IL-27 Inhibits Lymphatic Endothelial Cell Proliferation by STAT1-Regulated Gene Expression

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Abstract

Objective: IL-27 belongs to the IL-12 family of cytokines and is recognized for its role in Th cell differentiation and as an inhibitor of tumor angiogenesis. The purpose of this study was to investigate the effect of IL-27 on proliferation of lymphatic endothelial cells to gain insight into the interplay between the immune system and development of the lymphatic system.

Methods: IL-27-stimulated signal transduction in human dermal lymphatic endothelial cells was measured by western blotting and synthesis of CXCL10 and CXCL11 by use of RT-PCR and ELISA. Proliferation was measured using MTT and BrdU kits and the role of STAT1 and chemokines was determined by use of siRNA and recombinant proteins.

Results: Stimulation of lymphatic endothelial cell cultures with IL-27 induced JAK dependent phosphorylation of STAT1 and STAT3 and inhibited lymphatic endothelial cell proliferation and migration. Expression of CXCL10 and CXCL11, both STAT1 target genes, was profoundly up-regulated upon IL-27 stimulation, and recombinant CXCL10 and CXCL11 inhibited FGF-2-induced proliferation *in vitro*. siRNA targeting of STAT1 almost completely abrogated CXCL10 and CXCL11 expression as well as the proliferative effect of IL-27.

Conclusions: IL-27 function as an anti-lymphangiogenic regulator *in vitro* by up-regulating chemokines and interfering with the mitogenic effect of growth factors through STAT1 activation.

Key words: IL-27, lymphangiogenesis, proliferation, STAT1, CXCL10

Abbreviations used: BrdU, Bromo-deoxy Uridine; CXCL10/11, C-X-C motif chemokine ligand 10/11; EBI3, Epstein-Barr virus Induced gene 3; FGF-2, Fibroblast Growth Factor 2; gp130, Glycoprotein 130; HMVEC, Neonatal Human Dermal Lymphatic Microvascular Endothelial Cells; hTERT-HDLEC, Human Telomerase Reverse Transcriptase-Transfected Human Dermal Lymphatic Endothelial Cells; IL-27, Interleukin 27; INF-γ, Interferon gamma; JAK, Janus Kinase; STAT1/3, Signal Transducer and Activator of Transcription 1/3; WSX-1, IL-27 specific receptor chain.

Introduction

Pro- and anti-inflammatory cytokines influence various aspects of vascular biology, and the pathogenesis of several chronic inflammatory diseases as well as low grade inflammation coupled to aging is known to involve vascular remodeling. This includes the coordinated remodeling of lymphatic vessels. Research on lymphangiogenesis—defined as the formation of new lymphatic vessels from existing ones—has drawn considerable attention and recently a close relationship between inflammation and lymphangiogenesis has been established.

In an inflammatory environment, the key mediators of intercellular communication are interleukins and chemokines. Inflammatory cytokines, such as IL-1β, IL-7, and IL-20, are able to promote lymphangiogenesis [1,2,20,37], while other endogenous cytokines, such as TGF-β, IFN-γ and TNF-α, seem to have the opposite effect [3,5,10,29]. The balance between stimulatory and inhibitory cytokines together with other regulators of lymphangiogenesis such as VEGF-C and FGF-2 ultimately determines the potential for lymphatic neovascularization in the inflamed tissue.

The heterodimeric cytokine IL-27 belongs to the IL-12 family of cytokines, and consists of the Epstein–Barr virus Induced gene 3 (EBI3) and the p28 subunit [31]. The cytokine binds to the IL-27 receptor which is composed of two subunits, WSX-1, the IL-27 specific receptor chain, and gp130, the common receptor chain for IL-6 type cytokines.

IL-27 receptor activation initiates intracellular signaling primarily through the JAK/STAT signal transduction pathway, and both receptor subunits are necessary to trigger this signaling [24,30]. IL-27 is predominantly produced by APCs, including dendritic cells and macrophages [31] and studies of IL-27 have mainly focused on its effects in the context of inflammation. IL-27 function as a Th1 polarizing cytokine in addition to suppress Th17 and Th2 differentiation —thus, IL-27 serves complex functions by regulating the differentiation of T-helper cells [22,27,31,35,36,39]. Previous reports imply that IL-27 has antitumor activities and it is recognized for its anti-angiogenic effects *in vivo* [9,13,34,40]. However, the potential role of IL-27 in regulation of lymphangiogenic processes has not been studied.

In this study, we investigated the effects of IL-27 on the cell lines hTERT-HDLEC (human telomerase reverse transcriptase-transfected human dermal lymphatic endothelial cells) and HMVEC (Neonatal Human Dermal Lymphatic Microvascular Endothelial Cells). We show that IL-27 inhibits proliferation, a central lymphangiogenic process, via JAK/STAT1 activation and we also show an IL-27-mediated attenuation of the endothelial cell mobility. In addition, we identify CXCL10 and CXCL11 as STAT1 targets and as inhibitors of lymphatic endothelial cell proliferation.

Materials and Methods

Reagents

Recombinant human (rh)IL-27 and monoclonal anti-human CXCL10 and CXCL11 were obtained from R&D systems (Wiesbaden-Nordenstadt, Germany). rhFGF-2, siRNA targeting STAT1, scrambled siRNA and NTER transfecting medium were purchased from Sigma-Aldrich (Brøndby, Denmark).

Cell Lines and Cell Culture

hTERT-HDLECs, a human lymphatic endothelial cell line displaying longer lifespan [28] were cultured in gelatine-coated tissue culture flasks in endothelium cell growth medium MV2 (Promocell, Heidelberg, Germany) or EGM-2MV (Lonza, Basel, Switzerland) supplemented with 200 U/mL penicillin, 50 μg/mL streptomycin. The primary lymphatic endothelial cell line HMVEC was obtained from Lonza (Basel, Switzerland), and was cultured in collagen-coated flasks in MV2 or EGM-2MV medium.

Signal Transduction Assays

hTERT-HDLECs and HMVEC were grown to 90% confluency in gelatine-coated 60-mm dishes, washed with PBS and incubated for 60 minutes in serum-free medium (RPMI medium 1640) with or without 5 μM JAK Inhibitor I (Merck, Darmstadt, Germany) for 30 minutes before stimulation. After stimulation, medium was removed and cells were lysed in 100 μL 1% Igepal lysis buffer (Sigma-Aldrich) containing 1.25 μg/mL aprotinin, 1.25 μg/mL pepstatin, 1.25 μg/mL leupeptin, 1.25 mM sodium fluoride, 0.62 mM phenylmethanesulfonyl fluoride, and 1.25 mM sodium orthovanadate. Samples were mixed and centrifuged at 11,000 $\times$ *g*, 4°C for 10 minutes.

Pellets were discarded and equal amounts of protein samples were separated by SDS-PAGE (10% BIS–Tris gel) (Invitrogen, Carlsbad, CA, USA). Proteins were transferred to nitrocellulose membranes (Invitrogen), and membranes were blocked with 5% bovine serum albumin (BSA) in PBS with 0.1% Tween. Membranes were probed overnight at 4°C with antibodies diluted in PBS with 5% BSA and 0.1% Tween for detecting P-STAT1 (Y701), P-STAT3 (Y705), total STAT1 and STAT3 (Cell Signalling Technology, Danvers, USA), or GAPDH (Millipore, Billerica, MA, USA) as recommended by the manufacturer. This was followed by incubation for 1 hour in PBS with 1% BSA and 0.1% Tween with peroxidase-conjugated goat anti-rabbit immunoglobulin antibody diluted 1:1000 for P-STAT1, P-STAT3, total STAT1 and STAT3 and with peroxidase-conjugated goat anti-mouse immunoglobulin antibody diluted 1:10 000 for GAPDH (Dako, Glostrup, Denmark). Protein bands were detected using supersignal west pico chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL, USA) and visualized using the biospectrum AC imaging system (UVP, Cambridge, UK).

RT-PCR

Reverse transcription was performed using total RNA from hTERT-HDLEC and HMVEC and Omniscript Reverse Transcriptase kit (Qiagen Nordic, Copenhagen, DK). Complementary DNAs were amplified by PCR using the following primer pairs: 5′-ACGGCAGCATCAACCACAC-3′ (forward) and 5′-TGAAGCACCTCCATCTCTTTGTC-3′ (reverse) to generate a FGFR1 fragment of 274 bp; 5′-CGCTGTCTTTG-CATAGGCCCTGG-3′ and 5′-GCTGGACTCCTTTGGGC AGTGG-3′ to generate a CXCL11 fragment of 442 bp; 5′-TCTAAGTGGCATTCAAGGAGTACC-3′ and 5′-ACCTT CCTACAGGAGTAGTAGCAG-3′ to generate a CXCL10 fragment of 433 bp; 5′-GGAAGGTGAAGGTCGGAGTC AA-3′; 5′-GATCTCGCTCCTGGAAGATGGT-3′ to generate a GAPDH fragment of 340 bp; 5′-TGCTGATTGCAAAG-CAAAAC-3′ (forward) and 5′-CCCACTTGCTTCTTCACT-CC-3′ (reverse) to generate a gp130 fragment of 451 bp and 5′-TGGACTTTTCCGAGGATGAC-3′ (forward) and 5′-GGA GCAGCAGCAGGTAATTC-3′ (reverse) to generate a WSX-1 fragment of 452 bp.

Modified MTT Proliferation Assay

hTERT-HDLEC or HMVEC were seeded into 96-well plates coated with gelatin (hTERT-HDLEC) or collagen (HMVEC)

at 2000 cells per well in a total volume of 200 μL and grown in FGF- and VEGF-A depleted EGM-2MV medium (incomplete medium) containing 2% FCS and pen./strep. for 4 or 6 days. Cells were left untreated or were treated with IL-27 and FGF-2 in reduced medium at day 1 and renewed at day 3. siRNA targeting STAT1 (50 nM) or scrambled siRNA (50 nM) was added at day 1 and cells stimulated with IL-27 (100 ng/mL) and FGF-2 (10 ng/mL) at day 3. Proliferation was measured using the EZ4U-proliferation Kit (Biomedica Gruppe, Wien, Austria) based on the method of reduction of tetrazolium salt to colored formazan. Samples were incubated 3 hours with substrate before measurement of absorbance at 450 nm with 630 nm as background.

SYBR Green Proliferation Assay

hTERT-HDLEC were seeded in 24-well plates coated with gelatine at 12 000 cells per well in a total volume of 1 mL and grown in FGF-2 and VEGF-A depleted EGM-2MV medium containing 2% FCS and pen./strep. The cells were left untreated or were treated with various concentrations of IL-27 and/or FGF-2 at day 1. After 3 days, proliferation was measured by aspirating the medium and lyzing cells with 90 μL 1% Igepal buffer per well including 0,1% SYBR save (Invitrogen). Samples were incubated for 15 minutes before measurement of fluorescence (exc. 485 nm, em. 528 nm).

BrdU Proliferation Assay

hTERT-HDLEC and HMVEC were seeded into 96-well plates coated with gelatin (hTERT-HDLEC) or collagen (HMVEC) at 2000 cells per well in a total volume of 200 μL and grown in FGF- and VEGF-A depleted EGM-2MV medium (incomplete medium) containing 2% FCS (hTERT-HDLEC) or 3% FCS (HMVEC) and pen./strep. Cells were left untreated or were treated with IL-27, FGF-2, CXCL10, or CXCL11 in reduced medium at day 1 and were incubated with BrdU for 5 hours at day 2. Proliferation was measured using the BrdU Cell Proliferation Assay (Merck, Germany) according to the manufacturer's description. Absorbance was measured at 450 nm with 540 nm as background.

Transwell Migration Assay

In vitro migration of hTERT-HDLECs was studied using a transwell migration assay. hTERT- HDLECs were seeded in VEGF-A- and FGF-depleted 1% FCS MV2-medium at 30. 000 cells per 8.0 μm-pore diameter fluoroblok-insert (Becton Dickinson Labware, Franklin Lake, NJ, USA). Inserts were then placed in a 24-well plate, in 750 μL reduced medium, and incubated at 37°C in 5% CO2 for 1 hour. Stimulator and inhibitor were added to the bottom chamber, and cells were allowed to migrate for 6 hours at 37°C in 5% CO2 toward the stimulator. Migrated cells were incubated with 2 μM calcein in the lower chamber for 30 minutes, and each insert was subsequently washed $\times$5 in Krebs-Ringer buffer prior to measurements of fluorescence at 485/515 nm using a Synergy HT multi-detection microplate reader (BioTek Instruments, Winooski, VT, USA).

Elisa

Cell supernatant from proliferation experiments was stored at 20°C until quantification of chemokines. CXCL10 was quantified using Diaclone Elisa kits from Gene-Probe and CXCL11 using Raybio Elisa kit from Raybiotech (Norcross, GA, USA) according to the manufacturer descriptions.

Statistical Evaluations

Data are presented as means $\pm$ SE. Statistical evaluation of the results was made by two-tailed Student's *t*-test.

Results

IL-27 Receptor Expression and Activation of Cell Signaling

Expression of the IL-27 receptor subunits, gp130 and WSX-1 and fibroblast growth factor receptor 1 (FGFR1) in hTERT-HDLECs and in HMVECs was determined by RT-PCR (Figure 1A). These two cell lines were subsequently used as model systems to study IL-27-stimulated effects on lymphatic endothelial cells. Tyrosine phosphorylation is one of several essential STAT regulatory mechanisms (summarized in [16]). To identify intracellular signaling mediated by IL-27, we assayed the temporal- and concentration-dependent tyrosine phosphorylation of STAT1 and STAT3 in hTERT-HDLEC by immunoblotting. Using IL-27 concentrations between 0 and 100 ng/mL, we observed a marked phosphorylation of STAT1α(Tyr701) and STAT3α(Tyr705) (Figure 1B), and the following time-course experiment showed that phosphorylation of both STAT proteins reached maximal levels after 30–40 minutes incubation with IL-27 (Figure 1C and D). We further analyzed the effect of prolonged IL-27 stimulation on STAT phosphorylation and found that STAT1 and STAT3 phosphorylation was biphasic with a decline in the phosphorylation level after 1 hour followed by an increase from 12 to 24 hours that sustained within the measured time period (Figure 1E and F). These experiments also revealed that IL-27 augments STAT1α and STAT3α expression, measured as the total cellular content of the STAT proteins. In addition, IL-27 also induced expression and concomitant phosphorylation of the STAT1β isoform (Figure E, lower band). We also observed a solid phosphorylation of STAT1α, STAT1β, and STAT3α in the primary lymphatic cell line upon IL-27 stimulation for 25 minutes and 30 hours as well as increases in STAT1α, STAT1β, and STAT3α protein levels (Figure 1G). Thus,

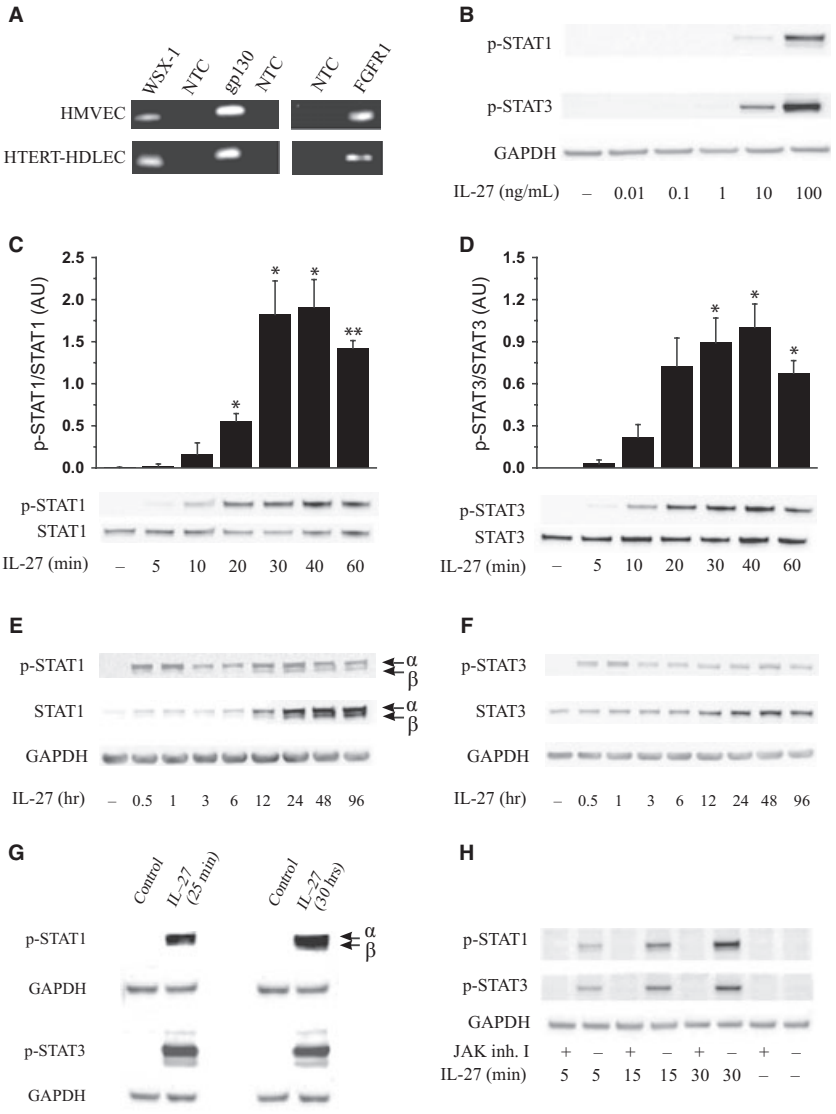

Figure 1. hTERT-HDLEC and HMVEC receptor expression and intracellular signaling. (**A**) Reverse transcription was performed using hTERT-HDLEC and HMVEC total RNA. Receptor expression was analyzed by PCR, and agarose gel electrophoresis, using specific primers for the two IL-27 receptor subunits, gp130 and WSX-1 and for FGFR1. (**B**–**H**) Total protein lysates of IL-27 treated hTERT-HDLEC and HMVEC were analyzed by immunoblotting and probed for p-STAT1 (Tyr701), p-STAT3 (Tyr705), total STAT1, and STAT3 as indicated. GAPDH was detected to verify equal sample loading. (**B**) hTERT-HDLECs were stimulated with increasing concentrations of IL-27 for 10 minutes. Representative of 3 experiments. (**C, D**) hTERT-HDLECs were stimulated with 20 ng/mL IL-27 for increasing time periods up to 60 minutes. The phosphorylation levels of STAT proteins are given by p-STAT/total STAT protein ratio signal in arbitrary units. Each bar represents the average of 3 individual experiments with error bars indicating SE. * $p < 0.05$, ** $p < 0.01$, (**E, F**) hTERT-HDLECs were stimulated with 20 ng/mL IL-27 for increasing time periods up to 96 hours. Data are representative of 3 individual experiments. G: HMVECs were stimulated with 100 ng/mL IL-27 for 25 minutes or 30 hours. H: hTERT-HDLECs were pre-treated with 5 nmol/mL JAK inhibitor I for 30 minutes and then stimulated with 20 ng/mL IL-27 for increasing time periods.

IL-27 induced both phosphorylation of the STAT proteins and augmented their expression levels. This was mediated through members of the JAK family, since we were able to completely abrogate STAT phosphorylation by pre-treating cells with a pan inhibitor of JAK kinases (Figure 1H). In addition, IL-27 stimulated phosphorylation of JAK1 (results not shown).

IL-27 Inhibits LEC Proliferation

Proliferation of endothelial cells is a fundamental requirement for lymphatic vessel formation. To clarify the possible effects of IL-27 on lymphatic endothelial cell proliferation, we tested the effect of IL-27 using the MTT assay. Stimulation of hTERT-HDLECs (Figure 2A) or HMVEC cells (Figure 2B) with FGF-2 significantly enhanced proliferation. IL-27 inhibited

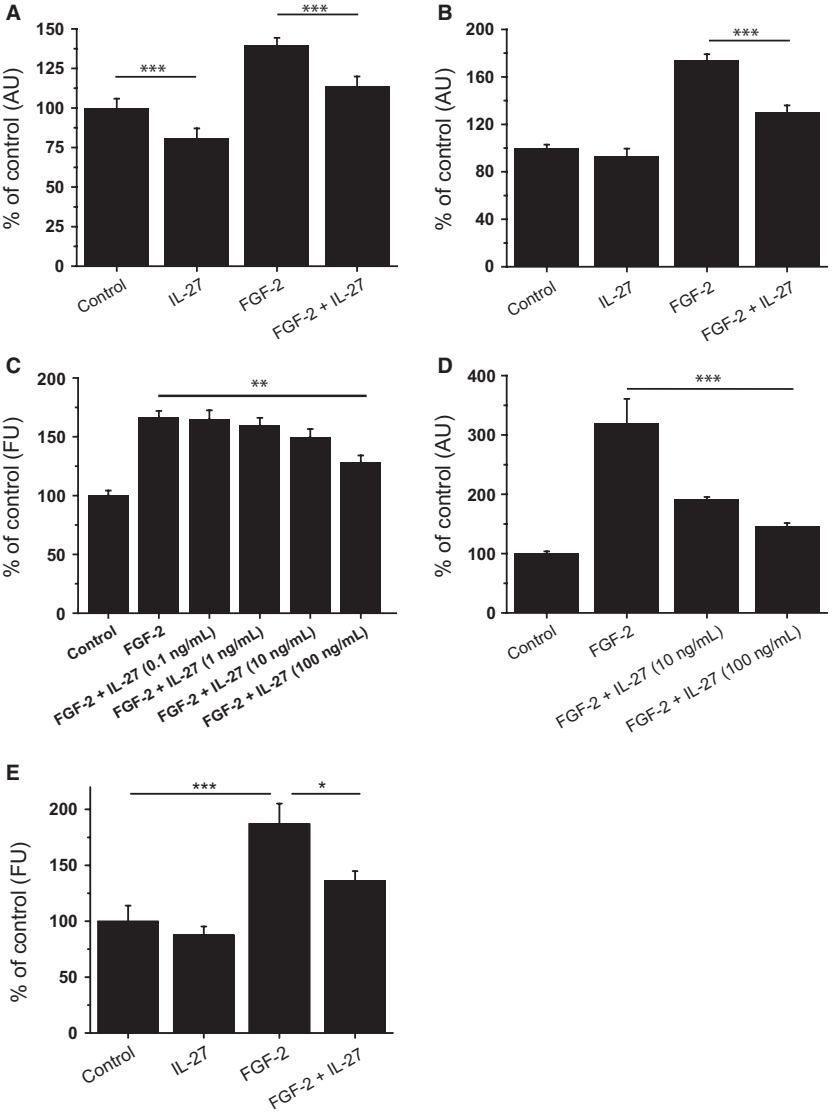

Figure 2. IL-27 inhibits LEC proliferation and migration. (**A, B**) hTERT-HDLEC and HMVEC plated in 96-well plates were stimulated with IL-27, FGF-2, or combinations of these, and proliferation was measured using MTT assay after 6 days. The results are presented as percentage of control, $n = 8$ for each condition. (**A**) hTERT-HDLECs stimulated with 100 ng/mL IL-27 and/or 50 ng/mL FGF-2 (representative of more than 10 experiments). Value of control in absorbance units 1.03 $\pm$ 0.06. (**B**) HMVEC stimulated with 100 ng/mL IL-27 and/or 50 ng/mL FGF-2. Value of control in absorbance units 0.56 $\pm$ 0.02 (**C**) Proliferation measured using a SYBR Green double stranded DNA assay. hTERT-HDLECs plated in 24-well plates were stimulated with various concentration of IL-27, FGF-2 (50 ng/mL) or both for 3 days. Cell proliferation was determined by measuring the fluorescence intensity of DNA-bound SYBR Green and results are presented as percentage of control. Each bar represents the average of 4 wells. (**D**) hTERT-HDLEC were plated in 96-well plates and stimulated with FGF-2 (10 ng/mL) in the presence or absence of IL-27 (10 ng/mL or 100 ng/mL). Cell proliferation rates were determined using an immunoassay for the incorporation of bromodeoxyuridine for 5 hours. BrdU was added 24 hours after stimulation. Value of control in absorbance units 0.17 $\pm$ 0.02. (**E**) Transwell migration. hTERT-HDLECs were stimulated with IL-27 (100 ng/mL) and/or FGF-2 (50 ng/mL) and allowed to migrate for 6 hours. The results are presented as percentage of control. Representative of 2 experiments, $n = 8$ for each condition). Error bars indicate SE, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FGF-2-induced proliferation of both cell lines profoundly, while endothelial cells receiving IL-27 alone tended to proliferate slightly less than control cells. The inhibitory effect of IL-27 on lymphatic endothelial proliferation under control conditions indicates that IL-27 has a general inhibitory effect on lymphatic endothelial cell proliferation rather than inhibiting effects of specific growth factors.

The MTT assay determines cell number indirectly by measuring mitochondria-induced reduction of tetrazolium salt to colored formazan. To verify that IL-27 reduces cell

proliferation using a second assay and to exclude that the effect of IL-27 is simply due to changes in mitochondria number or enzyme activity, we quantified the effect of IL-27 on FGF-2 -stimulated proliferation by measuring SYBR Green staining of cellular double stranded DNA after co-stimulation of hTERT-HDLEC with FGF-2 and various concentrations of IL-27. We observed an IL-27-mediated reduction of FGF-2-induced increase in double stranded DNA, and thus in cell number, thereby confirming our results from the modified MTT assay (Figure 2C). We also tested the effect of IL-27 on the lymphatic cell proliferation rate using a BrdU assay. BrdU incorporation over 5 hours was measured 24 hours after stimulation with FGF-2 in the presence or absence of 10 or 100 ng/mL IL-27. This snapshot view of proliferation clearly showed the anti-mitotic potential of IL-27 (Figure 2D).

Migration of the endothelial cells is another fundamental requirement for lymphatic vessel formation. To further test the possible effects of IL-27 on lymphangiogenic processes, we therefore tested hTERT-HDLEC motility in the presence of IL-27 by use of a transwell migration assay. Stimulation of hTERT-HDLEC with FGF-2 for 6 hours lead to increased migration compared to control. Cells receiving IL-27 migrated to the same extent as cells receiving no treatment; however, FGF-2-induced migration was significantly reduced in the presence of IL-27 (Figure 2E).

IL-27 Stimulates Expression of Anti-Angiogenic Chemokines

To gain insight into potential transcriptional changes underlying the inhibitory effect of IL-27 on cell proliferation, we performed a microarray analysis on hTERT-HDLEC mRNA isolated from cells stimulated for 0.5 and 4 hours. These time intervals were chosen in order to capture the primary as well as a secondary gene expression response, relevant for initiation and maintenance of IL-27 interference with proliferation. In accordance with the increased STAT1 activity (Figure 1), we observed a substantial up-regulation of anti-angiogenic and anti-tumorigenic factors within 4 hours of incubation (data not shown). We focused on two specific chemokines, CXCL10 and CXCL11, both of which were up-regulated in IL-27-treated LECs since activation of their receptor, CXCR3, blocks proliferation of blood endothelial cells, inhibits cell motility and tube formation as well as induces dissociation of newly formed blood vessels [7,8,26,32]. The expression of CXCL10 and CXCL11 by hTERT-HDLEC and HMVEC cells in response to IL-27 was validated by RT-PCR. As shown in Figure 3A and B, the transcriptional level of the chemokines in both hTERT-HDLEC and HMVEC cultures was increased after 4 hours of stimulation with IL-27. Increases in CXCL10 and CXCL11 protein levels secreted into the cell culture medium were analyzed by ELISA. While FGF-2 had no effect on the protein level of CXCL10 and CXCL11, IL-27 stimulation of hTERT-HDLEC resulted in a substantial rise in the concentration of the chemokines in the conditioned medium after 3 days (results not shown) and 6 days (Figure 3C and D).

While CXCL10 and CXCL11 are well-known angiostatic and anti-tumorigenic chemokines [7,8,26,32], the potential inhibitory effect of these chemokines on lymphangiogenesis has not been reported. In order to test whether IL-27 regulates lymphatic endothelial cell proliferation through expression of CXCL10 and CXCL11, we challenged HMVEC —with recombinant CXCL10 and CXCL11 proteins. The results (Figure 3E and F) showed that CXCL10, and to a smaller extent CXCL11, profoundly inhibited FGF-2-induced proliferation. In contrast, no significant decrease in FGF-2-induced migration was observed when treating cells with CXCL10 and CXCL11 (results not shown). The above results indicate a potential for IL-27 to regulate proliferation through an autocrine mechanism by expressing anti-angiogenic chemokines. However, our attempts to reverse the inhibitory effect of IL-27 by reducing synthesis of CXCL10 with siRNA failed, most likely because the anti-mitotic effect of IL-27 is coordinated by several factors, among which CXCL10 is one.

STAT1 Knock Out Reverses the Effect of IL-27 on FGF-2-Mediated Cell Proliferation

Besides regulating CXCL10 and CXCL11 expression, STAT1 is known to mediate cell cycle arrest by directly interacting with G1 cell cycle regulatory proteins [17]. This prompted us to investigate whether STAT1 was responsible for the inhibitory effect of IL-27 on lymphatic cell proliferation. Transfection of hTERT-HDLEC with siRNA targeting STAT1 reduced the protein level of STAT1 by 80% after 48 hours compared to control (Figure 4A). In addition, IL-27-induced CXCL10 and CXCL11 synthesis was also reduced by more than 80% (Figure 4B and C), thereby validating the effect of siRNA treatment on STAT1 activity as well as confirming CXCL10 and CXCL11 as STAT1 expression targets. Reduction of STAT1 protein almost completely reversed the inhibitory effect of IL-27 on FGF-2-mediated cell proliferation, revealing STAT1 activity as essential for the anti-mitotic effect of IL-27 (Figure 4D).

Discussion

The results of this study provide evidence that IL-27 inhibits proliferation of lymphatic endothelial cell lines through STAT1 signaling. As IL-27 interferes with an essential lymphangiogenic process, we hypothesize that IL-27 could be a critical participant in pathological lymphangiogenesis.

IL-27 induced the transcription of a defined set of genes in lymphatic endothelial cells. This process was dependent on STAT1 (Figure 3A and D and data not shown), and thus resembles the expression profile induced by IL-27 in blood

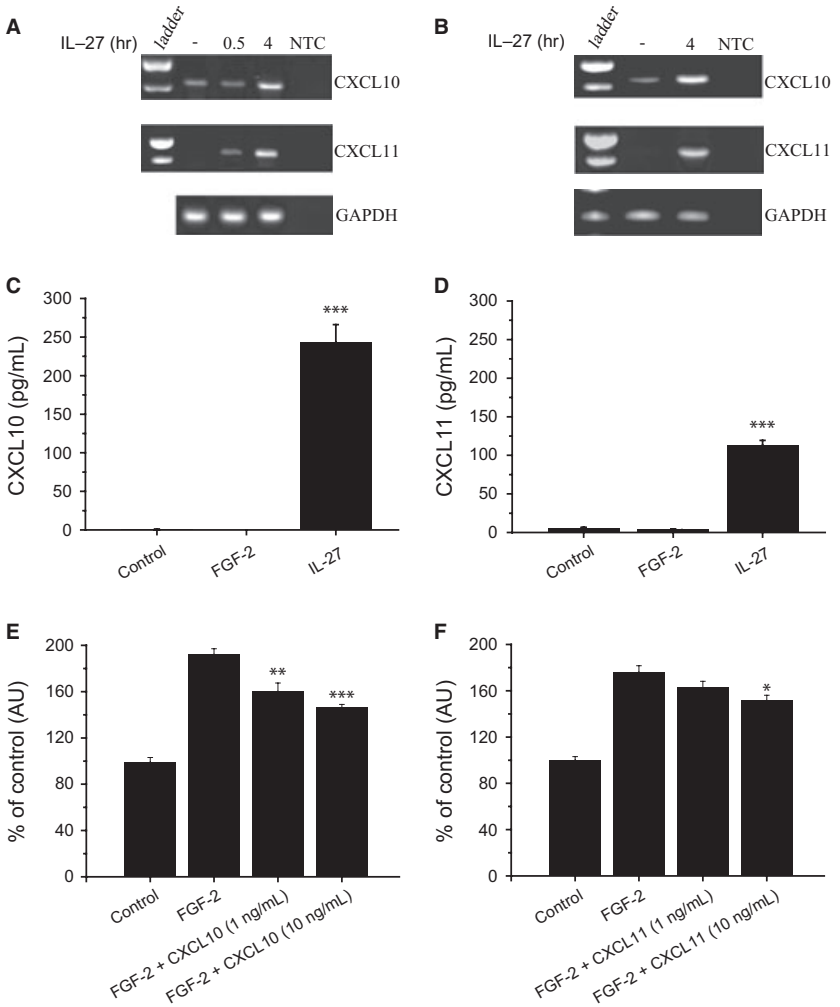

Figure 3. IL-27 inhibits LEC proliferation by expressing anti-angiogenic chemokines. (**A, B**) Total RNA was isolated from hTERT-HDLECs and HMVEC cells after IL-27 stimulation for 30 minutes and 4 hours and RT-PCR and agarose gel electrophoresis were performed to analyze expression levels of CXCL10 and CXCL11 in hTERT-HDLEC (**A**) and HMVEC (**B**). GAPDH expression verifies equal mRNA quantities. (**C, D**): CXCL10 and CXCL11 protein levels in the cell supernatant of HTERT-HDLEC treated with IL-27 (100 ng/mL) or FGF-2 for 6 days were measured by ELISA. Each bar represents the average of 4 wells with error bars indicating SE. (**E, F**): Cells plated in 96-well plates were stimulated with FGF-2 (10 ng/mL) in the presence or absence of human recombinant CXCL10 or CXCL11 (1 and 10 ng/mL) for 24 hrs. Cell proliferation rates were determined by using an immunoassay for the incorporation of bromodeoxyuridine for 5 hours. BrdU was added 24 hours after stimulation. Each bar represents the average of 12 wells with error bars indicating SE. Data are representative of 2 experiments. Value of control in absorbance units 0.34 $\pm$ 0.01 (**E**) and 0.35 $\pm$ 0.01 (**F**),* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

vascular endothelial cells [18,34]. Consistent with this, prolonged treatment of hTERT-HDLEC with IL-27 for more than 12 hours resulted in increased protein levels of both STAT1α and STAT1β, while expression of the STAT3 was less profound. Several studies have shown that transient STAT1 activity can lead to a large increase in STAT1 concentration that persists for days and regulates expression of a subset of genes [11, 12]. Thus, enhanced levels of STAT1α and STAT1β after long-lasting stimulation with IL-27 could prolong and direct STAT1-regulated activities toward an anti-proliferative phenotype at the expense of STAT3 signaling.

It is also possible that up-regulation of STAT1 expression is necessary to avoid sequestering of STAT1 by heterodimerization. In myeloid cells, over-expression of STAT3 favors the formation of STAT1/STAT3 heterodimers following IFNα stimulation, thereby preventing STAT1 homodimers [23]. Interestingly, tyrosine phosphorylation and translocation of STAT1 was not inhibited by over-expression of STAT3, but STAT1-regulated gene expression was attenuated and the IFNα response was shifted from a pro-inflammatory response to an anti-inflammatory response. Thus, the ratio between STAT1 and STAT3 protein might

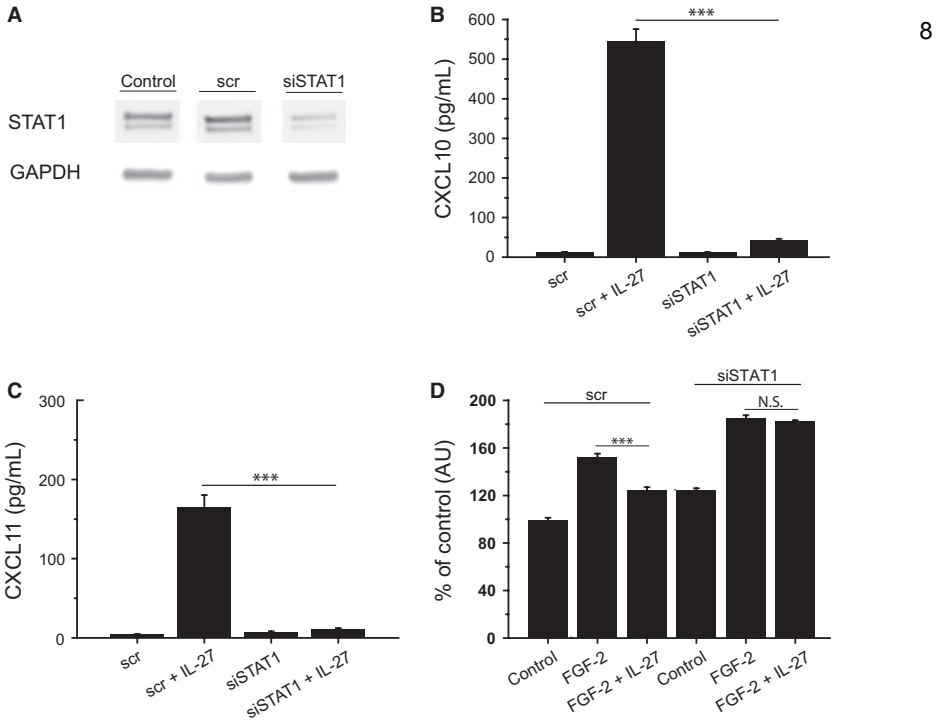

Figure 4. IL-27-regulated proliferation is STAT1 dependent. (**A**) hTERT-HDLEC were transfected with siRNA targeting STAT1 (siSTAT1) or scrambled siRNA (scr) and cultured for 48 hours. Lysates were analyzed using immunoblotting and probing for total STAT1. GAPDH was detected to verify equal sample loading. Data are representative of 3 experiments. B-C: CXCL10 (**B**) and CXCL11 (**C**) protein levels in the cell supernatant of siSTAT1 and scr transfected hTERT-HDLEC after stimulation with IL-27 (100 ng/mL) for 24 hours were measured by ELISA. Each bar represents the average of 6 wells with error bars indicating SE (representative of 3 experiments). (**C**) Proliferation measured by use of MTT assay. hTERT-HDLECs plated in 96-well plates and cultured for 48 hours after transfection with siSTAT1 or scr were stimulated with IL-27 (100 ng/mL), FGF-2 (10 ng/mL) or both for 3 days. Absorbance is shown in arbitrary units with control absorbance normalized to 100. Value of control in absorbance units 0.54 $\pm$ 0.04. Each bar represents average of 16 wells with error bars indicating SE (representative of 2 experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

influence the response to IL-27 in lymphatic endothelial cells. Clearly, further studies are needed to clarify this extra layer of complexity in STAT signaling.

SOCS3, a feedback inhibitor of cytokine signaling which has previously been shown to control the activation of IFN-induced gene expression mediated by the gp130 receptor and STAT3 [6,15,25], was strongly expressed in lymphatic endothelial cells stimulated with IL-27 (unpublished results). This is further consistent with STAT1 playing a dominant role in IL-27-regulated lymphatic endothelial cell biology.

IL-27 stimulation of hTERT-HDLEC and HMVEC strongly up-regulated the anti-angiogenic chemokines CXCL10 and CXCL11 (Figure 3A-D). These chemokines are both ligands for the CXCR3 receptor [26] and has previously been shown to block proliferation and migration of blood endothelial cells in addition to interfering with formation of blood vessels [7,8,26,32]. In addition, CXCL10 plays a role in the anti-tumor effect of IL-27 *in vivo* since neutralizing antibodies against CXCL10 partially abrogated the effect of IL-27 on melanoma tumor growth [34]. Consistent with the observed inhibitory effect on blood vessel formation, we show that CXCL10 and CXCL11 significantly reduced lymphatic endothelial cell proliferation, indicating that CXCL10 and CXCL11 can regulate lymphatic endothelial cell proliferation through autocrine signaling. The use of siRNA revealed STAT1 as an upstream regulator of CXCL10 and CXCL11 expression and as a key transcription factor in IL-27-regulated lymphatic endothelial cell proliferation (Figure 4).

IL-27 has been associated with several inflammatory disorders including psoriasis, eczematous skin lesions, periapical lesions, and osteoclastogenesis [14,19,33,38]. Our study indicates that lymphatic endothelial cells could actively participate in such inflammatory diseases. As we have shown that IL-27 significantly induces expression of CXCL10, it is interesting to note that patients with autoimmune diseases such as psoriatic arthritis and rheumatoid arthritis have elevated levels of serum or synovial fluid CXCL10 and that this high value is linked to an increased activation of Th1 cells [4,21]. This is in line with our results showing that IL-27 induces a transcription profile in lymphatic endothelial cells resembling that of IFN-γ, which is a classical Th1 cytokine.

Thus, IL-27 seems to promote transcription of genes supporting a Th1 response in lymphatic endothelial cells, and this effect could play a role in the initial pathogenesis of different inflammatory diseases.

This study identifies IL-27 as an anti-lymphangiogenic regulator which interferes with the mitotic effect of growth factors through STAT1 activity and up-regulation of CXCL10 and CXCL11. Conclusively, in inflamed tissue where cells are exposed to a complex milieu of cytokines, chemokines and growth factors, IL-27 might modulate the formation of new lymphatic vessels.

Perspectives

Lymphatic endothelial cells are challenged by a complex microenvironment of cytokines, chemokines, and growth factors at sites of chronic inflammation, but how these factors in concert influence the lymphatic system is poorly understood. This study provides evidence that IL-27-regulated chemokines have the capacity of inhibiting growth factor-induced proliferation. Elucidating the connection between lymphatic endothelial cell biology and the immune system is essential for understanding pathological processes associated with inflammation and cancer development.

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