human coronary artery and human umbilical vein endothelial cells in vitro as well as in human explanted atherosclerotic plaques ex vivo. ST2-fusion protein, but not IL-1 receptor antagonist, abolished these effects. IL-33 induced translocation of nuclear factor-κB p50 and p65 subunits to the nucleus in human coronary artery endothelial cells and human umbilical vein endothelial cells and overexpression of dominant negative form of IκB kinase 2 or IκBα in human umbilical vein endothelial cells abolished IL-33-induced adhesion molecules and monocyte chemoattractant protein-1 mRNA expression. We detected IL-33 and ST2 on both protein and mRNA level in human carotid atherosclerotic plaques.

Conclusion—We hypothesize that IL-33 may contribute to early events in endothelial activation characteristic for the development of atherosclerotic lesions in the vessel wall, by promoting adhesion molecules and proinflammatory cytokine expression in the endothelium. (Arterioscler Thromb Vasc Biol. 2011;31:2080-2089.)

Key Words: leukocyte adhesion | endothelial cells | IL-33 | ST2 | atherosclerosis

Cardiovascular disease is the leading cause of death in Western societies. Among cardiovascular pathologies atherosclerosis is thought to be the principal contributor to cardiovascular morbidity and mortality. Atherosclerosis is now generally thought to be a chronic inflammatory disorder. Leukocyte trafficking from bloodstream to tissue is important for rapid leukocyte accumulation at sites of inflammatory response or tissue injury. Thus it is evident that leukocyte extravasation is considered a key event in the pathogenesis of atherosclerosis. In fact, endothelial dysfunction, a hallmark in the early development of atherosclerosis is, besides impaired vasoconstriction function of the vessel wall, characterized by increased adhesiveness of the activated or injured endothelium for leukocytes at the site of developing and progressing atherosclerotic lesions. The process of leukocyte extravasation comprises a complex multistep cascade that is orchestrated by a tightly coordinated sequence of adhesive interactions of the leukocytes with vessel wall endothelial cells. Endothelial cells express an array of adhesion molecules that control processes such as leukocyte rolling along and attachment to the endothelium and transmigration of leukocytes into areas of inflammation. These leukocyte-endothelial interactions require the regulation of various adhesion molecules by endothelial cells such as intercellular adhesion molecule-1 (ICAM-1), vascular cell AM-1 (VCAM-1) and endothelial selectin (E-selectin). Several studies have demonstrated...
that these endothelial cell adhesion molecules are upregulated by inflammatory mediators such as interleukin-1 (IL-1) or tumor necrosis factor-α (TNF-α) and that their expression is increased in atherosclerotic lesions. In addition to this process, chemokines such as monocyte chemoattractant protein-1 (MCP-1) attract activated leukocytes to the inflammatory site.

Interleukin-33 (IL-33) is the most recently described member of the IL-1-family of cytokines, and it is a ligand of the ST2 receptor. According to the present knowledge, it is suggested that IL-33 is specifically released during necrotic cell death, which is thought to be associated with tissue damage during infection or trauma, but kept intracellular during apoptosis. Because of these properties, IL-33 was proposed to act as “alarmin,” as an endogenous “danger” signal to alert the immune system after infection or injury. Consequently, most studies investigating the biological role of IL-33 focus on its immunomodulatory functions. Thus, it was shown that IL-33 is involved in polarization of T-cells toward the T helper type 2 (Th2) cell phenotype as well as in activation of mast cells, basophils, eosinophils, and natural killer cells. IL-33 enhances adhesion, survival, and cytokine production of human mast cells, eosinophils, and basophils. Furthermore, IL-33 was shown to be involved in the modulation of inflammation as it can promote rheumatic and airway inflammatory diseases, anaphylactic shock, and inflammatory and fibrotic disorders of the gastro-intestinal tract. While the effects of IL-33 on the immune system have been extensively studied, the properties of this cytokine in the cardiovascular system are much less investigated. IL-33 and ST2 receptor are expressed in human vein endothelial cells and in coronary artery endothelium, as well as in the thoracic aorta of apolipoprotein E-deficient (ApoE−/−) mice. In such mice, IL-33 was also shown to inhibit the progression of high-fat diet-induced atherosclerosis and the accumulation of foam cells in the lesion. In human endothelial cells, however, IL-33 was shown to induce inflammatory activation as evidenced by increased vascular permeability, the increased production of inflammatory cytokines, and the stimulation of angiogenesis.

In this article, we provide evidence for yet another aspect of inflammatory activation of human endothelial cells by IL-33 by showing that this cytokine, which we found to be expressed in human atherosclerotic tissue, stimulates adhesion of leukocytes to the endothelium under both static and flow conditions and upregulates the expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin and of the chemokine MCP-1 in human endothelial cells in vitro. Furthermore, we demonstrate that the latter effects of IL-33 are also operative in explanted human atherosclerotic plaque tissue ex vivo.

**Methods**

**Cell Culture**

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords. Human coronary artery endothelial cells (HCAEC) were isolated from pieces of coronary arteries obtained from patients undergoing heart transplantation. Such endothelial cells were isolated by mild collagenase treatment, characterized and cultivated as described. For some experiments, HCAEC (Lonza, Verviers, Belgium) were used; cells were maintained in EGM-2 MV Bullet kit medium (Lonza) with 5% fetal calf serum (Lonza).

**Isolation of Human Polymorph-Nuclear Leukocytes and Monocytes**

Human polymorph-nuclear leukocytes (PMNL; 99% neutrophils and less than 1% eosinophils) were isolated from heparinized (100 U/mL) peripheral venous blood of healthy donors as described. For details, please see supplemental material. Monocytes were isolated from peripheral blood mononuclear cells by negative magnetic selection according to manufacturer’s instructions (Monocyte Isolation Kit II, MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the monocyte preparation was 97%.

**Adhesion Assay for PMNL and Monocytes Under Static and Flow Conditions**

PMNL adhesion to HCAEC or HUVEC under static conditions was measured as described. PMNL or monocyte adhesion to HCAEC under flow conditions was performed using VenAfux platform (Cellix, Dublin, Ireland). For details, please see online supplemental material.

**Tissue Sampling**

Atherosclerotic plaques were collected from 35 patients undergoing carotid endarterectomy. All subjects were Caucasian and did not suffer from acute infection or autoimmune or neoplastic disease. For details, please see online supplemental material. The study has been reviewed and approved by the Ethic Committee of the Medical University of Vienna, Austria, and all study subjects gave informed consent.

**Treatment of Cells**

HCAEC and HUVEC were incubated in minimum essential medium (M199; Sigma) containing 1.25% fetal calf serum (Lonza) without or with recombinant human IL-33 (R&D Systems, Minneapolis, MN) at concentrations between 100 ng/mL and 0.01 ng/mL for time periods between 1 and 48 hours. For soluble receptor inhibition experiments, IL-33 (1 ng/mL) was incubated with recombinant human ST2/IL-1 R4 Fc chimera (sST2-Fc; 5 µg/mL, R&D Systems) for 15 minutes at 37°C before addition to the cells for 24 hours, as described previously. In addition, recombinant human immunoglobulin G1 Fc (IgG1-Fc; 5 µg/mL, R&D Systems) was used as an isotype control. In order to determine whether the action of IL-33 is dependent on IL-1 effect, HUVEC were cultured for 6 hours in the presence of IL-33 (100 ng/mL) or recombinant human IL-1β (200 U/mL; R&D Systems) with or without IL-1 receptor antagonist (IL-1Ra) (10 µg/mL; R&D Systems). This concentration of IL-1Ra was shown previously to inhibit IL-1β-induced ICAM-1 and VCAM-1 expression in HUVEC. In additional experiments, the cells were preincubated for 1 hour with the mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor U0126 (Promega, Madison, WI), or the photophosphidininositol 3-kinase (PI3K) inhibitor LY-294002 (Calbiochem/Merck, Darmstadt, Germany) at the indicated concentrations that were similar to concentrations used by us and others in in vitro studies with endothelial cells. Thereafter, the cells were treated with IL-33 at a concentration of 100 ng/mL for 24 hours (for ICAM-1, VCAM-1, MCP-1 measurement) or 4 hours (for E-selectin determination). TNF-α (Roche Diagnostics, Indianapolis, IN) at 10 pM was used as a positive control in our experiments. The culture supernatants were collected followed by removal of cell debris by centrifugation and
stored at −80°C until used. The total cell number was counted with a hemocytometer after trypsinization.

**Flow Cytometry**
ICAM-1, VCAM-1, and E-selectin expressions at the cell surface were measured by means of flow cytometry (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ). For details, please see online supplemental material.

**Antigen Determination**
MCP-1, IL-6, and IL-8 antigen in cell culture supernatants or in supernatants from stimulated atherosclerotic plaque tissue was measured by specific ELISAs using monoclonal antibodies (all from Bender MedSystems, Vienna, Austria).

**Total RNA Purification and cDNA Preparation**
mRNA was isolated using High Pure RNA Tissue Kit (Roche). Reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche). For details, please see online supplemental material.

**RealTime Polymerase Chain Reaction**
RealTime-PCR was performed using LightCycler® TaqMan® Master (Roche) according to the manufacturer’s instructions. For details, please see online supplemental material.

**Nuclear Extraction and Analysis of NF-κB/DNA Binding**
Preparation of nuclear extracts was performed using a Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer’s instructions. For details, please see online supplemental material.

**NF-κB Translocation Staining**
HCAEC were treated with fresh M199 containing 1.25% fetal calf serum without or with 1, 10, or 100 ng/mL IL-33 for 1 hour and staining for NF-κB p50 and p65 subunits was performed. For details, please see online supplemental material.

**Immunofluorescence Analysis of IL-33 and ST2 in Human Atherosclerotic Tissue**
Human carotid endarterectomy tissues were fixed in 4% formaldehyde and embedded in paraffin. Sections (5 μm) were deparaffinized according to standard procedure and then boiled for antigen retrieval in citrate buffer (DAKO North America, Inc, CA). The following primary antibodies were used: mouse monoclonal antibody anti-IL33 (clone Nessy-1, 1:1000; Alexis Biochemicals, Enzo Life Sciences AG, Lausen, Switzerland), rabbit polyclonal antibody anti-ST2 (IL1RL1) (1:100 dilution; Sigma), and rabbit polyclonal antibody anti-von Willibrand factor (1:500 dilution; Dako). For details, please see online supplemental material.

**Adenoviral Infection**
HUVEC were infected with adenoviral vectors for overexpression of 1xBz (AdV-1xBz) or for overexpression of a mutant dominant negative IκB kinase 2 (AdV-dnIKK2), respectively, as described previously.43,44 For details, please see online supplemental material.

**Statistical Analysis**
ANOVA followed by Bonferroni-Holm multiple comparisons correction was carried out for experiments having more than 2 experimental groups. Dunnett’s post-hoc test was used to compare treated groups with the reference untreated group. Normally distributed data were analyzed with t tests in case of 2 groups. Mean concentrations of the respective protein for each plaque before and after stimulation with IL-33 were compared using Wilcoxon signed-rank test for nonparametric distribution. For mRNA correlation, a Spearman correlation for nonparametric variables was calculated using SPSS 16.0 (SPSS, Chicago, IL). Values are expressed as mean±SD. Values of P≤0.05 were considered significant.

**Results**
**IL-33 Promotes Adhesion of Human Leukocytes to the Monolayer of Endothelial Cells**
When HCAEC were pretreated with 100 ng/mL IL-33 for 4 hours, an increase in the number of PMNL adhering to the endothelial cell monolayer was seen already 5, 15, and, more prominently, 30 minutes after the addition of PMNL as compared to untreated control (Figure 1A and 1B). Similar results were also observed with HUVEC (control 5 minutes 2.4±1.7; IL-33 5 minutes 8.4±3.7, P≤0.05; control 15 minutes 4.8±2.3; IL-33 15 minutes 14.2±5.9, P≤0.05; control 30 minutes 5.2±2.6; IL-33 30 minutes 42.4±15.7, P≤0.05; values represent numbers of PMNL per 5×10^5 square (sq) microns and are given as mean±SD of 5 pictures, respectively).

Under flow conditions (0.5 dyne/cm² for 2 minutes and 20 seconds at 37°C), IL-33 at concentrations of 10 ng/mL and 100 ng/mL induced statistically significant (P≤0.05) adhesion of both PMNL and monocytes to monolayers of HCAEC (Figure 1C and 1D). TNF-α at 10 pM, used as a positive control, also significantly increased adhesion of both PMNL (control 8±4, TNF-α 107±26, P≤0.05) and monocytes (control 4±2, TNF-α 10±1, P≤0.05) to HCAEC under flow condition. To address which adhesion molecules might mediate the PMNL adhesion, we also performed additional experiments with blocking antibodies against E-selectin, ICAM-1, and VCAM-1 alone, or with all three blocking antibodies together. E-selectin and VCAM-1 antibodies significantly (P≤0.05) reduced PMNL adhesion to HCAEC monolayers which had been activated by 10 ng/mL of IL-33 (Figure 1E and 1F). ICAM-1 antibody also reduced IL-33–mediated PMNL adhesion, however, to a lesser extent than E-selectin or VCAM-1 antibodies (Figure 1E and 1F). If all 3 antibodies were applied simultaneously, IL-33-induced PMNL adhesion was reduced to the level of untreated endothelial cells (Figure 1E and 1F). Please see, also, respective movies in the supplemental data.

**IL-33 Stimulates VCAM-1, ICAM-1, E-Selectin, and MCP-1 Expression in Human Endothelial Cells via ST2 Receptor and in IL-1-, MEK-, and PI3K-Independent Manner**
When HUVEC were treated with IL-33 at concentrations from 0.01 to 100 ng/mL for 4, 16, 24, and 48 hours, a concentration-dependent upregulation of ICAM-1, VCAM-1, and MCP-1 protein production was observed at all time points (Figure 2A, B, D). E-selectin expression was also significantly increased in these cells, however, only after 4 hours of incubation (Figure 2C). TNF-α at 10 pM induced upregulation of these adhesion molecules and MCP-1 with similar kinetics in HUVEC (ICAM-1 [mean fluorescence intensity, MFI], control: 4 hours, 185±9; 16 hours, 239±3; 24 hours, 209±14; 48 hours, 169±9; TNF-α: 4 hours, 638±107; 16 hours, 2594±42; 24 hours, 2028±160; 48 hours, 1061±49. VCAM-1 [MFI], control: 4
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**Figure 1.** Interleukin (IL)-33 promotes adhesion of human leukocytes to monolayers of endothelial cells. A, Confluent monolayers of human coronary artery endothelial cells (HCAEC) were preincubated for 4 hours with IL-33 (100 ng/mL). HBSS (1.0 mL) containing 1 × 10^6/mL PMNL was then added to the endothelial cell monolayer for 5, 15, and 30 minutes. Respective photomicrographs are shown. B, Adhesion of polymorph-nuclear leukocytes (PMNL) to monolayers of HCAEC was determined as described in “Methods.” Experiments were performed twice. Values represent numbers of PMNL per 5 × 10^5 square (sq) microns and are given as mean ± SD of 40 pictures, respectively. Original magnification ×100. *P < 0.05 compared to control. C, HCAEC were grown on VenaEC biochips and preincubated for 4 hours with IL-33 (10 or 100 ng/mL). Endothelial monolayers were then superfused with suspensions of 3 × 10^6/mL PMNL or monocytes at 0.5 dyne/cm² for 2 minutes and 20 seconds at 37°C using Venaflux Nanopump. Part of the respective photomicrographs is shown. D, Adhesion of PMNL or monocytes to monolayers of HCAEC was determined as described in “Methods.” Experiments were performed 4 times, and 10 images were made at each experiment. Values represent numbers of PMNL or monocytes per 5 × 10^5 sq microns and are given as mean ± SD of 40 pictures, respectively. Original magnification × 100. *P < 0.05 compared to control for PMNL. §P < 0.05 compared to control for monocytes. E, Endothelial cell layers were preincubated for 4 hours with or without 10 ng/mL of IL-33. Afterward, the cells were incubated with blocking antibodies for E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), or all three antibodies together (5 µg/mL each), or with an isotype matched control antibody at the same concentration for 15 minutes at 37°C followed by superfusion with suspensions of 3 × 10^6/mL PMNL at 0.5 dyne/cm² for 2 minutes and 20 seconds at 37°C. Part of the respective photomicrographs is shown. F, Adhesion of PMNL to monolayers of HCAEC was determined as described in “Methods.” Experiments were performed 4 times, and 10 photos were made at each experiment. Values represent numbers of PMNL per 5 × 10^5 sq microns and are given as mean ± SD of 40 pictures, respectively. Original magnification × 100. *P < 0.05 compared to control. §P < 0.05 compared to IL-33 pretreated cells.

**Figure 2.** Effects of interleukin (IL)-33 on intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), endothelial selectin (E-selectin) and monocyte chemoattractant protein-1 (MCP-1) protein production by HUVEC and HCAEC. Confluent monolayers of HUVEC were incubated for 4 hours (224), 16 hours (Δ), or 48 hours (×) in the presence or absence of IL-33 from 0.01 ng/mL to 100 ng/mL. Confluent monolayers of HCAEC were incubated for 4 hours (□) in the presence or absence of IL-33 from 0.01 ng/mL to 100 ng/mL. A, E, ICAM-1, B, F, VCAM-1, and C, G, E-selectin expression at the cell surface was measured by means of flow cytometry as described in “Methods.” D, H, MCP-1 protein was determined in conditioned media by ELISA as described in Methods.” Each experiment was performed in triplicates. Values are given in mean fluorescence intensity (MFI) (A–C, E–G) or in pg/10⁴ cells (D, H) and represent mean values ± SD of 3 different experiments. The overall ANOVA comparing different concentrations for each tested molecule, and time point remained significant after correction for 6 comparisons (ICAM-1, VCAM-1, E-selectin, MCP-1, IL-6, IL-8). *P < 0.05 compared to control 4 hours; §P < 0.05 compared to control 16 hours; #P < 0.05 compared to control 24 hours; #P < 0.05 compared to control 48 hours.

hours, 97 ± 6; 16 hours, 102 ± 6; 24 hours, 91 ± 9; 48 hours, 110 ± 3; TNF-α: 4 hours, 505 ± 109; 16 hours, 412 ± 50; 24 hours, 256 ± 2; 48 hours 169 ± 13. E-selectin [MFI], control: 4 hours, 116 ± 5; 16 hours, 117 ± 2; 24 hours 109 ± 4; 48 hours, 109 ± 5; TNF-α: 4 hours, 1441 ± 318; 16 hours, 124 ± 14; 24 hours, 103 ± 3; 48 hours, 139 ± 6. MCP-1 [pg/10⁶ cells], control: 4 hours, 715 ± 59; 16 hours 1039 ± 119; 24 hours 1458 ± 102; 48 hours 3988 ± 507; TNF-α 4 hours, 11 760 ± 981; 16 hours, 54 094 ± 7437; 24 hours, 67 730 ± 1463; 48 hours, 85 696 ± 7437.
IL-33 Induces NF-κB Nuclear Translocation and IL-33 Effects on Endothelial Cells are NF-κB-Dependent

As can be seen in Figure 3, IL-33 at 100 ng/mL induced statistically significant (P<0.05) nuclear translocation of NF-κB subunits p50 (panel A) and p65 (panel B) in human endothelial cells 15, 30, and 60 minutes after incubation. In HCAEC, IL-33 at 1 ng/mL (panel D), 10 ng/mL (panel E), and 100 ng/mL (panel F) induced nuclear translocation of p50 and p65 as compared to untreated cells (panel C). Adenoviral overexpression of dnIκK2 or IκBα in HUVEC abolished the IL-33-induced mRNA expression specific for ICAM-1, VCAM-1, E-selectin, and MCP-1 as compared to control adenovirus (AdV-GFP) (Table).

IL-33 and ST2 Protein and mRNA Are Expressed in Human Atherosclerotic Plaque and Recombinant IL-33 Induces a Proadhesive and Proinflammatory State in Explanted Atherosclerotic Plaque Tissue

As shown on Figure 4 using fluorescence immunohistochemistry, we detect both IL-33 and ST2 protein in human carotid endarterectomy specimens. IL-33 protein is expressed by endothelial cells as shown by colocalization with von Willebrand factor (Figure 4A). Nuclear IL-33 expression and membrane-bound ST2 expression was found on the same cells (Figure 4B and 4C). Using RealTime-PCR, we detected also mRNA specific for IL-33 and ST2 in human carotid plaques samples. Moreover, we found a positive correlation between IL-33 mRNA and ST2 mRNA expression (r=0.566; P=0.044; Figure 4D) in atherosclerotic tissue. In order to determine whether the effect of IL-33 on the expression of adhesion molecules and MCP-1 seen in vitro is reproducible in an ex vivo situation, we treated explanted human carotid atherosclerotic plaques with IL-33 for 24 hours (n=12) to determine MCP-1, IL-6, and IL-8 protein, or for 6 hours (n=7) to assess ICAM-1, VCAM-1, E-selectin, MCP-1, IL-6, and IL-8 mRNA. As shown in Figure 5, IL-33 significantly increased ICAM-1 mRNA (P=0.018), VCAM-1 mRNA (P=0.028), E-selectin mRNA (P=0.018), MCP-1 mRNA (P=0.018), and IL-6 mRNA (P=0.018) expression as well as MCP-1 protein (P=0.031), IL-6 protein (P=0.00006) and IL-8 protein (P=0.003). IL-8 mRNA level also increased. However, the difference did not reach statistical significance (P=0.063, data not shown).

Discussion

Leukocyte interaction with vascular endothelial cells is a pivotal event in the inflammatory response characteristic for many pathologies including atherosclerosis. Circulating leukocytes adhere poorly to healthy endothelium under physiological conditions. When the endothelium becomes activated, however, it expresses adhesion molecules that bind cognate ligands on leukocytes. This switch from a resting, antiadhesive to an inflammatory activated, adhesive endothelium is a key event in the development of endothelial dysfunction, which is considered a hallmark in the early pathogenesis of
atherosclerosis. Selectins are involved in the rolling and tethering of leukocytes on the vascular wall. ICAM-1 and VCAM-1 induce firm adhesion of inflammatory cells at the vascular surface. Chemokines such as MCP-1 provide a chemotactic stimulus to the adherent leukocytes, directing their diapedesis and migration into the vessel wall. Classical inflammatory mediators such as IL-1 and TNF-α were shown to induce VCAM-1, ICAM-1, and E-selectin in human endothelial cells. Here, we show for the first time that the novel IL-1 cytokine family member, IL-33, induces rapid adhesion of leukocytes to monolayers of human endothelial cells isolated from coronary artery and umbilical vein. On further analysis, we could show upregulation of the production of the adhesion molecules VCAM-1, ICAM-1, E-selectin, and the chemokine MCP-1 in these endothelial cells by IL-33. The upregulation of these adhesion molecules was concentration-dependent with a significant increase seen at concentrations between 1 ng/mL and 100 ng/mL of IL-33. In agreement with a recently published study, we found that IL-33 also upregulated the inflammatory cytokines IL-6 and IL-8 in human endothelial cells.

Table. AdV-dnIKK2 and AdV-IκBα Inhibited Interleukin-33-Induced ICAM-1, VCAM-1, E-Selectin, and MCP-1 mRNA Expressions in HUVEC

<table>
<thead>
<tr>
<th></th>
<th>ICAM-1</th>
<th>VCAM-1</th>
<th>E-Selectin</th>
<th>MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>No virus</td>
<td>3.4±0.7</td>
<td>9.2±3.8</td>
<td>17.1±0.9</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td>AdV-GFP</td>
<td>5.2±1.0</td>
<td>10.1±1.5</td>
<td>17.2±5.4</td>
<td>2.5±0.01</td>
</tr>
<tr>
<td>AdV-dnIKK2</td>
<td>1.2±0.1</td>
<td>2.8±0.3</td>
<td>9.3±3.7</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>AdV-IκBα</td>
<td>1.1±0.5</td>
<td>1.6±0.1</td>
<td>4.7±1.5</td>
<td>0.8±0.3</td>
</tr>
</tbody>
</table>

HUVEC were left uninfected or were infected with a recombinant adenovirus expressing dnIKK2 (AdV-dnIKK2), IκBα (AdV-IκBα) or a control adenovirus (AdV-GFP) for 4–6 h. 48-h post infection cells were stimulated with Interleukin-33 (100 ng/mL) for 6 h whereas control cells were left untreated. mRNA was prepared and RealTime-PCR with primers specific for intercellular adhesion molecule-1 (ICAM-1), vascular cell AM-1 (VCAM-1), endothelial selectin (E-selectin), monocyte chemoattractant protein-1 (MCP-1), and GAPDH was performed as described in “Methods.” Each experiment was performed in triplicates. Adhesion molecules and MCP-1 mRNA levels were normalized according to the respective GAPDH mRNA level. Values are given as x-fold of control, which was set as 1 and represent mean values±SD of 2 different experiments. The overall ANOVA for each tested molecule remained significant after correction for 4 comparisons.

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Figure 3. Interleukin (IL)-33 induces NF-κB p50 and p65 subunit nuclear translocation in human coronary artery and umbilical vein endothelial cells. Confluent monolayers of human umbilical vein endothelial cells were incubated for 15, 30, or 60 minutes in the absence or presence of IL-33 at 100 ng/mL. Preparation of nuclear extracts and quantification of (A) p50 and (B) p65 NF-κB subunits were performed as described in “Methods.” Each experiment was performed in triplicates. Values are given as OD492 nm and represent mean values±SD of 2 different experiments. C–F. Confluent monolayers of human coronary artery endothelial cells were incubated for 1 hour in the absence (C) or presence of IL-33 at 1 (D), 10 (E), or 100 ng/mL (F). p50 (in green), p65 (in red) and nuclei (in blue) staining was performed as described in “Methods.” Original magnification ×1000. A representative experiment is shown. Experiments were performed 2 times.
exerted direct proinflammatory properties on human endothelial cells without a requirement for intermediate autocrine or juxtacrine action of IL-1β, as the natural antagonist IL-1Ra, which inhibits IL-1β action by preventing its binding to specific receptors, did not inhibit IL-33-induced upregulation of ICAM-1, VCAM-1, E-selectin, or MCP-1 specific mRNA in these cells. We also show that IL-33 induces nuclear translocation of NF-κB p50 and p65 subunits in both types of endothelial cells suggesting that the effects of IL-33 are mediated via the NF-κB pathway. This notion is further supported by our finding that the stimulatory effect of IL-33 on adhesion molecule and MCP-1 expression is abolished by adenoviral overexpression of IκB and IkB kinase in these cells. In agreement with our observations, IL-33 has been shown to activate NF-κB in various other cell types such as mast cells,13,20,46 eosinophils,47 basophils,18 CD4+ T-cells48 and rat neonatal cardiac myocytes and fibroblasts.25 Although IL-33 was previously shown to activate Erk1/2 and Akt pathways,27,31 MEK inhibitor U0126 or PI3K inhibitor LY-294002 did not abrogate induction by IL-33 of any of the proteins tested in our study.

Moreover, we found that IL-33 and ST2 are expressed in human atherosclerotic tissue. Our study is the first that demonstrated expression of both IL-33 and ST2 in human atherosclerotic tissue. IL-33 protein is localized to endothelial cells in human atherosclerotic tissue. It should be emphasized that a previous study demonstrated increased IL-33 expression in the atherosclerotic aorta of ApoE−/− mice fed a high-fat diet as compared to ApoE−/− mice fed a normal diet and to wild-type mice.28 Here, we describe that in human atherosclerotic plaques nuclear IL-33 and membrane-bound ST2 protein are expressed by the same cells, namely by endothelial cells, and that IL-33 mRNA significantly correlates with ST2 mRNA expression in carotid atherosclerotic tissue, suggesting that both proteins are highly coregulated in this tissue. Furthermore, ex vivo treatment of atherosclerotic tissue samples with IL-33 increased the expression of ICAM-1, VCAM-1, E-selectin, and MCP-1 in these tissue specimens.

Since the discovery of IL-33 in 2005, numerous immunomodulator effects of this cytokine were described in different cells. IL-33 enhances adhesion and survival of
mast cells, eosinophils and basophils, as well as release of different cytokines from these cells, and is a chemoattractant for Th2 cells in vitro.19–22,49 Furthermore, it was shown recently that IL-33 upregulated cell surface expression of the adhesion molecule ICAM-1 on eosinophils, but it suppressed that of ICAM-3 and L-selectin.47 In an in vivo experimental sepsis model, IL-33 treatment increased neutrophil influx into the peritoneal cavity and induced more efficient bacterial clearance, which was associated with reduced mortality.50 In a murine model of collagen-induced arthritis, IL-33 treatment markedly exacerbated neutrophilic and polymorphonuclear cell infiltration into the joint which was associated with disease progression.51 Taken together, IL-33 appears to be an important immune regulator that plays a role in inflammation, especially in the rapid recruitment of certain effector cells into sites of ongoing inflammation.

Compared to these effects of IL-33 on the immune system, data on its contribution to cardiovascular pathology is scarce. 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.27,31 A possible role for IL-33 in the development and progression of atherosclerosis is suggested but not well characterized yet. IL-33 was shown to be expressed in cells that are known to be present in atherosclerotic lesions and to activate such cells.23 For example, IL-33 was found in coronary artery endothelium,29 and was shown to induce the production of proinflammatory cytokines from human mast cells and T-cells and to enhance lipopolysaccharide (LPS)-induced TNF-α, IL-6, and IL-1β production from macrophages.24,52,53 It should be noted that recently generated IL-33-deficient mice showed a substantially diminished LPS-induced systemic inflammatory response.54 Furthermore, IL-33 mRNA expression was found in human endothelial cells, coronary artery smooth muscle cells, and peripheral blood monocytes and leukocytes.13,28,29,55 In contrast to our data presented here, which suggest a detrimental contribution of IL-33 to the early development of atherosclerosis, in ApoE−/− mice fed on a high-fat diet treatment with IL-33 had reduced atherosclerotic lesions in the thoracic aorta via increased IL-5 and oxidized low density lipoprotein auto-antibody production as well as by decreased macrophage foam cell formation.28,30 It should be noted, however, that in these latter studies, which showed antiatherosclerotic effects of long-term administration (6 weeks) of IL-33, 1 μg/injection of the cytokine was administered into ApoE−/− mice that already had developed atherosclerosis.28,30 IL-33 administration increased Th2 cytokines, IL-4, IL-5, and IL-13, and reduced the Th1 cytokine IFN-γ by lymph node cells in vitro and in serum ex vivo.28 The level of the Th17 cytokine IL-17 was not different in lymph node cells between IL-33- and PBS-treated animals, and IL-17 and IL-10 serum levels were not detectable. Local cytokine expression in the vasculature ex vivo was not investigated in this study. There is strong evidence supporting a proatherogenic role of Th1 cells, whereas a possible contribution of the Th2 subset in atherosclerotic lesion formation is controversially discussed.45,56 The role of the Th2 pathway in the development of atherosclerosis seems to be depending on the stage and site of the lesion, and on the experimental model.57 Both anti- and proatherogenic effects of IL-4, another prototypical Th2 cytokine, and IL-17, a cytokine produced by Th17 cells, were shown in different studies using different experimental approaches.57 It should be noted that Th2 cell induction is promoted in severe hyperlipidemia,58 and under these conditions Th2 cytokines could be a target for IL-33 as shown in this experimental mouse model of atherosclerosis.28,30

These contradictory findings could be also explained on the basis of different roles for IL-33 and ST2 in distinct ST2-expressing cells. In support of that concept, IL-33 is able to enhance the production of the Th1 cytokine IFN-γ by natural killer cells and invariant cells,22,59 which are found in atherosclerotic lesions and are thought to be involved in the pathogenesis of atherosclerosis.60 It is of interest that in our study recombinant human IL-33 at 100 ng/mL did not affect the production of the Th2 cytokines IL-4 and IL-5 and the T regulatory cytokine IL-10 in human coronary artery endothelial cells.

Upregulation of adhesion molecules and chemokines in endothelial cells as shown here by us is, besides an impaired vasomotor function, a key event in endothelial dysfunction that is considered to be an early hallmark in the pathogenesis of atherosclerosis.2–3 It should also be emphasized that in one of the articles mentioned above IL-33 injection into the mice enhanced their IL-6 serum levels.28 IL-6 has been shown to contribute to development of atherosclerotic lesions, and its serum levels correlate with poor prognosis in humans with cardiovascular disease and are an independent predictor of sudden death in asymptomatic men.51–63 Furthermore, it should be noted that IL-33 was shown to be associated with other human inflammatory pathologies such as rheumatoid arthritis, systemic sclerosis, inflammatory bowel disease, chronic pancreatitis, asthma, psoriasis, and anaphylactic shock.23,24 However, the mechanisms of the deleterious effects of IL-33 in these disorders are not completely understood yet.

In conclusion, we provide evidence for the first time that the latest member of the IL-1 cytokine family, the “alarmin” IL-33,14,16 is present in human atherosclerotic lesion and stimulates the expression of the adhesion molecules ICAM-1, VCAM-1, E-selectin, and the chemokine MCP-1 in atherosclerotic tissue ex vivo and in human coronary artery and umbilical vein endothelial cells in an IL-1, MEK- and PI3K-independent but ST2- and NF-κB-dependent manner in vitro. Given the fact that the expression of ICAM-1, VCAM-1, E-selectin, and MCP-1 in atherosclerotic lesions has been reported to increase during atherogenesis and that this expression seems to be directly associated with plaque progression,2,3,10,11 we hypothesize that IL-33, by promoting adhesion molecules and proinflammatory cytokine expression in the endothelium as well as adhesion of blood cells to the endothelium, may contribute to early events in endothelial dysfunction characteristic for the development of atherosclerotic lesions in the vessel wall.
Sources of Funding

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Disclosures

None.

References


Interleukin-33 Induces Expression of Adhesion Molecules and Inflammatory Activation in Human Endothelial Cells and in Human Atherosclerotic Plaques
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Supplemental Material

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- Supplemental Methods
- Supplemental Tables I, II, III
- Supplemental Figures I, II, III

Supplemental Methods

Isolation of human polymorph-nuclear leukocytes (PMNL)

Human polymorph-nuclear leukocytes (PMNL; 99% neutrophils, less than 1% eosinophils) were isolated from heparinized (100 U/mL) peripheral venous blood of healthy donors as described. Red blood cells were sedimented with 6% dextran (Pharmacia, Uppsala, Sweden) in Hank’s balanced salt solution (HBSS; Sigma, St. Louis, MO, USA) for 60 min at room temperature, leukocyte-rich plasma was collected and PMNL were then isolated by centrifugation, washed and resuspended to $1 \times 10^6$ cells/mL in RPMI-1640 (Sigma) with 10 mmol/L HEPES (Sigma).

Adhesion assay under static conditions

PMNL adhesion to HCAEC or HUVEC was measured as described. Briefly, endothelial cells were plated in gelatine coated 24-well plates and allowed to grow to confluence. The medium was removed and cells were then incubated with 1.0 mL/well of minimum essential medium (M199; Sigma) containing 20% fetal calf serum (FCS, Lonza, Verviers, Belgium) with or without recombinant human (rh) IL-33 (R&D Systems; Minneapolis, MN). After 4 h of incubation the culture supernatant of such treated cells was removed and the cells were gently washed three times with M199. 1.0 mL of HBSS containing $1 \times 10^6$ PMNL was then added to the endothelial cell monolayer in each well. The binding phase of the assay was performed at
37°C in a 5% CO₂ atmosphere for 5, 15 and 30 min. Thereafter, the wells were washed with HBSS (1.0 mL/well) three times and all wells were examined under microscope in order to determine whether loss of endothelial cells had occurred during incubation or washing. An Olympus IMT 2 microscope was used for light microscopy with 10x lens with numeric apertures of 0.30. A Canon EOS-400 camera was used to acquire images.

**Adhesion assay under flow conditions**

HCAEC (4.3×10⁵/substrate) were grown on VenaEC biochips (Cellix, Dublin, Ireland). After reaching confluence (usually within two days) endothelial layers were incubated with fresh M199 (Sigma) containing 20% FCS (Lonza) with or without rh IL-33 (10 or 100 ng/mL) for 4 h. Endothelial monolayers were superfused with suspensions of 3×10⁶ /mL PMNL or monocytes at 0.5 dyne/cm² for 2 min and 20 seconds at 37°C in the OKOLAB H201-T1 heated cage. PMNL and monocytes were preconditioned in endothelial cells media for 5 min at 37°C prior to the flow experiment. In some experiments the endothelial layers were pre-incubated with blocking antibodies for E-selectin, VCAM-1, ICAM-1 (10 µg/mL each; R&D Systems) or all three antibodies together (5 µg/mL each) or with an isotype matched control antibody at the same concentrations for 15 min at 37°C. Cell adhesion was monitored by phase contrast on a Zeiss Axiovert 40 CFL microscope and a Zeiss A-Plan 10x/0.25 Ph1 lens, using Hamamatsu ORCA-03G digital camera and VenaFlux software (Cellix). Computerized image analysis was performed by DucoCell analysis software (Cellix), where adherent cells were quantified on each single image.

**Tissue sampling**

Atherosclerotic plaques were collected from 35 patients undergoing carotid endarterectomy. All subjects were Caucasian and did not suffer from acute infection, autoimmune or neoplastic disease. The carotid endarterectomy samples for IL-33 mRNA and ST2 mRNA
analysis (n=13, 85% male, mean age 70 years, 38% symptomatic) were snap-frozen in liquid nitrogen in the surgery room and stored at -80°C until RNA extraction. The carotid endarterectomy tissue for immunofluorescence analysis (n=3, 67% male, mean age 72 years, 33% symptomatic) were fixed in 4% formalin and embedded in paraffin. The carotid endarterectomy samples (n=19), which were used for culture experiments, were collected in sterile tubes with M199 containing 5% FCS. These fresh tissues were cut into small pieces, and for each plaque, equal numbers of nonadjacent plaque pieces were randomly distributed into wells of a 24-well plate filled with M199 that contained 5% FCS as described previously.\textsuperscript{5} They were stimulated with 100 ng/mL rh IL-33 for 24 h (n=12, 67% male, mean age 65 years, 42% symptomatic) for MCP-1, IL-6 and IL-8 protein determination or for 6 h (n=7, 100% male, mean age 69 years, 17% symptomatic) for ICAM-1, VCAM-1, E-selectin, MCP-1, IL-6 and IL-8 mRNA measurement. Stimulations were done in triplicates. After incubation, tissue was shock-frozen for RNA isolation or culture supernatants were collected for further analysis by ELISA.

Flow cytometry

ICAM-1, VCAM-1 and E-selectin expressions at the cell surface were measured by means of flow cytometry (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ, USA). HUVEC and HCAEC were gently detached by detachment buffer (25 mM HEPES (Boehringer Mannheim GmbH, Germany), 10 mM EDTA (Pierce, Rockford, IL) in Dulbecco’s PBS without calcium and magnesium (PAA, Pasching, Austria)). Endothelial cells were incubated for 30 min at 4°C in dark with primary antibodies against ICAM-1 FITC (mouse anti-human CD54; Beckman Coulter, Brea, CA, USA), VCAM-1 (PE-Cy\textsuperscript{TM}5 mouse anti-human CD106; BD Pharmingen, San Jose CA, USA) and E-selectin (PE mouse anti-human CD62E; BD Pharmingen) which were diluted 1:40 or with respective isotype-matched control antibodies in antibody diluents solution (DAKO North America, Inc., CA, USA; catalogue number
S3022). After the cells were washed once with PBS and were resuspended in fixative solution (FACS flow solution, distilled water, BD CellFix™), flow cytometric analysis was performed by FACS Diva software (Becton Dickinson). For IL-4, IL-5, IL-10 determination supernatants were collected from HCAEC incubated in the absence or presence of 100 ng/ml IL-33 for 4, 16 and 24 h, respectively. Supernatants were processed per manufacturer’s instruction (BD Cytometric Bead Array, Human Th1/Th2 cytokine kit). In detail, 25µl conditioned supernatant was added to 25µl mixed beads (each specific for IL-4, IL-5, IL-10) and 25µl PE detection reagent. The mixture was incubated for 3 h at room temperature in the dark. The beads were washed and analyzed immediately by flow cytometry. Mean fluorescence intensities (MFI) for treated endothelial cells were compared to the MFI of unstimulated cells.

**Total RNA purification and cDNA preparation**

Cells were treated as described, supernatants were removed and total cellular RNA was isolated using High Pure RNA Isolation Kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. For IL-33 mRNA and ST2 mRNA measurement, three representative samples (each 25 mg of wt) were collected from each carotid artery lesion. Frozen tissue was homogenized using a ball mill (Retsch, Haan, Germany), and mRNA was isolated using High Pure RNA Tissue Kit (Roche). Reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche).

**RealTime Polymerase Chain Reaction**

RealTime-PCR was performed using LightCycler® TaqMan® Master (Roche) according to the manufacturer’s instructions. Primers were designed using the Roche Universal ProbeLibrary Assay Design Centre (http://www.universalprobelibrary.com/): GAPDH (forward primer: 5’-agccacatcgctcagacac-3’, reverse primer: 5’-gcccaatacgaccaaatcc-3’, UPLprobe #60; Amplicon Size [bp] 66) – ST2 (forward primer: 5’-ttgtcctaccattgacctata-3’,
reverse primer: 5’-gatccttggaagacgctgacaa-3’, UPL probe #56; Amplicon Size [bp] 75 – IL-33
(forward primer: 5’-agcaagttgaagaaccacga-3’, reverse primer: 5’-ctctctctttggtcttg-3’, UPL probe #33, Amplicon Size [bp] 74 – VCAM-1 (forward primer: 5’-tgtaaatggaaattggagaaga-3’, reverse primer: 5’-tgtaaatgccttggattgagga-3’, UPL probe #39; Amplicon Size [bp] 69) – ICAM-1 (forward primer: 5’-ccctctccacgtgtgactgg-3’, reverse primer: 5’-acgctagggtaagctttgac-3’, UPL probe #71; Amplicon Size [bp] 90) – E-selectin (forward primer: 5’ accagggccaggtgtgaag-3’, reverse primer: 5’ ggtggagaaggcgtgc-3’, UPL probe #86; Amplicon Size [bp] 89) – MCP-1 (forward primer: 5’-ttctgctgctgtgctcat-3’, reverse primer: 5’-ggtggagaaggcgtgc-3’, UPL probe #83; Amplicon Size [bp] 73) – IL-6 (forward primer: 5’-gatgagttggagagctgatg-3’, reverse primer: 5’-ctgcaagcccctggttctgt-3’, UPL probe #40; Amplicon Size [bp] 130) – IL-8 (forward primer: 5’-agacagccagagcacaac-3’, reverse primer: 5’-atggtccctccggtggt-3’, UPL probe #72; Amplicon Size [bp] 62). The amplification conditions consisted of an initial incubation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec, 63°C for 20 sec and 72°C for 6 sec and a final cooling to 40°C. Data was analysed using LightCycler Software Version 3.5 (Roche).

**Nuclear extraction and analysis of NF-κB/DNA binding**

Endothelial cells were incubated for 15, 30 or 60 min in 1.25% FCS with or without rh IL-33 at a concentration of 100 ng/mL. Preparation of nuclear extracts was performed using a Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer’s instructions. Quantitation of p50 and p65 NF-κB subunits in nuclear extracts of such treated cells was performed using the ELISA-based TransAM™ NF-κB Family kit (Active Motif, Rixensart, Belgium) as described previously.6
NF-κB translocation staining

HCAEC were seeded on Permanox chamber slides (Nunc Inc., Naperville, IL, USA). Confluent monolayers were treated with fresh M199 containing 1.25% FCS without or with 1, 10 or 100 ng/mL IL-33 for 1 h. Monolayers were washed with PBS and fixed with 3.7% paraformaldehyde. The cells were washed again and then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 20 min at room temperature, washed in PBS, and blocked with 5% bovine serum albumin (BSA; Sigma) in PBS for 30 min. Subsequently, cells were incubated for 1 h at room temperature with a goat polyclonal anti-human p50 antibody (Santa Cruz, CA, USA) at a dilution of 1:100 or goat IgG (R&D Systems) as an isotype control in diluent solution for primary antibody (DAKO, catalogue number S3022). After washing, a 1:200 dilution of afluorescein (FITC)-conjugated mouse anti-goat antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in diluent solution for secondary antibody (DAKO, catalogue number S0809) was incubated with the cells for 1 h at room temperature in the dark. After washing, the cells were incubated for 1 h with a rabbit polyclonal anti-human NF-κB p65 (Santa Cruz) at 1:100 or rabbit IgG (R&D Systems) followed by washing step and 1 h incubation with Texas Red dye-conjugated donkey anti-rabbit antibody (Jackson) at 1:200 in the dark. After final washes, cells were mounted using Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA) and sealed with nail polish. Fluorescence was assessed by microscopy with Zeiss Axio Imager.M2 with 100x lens (numeric aperture 0.9) with immersion oil (Imersol™, Zeiss, Oberkochen, Germany) using AxioVision Rel. 4.8 software.

Immunofluorescence analysis of IL-33 and ST2 in human atherosclerotic tissue

Primary antibodies, mouse monoclonal anti-IL33 antibody (clone Nessy-1, 1:1000 dilution; Alexia Biochemicals, Enzo Life Sciences AG, Lausen, Switzerland), rabbit polyclonal anti-
ST2 antibody (IL1RL1) (1:100 dilution; Sigma) and rabbit polyclonal antibody anti-von Willebrand factor (1:500 dilution; Dako), were incubated overnight at 4°C. After extensive washing in PBS, slides were incubated with secondary antibodies for 2 h at room temperature in the dark. Secondary antibodies were Alexa Fluor-488 goat anti-mouse IgG (Invitrogen-Molecular Probes), Alexa Fluor-546 goat anti-rabbit IgG (Invitrogen-Molecular Probes) and Cy 5 goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). All antibodies were diluted in PBS containing 0.05% Tween-20, 3% BSA for blocking and 0.1% Triton X-100 for permeabilization. Nuclear counter staining was performed with DAPI (1µg/mL; Sigma) for 10 min at room temperature. Tissue sections were analyzed with a confocal laser scanning microscope (LSM-700; Carl Zeiss) with 20x lens (numeric aperture 0.8) or 63x lens with oil (numeric aperture 1.4) using ZEN 2009 software. Tissue sections of human normal tonsil or human colon from Crohn's disease patients, obtained from the Department of Pathology, Medical University of Vienna, Austria, were used as a positive control for IL-33 or ST2 staining, respectively.7-10

**Adenoviral infection**

To study a potential NF-κB-dependent effect on endothelial cells upon IL-33 stimulation, HUVEC were infected with adenoviral vectors for overexpression of IκBα (AdV-IκBα) or for overexpression of a mutant dominant negative IκB kinase 2 (AdV-dnIKK2), respectively, as described previously.11, 12 Infection was performed in M199 supplemented with 20% FCS, 2mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 5 U/mL heparin, and 25 µg/mL endothelial cell growth supplement (Promocell, Heidelberg, Germany) for 4-6 h with the AdV-IκBα, the AdV-dnIKK2 or control adenovirus (AdV-green fluorescent protein (GFP))13 at a multiplicity of infection of 100. 48 h post infection cells were stimulated with IL-33 (100 ng/mL) for 6 h.
References


Supplemental Table I. IL-1 mRNA is up-regulated by IL-33 in HUVEC and HCAEC.

<table>
<thead>
<tr>
<th></th>
<th>1 h</th>
<th>3 h</th>
<th>6 h</th>
<th>9 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVEC</td>
<td>30.3±7.7*</td>
<td>20.8±0.6*</td>
<td>68.2±15.3*</td>
<td>19.8±0.4*</td>
<td>5.3±0.6*</td>
</tr>
<tr>
<td>HCAEC</td>
<td>3.5±0.7*</td>
<td>7.5±2.2*</td>
<td>6.9±2.6*</td>
<td>5.5±2.9*</td>
<td>3.8±0.8*</td>
</tr>
</tbody>
</table>

Confluent monolayers of HUVEC or HCAEC were incubated for 1, 3, 6, 9 or 24 h in the absence or presence of IL-33 (100 ng/mL). mRNA was prepared and RealTime-PCR with primers specific for IL-1 was performed as described in “Methods”. IL-1 mRNA levels were normalized according to the respective GAPDH mRNA level. Each experiment was performed in triplicates. Values are given as x-fold of control, which was set as 1 and represent mean ± SD of 2 different experiments.*p≤0.05 as compared to respective unstimulated control.
Supplemental Table II. IL-1 receptor antagonist does not counteract IL-33-induced ICAM-1, VCAM-1, E-selectin and MCP-1 mRNA expressions in HUVEC.

<table>
<thead>
<tr>
<th></th>
<th>IL-33</th>
<th>IL-33 + IL-1Ra</th>
<th>IL-1β</th>
<th>IL-1β+ IL-1Ra</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>55.8±8.7</td>
<td>54.1±5.7</td>
<td>20.3±1.0</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>72.6±2.2</td>
<td>76.8±3.1</td>
<td>33.2±5.7</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>E-selectin</td>
<td>1032.1±99.2</td>
<td>1021.8±17.1</td>
<td>343.3±5.7</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>MCP-1</td>
<td>73.3±0.7</td>
<td>65.2±9.2</td>
<td>20.4±2.2</td>
<td>1.2±0.1</td>
</tr>
</tbody>
</table>

Confluent monolayers of HUVEC were incubated for 6 h in the absence or presence of IL-33 (100 ng/mL) or IL-1β (200 U/mL) with or without IL-1Ra (10 µg/mL). mRNA was prepared and RealTime-PCR with primers specific for ICAM-1, VCAM-1, E-selectin and MCP-1 was performed as described in “Methods”. Adhesion molecules and MCP-1 mRNA levels were normalized according to the respective GAPDH mRNA level. Each experiment was performed in triplicates. Values are given as x-fold of control, which was set as 1 and represent mean ± SD of 2 different experiments. The overall ANOVA for each tested molecule remained significant after correction for 4 comparisons.
Supplemental Table III. Mitogen-activated protein/extracellular signal regulated kinase (MEK) inhibitor U0126 or phosphoinoside-3-kinase (PI3K) inhibitor LY-294002 do not abrogate IL-33 induced ICAM-1, VCAM-1, E-selectin or MCP-1 protein in HUVEC.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IL-33</th>
<th>U+IL-33</th>
<th>LY+ IL-33</th>
<th>U</th>
<th>LY</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM</td>
<td>297±50</td>
<td>5338±799</td>
<td>4886±959</td>
<td>5354±983</td>
<td>304±61</td>
<td>254±44</td>
</tr>
<tr>
<td>VCAM</td>
<td>177±63</td>
<td>547±81</td>
<td>591±121</td>
<td>318±80</td>
<td>170±56</td>
<td>182±49</td>
</tr>
<tr>
<td>E-selectin</td>
<td>151±33</td>
<td>4214±434</td>
<td>3666±754</td>
<td>4447±857</td>
<td>156±42</td>
<td>143±40</td>
</tr>
<tr>
<td>MCP-1</td>
<td>2565±44</td>
<td>41945±6670</td>
<td>46288±7007</td>
<td>36147±8406</td>
<td>2496±65</td>
<td>2201±78</td>
</tr>
</tbody>
</table>

Confluent monolayers of HUVEC were pre-incubated for 1 h with the MEK inhibitor U0126 (U) at 1 µM or the PI3K inhibitor LY-294002 (LY) at 5 µM. Thereafter, the cells were treated with or without IL-33 at a concentration of 100 ng/mL for 24 h (for ICAM-1, VCAM-1, MCP-1 measurement) or 4 h (for E-selectin determination) or left untreated (control). Cells were gently detached by detachment buffer and ICAM-1, VCAM-1 and E-selectin expressions at the cell surface were measured by means of flow cytometry as described in “Methods” and MCP-1 protein was determined in conditioned media by ELISA as described in “Methods”. Each experiment was performed in triplicates. Values are given in mean fluorescence intensity (MFI) for ICAM-1, VCAM-1, E-selectin or in pg/10^4 cells for MCP-1 and represent mean values ± SD of 3 different experiments. Post hoc analyses indicated significant differences between IL-33 stimulations and controls for each molecule but no significant differences between IL-33 stimulations in the presence and absence of the MEK inhibitor U0126 or the PI3K inhibitor LY-294002. The overall ANOVA was not affected by correction for 4 comparisons.
Supplemental Figure I

Supplemental Figure I. Soluble ST2 fusion protein (sST2-Fc) abolished IL-33 effects in human endothelial cells. IL-33 (1 ng/mL) was incubated with rh sST2-Fc (5 µg/mL) or with IgG1-Fc (5 µg/mL) for 15 min at 37°C before addition to confluent monolayers of HUVEC for 24 h (for ICAM-1, VCAM-1, MCP-1 determination) or 4 h (for E-selectin measurement). Cells were gently detached by detachment buffer and (A) ICAM-1, (B) VCAM-1 and (C) E-selectin expressions at the cell surface were measured by means of flow cytometry as described in “Methods” or (D) MCP-1 protein was determined in conditioned media by ELISA as described in “Methods”. Each experiment was performed in triplicates. (A, B, C) Values are given in mean fluorescence intensity (MFI) or (D) in pg/10^4 cells and represent mean values ± SD of 3 different experiments. The overall ANOVA for each tested molecule remained significant after correction for 4 comparisons.
Supplemental Figure II. Effects of IL-33 on ICAM-1, VCAM-1, E-selectin and MCP-1 mRNA expression by HUVEC. Confluent monolayers of HUVEC were incubated for 1, 3, 6, 9 or 24 h in the absence or presence of IL-33 (100 ng/mL). mRNA was prepared and RealTime-PCR with primers specific for (A) ICAM-1, (B) VCAM-1, (C) E-selectin or (D) MCP-1 was performed as described in “Methods”. Adhesion molecules and MCP-1 mRNA levels were normalized according to the respective GAPDH mRNA level. Each experiment was performed in triplicates. Values are given as x-fold of control, which was set as 1 and represent mean ± SD of 2 different experiments. The overall ANOVA for each tested molecule remained significant after correction for 4 comparisons.
Supplemental Figure III

A

Supplemental Figure III. IL-33 did not modulate Th2 and T regulatory cytokines in HCAEC. Confluent monolayers of HCAEC were incubated for 4, 16, or 24 h in the absence or presence of IL-33 (100 ng/mL). Supernatants were processed per manufacturer’s instruction as described in "Methods". (A) IL-4, (B) IL-5 and (C) IL-10 were measured by means of flow cytometry. Each experiment was performed in triplicates. Values are given in pg/mL and represent mean values ± SD of 3 different experiments. The overall ANOVA for each tested molecule was ≥0.05.