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ORIGINAL RESEARCH

# IL1RAP Expression in Human Atherosclerosis: A Target of Novel Antibodies to Reduce Vascular Inflammation and Adhesion

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**BACKGROUND:** Blockade of IL1RAP (interleukin 1 receptor associated protein) was recently shown to reduce atherosclerosis in mice, but the effect on human vascular cells is largely unknown. Targeting the IL1RAP coreceptor represents a novel strategy to block the IL1RAP-dependent cytokines IL (interleukin)-1, IL-33, and IL-36. In the present study, we aimed to evaluate the role of novel antibodies targeting IL1RAP to reduce the effects of IL-1β, IL-33, or IL-36γ in human vascular cells.

**METHODS:** Expression of IL1RAP was observed in human atherosclerotic plaques by immunohistochemistry and microarray and in endothelial cells by flow cytometry. Endothelial cells were cultured with IL-1β, IL-33, or IL-36γ cytokines with or without IL1RAP antibodies and analyzed with Olink proteomics, ELISA, Western blot, and real-time quantitative polymerase chain reaction. The functional effect of IL1RAP antibodies on endothelial cells were analyzed with adhesion and permeability assays.

**RESULTS:** Olink proteomics showed inhibition of the inflammatory proteins LIF (leukemia inhibitory factor), OPG (osteoprotegerin), CCL4 (C-C motif chemokine ligand 4), and MCP-3 (monocyte chemoattractant protein 3) by IL1RAP-blockade in endothelial cells after IL-1β stimulation. In addition, the IL1RAP antibodies inhibited IL-1β, and IL-33 induced IL-6 and IL-8 secretion. Secretion of MCP-1 (monocyte chemoattractant protein 1) was induced by IL-1β, IL-33, and IL-36γ, and subsequently was inhibited by IL1RAP antibodies. Similar effects were found on mRNA expression level. Endothelial expression of the adhesion markers *ICAM1*, *VCAM1,* and *SELE* were significantly reduced by IL1RAP antibodies, and neutrophil adhesion to endothelial cells induced by IL-1β and IL-33 was reduced by IL1RAP blockade. In human atherosclerotic lesions, *IL1RAP* expression correlated with markers of inflammation like *IL6*, *IL8,* and *MCP1*.

**CONCLUSIONS:** IL1RAP-targeting antibodies can reduce the expression of inflammatory cytokines and markers of adhesion in endothelial cells, which may be of importance for future putative targeted treatments against cardiovascular disease.

**Key Words:** endothelial cells ■ HUVECs ■ IL-1 ■ IL-33 ■ IL-36

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Atherosclerosis is the underlying cause of cardiovascular diseases (CVDs), and inflammation is a central hallmark of the disease. The formation of atherosclerotic lesions is facilitated by dyslipidemia, hypertension, and proinflammatory mediators, beginning with endothelial activation via expression of adhesion molecules such as E-, P-, or L-selectins and integrins like ICAM (intercellular adhesion molecule) and VCAM (vascular cell adhesion molecule).[1,2] The expression of adhesion molecules facilitates the attraction and

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## RESEARCH PERSPECTIVE

### What New Question Does This Study Raise?

- Targeting the IL1RAP (interleukin 1 receptor associated protein) coreceptor in vascular inflammation represents a novel strategy to block the IL1RAP-dependent cytokines IL (interleukin)-1, IL-33, and IL-36 simultaneously.
- The IL1RAP antibodies in this study affect the proinflammatory effects of IL-1β, IL-33, and IL-36γ in human endothelial cells by reducing cytokines, chemokines, and markers of adhesion, which are central in the development of atherosclerosis.

### What Question Should Be Addressed Next?

- Could IL1RAP blockade reduce vascular inflammation in patients with cardiovascular disease?

### Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **ATH** | atheroma plaque |
| **CANTOS** | Canakinumab Anti-Inflammatory Thrombosis Outcome Study |
| **CCL** | C-C motif chemokine ligand |
| **CD** | cluster of differentiation |
| **CXCL** | C-X-C motif chemokine ligand |
| **FACS** | fluorescence activated cell sorting |
| **HUVEC** | human umbilical vein endothelial cell |
| **ICAM** | intercellular adhesion molecule |
| **IL1R3** | interleukin-1 receptor 3 |
| **IL1RAcP** | interleukin-1 receptor accessory protein |
| **IL1RAP** | interleukin 1 receptor associated protein |
| **IL-36R** | interleukin-36 receptor |
| **LIF** | leukemia inhibitory factor |
| **MAPK** | mitogen activated protein kinase |
| **MCP** | monocyte chemoattractant protein |
| **MIT** | macroscopically intact carotid tissue |
| **NF-κB** | nuclear factor κB |
| **OPG** | osteoprotegerin |
| **SELE** | E-selectin |
| **IL-33R** | IL-33 receptor |
| **VCAM** | vascular cell adhesion molecule |

migration of leukocytes into the atherosclerotic lesion, followed by production of proatherogenic cytokines and chemokines, changes in the extracellular matrix, plaque disruption, and ultimately thrombosis.[1]

One of the key drivers of inflammation in atherosclerosis is the IL (interleukin)-1 family of cytokines.[3,4] The IL-1 family of cytokines is a broad group of 11 different cytokines involved in both innate and adaptive immune defense.[5] The IL-1 family of cytokines acts via receptor binding and recruitment of a coreceptor, whereof IL1RAP (IL-1 receptor associated protein), also called IL1RAcP (IL-1 receptor accessory protein) or IL1R3 (IL-1 receptor 3), is crucial for IL-1, IL-33, and IL-36 signaling.[4] Upon IL-1 signaling, IL1RAP associates with the IL-1R1 receptor, whereas in the cases of IL-33 or IL-36 signaling, IL1RAP binds to ST2 (supression of tumorigenicity 2; also called the IL-33 receptor or IL-1 receptor 4) or IL-36R (IL-36 receptor; also called IL-1receptor 6 or IL-1 Receptor-Related Protein 2), respectively.[5–7] Cytokine signaling is followed by activation of intracellular pathways such as MAPKs (mitogen activated protein kinases) and NF-κB (nuclear factor κB) and induction of genes encoding proinflammatory cytokines such as TNF-α (tumor necrosis factor α), IL-6, IL-8, IL-12, and several chemokines like MCP-1 (monocyte chemoattractant protein 1).[5,6,8,9]

In CVDs, the role of the IL1RAP-dependent cytokine IL-1β has been extensively studied. Expression of IL-1β has been observed in atherosclerotic lesions and genetic or pharmacological inhibition of IL-1β ameliorated atherosclerosis in *ApoE*$^{-/-}$ (apolipoprotein E) mice or mice fed with an atherogenic diet.[10–12] Moreover, the CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcome Study) trial showed that administration of the anti-IL-1β antibody canakinumab to patients with previous myocardial infarction resulted in reduced C-reactive protein levels and lower frequency of recurrent events.[13] On the other hand, the roles of the additional IL1RAP-dependent cytokines IL-33 and IL-36 in atherosclerosis is not as well described. Expression of IL-33 has been observed in atherosclerotic lesions,[14] and pharmacological delivery of IL-33 resulted in reduced development of atherosclerosis in *ApoE-/-* mice,[15] although genetic deficiency of IL-33 did not affect the severity of atherosclerosis in *ApoE*$^{-/-}$ mice.[16] There are only a few studies to date that implicate a role for IL-36γ in CVDs, and expression of IL-36γ has been identified in aortic arches and early atherosclerotic lesions from *ApoE*$^{-/-}$ mice.[17] Furthermore, IL-36γ administration exacerbates atherosclerosis in *ApoE*$^{-/-}$ mice and elevated levels of IL-36γ have been determined in plasma from patients with coronary artery disease compared with healthy controls.[17]

Simultaneous inhibition of IL1RAP-dependent cytokines could be favorable in diseases driven by this group of cytokines.[18–21] Targeting the coreceptor IL1RAP represents a novel strategy to reduce the effects of IL-1-, IL-33-, and IL-36-driven inflammation in CVDs and a possibility for simultaneous inhibition of these cytokines.[21] A recent study demonstrated

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reduced atherosclerosis and plaque inflammation after IL1RAP blockade in *ApoE*-deficient mice,[20] but the knowledge about the role of targeting IL1RAP in human vascular cells is limited.

In the present investigation, we aimed to identify IL1RAP in human atherosclerotic lesions and to characterize 3 of the IL1RAP dependent cytokines, IL-1β, IL-33, and IL-36γ, and their inhibition via novel antibodies targeting IL1RAP in endothelial cells. Investigating the effects of IL1RAP inhibition in human endothelial cells is of importance for future putative targeted therapy against IL1RAP in patients with CVD.

# METHODS

## Ethics and Data Availability

All experiments using human material were conducted in accordance with the ethical guidelines of both the declaration of Helsinki and Swedish National Board of Health and Welfare. Ethical permission was acquired for blood withdrawal from healthy volunteers at Örebro University Hospital (2015-543; Swedish Ethical Review Authority, Uppsala), and for the use of human carotid plaque tissue from the ALIPACA (Atherosclerosis – Lipids and Inflammation in Peripheral Arterial Disease and Carotid Artery Disease) cohort (2018-207; Swedish Ethical Review Authority, Uppsala), and the study subjects gave informed consent. The microarray study was ethically approved as previously described.[22] The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Immunohistochemistry of Atherosclerotic Plaque

Immunofluorescent staining of IL1RAP in a human carotid atherosclerotic lesion was obtained by anti-IL1RAP (N1N3) (GTX104513; GeneTex International, Hsinchu City, Taiwan; 1:500). Cellular markers for endothelial cells (anti-human von Willebrand factor; M0616; Dako, Denmark; 1:200), smooth muscle cells (anti-human smooth muscle actin; M0851, Dako; 1:150), and macrophages (anti-CD68 [cluster of differentiation 68]; Novocastra, Leica Biosystems, Newcastle, UK; 1:10) were used in parallel. All antibodies were diluted in da Vinci Green Diluent (Biocare Medical, Pacheco, CA). Sections (4 μm) of formalin-fixed and paraffin-embedded tissues were deparaffinized and rehydrated in Tissue Clear and xylene, decreasing series of ethanol, and lastly in deionized $H_2O$. Antigens were retrieved in Diva decloaker buffer (Biocare Medical) using a decloaking chamber (Biocare Medical) at 110 °C for 10 minutes. The tissues were washed in PBS with 0.1% TritonX before blocking with Background Sniper (Biocare Medical) for 60 minutes. The tissues were incubated with primary antibodies for 60 minutes, followed by secondary antibodies (Cy3 AffiniPure Donkey Anti-Rabbit IgG or CY5-AffiniPure Donkey Anti-Mouse IgG, both from Jackson ImmunoResearch Europe, Cambridge, UK; 1:400) for 120 minutes. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; 5 μg/mL, Thermo Fisher Scientific, Waltham, MA) for 10 minutes and the tissues mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific). Tissues were also stained with only secondary antibodies and used as negative controls. All incubations and rinsing steps using PBS with 0.1% Tween 20 were performed at room temperature, and tissues were kept from light. The stainings were digitalized using Panoramic MIDI digital slide scanner (3DHistech Ltd, Budapest, Hungary), and images were captured in the software CaseViewer version 2.3 (3DHistech Ltd) in ×50 magnification. Brightness and contrast were adjusted and scalebars assembled in ImageJ (ImageJ i.52i, Fiji).

## Flow Cytometry Analysis of IL1RAP-Dependent Cytokine Receptors

For flow cytometry, 100 000 cells per well were added to a V-bottom 96 well plate. The plate was centrifuged at 360*g* for 3 minutes. Supernatant was discarded, and cells were washed with fluorescence activated cell sorting (FACS) buffer (2% FBS and 2 mM ethylenediaminetetraacetic acid (EDTA) in PBS). Primary antibody (anti-IL1RAP_hIgG1, anti-IL-33R_BM1-hIgG2, anti-IL-36R_BM2-hIgG4-A647, produced by Innovagen, Lund, Sweden) at 1, 3, or 10 μg/mL or diluted 1:30 of IL-1R1-PE and isotype control goat IgG-PE (R&D Systems) in FACS buffer were added, and cells were incubated for 30 minutes at 4 °C in the dark. Cells were washed twice with 200 μL FACS buffer per well. For nonlabeled antibodies, cells were stained with secondary anti-human IgG1 APC (Jackson ImmunoResearch, Ely, UK) diluted 1:200 in FACS buffer for 20 minutes at 4 °C in the dark. Cells were washed twice with 200 μL FACS buffer before being analyzed on an Attune NxT flow cytometer (Thermo Fisher Scientific). Histogram and dot plots were generated using FlowJo version 10.9.

## Cell Culture of Endothelial Cells

Human umbilical vein endothelial cells (HUVECs; Thermo Fisher Scientific) from pooled donors were cultured in the endothelial medium VascuLife basal medium supplemented with VEGF LifeFactors kit (LifeLine Cell Technology, Frederick, MD), including antibiotics (10 U/mL penicillin and 10 μg/mL streptomycin; Gibco/Thermo Fisher Scientific) at 37 °C in a humidified incubator with 5% $CO_2$. The HUVEC medium was replaced every 48 to 72 hours, and cells between passage 4 and 10 were used for the experiments.

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## IL1RAP Inhibiting Antibodies

It was previously reported that antibodies specific for IL1RAP have different IL-1 family cytokine inhibition profiles depending on the IL1RAP epitope they engage.[21,23] Two different monoclonal antibodies targeting IL1RAP, developed by Cantargia AB (Lund, Sweden), were used to inhibit IL-1β, IL-33, and IL-36γ signaling. The antibody 3F8 is a potent blocker of IL-1 signaling and was used to block IL-1β, whereas the antibody 3G5 blocks IL-33 and IL-36 signaling more effectively and was used in experiments where endothelial cells were treated with these cytokines.[21]

## Treatments of Endothelial Cells With IL-1β, IL-33, or IL-36γ and Antibodies Against IL1RAP

The HUVECs were seeded in technical duplicates at a density of 10 000 cells per well in 96-well plates, 60 000 cells per well in 24-well plates, or 300 000 cells per well in 6-well plates and allowed to grow overnight to attain adherence and confluency. Dose–response experiments were performed on endothelial cells with IL-1β, IL-33, and IL-36γ, respectively, to identify ideal concentrations for each cytokine (data not shown). Cells were preincubated with 20 μg/mL of anti-IL1RAP (Cantargia AB) or a corresponding mouse IgG2a κ isotype control antibody (eBM2a, catalog number 14-4724-82; eBioscience/Invitrogen/Thermo Fisher Scientific) for 1 hour, followed by treatment with 5 ng/mL mature IL-1β (PHC0814; Thermo Fisher Scientific), 100 ng/mL IL-33 (R&D Systems), or 100 ng/mL IL-36γ (R&D Systems) for 30 minutes to 24 hours, depending on the readout. The culture media was collected and stored at −80 °C until ELISA or Olink proteomics analysis, cells were stored in −80 °C until RNA extraction for real-time quantitative polymerase chain reaction (PCR) analysis or protein expression analysis by Western blot was performed.

## Protein Release From Endothelial Cells

The culture medium was analyzed using the inflammation panel from Olink proteomics (Uppsala, Sweden). The panel contains an array of 92 established protein biomarkers for inflammation (https://www.olink.com/), using the Olink Proseek Multiplex Assay using proximity extension assay technology.[24,25] Briefly, pairs of oligonucleotide-linked antibodies bind to a target protein resulting in proximity of the probes to facilitate hybridization, and this serves as a DNA amplicon that is subsequently detected and quantified by real-time PCR. Protein quantities were log2-transformed using Olink Wizard GenEx (MultiD Analyses, Gothenburg, Sweden). Data are shown as fold changes of normalized protein expression units, which is an arbitrary unit in log2 scale for relative quantification.

Selected cytokines of interest were measured with ELISA in the medium from HUVECs treated with cytokines, antibodies against IL1RAP, or corresponding isotype control for 24 hours. In short, levels of IL-6, IL-8, and MCP-1 were spectrophotometrically measured using DuoSet ELISA kits (R&D Systems) at 450 nm, and protein concentrations were calculated from a standard curve of each protein according to the manufacturer's instructions.

## RNA Extraction, cDNA Preparation, and Quantitative Real-Time PCR

Total RNA was extracted from HUVECs using the E.Z.N.A total RNA kit (Omega Bio-Tek, Norcross, GA) in accordance with the manufacturer's instructions. The cDNAs were prepared using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) and analyzed for the expression of selected cytokines and chemokines *IL6* (Hs00174131), *IL8* (Hs00174103), *MCP1* (Hs00234140), *ICAM1* (Hs00164932), *VCAM1* (Hs01003372), and *SELE* (Hs00174057) using TaqMan universal PCR master mix, TaqMan primers and probes using a QuantStudio 7 Flex real-time PCR system (Thermo Fisher Scientific) according to the manufacturer's instructions. Data were normalized relative to *PPIB* (peptidylprolyl isomerase B; Hs00168719) expression, which was used as a housekeeping gene.

## Gene Expression of Inflammatory Markers and Markers Associated With Adhesion in Human Atherosclerotic Lesions

In the present study, an openly available data set GSE43292[22] of microarray data on 32 atheroma plaques (ATHs) and paired macroscopically intact carotid tissues (MITs) adjacent to the ATHs from the Gene Expression Omnibus database was used. In brief, the material originated from patients with hypertension undergoing carotid endarterectomy at the University Hospital of Lyon (Hôpital Edouard Herriot) and contained samples from media and neointima without adventitia. Samples were hybridized to Affymetrix Human GeneChip Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol. A detailed description was previously published.[22]

In the analysis, data from ATHs and MITs were used to compare the expression in plaque versus intact carotid tissue. Furthermore, we correlated the *IL1RAP* expression in ATHs with (1) significantly affected markers of inflammation evident in the ELISA and Olink proteomics data from HUVECs evaluated in the present study *IL6*, *IL8*, *MCP1*, *LIF*, *OPG* (in the Gene Expression Omnibus database known as *TNFRSF11B*), *CCL4*, *MCP-3*, and (2) adhesion markers *ICAM1*, *VCAM1*, and *SELE*.

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## Neutrophil Adhesion Assay

Peripheral blood from healthy blood donors was collected in EDTA tubes. Human polymorphonucleated leukocytes were isolated from whole blood using Polymorphprep (Axis-Shield PoC AS, Oslo, Norway) and Lymphoprep (Axis-Shield PoC AS) by density gradient centrifugation. The polymorphonucleated leukocytes were suspended in endothelial medium after lysis of the remaining red blood cells using hypotonic shock. Neutrophils at a density of $5\times10^6$ cells/mL were labeled with 50 μg of calcein AM (Thermo Fisher Scientific) for 30 minutes at 37 °C. Cells were washed with PBS and resuspended in endothelial medium.

For the neutrophil adhesion assay, HUVECs were grown to confluence in 96-well microtiter plates in endothelial medium for 24 hours. The HUVECs were treated with cytokines and anti-IL1RAP antibodies for 24 hours. The wells were aspirated and washed with PBS, followed by incubation with calcein AM-labeled cells for 30 minutes at 37 °C in 5% $CO_2$. The wells were aspirated, washed, and fixed with 4% paraformaldehyde. Fluorescence was quantified with a Cytation 3 imaging reader (BioTek/Agilent, Santa Clara, CA) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Cell count and image statistics were performed using Cytation 3 imaging reader (BioTek/Agilent) and Gen5 Image+ software (BioTek/Agilent).

## Permeability Assay

The HUVECs at a density of 200 000 cells per well were allowed to grow on collagen-coated inserts (ECM644; EMD Merck Millipore) for 72 hours to form a monolayer. The cell monolayers were treated with IL-1β and anti-IL1RAP antibodies for 24 hours, followed by addition of fluorescein isothiocyanate (FITC)-dextran (1:40) to the inserts for 20 minutes at 37 °C in 5% $CO_2$. Fluorescence intensities were measured from the culture medium of the bottom chambers with Cytation 3 imaging reader (BioTek/Agilent) using an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

## Western Blot

Western blotting was used to evaluate whether HUVEC intracellular signaling was affected by the anti-IL1RAP treatment. Cells were lysed with ice-cold radioimmunoprecipitation buffer (Merck Millipore, Burlington, MA), and total protein was quantified by a Micro Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Absorbance was measured at 540 nm using Cytation 3 imaging reader (BioTek/Agilent). Cell lysates were mixed with sodium dodecyl sulfate (Sigma-Aldrich) and denatured at 95 °C for 5 minutes. For analyses of IL-1β-induced NF-κB signaling, proteins (10–15 μg) were loaded onto 8%-16% Criterion TGX Stain-Free Precast gels, and protein separation was performed using ×10 Tris/Glycine/sodium dodecyl sulfate running buffer (BioRad, Hercules, CA). Precision Plus Protein Kaleidoscope Prestained Protein Standards (BioRad) was used to determine the molecular mass of the protein. Proteins were blotted on to nitrocellulose membranes and were visualized using a reversible protein stain (Thermo Fisher Scientific). The membranes were blocked using 5% skim milk in TBS-T (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween-20) for 1 hour and probed with the primary antibodies IκBα (44D4) (Cell Signaling Technology, Danvers, MA; #4812, 1:1000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnologies, Dallas, TX; number 47724, 1:5000) for loading control. Primary antibodies incubated overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit Ig (immunoglobulin) Gs (Cell Signaling Technology; number 7074; 1:2000) or horse anti-mouse IgGs (Cell Signaling Technology; number 7076; 1:2000). Proteins were visualized using enhanced chemiluminescence reagent Western-Ready Enhanced Chemiluminescence Substrate Premium Kit (BioLegend, San Diego, CA; number 426319), and chemiluminescence was detected by ChemiDoc MP Imaging system (BioRad).

For analyses of IL-33- and IL-36γ-induced NF-κB signaling, proteins (10 μg) were loaded onto NuPAGE 4%-12% Bis-Tris Gels (Thermo Fisher Scientific), and separation was performed at 140 V in NuPAGE MOPS SDS running buffer (Thermo Fisher Scientific). Magic Mark XP and Novex Sharp prestained protein standards (both from Thermo Fisher Scientific) were used to determine molecular weight. Proteins were blotted on a Immobilion FL transfer membrane (Merck Millipore) and blocked with 5% bovine serum albumin in TBS-T. Membranes were probed with the same primary and secondary antibodies as for IL-1β analyses (see above). Proteins were visualized using Immobilon Western Chemiluminescent horseradish peroxidase substrate solution (Merck Millipore), and chemiluminescence was detected by a LI-COR Odyssey Fc imager and analyzed with Image Studio Software (both from LI-COR Biosciences, Lincoln, NE).

## Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 5.01; GraphPad Software, San Diego, CA), and $P<0.05$ was considered statistically significant. Statistical comparisons between 2 or more groups were analyzed by one-way ANOVA with Bonferroni post hoc corrections. Statistical difference between groups and over time were analyzed by two-way ANOVA with Tukey post hoc corrections. A *t* test was performed on proteomics data from Olink, followed

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by multiple corrections by Benjamini-Hochberg with false discovery rate <0.1. Olink data are shown as fold change of normalized protein expression units (NPX; log2) between cytokine treatment alone or in combination with the IL1RAP blocking antibodies. Pearson's correlation analysis was used for microarray data, followed by multiple Benjamini-Hochberg corrections with a false discovery rate <0.1.

# RESULTS

## Expression of IL1RAP-Dependent Cytokine Receptors in Human Atherosclerotic Lesions and Endothelial Cells

In human atherosclerotic plaque tissue, IL1RAP was detected in endothelial cells (von Willebrand factor-positive) lining the artery lumen (Figure 1A). Below the endothelial lining, IL1RAP did not coexpress with smooth muscle actin, but at locations with high inflammatory cell infiltration (CD68-positive), IL1RAP was detected (Figure 1A).

In addition to immunohistochemistry, we used an open available microarray data set of ATHs and paired MITs to study the levels of IL1RAP-dependent cytokine receptors in atherosclerotic lesions. Interestingly, we observed significantly increased levels of the IL-33 receptor *ST2* in ATHs compared with paired MITs (Figure 1B), whereas *IL1R1*, *IL1RAP*, and *IL36R* were expressed at similar levels in plaque and intact carotid tissue.

To further evaluate the expression of IL1R1, IL1RAP, ST2, and IL36R on endothelial cells, the expression was investigated on HUVECs using flow cytometry. The HUVECs displayed cell surface expression of all receptors, with the highest intensity for IL1RAP (Figure 1C). This suggests that HUVECs can be used as a cellular model to further investigate the role of IL1RAP in the vasculature and the potential effects of targeting this coreceptor by novel IL1RAP targeting antibodies.

## Release of Inflammatory Mediators by IL-1β, IL-33, and IL-36γ Apolymorphonucleated Leukocytes are Inhibited by IL1RAP Targeting Antibodies in Endothelial Cells

Novel antibodies targeting IL1RAP were used to investigate the effects of IL1RAP-blockade on cytokine and chemokine release from endothelial cells via Olink proteomics. The cytokine IL-1β induced release of several proinflammatory mediators, such as CCL20 (C-C motif chemokine ligand 20), CXCL5 (C-X-C motif chemokine ligand 5), CXCL10 (C-X-C motif chemokine ligand 10), CXCL6 (C-X-C motif chemokine ligand 6), and LIF (leukemia inhibitory factor) (Table S1). The levels of 4 proteins were significantly downregulated by anti-IL1RAP treatment: LIF, OPG (osteoprotegerin), CCL4 (C-C motif chemokine ligand 4), and MCP-3 (monocyte chemoattractant protein 3) (Figure 2A).

Treatment with IL-33 resulted in a trend of upregulation for CCL20, CXCL6, CXCL5, IL-6, and CXCL10. Only a slight trend of upregulation after IL-36γ treatment was observed for CCL20, CXCL10, CXCL6, and CXCL5 (Table S1). Anti-IL1RAP did not show any significant changes in protein release after IL-33 or IL-36γ treatment analyzed by the Olink proteomic analysis (data not shown).

To further investigate a selection of relevant cytokines and chemokines that were highly expressed in the Olink proteomics analysis, we further diluted samples to analyze IL-6, IL-8, and MCP-1 by ELISA. Treatment with IL-1β and IL-33 induced the release of IL-6 and IL-8 from HUVECs cultured for 24 hours, and anti-IL1RAP inhibited the IL-1β- and IL-33-induced release of IL-6 and IL-8 (Figure 2B, 2C, 2E, and 2F). Treatment with IL-36γ did not induce release of IL-6 or IL-8 (Figure 2H and 2I). All 3 cytokines, IL-1β, IL-33, and IL-36γ, induced release of MCP-1, and the release of MCP-1 was inhibited by anti-IL1RAP (Figure 2D, 2G, and 2J).

## Antibodies Targeting IL1RAP Inhibit Cytokine-Induced Expression of Inflammatory Mediators in Endothelial Cells

Next, we investigated the effects of IL1RAP inhibition on *IL6*, *IL8*, and *MCP1* expression in endothelial cells via real-time PCR at 4, 8, 12, and 24 hours. The expression was mostly pronounced at 4 hours, whereas at 8, 12, and 24 hours there were similar expression patterns only by IL-1β treatment (Figure S1A through S1C). Treatment by IL-33 only induced gene expression of the inflammatory mediators at 4 hours (Figure S1D through S1F). Treatment with IL-36γ did not induce the gene expression of *IL6*, *IL8*, or *MCP1* at any of the time points investigated (Figure 3G through 3I; Figure S1G through S1I).

At 4 hours, treatment with IL-1β and IL-33 upregulated *IL6*, *IL8*, and *MCP1* gene expression. In accordance with protein data, anti-IL1RAP inhibited the IL-1β- and IL-33-induced upregulation of *IL6*, *IL8*, and *MCP1* expression (Figure 3A through 3F).

## IL1RAP Expression Correlates to Markers of Inflammation in Human Atherosclerotic Plaques

We used microarray data from human carotid plaques included in the Gene Expression Omnibus database to study the correlation between IL1RAP and cytokines/

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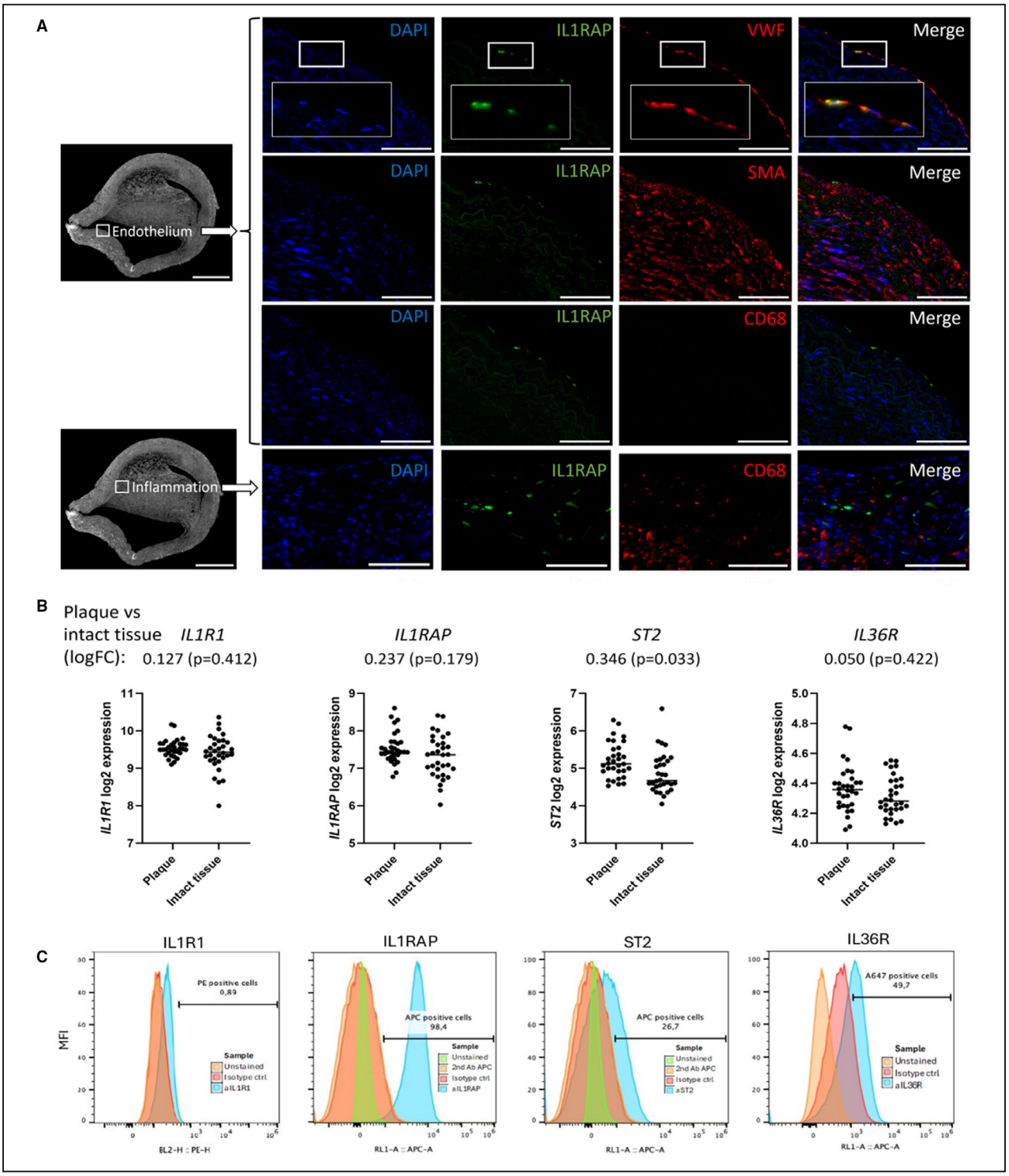


**Figure 1. Expression of IL-1 receptor complex components in human atherosclerotic plaque and endothelial cells.**
**A**, Immunofluorescent staining depicts IL1RAP (green) alongside VWF (red), SMA (red), and the macrophage marker CD68 (red) within different areas of a human atherosclerotic plaque (in gray). All scalebars=100 μm except for whole plaque images, where the scalebar=1 mm. **B**, Expression of *IL1R1*, *IL1RAP*, *ST2*, and *IL36R* in human atherosclerotic plaques compared with donor-matched macroscopically intact carotid tissue (n=32 biological replicates in each group), from the Gene Expression Omnibus database of microarray data. **C**, Expression of IL1R1, IL1RAP, ST2, and IL36R in human umbilical vein endothelial cells detected by flow cytometry shown as mean fluorescence intensity of fluorophore (APC, PE, A647)-conjugated antibodies (Ab) towards the receptor components (IL1R1, IL1RAP, ST2, IL36R). CD68 indicates cluster of differentiation 68; DAPI, 4′,6-diamidino-2-phenylindole; FC, fold change; IL-1, interleukin 1; IL1R1, IL-1 receptor 1; IL1RAP, interleukin 1 receptor associated protein; IL36R, IL-36 receptor; SMA, smooth muscle actin; ST2, supression of tumorigenicity 2; and VWF, von Willebrand factor

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| OLINK | Protein expr. (NPX) FC by anti-IL1RAP + IL-1β |
|---|---|
| LIF | -4,74689 |
| OPG | -2,29076 |
| CCL4 | -2,28000 |
| MCP-3 | -1,29192 |

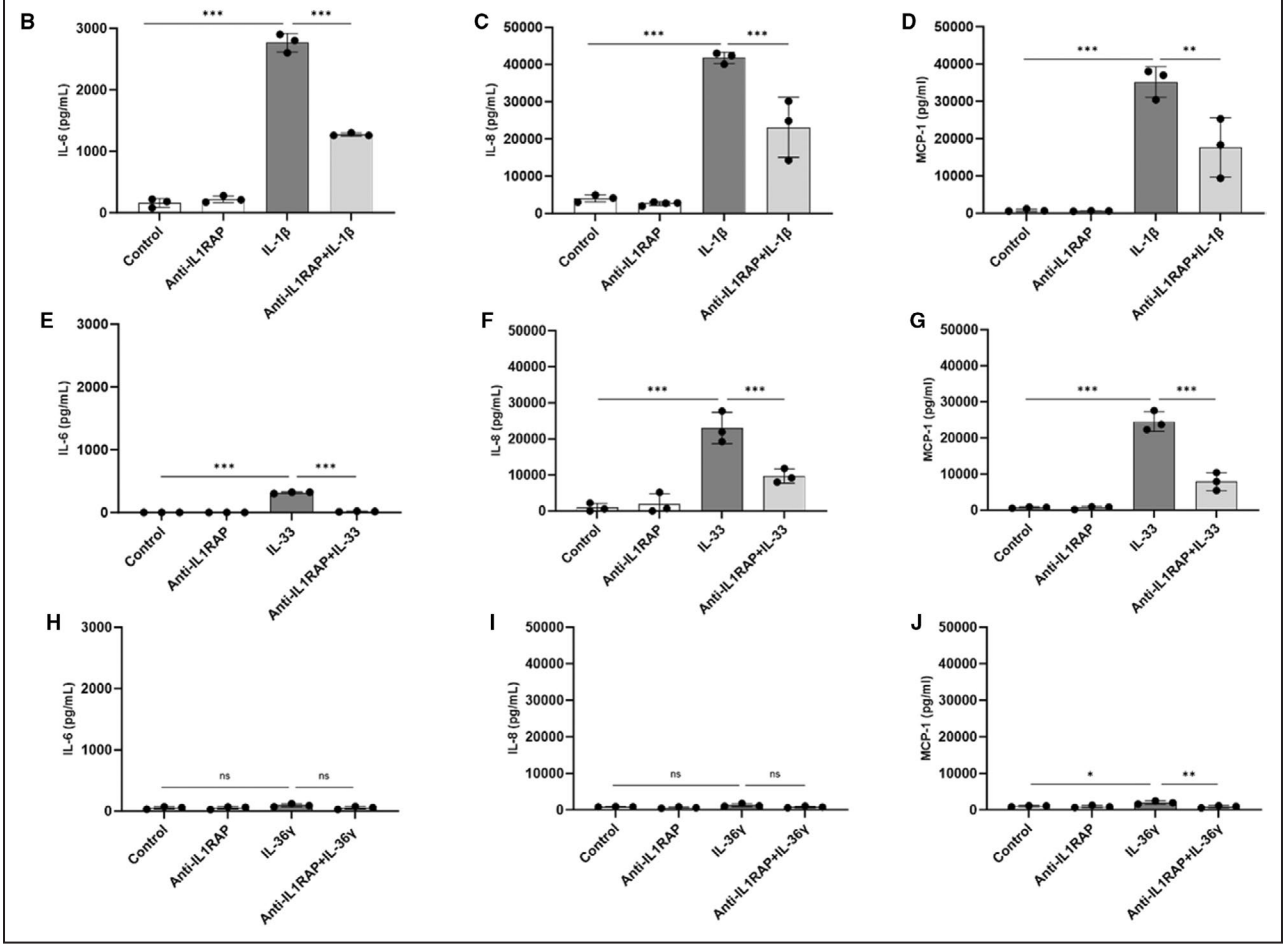


**Figure 2. Inhibitory effect of IL1RAP antibodies on release of inflammatory proteins from human umbilical vein endothelial cells.**

Protein content in cell culture media after 24-hour treatment with IL-1β (5 ng/mL) (**A** through **D**), IL-33 (100 ng/mL) (**E** through **G**), or IL-36γ (100 ng/mL) (**H** through **J**) in the presence of anti-IL1RAP (20 μg/mL) or matching isotype control antibody. In (**A**), a proximity extension assay (Olink proteomics) using the inflammation panel was analyzed. Data are shown as FC of NPX units (log2) by anti-IL1RAP compared with cytokine treatment alone and represents proteins with significant downregulation ($P$<0.05), FC >0.58, and Benjamini–Hochberg FDR <0.1 (n=3). ELISA was used to detect IL-6 in (**B**), (**E**), and (**H**); IL-8 in (**C**), (**F**), and (**I**); and MCP-1 in (**D**), (**G**), and (**J**). Data are shown as mean±SD (n=3), ANOVA *$P$<0.05, **$P$<0.01, ***$P$<0.001. CCL4 indicates C-C motif chemokine 4; expr, expression; FC, fold change; FDR, false discovery rate; IL, interleukin; IL1RAP, interleukin 1 receptor associated protein; LIF, leukemia inhibitory factor; MCP-3, monocyte chemotactic protein 3; NPX, normalized protein expression; ns, not significant; and OPG, osteoprotegerin.

chemokines in atherosclerosis for markers that were significantly affected in the Olink proteomics data and ELISA analysis. In line with the HUVEC results, we observed a positive correlation between the expression of *IL1RAP* and *IL6*, *IL8*, and *MCP-1*, whereas a weak, but significant, correlation was observed for *LIF*

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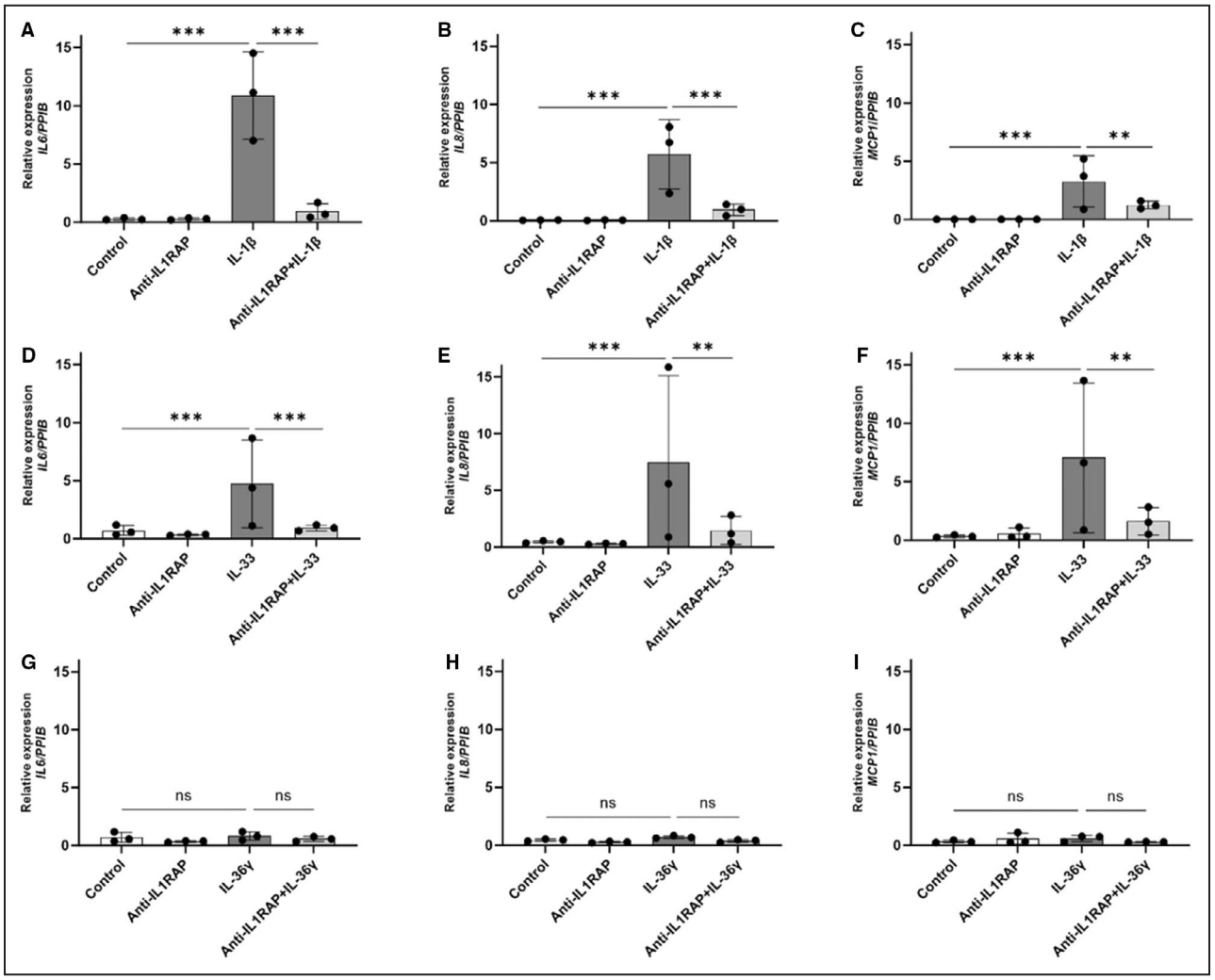


**Figure 3. Effect of IL1RAP-blocking antibodies on gene expression of inflammatory cytokines in endothelial cells.** HUVECs were treated for 4 hours with (**A** through **C**) IL-1β (5 ng/mL), or (**D** through **F**) IL-33 (100 ng/mL), or (**G** through **I**) IL-36γ (100 ng/mL) in the presence of anti-IL1RAP (20 μg/mL) or matching isotype control antibody. The mRNA expression was measured by RT-qPCR of *IL6* in (**A**), (**D**), and (**G**); *IL8* in (**B**), (**E**), and (**H**); and *MCP1* in (**C**), (**F**), and (**I**). Data are shown as mean±SD (n=3), ANOVA **P*<0.05, ***P*<0.01, ****P*<0.001. HUVECs indicates human umbilical vein endothelial cells; IL, interleukin; IL1RAP, interleukin 1 receptor associated protein; ns, not significant; and RT-qPCR, real-time quantitative polymerase chain reaction.

and *MCP-3*, in atherosclerotic plaque tissue (Figure 4A through 4D and 4G). A nonsignificant but borderline weak correlation was observed for *CCL4* (Figure 4F), whereas *OPG* was not correlated to *IL1RAP* in atherosclerotic plaque tissue (Figure 4E). Together, this indicates that IL1RAP may be involved in an upregulated inflammatory process in the vascular wall during atherosclerosis.

## Antibodies Targeting IL1RAP Inhibit Cytokine-Induced Adhesion in HUVECs

Because vascular endothelial cells positioned at the lining of blood vessel walls are important for barrier function and cell infiltration between the circulating blood and the surrounding tissue, we further investigated if cell adhesion to HUVECs was affected by anti-IL1RAP treatment.

The gene expression of *ICAM1*, *VCAM1*, and *SELE* (also known as E-selectin), was analyzed after 4, 8, 12, and 24 hours (Figure S2A through S2I), but because our results indicated a quick upregulation of gene expression, we decided to focus on the expression at the 4-hour time point. The expressions of *ICAM1*, *VCAM1*, and *SELE* were all upregulated by all IL1RAP-dependent cytokines (Figure S2A through S2I).

Anti-IL1RAP inhibited the upregulated expression of *ICAM1*, *VCAM1*, and *SELE* by IL-1β, IL-33, and IL-36γ (Figure 5A through 5C, 5E through 5G, and 5I through 5K). To test whether the effect of anti-IL1RAP on adhesion molecules reflects the ability to inhibit the adhesion of neutrophils to endothelial cells, an *in vitro* coculture

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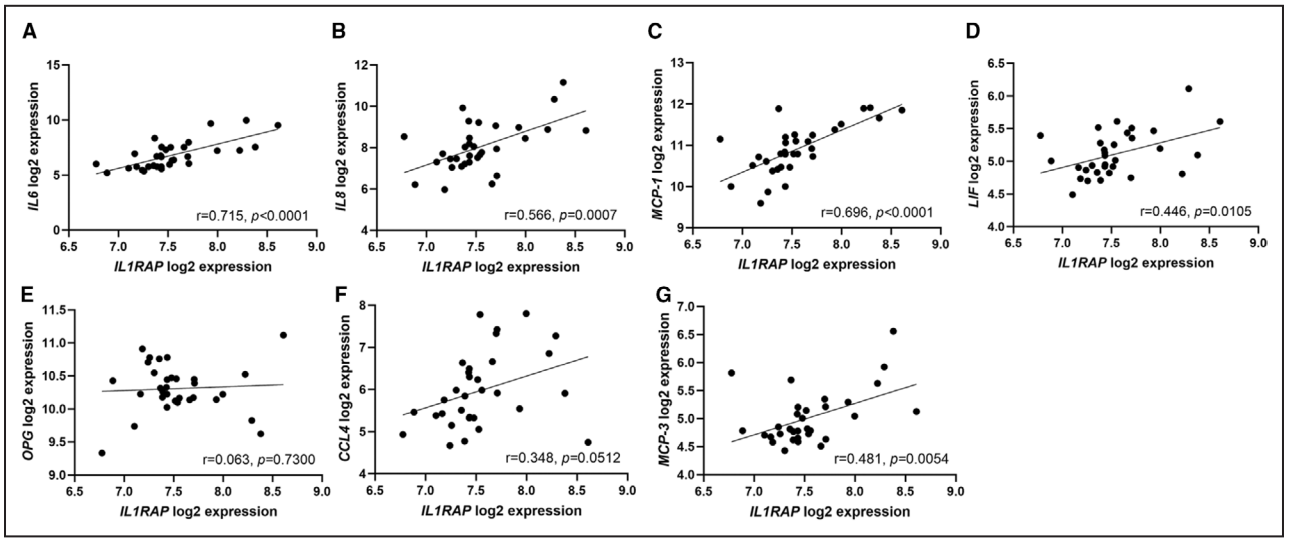


**Figure 4.** ***IL1RAP*** **expression correlates to markers of inflammation in human atherosclerotic plaques.**
Microarray data from the Gene Expression Omnibus database were used for correlation analysis of *IL1RAP* and (**A**) *IL6*, (**B**) *IL8*, (**C**) *MCP-1*, (**D**) *LIF*, (**E**) *OPG*, (**F**) *CCL4*, and (**G**) *MCP-3* in human atherosclerotic plaque tissue (n=32 biological replicates).

of these cells was established. The cytokines IL-1β and IL-33, but not IL-36y, induced an increased adhesion of neutrophils to HUVECs (Figure 5D, 5H, and 5L). Treatment by anti-IL1RAP significantly reduced the IL-1β- and IL-33-induced neutrophil adhesion.

## IL1RAP Expression Correlates to Markers of Adhesion in Human Atherosclerotic Lesions

Furthermore, we used microarray data to study the correlation between *IL1RAP* and adhesion markers in atherosclerotic lesions. A weak, but statistically significant, correlation between *IL1RAP* and *ICAM1* in human atherosclerotic lesions was observed (Figure 5M). In addition, we observed a trend to a weak correlation between *IL1RAP* and *VCAM1* ($P$=0.056; Figure 5N). There was no significant correlation between *IL1RAP* and *SELE* (Figure 5O).

## Antibodies Targeting IL1RAP Inhibit IL-1β-Induced Permeability and NF-κB Signaling in HUVECs

As an example of IL1RAP-dependent signaling, we next selected IL-1β to study whether IL1RAP targeting antibodies also affect endothelial permeability and NF-κB signaling in HUVECs. Treatment with an anti-IL1RAP antibody significantly reduced the IL-1β-induced endothelial permeability (Figure 6A). Also, NF-κB signaling induced by IL-1β was significantly reduced by IL1RAP blockade, shown as restored IκBα protein expression (Figure 6B and 6C). Data of IL-33-induced NF-κB signaling indicate a similar effect of anti-IL1RAP antibodies as with IL-1β (Figure 6D). The NF-κB signaling was not as prominent in response to IL-36γ as IL-1β or IL-33 (Figure 6E).

# DISCUSSION

The IL-1 signaling pathway is central in the pathogenesis of inflammatory and autoinflammatory diseases, including CVDs.[26] IL-1β induces inflammatory functions in the endothelial cells and affects recruitment of leukocytes via stimulation of adhesion molecules such as ICAM and VCAM,[1] whereas the roles of IL-33 and IL-36 are not as well described. Targeted therapeutic options against the IL-1 pathway exist, such as anakinra (Kineret/Sobi), mimicking the naturally occurring IL-1Ra (IL-1 receptor antagonist) and the IL-1β neutralizing antibody canakinumab (Ilaris/Novartis), where the latter was previously evaluated in the CANTOS trial.[13] However, targeting the coreceptor IL1RAP represents a unique opportunity to reduce the broad effects of the IL1RAP-dependent cytokines IL-1α, IL-1β, IL-33, and IL-36α/β/γ simultaneously.[18]

In the present study, we have evaluated the expression and release of cytokines and chemokines after IL1RAP blockade and the effects of anti-IL1RAP treatment on cytokine-induced neutrophil adhesion and permeability to endothelial cells. Expression of IL1RAP has previously been shown in several different tissues such as brain, lungs, appendix, lymph nodes, tonsils, and bone marrow[27] (www.proteinatlas.org). In the present study, we detected IL1RAP expression in the endothelial layer of a human atherosclerotic lesion. Microarray data of ATHs and MITs show expression of *IL1R1*, *IL1RAP*, *ST2*, and *IL36R*, which together could facilitate signaling by the IL-1 family cytokines IL-1,

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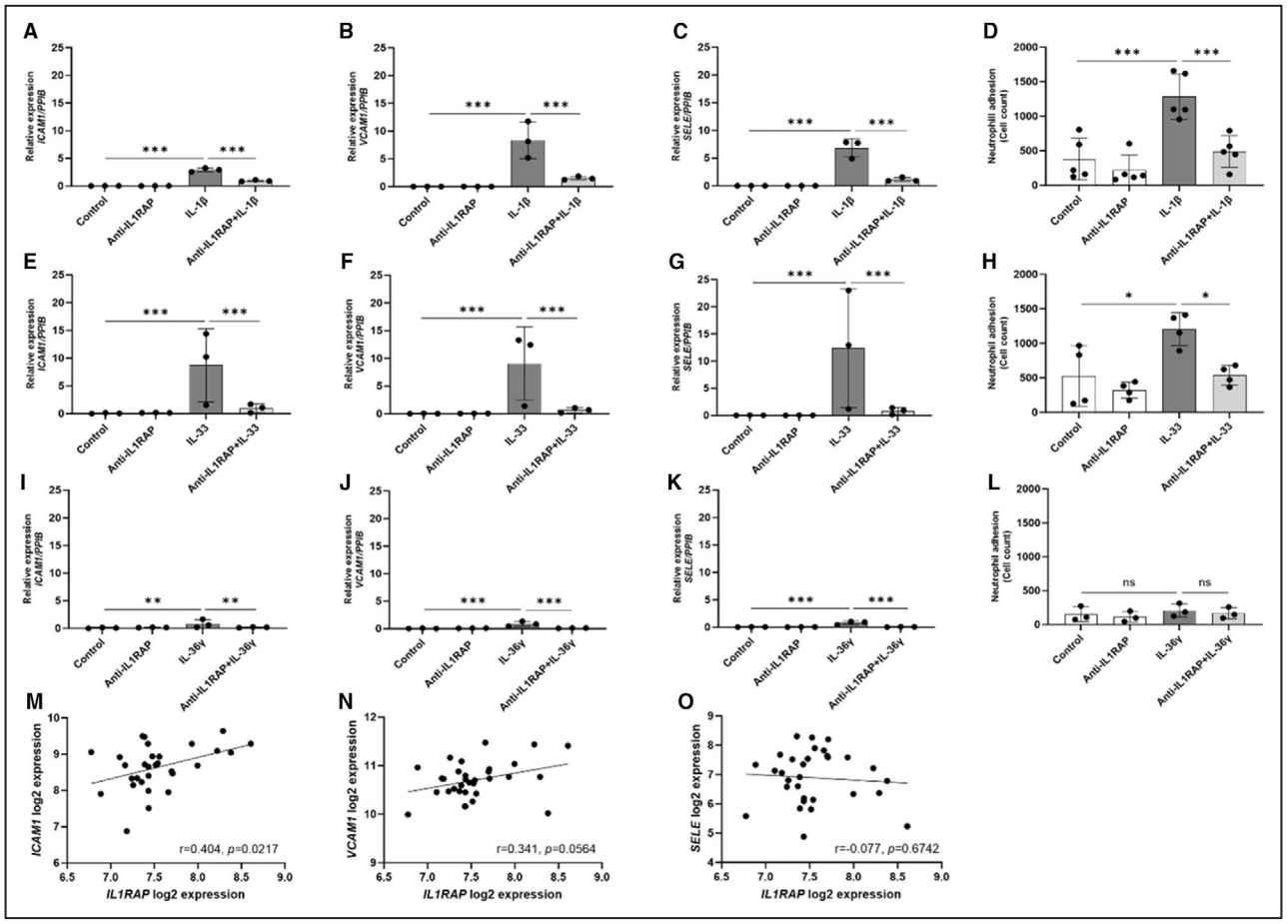


**Figure 5. Endothelial adhesion markers and neutrophil adhesion to endothelium are reduced by IL1RAP blocking antibodies.**

HUVECs were treated with (**A** through **D**) IL-1β (5 ng/mL), or (**E** through **H**) IL-33 (100 ng/mL), or (**I** through **L**) IL-36γ (100 ng/mL) in the presence of anti-IL1RAP (20 μg/mL) or matching isotype control antibody. The mRNA expression was measured after 4 hours of treatment by RT-qPCR of *ICAM1* in (**A**), (**E**), and (**I**); *VCAM1* in (**B**), (**F**), and (**J**); and *SELE* in (**C**), (**G**), and (**K**). HUVECs treated for 24 hours ([**D**], [**H**], and [**L**]) were coincubated with calcein-AM-labeled neutrophils obtained from human polymorphonucleated leukocytes isolation. Neutrophil adhesion was detected as number of fluorescent neutrophils attached to HUVECs after 30 minutes of coincubation at 37 °C in 5% $CO_2$ (n=3–5). Data are shown as mean±SD, ANOVA **P*<0.05, ***P*<0.01, ****P*<0.001. Correlation analysis of microarray data of *IL1RAP* and (**M**) *ICAM1*, (**N**) *VCAM1*, and (**O**) *SELE* from the Gene Expression Omnibus database of human atherosclerotic plaques (n=32 biological replicates). HUVECs indicates human umbilical vein endothelial cells; IL, interleukin; IL1RAP, interleukin 1 receptor associated protein; ns, not significant; and RT-qPCR, real-time quantitative polymerase chain reaction.

IL-33, and IL-36. However, although an elevated expression of *ST2* was identified in ATHs compared with MITs, no differential expression between ATHs and MITs was found for *IL1R1*, *IL1RAP*, or *IL36R* by microarray. The reason for this, however, is unclear. Similar expression in ATHs compared with MITs could be due to infiltration of inflammatory cells in the MITs, and elevated expression in the MITs may therefore not necessarily reflect the processes occurring in normal healthy tissue. On the other hand, differential expression was observed for *ST2*, but the reason for this remains to be elucidated.

Additionally, we show expression of the IL1RAP-dependent cytokine receptors in cultured human endothelial cells, where the expression of IL1RAP was the most prominent. This expression patterns in atherosclerotic plaque tissues and endothelial cells indicate that IL1RAP blockade could be beneficial in the prevention of inflammatory progression in the human vascular wall.

Recently, IL1RAP blockade was evaluated in *ApoE*$^{-/-}$ mice and showed a 20% reduction in subvalvular lesion size and reduced accumulation of neutrophils and macrophages in the atherosclerotic lesions,[20] which indicate a beneficial role of IL1RAP blockade *in vivo*. To our knowledge, the present investigation represents the first study using IL1RAP-targeting antibodies to reduce vascular inflammation and adhesion in the human setting. The IL1RAP-targeting antibodies were studied in response to IL-1β, IL-33, or IL-36γ treatment, all of

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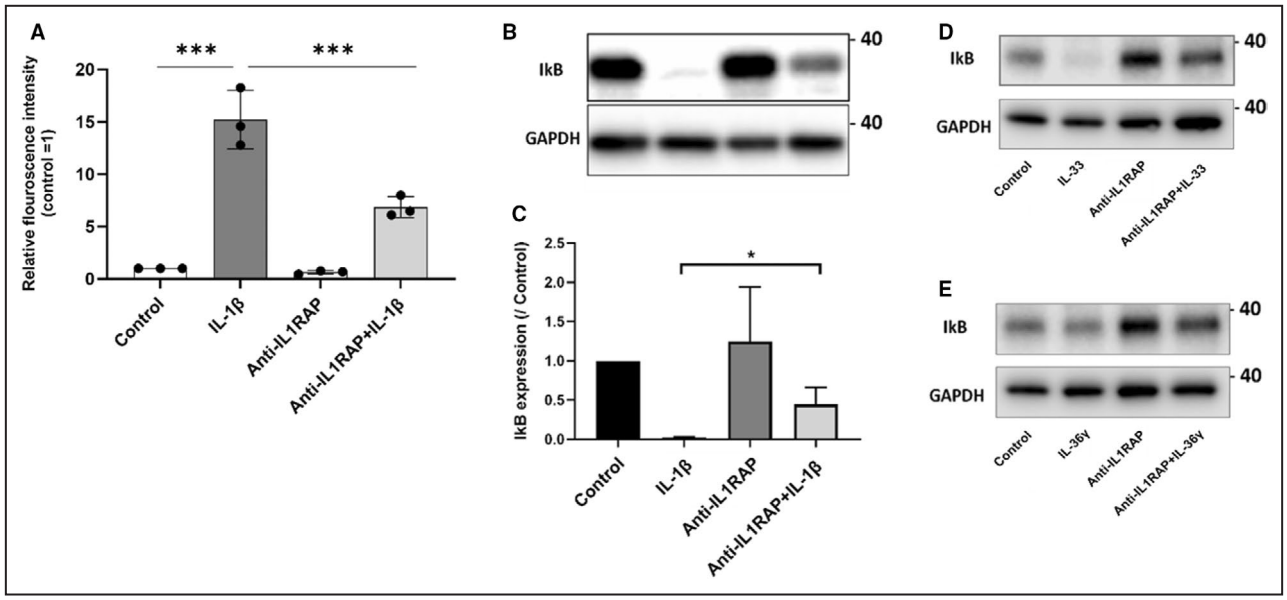


**Figure 6. Reduction of IL-1β-induced endothelial permeability and NF-κB signaling by IL1RAP blocking antibodies.**
HUVECs were treated with (**A** through **C**) IL-1β (5 ng/mL), or (**D**) IL-33 (100 ng/mL, n=1), or (**E**) IL-36γ (100 ng/mL, n=1) in the presence of anti-IL1RAP (20 μg/mL) or a matching isotype control antibody. **A**, Permeability assay of FITC-dextran on HUVECs. Permeability through the endothelial monolayer after 24 hours of treatment was measured as fluorescence intensity relative to control (n=3). **B** through **E**, IκBα protein expression was measured after 30 minutes of treatment by Western blot. GAPDH protein expression was analyzed as a loading control. **B**, Representative blots of (**C**) that show quantification and treatments (n=3). **A** and **C**, Data are shown as mean±SD, ANOVA **P*<0.05, ***P*<0.01, ****P*<0.001. FITC indicates fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVECs, human umbilical vein endothelial cells; IL, interleukin; IL1RAP, interleukin 1 receptor associated protein; IκBα, inhibitory κBα; and NF-κB, nuclear factor κB.

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which are putative agonists for the coreceptor IL1RAP in association with their specific receptors. Here, we show inhibitory effects of IL1RAP-targeting antibodies with various signatures on cytokine and chemokine release in human endothelial cells. For IL-1β and IL-33 stimulation, our data indicate an inhibitory effect of IL1RAP antibody treatment *in vitro* on the release of several chemokines and cytokines investigated via Olink proteomics and ELISA. Via Olink proteomics, we observed that the 4 cytokines and chemokines LIF, OPG, CCL4, and MCP-3 were reduced by anti-IL1RAP after IL-1β stimulation. Previous studies have shown that these chemokines are involved in the atherosclerotic process and can act as mediators of inflammatory response, chemoattractants, calcification of atherosclerotic lesions, or plaque stability.[28–31] On the other hand, in the proteomics analysis of IL-33- and IL-36γ-stimulated endothelial cells treated with anti-IL1RAP, no proteins were found significantly altered after IL-33 or IL-36γ stimulation and anti-IL1RAP treatment, although a trend of upregulation was observed for some cytokines and chemokines. Future studies on other cell types with more prominent IL-33 and IL-36γ signaling pathways may add information on the impact of IL1RAP antibody treatment against these cytokines.

In the Olink proteomics analysis, we observed that proteins with high normalized protein expression values did not reach significance, possibly due to high concentrations, and that the dilution of samples in Olink are not optimized for each protein to detect. Small inhibitory effects could be challenging to detect for highly expressed proteins on the Olink platform. We therefore decided to perform ELISA on optimized diluted samples for 3 central cytokines/chemokines in inflammation and atherosclerosis, IL-6, IL-8, and MCP-1, and observed that anti-IL1RAP inhibits IL-1β- and IL-33-induced IL-6, IL-8, and MCP-1 release, respectively. Only MCP-1 release was significantly upregulated when stimulated with IL-36γ, which was also inhibited by anti-IL1RAP. The lack of effect on IL-6 and IL-8 might be explained by the relatively low induction of cellular and molecular responses we got from the endothelial cells when treated with IL-36γ. The specific reason for this observation is not known, although it may indicate differences in responsiveness of endothelial cells to the IL1RAP dependent cytokines. This was further supported by the recovered IκBα protein expression by anti-IL1RAP antibodies, which indicates that NF-κB signaling is important for IL1RAP-mediated responses in endothelial cells. The effects of inflammatory markers, such as IL-6, IL-8, and MCP-1 are NF-κB mediated.[32,33] In atherosclerotic lesions, expression of IL-6, IL-8, and MCP-1 have been observed and associated with several central stages in the development of

atherosclerosis.[34,35] For example, IL-6 is an important mediator of the inflammatory response in atherosclerosis, smooth muscle cell proliferation, migration, and endothelial dysfunction, which results in the development of atherosclerotic lesions and destabilization of plaque.[36] Moreover, MCP-1 has been associated with the vulnerability of human atherosclerotic plaque and has a proatherogenic effect in ApoE-deficient mice.[37,38] In addition, both IL-8 and MCP-1 are involved in adhesion between monocytes and endothelial cells expressing E-selectin.[39] Collectively, our data show that IL1RAP-targeting antibodies inhibit IL-1β, IL-33, and IL-36γ signaling and reduces the increased levels of several cytokines and chemokines, although future *in vivo* studies in the human setting are warranted.

In atherosclerotic lesions, we evaluated the correlation of significantly altered cytokines and chemokines from the Olink proteomics and ELISA data with the expression of *IL1RAP* in microarray data from the GSE43292 data set.[22] Of these, positive correlation was observed between *IL1RAP* and *IL6*, *IL8*, *MCP-1*, and to a lesser extent with *LIF* and *MCP-3*. Collectively, our ELISA, Olink proteomics, and microarray data show a potential regulatory role of IL1RAP on these cytokines and chemokines in endothelial cells and atherosclerotic lesions. This indicates that IL1RAP-targeting antibodies affect several pathways downstream of the IL-1 family of cytokines, although further analysis in the human setting is warranted.

As described, our ELISA and Olink proteomics data showed that chemokines in the C-C and C-X-C families were reduced by anti-IL1RAP treatment. As a functional aspect of IL1RAP-targeting antibodies, we investigated neutrophil adhesion to endothelial cells, because chemokines in the C-C and C-X-C families are involved in the recruitment of leukocytes to atherosclerotic lesions.[40] Adhesion is a central mechanism in the development of atherosclerosis, and ICAM, VCAM, and E-selectin are central mediators of endothelial dysfunction and monocyte infiltration in the lesion and have all been found expressed in atherosclerotic lesions.[41–44] Furthermore, the role of adhesion markers has previously been studied by genetic inhibition of *Icam1* or *Vcam1* in *ApoE*$^{-/-}$ mice, and mice with deficiency of these adhesion markers had less severe atherosclerotic lesions.[45,46] In the present study, we found a lower neutrophil adhesion in IL-1β- and IL-33-stimulated endothelial cells treated with IL1RAP antibodies, in addition to reduced expression of the 3 adhesion markers, *ICAM1*, *VCAM1*, and *SELE* in endothelial cells. In atherosclerotic lesions, a weak positive correlation between *IL1RAP* expression and *ICAM1* was observed in microarray data from human atherosclerotic lesions. Furthermore, we observed reduced IL-1β-induced permeability after anti-IL1RAP treatment. Our data suggest that treatment with IL1RAP inhibiting antibodies may be favorable in the treatment against CVDs by reducing permeability and markers of adhesion. This is supported by data from a recent study on *ApoE*-deficient mice, where a reduction of the adhesion molecules *Icam1* and *Vcam1* and a trend of reduced neutrophil infiltration was observed.[20] It has also been suggested that modulation of markers of adhesion in atherosclerosis, either directly via targeting the adhesion molecule itself or indirectly via targeting upstream cytokines, represents a novel putative therapeutic strategy against atherosclerosis.[41] In our neutrophil adhesion assay, no significant induction of adhesion was observed after IL-36γ treatment, although the mRNA expression of *ICAM1*, *VCAM1*, and *SELE* were induced by IL-36γ and suppressed by anti-IL1RAP. The reason for the lack of significance in the functional assay is not known but is likely to be a consequence of the low levels of mRNA expression of the adhesion molecules induced by IL-36γ in our study. This could be further evaluated in arterial endothelial cells with flow conditions, although HUVECs are a frequently used model for vascular research. Collectively, our data indicate that among the IL1RAP-regulated proteins, there are indications that inhibition of IL1RAP is favorable in atherosclerosis via its impact on downstream target genes related to neutrophil adhesion to the endothelium.

In summary, our data indicate that IL-1β-, IL-33-, or IL-36γ-induced pathological endothelial inflammation and adhesion to the vascular wall can be reduced by novel antibodies targeting IL1RAP. This represents a novel tool to reduce the pleiotropic effects of IL1RAP-mediated signaling in atherosclerosis. This knowledge is of importance for the development of future putative targeted therapy to prevent progression of CVDs.

## ARTICLE INFORMATION

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Author contributions: Conceived and designed the study: K.F., D.L. Performed the experiments: M.L., A.G.E., G.V.P, S.A.H., C.G. Analyzed and interpreted the data: M.L., A.G.E., G.V.P., S.A.H., A.S., K.F., C.R.M., S.R., C.G., D.L. Contributed to reagents/materials/analysis tools: K.F., A.S., C.R.M., S.R., C.G., D.L. Coordinator of the study: K.F. Wrote the article: K.F., M.L. Contribution of writing the article: G.V.P., A.G.E. Editing the article: M.L., A.G.E., G.V.P., C.R.M., S.R., C.G., D.L., A.S., K.F. All authors read and approved the final version of the article.

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## Disclosures

C.R.M., S.R., C.G., and D.L. are employees and hold stocks or options in Cantargia AB. C.R.M., S.R., C.G., and D.L. are listed as inventors on patents in regard to IL1RAP. No conflicts of interest: M.L., A.G.E., G.V.P., S.A.H., A.S., and K.F.

## Supplemental Material

Table S1
Figures S1–S2
Data S1

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