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Identification of IL-23p19 as an endothelial proinflammatory peptide that promotes gp130-STAT3 signaling

Georgina Espígol-Frigolé^{#1,2,†}, Ester Planas-Rigol^{#1,2}, Hidetaka Ohnuki¹, Ombretta Salvucci¹, Hyeonil Kwak¹, Sarangan Ravichandran³, Brian Luke³, Maria C. Cid², and Giovanna Tosato^{1,†}

¹Laboratory of Cellular Oncology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA.

²Department of Systemic Autoimmune Diseases, Clinical Institute of Medicine and Dermatology, Hospital Clinic, University of Barcelona, IDIBAPS-CRB CELLEX, Barcelona 08036, Spain.

³Advanced Biomedical Computing Center, Leidos Biomedical Research Inc., Frederick National Laboratory for Cancer Research, Frederick, MD 21702, USA.

These authors contributed equally to this work.

Abstract

Interleukin-23 (IL-23), a heterodimeric cytokine composed of the unique p19 peptide (IL-23p19) and a peptide called IL-12p40, which is shared with IL-12, is implicated in Crohn's disease, rheumatoid arthritis, psoriasis, and other immune-mediated inflammatory diseases. Endothelial cells produce the IL-23p19 peptide in the absence of the IL-12p40 chain and thus do not make heterodimeric IL-23. We found that intracellular IL-23p19 increased the cell surface abundances of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, which enhanced the attachment of leukocytes and increased their transendothelial migration. Intracellular p19 associated with the cytokine receptor subunit gp130 and stimulated the gp130-dependent activation of signal transducer and activator of transcription 3 (STAT3) signaling. Proinflammatory factors promoted the generation of IL-23p19 in endothelial cells. The adventitial capillaries of inflamed temporal arteries in patients with giant-cell arteritis (GCA) had endothelial p19 protein associated with gp130, but did not contain the IL-12p40 chain. Because adventitial capillaries are essential for the entry of inflammatory cells into arterial walls, these data suggest that p19 may contribute to GCA disease and could represent a therapeutic target. Our results provide evidence that IL-23p19 is a previously unrecognized endothelial proinflammatory peptide that promotes leukocyte transendothelial migration, advancing our current understanding of the complexities of inflammatory responses.

[†]Corresponding author. georginaespigol@gmail.com (G.E.-F.); Tosatog@mail.nih.gov (G.T.).

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INTRODUCTION

Inflammatory cells and inflammatory mediators play critical roles in the pathogenic cascade, leading to vascular lesions that characterize different types of vasculitis. Giant-cell arteritis (GCA) is an inflammatory vasculitis that typically involves medium and large arteries, predominantly branches of the aortic arch (1). The condition is associated with systemic inflammatory symptoms (1) and is generally long-lasting (2). Luminal occlusion from intimal hyperplasia can lead to ischemic complications, including loss of vision (3), and vascular wall injury may lead to aortic aneurysm or dissection (4). The cause of GCA is unknown. Mechanistic studies have outlined activation of vessel wall-resident dendritic cells and the recruitment of lymphocytes, monocytes, or both to the vessel wall as key pathogenic events (2, 5). The adventitial capillaries (vasa vasorum) of GCA arteries are believed to be the port of entry for inflammatory cells because they abnormally express various adhesion molecules that promote leukocyte adhesion to the endothelium and transendothelial migration (6). A key feature of GCA lesions is the abundance of various inflammatory cytokines, chemokines, and other mediators, which produce systemic symptoms, amplify local inflammation, and produce vascular pathology by targeting the endothelium and vascular smooth muscle cells and fibroblasts (2).

Interleukin-23 (IL-23), a heterodimeric cytokine composed of the unique p19 peptide (also known as IL-23A) and the shared IL-12p40 peptide (also known as the IL-12 β chain) (7), promotes the development of a population of T helper (T_H) cells, designated T_H17 cells because they produce IL-17, which are implicated in Crohn's disease, rheumatoid arthritis, psoriasis, and other immune-mediated inflammatory diseases (8). Macrophages and dendritic cells, which produce p19 and p40, secrete IL-23, whereas endothelial cells and certain T cell subsets express *IL23A* (which encodes p19), but not *IL12B* (which encodes p40), and thus do not secrete IL-23 (7).

There is evidence for the involvement of IL-23 in GCA because T_H17 cells are abnormally increased in number in the blood of untreated GCA patients, and circulating monocytes have abnormally increased amounts of mRNA for *IL23A* and *IL12B* (9). GCA vascular lesions have abnormally increased amounts of *IL17A* mRNA (10), and pretreatment GCA tissue specimens contain IL23A mRNA, which decreases in abundance after treatment (11). However, the role of IL-23 in GCA has not been fully investigated. Here, we discovered a previously unrecognized role for IL-23p19 as a proinflammatory peptide that is produced by the endothelium.

RESULTS

IL-23p19 is detected in inflamed temporal arteries

Normal superficial temporal arteries displayed the characteristic central lumen limited by the endothelium of the tunica intima, the internal elastic membrane, the tunica media, and the adventitia (Fig. 1A). In GCA, diseased superficial temporal arteries were variously disrupted (Fig. 1B): the lumen was narrowed, the intima was thickened, the internal elastic membrane was fragmented, and the tunica media contained inflammatory cells and occasional multinucleated giant cells (2). The blood capillaries (vasa vasorum), which are normally

restricted to the adventitia (2), were also found in the tunica media of a GCA temporal artery.

The endothelial cell marker CD31 identified the endothelium limiting the lumen of the normal temporal artery and the narrowed lumen of the GCA temporal artery (Fig. 1, C and D). CD31 additionally identified an expansive network of adventitial neoangiogenic capillaries in the GCA vessel wall and scattered other cells (Fig. 1D) that were missing from the normal artery (Fig. 1C). We found that IL-23p19 (henceforth p19) was present in the CD31⁺ pathological capillary network of GCA (Fig. 1E). By contrast, IL-23p40 (henceforth p40) was generally absent from CD31⁺ cells but was detected in occasional CD31⁻ cells (Fig. 1E). The presence of CD31 and p19 without p40 was confirmed in two additional GCA temporal arteries. Consistent with a previous study of cultured endothelial cells that showed that this cell population does not express p40 (7), our results demonstrate that p19, but not p40, is generally present in the capillary endothelium lining the pathological vasa vasorum of inflamed temporal arteries from GCA patients. Because previous studies showed that p19 is only secreted extracellularly as a complex with p40 to form the cytokine IL-23 (7), our findings are consistent with p19 being an intracellular peptide in the GCA endothelium.

Inflammatory signals induce p19 production in endothelial Cells

Because the serum and tissue concentrations of tumor necrosis factor- α (TNF- α), IL-6, and IL-1 β are often abnormally increased in active GCA patients (5), we tested whether proinflammatory signals promoted p19 production in primary endothelial cells. TNF- α and lipopolysaccharide (LPS) induced *IL23A* mRNA expression in primary human umbilical vein endothelial cells (HUVECs) and in human dermal microvascular endothelial cells (HDMECs) (Fig. 2A), but they did not induce the expression of *IL12B* mRNA (Fig. 2B). In contrast, LPS expectedly induced the expression of both *IL23A* and *IL12B* mRNAs in normal peripheral blood mononuclear cells (PBMCs) (Fig. 2, A and B). Western blotting analysis showed that LPS and TNF- α increased the abundance of p19 protein in HUVEC and HDMEC lysates (Fig. 2C). Western blotting (Fig. 2C) and a sensitive enzyme-linked immunosorbent assay (ELISA) (Fig. 2D) failed to detect p19 protein in the cell culture medium of endothelial cells even after stimulation with LPS or TNF- α , which suggests that endothelial cells secrete little or no p19 protein into the extracellular environment. Instead, p19 was detected in the cell culture medium of LPS-activated PBMCs (Fig. 2D). Thus, consistent with earlier studies (7), activated endothelial cells fail to secrete IL-23 because they do not produce p40, but they also do not secrete p19.

To investigate potential functions of p19 in endothelial cells, we took advantage of a lentiviral vector (LV) for the stable expression of p19 in these cells. After selection in puromycin-containing medium, HUVECs consistently expressed *IL23A* mRNA (Fig. 2E, top) and p19 protein (Fig. 2E, bottom). The exogenous p19 was detected in the cytoplasm of the HUVECs (Fig. 2E, right). We considered the possibility that p19 might promote the expression of *IL12B* and *IL23R*; however, we found no increase in the abundances of *IL12B* and *IL23R* mRNAs in p19-expressing HUVECs (Fig. 2F). Multiple attempts at recovering p19 protein from the cell culture medium of the lentiviral transduced HUVECs were unsuccessful despite efforts to concentrate the culture medium (Fig. 2G).

p19 induces the cell surface expression of adhesion molecules in endothelial cells

Because the vascular endothelium critically regulates the crawling, adhesion, and transendothelial migration of circulating leukocytes, we investigated whether p19 modulated the endothelial cell surface expression of adhesion molecules that mediate these processes (12). We found that p19-expressing endothelial cells had greater amounts of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) mRNA and protein than did control cells (Fig. 3, A and B). The amount of platelet endothelial cell adhesion molecule-1 (PECAM-1), a member of the immunoglobulin (Ig) superfamily prominently found in endothelial intercellular junctions, minimally differed between p19-expressing cells and control cells (Fig. 3, A and B). However, unlike LPS or the combination of interferon- γ (IFN- γ) and TNF- α , the presence of p19 induced minimal expression of CXCL9, CXCL10, and CXCL11 mRNA and protein and *IL6* mRNA in HUVECs (fig. S1).

The endothelial adhesion molecules VCAM-1 and ICAM-1 promote leukocyte adhesion to the endothelium by engagement of the integrins very late antigen-4 (VLA-4) and leukocyte function-associated antigen-1 (LFA-1), respectively (13), and they stimulate critical signals to enhance transendothelial migration together with endothelial-derived inflammatory chemokines (13). We examined whether the endothelial cell expression of p19 altered leukocyte attachment to the cells and transendothelial migration. In cell attachment assays, we found that normal PBMCs attached substantially more to a monolayer of p19-expressing HUVECs than to a monolayer of control HUVECs over a wide range of cell densities (Fig. 3C). In cell transmigration assays, we observed that p19-expressing endothelium sustained greater leukocyte transmigration than did control endothelium after activation with LPS or with both TNF- α and IFN- γ (Fig. 3D).

p19 induces autocrine signaling in endothelial cells

We examined the potential mechanisms underlying the ability of p19 to activate the transcription of genes encoding adhesion molecules. We found that p19 did not induce the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), Akt, signal transducer and activator of transcription 1 (STAT1), or the nuclear factor κ B (NF- κ B) subunit p65 in HUVECs (fig. S2A). In contrast, we found that p19-expressing HUVECs displayed statistically significantly increased ($P < 0.05$) amounts of phospho-phosphorylated STAT3 (pSTAT3; Tyr⁷⁰⁵) compared to that in control cells (Fig. 3E). Selective activation of STAT3, but not STAT1, has previously been attributed to differences among activating signals, engagement of suppressor of cytokine signaling (SOCS) inhibitors, and other factors (14). Consistent with the p19-dependent activation of STAT3, p19-expressing HUVECs displayed nuclear localization of STAT3, whereas p19-negative cells did not (fig. S2B).

Because IL-6 and the IL-6 receptor (IL-6R) stimulate gp130-dependent STAT3 activation from the cell surface (15), whereas p19 is an intracellular peptide in endothelial cells, we measured pSTAT3 abundance in p19-expressing HUVECs activated by IL-6 together with IL-6R. We found that IL-6 and soluble IL-6R time-dependently induced substantially more STAT3 phosphorylation in p19-expressing HUVECs than in control HUVECs at all time points (Fig. 3G). These results provide evidence that p19 promotes STAT3 activation in endothelial cells. In addition, because *ICAM1* and *VCAM1* are transcriptional targets of

IL-6:IL-6R-gp130-pSTAT3 signaling (16), these results suggest the possibility that p19 promotes the transcriptional activation of *ICAM1* and *VCAM1* by inducing gp130-pSTAT3 signaling.

There is structural similarity (~16% sequence identity and 34.3% sequence similarity) between p19 and vIL-6 (7), a viral cytokine product of human herpesvirus 8 (HHV-8, also known as KSHV) (7, 17). Intracellular vIL-6 binds to intracellular gp130 and promotes gp130 signaling from an intracellular location (18, 19). We hypothesized that intracellular p19 also bound to intracellular gp130 and activated gp130-STAT3 signaling. The experimental structure of the tetrameric complex of vIL-6 and the extracellular domain of gp130 (20) (Fig. 4A, left) was used as a basis to evaluate possible p19-gp130 interactions. On the basis of the conservation of key binding residues (fig. S3, A to C, and see Materials and Methods), we concluded that p19 has a high probability of forming a complex with gp130. We generated a model for a predicted tetrameric structure of p19-gp130 (Fig. 4A, right), which we compared to the experimental structure of vIL-6-gp130 (Fig. 4A, left).

We tested for the possibility that p19 physically associates with gp130. First, we looked for binding of recombinant p19 protein to immobilized recombinant gp130-Fc in a cell-free system. We found that p19 dosedependently bound to gp130-Fc, as did control vIL-6, although at higher protein concentrations (Fig. 4B). Second, we used murine BAF-130 cells, which express human gp130, to confirm the binding of p19 to gp130 in cells. We found that recombinant p19 time-dependently activated gp130 (Fig. 4C) and STAT3 (Fig. 4D) in BAF-130 cells, but to a lower degree than did human IL-6 and IL-6R. Third, we tested whether endogenous gp130 associated with p19 in p19-expressing HUVECs. We found that gp130-specific antibodies specifically immunoprecipitated p19 from lysates of p19-expressing HUVECs with or without preactivation with IL-6 and IL-6R (Fig. 4E, left), which promotes *IL6ST/GP130* expression (21). Further Western blotting analysis confirmed the specific immunoprecipitation of gp130 (Fig. 4E, right).

Fourth, we used a proximity ligation assay (PLA) to visualize the colocalization of p19 and gp130 in HUVECs. This assay generates dots that mark the colocalization of two molecules when they are <40 nm apart. We tested p19-expressing and control HUVECs before and after a 15-min activation with IL-6 and IL-6R, which induces gp130 internalization and reexpression on the cell surface (15). Because previous results have shown that vIL-6 associates with gp130 in the endoplasmic reticulum (19) and our current results suggested that vIL-6 and p19 similarly engage gp130, we reasoned that activation with IL-6 and IL-6R would result in increased colocalization of p19 and gp130. Consistent with this, PLA detected the specific association of p19 with gp130 in p19-expressing HUVECs before and, to a greater degree, after 15 min of activation with IL-6 and IL-6R (Fig. 5, A and B). Control HUVECs also displayed a low-level specific (not detected in control PLA) association between p19 and gp130, likely reflecting the reduced abundance of p19 in endothelial cells (Fig. 2C) and the high sensitivity of the PLA-based detection of the p19-gp130 association. Together, these results suggest that intracellular p19 associates with gp130, and suggest that gp130 serves as a mediator of p19-induced signaling in endothelial cells.

Because the phosphorylation of specific tyrosine residues in gp130 initiates STAT3 signaling, we tested whether p19 modulated the tyrosine phosphorylation of gp130. Through a quantitative ELISA, we found that there was substantially greater tyrosine phosphorylation of gp130 in the lysates of p19-expressing HUVECs than in the lysates of control cells (Fig. 5C). Similarly, IL-6 and IL-6R induced greater tyrosine phosphorylation of gp130 in p19-expressing HUVECs than in control cells (Fig. 5C). These results suggest that p19 associates with gp130 and promotes its tyrosine phosphorylation.

p19 specifically associates with gp130 in temporal arteries from GCA patients

The experiments in vitro predicted that p19 associated with gp130 in endothelial cells within GCA lesions. We used PLA to visualize the colocalization of p19 and gp130, as well as CD31 immunostaining to mark endothelial cells (Fig. 6). Specificity controls, including PLA in a control, GCA-negative temporal artery (fig. S4A, temporal artery from an elderly patient suffering from headaches of unknown etiology), and PLA with no primary antibody in a GCA-positive temporal artery biopsy (fig. S4B), showed the specificity of PLA for the p19-gp130 association. As reflected by the specific PLA staining, p19 and gp130 colocalized in a proportion of CD31⁺ cells scattered in the GCA vessel wall (Fig. 6 and figs. S5 and S6). Little PLA signal was detected in the endothelium limiting the central lumen (fig. S6). PLA signal was also detected in a proportion of CD31⁻ cells, possibly T lymphocyte subsets (Fig. 6 and fig. S6), which express p19 (7) and gp130 (22). Thus, p19 and gp130 colocalized in CD31⁺ endothelial cells lining the vasa vasorum of GCA-affected arteries, which could provide an opportunity for p19-gp130 signaling at these sites.

DISCUSSION

These results suggest that IL-23p19, which is considered a biologically inactive intracellular peptide (7), plays a previously unrecognized role as an endogenous activator of endothelial inflammation, promoting leukocyte adhesion to endothelial cells and transendothelial migration. This proinflammatory function of p19 is independent of IL-23 and may contribute to the pathogenesis of GCA because p19 is broadly expressed in the GCA adventitial capillaries, which are the principal port of entry for inflammatory cells into the vessel wall (2, 6). Mechanistically, by stimulating the cell surface expression of VCAM-1 and ICAM-1 on endothelial cells, p19 would be expected to promote the adventitial accumulation of leukocytes, which preferentially express VLA-4 and LFA-1, the cognate ligands for VCAM-1 and ICAM-1, respectively (6). Because inflamed endothelium is common to a number of inflammatory disorders, the current results suggest a broader proinflammatory role for p19 in other forms of vasculitis. In addition, besides endothelial cells, polarized T lymphocytes express p19, but not p40 (7); however, a potential function of p19 in these cells has not been fully investigated. Furthermore, *IL23A* mRNA is relatively more abundant than *IL12B* mRNA in inflammatory macrophages (7), raising the possibility that p19 may play independent functional roles even in cells that secrete IL-23. One of the limitations of our study is that the relative contribution of p19 to tissue inflammation could not be fully assessed, because we have focused on endothelial cells to avoid confounding phenotypes associated with IL-23 secretion. Nonetheless, the identification of p19 as a

previously unrecognized intracellular proinflammatory peptide presents an advance and offers a new potential therapeutic target.

p19-transgenic mice develop severe inflammation in many tissues, which is associated with swollen abdomen, runting, and early death despite the mice having normal amounts of circulating IL-12p40, supporting the possibility that p19 may be biologically active independently of p40 when expressed in certain cells (23). Consistent with this, IL-23 did not reproduce the phenotype of p19-transgenic mice when expressed systemically in mice at high amounts over a prolonged period, promoting instead specific inflammation at the tendon-bone insertion (24). Although these phenotypic differences may reflect a spectrum of IL-23–related functions, they raise the possibility that the more severe phenotype of the p19-transgenic mice is attributable in part to the activity of p19 alone.

The viral cytokine vIL-6 and the p19 peptide share about 16% sequence identity and are mostly intracellular proteins. Similar to vIL-6, which forms a tetrameric intracellular complex with the signaling molecule gp130 that is composed of two gp130 chains and two vIL-6 molecules (gp130₂–vIL-6₂) (25, 26), we found that intracellular p19 bound to gp130 and activated gp130-STAT3 signaling. Human IL-6 and vIL-6 share about 25% sequence identity, but human IL-6 forms hexameric signaling complexes at the cell surface, which include two IL-6R subunits in addition to two gp130 chains and two IL-6 chains (IL-6R₂–gp130₂–human IL-6₂). Nonetheless, human IL-6 and vIL-6 initiate similar signaling from phosphorylated gp130 (19, 27). Thus, p19, initially discovered through computational analysis as a member of the IL-6 family of cytokines (7), is now found to share gp130 signaling with a member of this family.

Most patients with GCA are treated effectively with steroid therapy, which improves systemic inflammatory symptoms, but steroids do not control all of the inflammatory pathways active in GCA (2). Disease flares are frequent after glucocorticoids are tapered, and prolonged use of glucocorticoids is associated with various complications; thus, new drugs are needed. The current disclosure of a previously unrecognized proinflammatory role for intracellular p19 unveils a new potential therapeutic target in GCA and other inflammatory diseases in which p19 is found in the absence of p40. Neutralizing antibodies to p19, which hold promise as a treatment for psoriasis (28), are not likely to be effective against intracellular p19. Rather, Janus kinase (JAK) inhibitors (29) and Src homology 2 (SH2) domain–containing protein-tyrosine phosphatase (SHP) inhibitors (30) should be explored for pharmacologic targeting of p19 signaling in inflammatory diseases such as GCA.

MATERIALS AND METHODS

Cells, reagents, and cell culture

HUVECs were derived and propagated as described previously (31). HDMECs (Lonza) were maintained in endothelial basal medium-2 (EBM-2) supplemented with endothelial growth medium-2 microvascular (EGM-2MV BulletKit, Clonetics). BAF-130 cells (murine BAF-B03 cells stably expressing human gp130) were previously reported (27). PBMCs were obtained from volunteer donors according to an approved protocol. Cells were stimulated

with PMA (10 ng/ml; R&D Systems) or LPS (1 µg/ml; Sigma) or with human TNF-α (20 ng/ml; R&D Systems) and human IFN-γ (1 or 10 ng/ml; R&D Systems) for 24 hours in complete culture medium. To activate gp130-pSTAT3 signaling, HUVECs were stimulated (0 to 45 min) with p19 (1 µg/ml; Abnova) or with human IL-6 (200 to 1000 ng/ml; R&D Systems) and IL-6R (200 to 2500 ng/ml; R&D Systems). In selected experiments, IL-6 and IL-6R were used to preactivate HUVECs for 18 to 24 hours. For production of serum-free cell culture medium, endothelial cells were cultured in serumfree M199 complete medium for 24 to 72 hours. TCA was used to precipitate proteins from the culture medium. To reduce background activation, HUVECs were cultured for 18 hours in M199 medium containing 0.5% bovine serum albumin (BSA).

Transduction of cells with lentivirus encoding IL-23p19

The lentiviral vector for expression of human IL-23p19 was from Gene-Copoeia (catalog no. EX-U1167-Lv105); the pLVX-Puro vector (31) was used as a control. Infection of cells and the selection of transduced cells in puromycin were performed as described previously (31).

Real-time reverse transcription PCR analysis

RNA was extracted from cells with TRI Reagent (Molecular Research Center). Complementary DNA (cDNA) was synthesized from 1 mg of total RNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). The relative amount of mRNA was measured by real-time PCR assay with 1 ml of cDNA and TaqMan PCR Universal Master Mix (Applied Biosystems). Human TaqMan gene expression probes included *IL23A*, *IL12B*, *ICAM1*, *VCAM1*, *PECAM1*, *CXCL9*, *CXCL10*, *CXCL11*, *IL6*, and *GAPDH* (Applied Biosystems). The PCR conditions used were those recommended by the manufacturer.

Enzyme-linked immunosorbent assays

The human IL-23p19 ELISA kit was obtained from Abcam; CXCL9, CXCL10, and CXCL11 Quantikine ELISA kits were purchased from R&D Systems. Human phosphorylated gp130 was measured in cell lysates (25 and 12.5 mg per well) with a sandwich ELISA (R&D Systems) according to the manufacturer's instructions. The binding of p19 and vIL-6 to gp130 was measured through the modification of a reported method (27). Recombinant human soluble gp130 (R&D Systems) was immobilized on ELISA plate wells (4HBX, Immulon) at 5 µg/ml in phosphate-buffered saline (PBS). After blocking (SuperBlock, Life Technologies), maltose-binding protein (MBP) fused to vIL-6 (MBP-vIL-6) (27) or p19 (IL-23A, Abnova) was applied at various concentrations in PBS, 1% BSA, and the mixtures were incubated for 4 hours at room temperature. Bound protein was detected with polyclonal rabbit IgG against vIL-6 (27) or goat IgG against p19 (AF1716, R&D Systems; both at 1 µg/ml), followed by horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (NA934V, GE Healthcare Life Sciences) or rabbit anti-goat IgG (401504, Calbiochem) at a 1:3000 dilution in PBS, 0.05% Tween 20. Reactions were visualized with tetramethoxybenzene peroxidase substrate, followed by 2NH₂SO₄. Plates were read at 450 nm with a microplate reader.

Immunoprecipitations

Protein extracts were prepared from p19-expressing HUVECs, which were untreated or treated with human IL-6 (200 to 1000 ng/ml; R&D Systems) and soluble IL-6R (200 to 2500 ng/ml; R&D Systems), and from human gp130-expressing BAF-130 cells, which were untreated or treated with human IL-6 (200 to 1000 ng/ml), soluble IL-6R (200 to 2500 ng/ml), or p19 protein (1 µg/ml; Abnova), in lysis buffer containing 10 mM tris-HCl (pH 7.5), 150 mM NaCl, 0.2% NP-40 (Abcam), 1 mM NaF, 1 mM EDTA, 10% glycerol, complete protease inhibitor cocktail (Roche), 2 mM sodium orthovanadate (Sigma-Aldrich), pepstatin A (1 mg/ml; Sigma-Aldrich), leupeptin hemisulfate salt (1 mg/ml; Sigma-Aldrich), and aprotinin (1 µg/ml; Sigma-Aldrich). Rabbit polyclonal IgG against human gp130 (Millipore) and control rabbit polyclonal IgG (Jackson ImmunoResearch Laboratories) were individually incubated (4 µg) with washed protein G Dynabeads (50 µl; Life Technologies) and incubated for 10 min at room temperature. After magnetic separation, the antibody-bead complex was added to the cell lysates (500 mg) followed by incubation for 18 hours at 4°C with rotation. Bead-antibody-protein complexes were recovered by magnetic separation. Target antigen was eluted by heat treatment (for 10 min at 70°C).

Western blotting

Immunoprecipitates and protein extracts prepared in SDS lysis buffer with Protease Inhibitor Cocktail Set III (Calbiochem), 50 mM NaF, and 1 mM sodium orthovanadate were resolved through NuPAGE 4 to 12%, bis-tris, or 6% tris-glycine gels (Invitrogen) and transferred onto nitrocellulose membranes. After blocking, the membranes were incubated with the following antibodies: polyclonal rabbit antibody against human IL-23p19 (Sigma-Aldrich), polyclonal goat IgG against human IL-23p19 (R&D Systems), mouse monoclonal antibody against human gp130 (R&D), rabbit polyclonal antibody against human gp130 (Millipore), sheep polyclonal antibody against human VCAM-1 (R&D Systems), rabbit polyclonal antibody against human ICAM-1 (Cell Signaling Technology), mouse monoclonal antibody against CD31/PECAM-1 (BD Pharmingen), sheep polyclonal antibody against CD31/PECAM-1 (R&D Systems), rabbit polyclonal against STAT1, rabbit monoclonal antibody against pSTAT1 (Tyr701), rabbit monoclonal antibody against STAT3, rabbit polyclonal antibody against pSTAT3 (Tyr705), rabbit polyclonal antibody against p44/42 mitogen-activated protein kinase (MAPK; ERK1/2), rabbit monoclonal antibody against phosphorylated p44/42 MAPK (ERK1/2) (Thr²⁰²/Tyr²⁰⁴), rabbit monoclonal antibody against NF-κB p65, rabbit monoclonal antibody against NF-κB phospho-p65 (Ser⁵³⁶), rabbit polyclonal antibody against Akt, rabbit monoclonal antibody against pAkt (Ser⁴⁷³) (all from Cell Signaling Technology), mouse monoclonal antibody against phosphotyrosine residues (4G10, Millipore), and goat antibody against b-actin (Santa Cruz Biotechnology). HRP-conjugated donkey anti-mouse IgG, anti-rabbit IgG, and anti-sheep IgG were from Amersham Pharmacia Biotech. Bound secondary antibodies were visualized by enhanced chemiluminescence (Amersham). The membranes were stripped and restained.

Tissues

Biopsies of temporal arteries were obtained for diagnostic purposes from eight patients with suspected GCA participating in institutionally approved study protocols of the Vasculitis

Research Unit, Department of Autoimmune Diseases, “Hospital Clinic of Barcelona” (Spain). All participants in the study provided written informed consent after the nature and possible consequences of participation in the study were explained. Five of the patients had histological features of GCA in the biopsy (table S1), and three of the patients did not have histological features of tissue inflammation and served as controls (table S2).

Immunofluorescence imaging

HUVECs were cultured on Lab-Tek chamber slides (Nunc, Thermo Scientific), fixed in cold 4% paraformaldehyde (PFA) in PBS, and stained with antibodies against IL-23p19 (rabbit polyclonal, Abcam), IL-23p19 (goat IgG polyclonal, R&D Systems), STAT3 (rabbit monoclonal; Cell Signaling Technology), and pSTAT3 (Tyr⁷⁰⁵) (rabbit monoclonal; Cell Signaling Technology). Secondary antibodies were Alexa Fluor–conjugated anti-goat or anti-rabbit antibodies (Molecular Probes, Life Technologies). Nuclei were stained with DAPI. The fluorescence intensity of pSTAT3 from confocal imaging (Carl Zeiss LSM 780 confocal microscope) of HUVECs was measured by ImageJ and was subsequently normalized by DAPI fluorescence intensity in each region to adjust for different cell densities. Temporal artery tissues were fixed in cold 4% PFA in PBS, cryoprotected in 15 and 30% sucrose, embedded in optimum cutting temperature compound, and processed for histology. For staining, slides were further fixed in 4% PFA for 10 min at room temperature, washed in PBS, and soaked in PBS, 0.1% Triton, 1% BSA, and 5% donkey serum (Sigma-Aldrich) for 1 hour at 4°C. The slides were incubated overnight at 4°C with the following primary antibodies: human CD31 (mouse monoclonal antibody, DAKO), human IL-23p19 (rabbit polyclonal, Abcam), and human IL-12p40 (goat polyclonal antibody, Santa Cruz Biotechnology). Slides were washed in PBS (three times for 5 min each), followed by incubation with secondary antibodies (spectrally distinct Alexa Fluor–conjugated antibodies against goat, mouse, and rabbit IgG; Molecular Probes, Life Technologies). Nuclei were visualized with DAPI. Images were obtained with a laser-scanning confocal Leica TCS SP5 microscope (Leica Microsystems). Images were processed with Leica Confocal software (LAS-AF Lite) and ImageJ software (Wayne Rasband).

Proximity ligation assay

PLA was used to visualize the proximate colocalization (<40 nm distance) of p19 and gp130 in HUVECs and tissue biopsies of temporal arteries with the Duolink detection kit (Olink Bioscience). HUVECs were fixed in 4% PFA for 20 min at room temperature and stored in 50% glycerol. After blocking for 1 hour in blocking buffer at room temperature, the cells were incubated overnight at 4°C with rabbit polyclonal anti-human p19 antibody (2 µg/ml; Sigma) and mouse anti-human gp130 IgG1 monoclonal antibody (10 µg/ml; R&D Systems) in blocking buffer. After being washed twice for 5 min in wash buffer [0.1 M Tris-HCl (pH 7.5), 0.5 M NaCl, 5% Tween 20 in ultrapure water], the cells were incubated for 30 min at 37°C with PLA probe solution containing anti-rabbit MINUS and anti-mouse PLUS Duolink PLA probes. After washing and circularization and ligation of the oligonucleotides in the probes, an amplification step was performed with polymerase solution for 100 min at 37°C. After washing, refixation in 4% PFA, and additional washing, the cells were stained with Alexa Fluor 488–Zenon–labeled mouse anti-human CD31 monoclonal antibody (Covance/ EMD Millipore) or with fluorescein isothiocyanate–conjugated mouse

monoclonal antibody against human CD31 (ImmunoTools). After washing and refixation in 4% PFA, the slides were mounted with Duolink II mounting medium containing DAPI. Images were obtained with a Carl Zeiss LSM 780 confocal microscope with ZEN software (Carl Zeiss). Quantification of the PLA signal was calculated as the mean ($\pm$ SD) number of dots per cell. The total number of dots per area and the total DAPI signal per area were measured by ImageJ software over multiple fields including at least 200 cells.

Leukocyte-endothelium adhesion assay

Leukocyte adhesion to endothelial cells was evaluated with the CytoSelect Leukocyte-Endothelium Adhesion assay kit according to the manufacturer's instructions. Briefly, confluent monolayers of control or p19-expressing HUVECs were generated in 96-well plates precoated with gelatin by culturing the cells (at 25,000 cells per well) for 3 days. Human PBMCs isolated from the peripheral blood of volunteer donors (1×10^6 cells/ml) were labeled with LeukoTracker solution for 1 hour at 37°C. After washing and suspension in serum-free medium, the labeled PBMCs (2×10^5 cells per well in 200 μ l) were incubated on the prewashed HUVEC monolayer and incubated for 90 min at 37°C. After removal of nonadherent cells and washing three times in wash buffer, 150 μ l of lysis buffer (from the kit) was added to each well and incubated for 10 min with shaking. Fluorescence in the solution (100 μ l) was measured at 480/520 nm. The results are expressed as mean relative fluorescence units ($\pm$ SD) of triplicate cultures.

Transendothelial migration assay

The assay was performed essentially as described previously (32). Control and p19-expressing HUVECs were seeded (at 40,000 cells per well) onto the membrane (precoated with 0.1% gelatin) separating the top from the bottom chamber of a 96-well Transwell plate (5- μ m pore size; Costar, Fisher Scientific) to generate a monolayer. Once formed, the monolayer was incubated overnight in complete medium alone, complete medium containing LPS (1 μ g/ml), or complete medium containing TNF- α and IFN- γ . Transendothelial migration of PBMCs (1×10^6 cells per well) was performed in RPMI 1640 medium containing 0.5% BSA over a 6-hour incubation period at 37°C. Viable cells in the lower chamber were collected and counted. The results of triplicate cultures are expressed as means $\pm$ SD.

Molecular modeling

The experimental structural complex of vIL-6:gp130 (20) (PDB ID: 1I1R) was used as the template to model potential binding interactions between human p19 and gp130. Multiple sequence alignments of vIL-6, human p19, and human IL-6 were used as guidance to overlay the experimental structures of human p19 (PDB ID: 3D87) onto the structure of vIL-6-gp130 (PDB ID: 1I1R) to generate a tetrameric p19-gp130 model complex. Sequence alignments were created with T-COFFEE software (online version_10.00.r1613; www.tcoffee.org/) in the Expresso mode, which aligns protein sequences with structural information. The following T-COFFEE methods were chosen to compute the Expresso library: sap_pair, muscle_msa, and t_coffee_msa. The option to automatically fetch PDB templates was turned on, and all of the remaining options were kept at the default mode. The validity of human p19-gp130 model and its binding interface compatibility were evaluated

by comparing the key contact surface residues of vIL-6:gp130. The nonsimilar residues in the binding interface and their compatibility with the generated model were evaluated at the sequence and structural levels with the National Center for Biotechnology Information (NCBI) tool Amino Acid Explorer (33) and molecular modeling software (Accelrys Discovery Studio Visualizer 3.5).

Statistical analysis

The results are presented as means $\pm$ SD. The statistical significance of differences between two groups was calculated with a two-tailed Student's *t* test. The results are provided as *P* values, where *P* < 0.05 is considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. IL-23p19 is found in GCA temporal arteritis.

(A and B) Histological analysis of normal (A) and GCA (B) temporal arteries, showing narrowing lumen, inflammatory infiltrates in the vessel wall, and disrupted internal elastic membrane (B). Hematoxylin and eosin staining (left) and 4',6-diamidino-2-phenylindole (DAPI) staining of nuclei (right) were performed. L, lumen; I, tunica intima; M, tunica media; Adv, adventitia; E, internal elastic membrane. (C and D) CD31 immunostaining (red) in sections of normal temporal artery (C), showing the selective presence of CD31 in the endothelium lining the temporal artery, and in sections of GCA temporal artery (D), showing

CD31 (red) in the endothelium lining the narrow lumen and in cells scattered throughout the vessel wall. Nuclei were stained with DAPI (blue). (E) CD31 (red), p19 (green), and p40 (white) immunostaining in the adventitia of the GCA temporal artery [magnified views of the boxed areas (i) to (iii) in (D)] showing the colocalization of CD31 and p19 (yellow, indicated by arrows) in capillary structures and other isolated cells. Boxed areas (bottom) limit p40⁺ (white) cells. Results are representative of three arteries from three GCA patients, and at least three sections per sample were evaluated.

Fig. 2. Inflammatory signals induce p19 production in endothelial cells.

(A and B) Effects of phorbol 12-myristate 13-acetate (PMA), LPS, and TNF- α on the abundances in endothelial cells (HUVECs and HDMECs) of *IL23A* (p19) (A) and *IL12B* mRNA (p40) (B). Results are from real-time polymerase chain reaction (PCR) analysis and show the fold increase in mRNA relative to that in cells treated with medium alone after 24 hours of culture with the indicated stimuli. Data are means $\pm$ SD of three experiments, each performed in triplicate. (C) Western blotting analysis of p19 abundance in HUVECs and HDMECs that were left untreated or were treated with LPS or TNF- α . No p19 was detected

in trichloroacetic acid (TCA) precipitates of serum-free culture medium of HDMECs (SN; 20 ml). Lysates of LPS-treated PBMCs were used as a control. Western blots are representative of three experiments. **(D)** PBMCs ($2 \times 10^6/\text{ml}$), HUVECs ($1 \times 10^6/\text{ml}$), and HDMECs ($1 \times 10^6/\text{ml}$) were left untreated or were treated with PMA, LPS, or TNF- α for 24 hours. Cell culture medium was then analyzed by ELISA to detect p19 protein. Data are means $\pm$ SD of four experiments, each performed in triplicate. **(E)** Untreated HUVECs and HUVECs transduced with lentivirus expressing p19 (p19LV) or with control lentivirus (control) were subjected to real-time PCR analysis and Western blotting analysis to detect p19 Mrna (top left) and protein (bottom left), respectively. Results from the PCR analysis are given as the fold increase ($\times 10^3$) in *IL23A* (p19) mRNA abundance. Data are means $\pm$ SD of five experiments, each performed in triplicate. Western blots are representative of six experiments. p19-expressing (bottom right) and control (top right) HUVECs were also analyzed by immunofluorescence microscopy to visualize p19. Percentage of p19-expressing HUVECs with cytoplasmic p19: $92 \pm 6.3\%$ ($n = 6$ experiments). **(F)** Uninfected HUVECs, control HUVECs, p19-expressing HUVECs, and LPS-treated PBMCs were analyzed by real-time PCR to determine the abundances of *IL12B* (p40) and *IL23R* mRNAs. Results are expressed as the fold increase in the indicated mRNAs relative to those in untransduced HUVECs. Data are means $\pm$ SD of three experiments performed in triplicate. **(G)** LPS-activated PBMCs and p19-expressing HUVECs ($1 \times 10^6/\text{ml}$) were cultured in serum-free medium for 24 and 72 hours before the conditioned cell culture medium was treated with TCA to precipitate proteins. Precipitated samples (SN) were analyzed by Western blotting to detect p19. One hundred milliliters of culture medium from p19-expressing HUVECs and 20 ml of culture medium from LPS-activated PBMCs were used for the analysis. Lysates of cells cultured for 72 hours were used as controls. Data are representative of three experiments.

Fig. 3. p19 induces the production of adhesion molecules and STAT3 phosphorylation in endothelial cells.

(A and B) Control and p19-expressing HUVECs were analyzed by real-time PCR (A) and Western blotting (B) to determine the relative abundances of ICAM-1, VCAM-1, and PECAM-1 mRNA and protein, respectively. (A) Data show the fold increase in mRNA abundance relative to that in control cells and are means $\pm$ SD of three experiments, each performed in triplicate. (B) Left: Western blots are representative of three experiments. Right: Densitometric analysis of the indicated band intensities relative to those of β -actin. Data are means $\pm$ SD of three experiments. (C) PBMCs were incubated on monolayers of control HUVECs or p19-expressing HUVECs, and the extent of their attachment was measured as described in Materials and Methods. Data are means $\pm$ SD of triplicate samples in a single experiment and are representative of five independent experiments. (D) PBMCs were incubated on monolayers of control HUVECs or p19-expressing HUVECs that had been cultured in medium only or were treated with LPS or with both TNF- α and IFN- γ . The

numbers of PBMCs that underwent transendothelial migration were then determined. Data are means $\pm$ SD of three experiments; each experiment included PBMCs from three or four donors. **(E)** Control and p19-expressing HUVECs were analyzed by Western blotting to determine the extent of STAT3 phosphorylation. Left: Western blots were analyzed with antibodies against the indicated proteins and are representative of three experiments. Right: Densitometric analysis of pSTAT3 band intensity relative to those of total STAT3 and β -actin. Data are means $\pm$ SD of three experiments. **(F)** Right: Control and p19-expressing HUVECs were treated with IL-6 and IL-6R for the indicated times. After immunostaining for pSTAT3 and visualization of nuclei with DAPI, immunofluorescence was quantified in at least 500 cells from 10 fields at each time point. Left: Bar graph shows pSTAT3 fluorescence intensity at time 0 with an expanded scale. $**P < 0.01$, $*P < 0.05$ by two-tailed Student's t test.

Fig. 4. p19 binds to gp130.

(A) Structural similarity of the vIL-6-gp130 and p19-gp130 tetrameric complexes. The vIL-6-gp130 complex representation (left) is based on the published experimental structure [Protein Data Bank (PDB) ID: 1I1R]; the p19-gp130 complex representation (right) is based on modeling predictions (see Materials and Methods). (B) p19 and vIL-6 bind to immobilized gp130. Purified soluble gp130 immobilized on plastic wells was incubated with serial dilutions of recombinant human p19 (78 to 250 ng/ml) or vIL-6 (1.5 to 50 ng/ml). Bound proteins were detected with specific antibodies. Data are means of triplicate

measurements and are representative of two independent experiments. **(C)** Mouse BAF-130 cells stably expressing human gp130 were left untreated or were treated with recombinant human p19 (1 µg/ml) for the indicated times. In one set of samples, human IL-6 (1 µg/ml) and IL-6R (2.5 µg/ml) were added to the BAF-130 cells for 15 min. Cell lysates were immunoprecipitated (IP) with rabbit anti-gp130 antibody or rabbit IgG. The immunoprecipitates were analyzed with anti-phosphotyrosine antibody and, after stripping, with anti-gp130 antibody. Western blots are representative of three experiments. p-gp130, phosphorylated gp130. **(D)** Mouse BAF-130 cells stably expressing human gp130 were incubated with recombinant human p19 protein (1 µg/ml) or with buffer alone for the indicated times. As a positive control, the cells were incubated with vIL-6 (1 µg/ml) or with IL-6 (1 µg/ml) and IL-6R (2.5 µg/ml) (C). Cell lysates were analyzed by Western blotting with antibodies against the indicated proteins. Western blots are representative of three experiments. **(E)** p19-expressing HUVECs were left untreated (lanes 1 and 3) or were treated for 18 hours with IL-6 and IL-6R (lane 2) and then immunoprecipitated with rabbit anti-human gp130 IgG (lanes 1 and 2) or rabbit IgG control (sample 3). Samples were then analyzed by Western blotting (IB) with antibodies against p19 and gp130, whose positions on the blots are marked by arrows. Western blots are representative of three experiments.

Fig. 5. p19 colocalizes with gp130 in endothelial cells and activates gp130.

(A) Right: Control HUVECs and p19-expressing HUVECs were left untreated (0 min) or were treated for 15 min with IL-6 and IL-6R before being analyzed by PLA. The red dots mark the colocalization (<40 nm) of p19 and gp130. CD31 immunostaining (green) marks the endothelial cell surface membrane. Nuclei are identified by DAPI staining (blue). Representative images of five larger fields per condition are shown. Left: Representative images from control PLAs documenting PLA specificity. (B) Quantitation of the colocalization of p19 and gp130 in control HUVECs and p19-expressing HUVECs that were

treated as described in (A). Data are mean ($\pm$ SD) number of dots per cell from the analysis of at least 200 cells from five fields per condition. (C) Control HUVECs and p19-expressing HUVECs were left untreated (0 min) or were treated for 5 min with IL-6 and IL-6R. Cells were then lysed and analyzed by ELISA to detect the relative amounts of tyrosinephosphorylated gp130 (pTyr-gp130). Data are means $\pm$ SD of triplicate measurements from a single experiment and are representative of three experiments.

Fig. 6. Endothelial colocalization of p19 and gp130 in GCA temporal artery.

The adventitia of a temporal artery affected with GCA were subjected to PLA to determine the colocalization of p19 and gp130 in CD31⁺ vascular structures. The red staining marks the colocalization (<40 nm) of p19 and gp130 in the tissue. The green staining marks CD31, and blue (DAPI) marks the cell nuclei. White arrows point to the PLA signal (p19 + gp130, red) and the corresponding CD31⁺ signal (green). Top left: Low-magnification image. The two boxed areas [labeled (i) and (ii)] are magnified on the right [three panels, corresponding

to box (i)] and below [three panels, corresponding to box (ii)]. The results are representative of three patients with GCA; at least three sections per patient were evaluated.

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