

Angiostatic activity of the anti-tumor cytokine interleukin-21

Karolien Castermans¹, Sebastien P. Tabruyn¹, Rong Zeng², Judy R. van Beijnum, Cheryl Eppolito³, Warren J. Leonard², Protul A. Shrikant³, Arjan W. Griffioen¹

¹*Angiogenesis Laboratory, School for Oncology and Developmental Biology (GROW), Depts. of Pathology and Internal Medicine, Maastricht University and University Hospital, Maastricht, The Netherlands.*

²*Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, National Institute of Health, Bethesda, MD 20892-1674*

³*Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA*

Running title: Anti-angiogenesis activity of IL-21

Key words: Angiogenesis, anti-angiogenesis, endothelial cells, immunotherapy, IL-2, IL-21, signal and activator of transcription (Stat), Stat3.

Scientific category: Thrombosis and vascular biology

Abstract: 198 words

Text: 4043

References: 50

Correspondence to:

Dr. Arjan W. Griffioen, Angiogenesis Laboratory, Dept. of Pathology, University Hospital Maastricht, P.O.Box 5800, 6202AZ Maastricht, Phone +31-43-3876543, fax +31-43-3876613, Email: aw.griffioen@path.unimaas.nl

Abstract

Interleukin (IL)-21 is a recently described immunoregulatory cytokine. It has been identified as a very potent immunotherapeutic agent in several cancer types in animal models and clinical studies are ongoing. IL-21 belongs to the type-I cytokine family of which other members, i.e. IL-2, IL-15 and IL-4, have been shown to exert activities on vascular endothelial cells (EC). We hypothesized that IL-21, in addition to inducing the anti-tumor immune response, also inhibits tumor angiogenesis. *In vitro* experiments showed a decrease of proliferation and sprouting of activated EC after IL-21 treatment. We found that the IL-21 receptor is expressed on vascular EC. Furthermore, *in vivo* studies in the chorioallantoic membrane of the chick embryo and in mouse tumors demonstrated that IL-21 treatment disturbs vessel architecture and negatively affects vessel outgrowth. Our results also confirm the earlier suggested angiostatic potential of IL-2 *in vitro* and *in vivo*. The angiostatic effect of IL-21 is confirmed by the decrease in expression of angiogenesis related genes. Interestingly, IL-21 treatment of EC leads to a decrease of Stat3 phosphorylation. Our research shows that IL-21 is a very powerful anti-tumor compound which combines the induction of an effective anti-tumor immune response with inhibition of tumor angiogenesis.

Introduction

Interleukin (IL)-21, the most recently described member of the common gamma-chain cytokine family, is found to be a potent immunoregulatory cytokine¹. This cytokine is produced by activated CD4⁺ T cells and targets several immune cells²⁻⁵ including NK cells^{3,4,6-8}, dendritic cells, CTLs^{3,4,9} as well as B cells¹⁰⁻¹³. Structurally, IL-21 is considered to be a member of the type-I-cytokine family, which includes IL-2, IL-4 and IL-15^{2,14-16}. The receptors for all these type I cytokines contain the common gamma-chain^{2,14-16}. In leukocytes, type-I cytokines activate the Janus family tyrosine kinases, JAK1 and JAK3², which in turn regulate activation of signal transducer and activator of transcription 1 (Stat1), Stat3 and, Stat5a and Stat5b^{2,17-19}. IL-2 and IL-15 mainly activate STAT5a and STAT5b, whereas IL-4 primarily activates Stat6². IL-21 is demonstrated to activate Stat1, Stat3, Stat5a and Stat5b. The activation of Stat5a and Stat5b by IL-21 is weak and transient whereas the activation of Stat3 is the most sustained. Stat3 appears to be the most important STAT protein for IL-21 signaling²⁰. This activation of Stats is cell-type specific. Interestingly, in endothelial cells, Stat3 phosphorylation has been associated with induction of angiogenesis^{21,22}. Recent studies suggest that IL-21 can be used as an immunomodulatory compound^{23,24}, and its use in the clinical setting is underway²⁵.

The process of new blood vessel formation or angiogenesis is essential for tumor growth and metastasis, as it is critical for supply of oxygen and nutrients. Inhibition of angiogenesis is therefore a promising strategy for treatment of cancer^{26,27}. Several anti-angiogenesis compounds have been developed and tested based on their ability to inhibit pro-angiogenic growth factors such as VEGF [SU6668, SU11248 and bevacizumab²⁸⁻³⁰] or intervention in signal transduction in endothelial cells directly [TNP-470, endostatin and anginex³¹⁻³⁴]. Members of the type-I-cytokine family, i.e. IL-2, -4, are suggested to affect the formation of new blood vessels³⁵⁻³⁷, but their ability to block angiogenesis has not been carefully tested. In analogy to IL-2 and IL-4, we hypothesized that IL-21 inhibits angiogenesis and thus mediates anti-tumor efficacy partly due to its effects on endothelial growth and sprouting. These properties along with its ability to regulate various immune cells endow IL-21 with the potent ability to promote tumor regression.

To test this notion, we conducted experiments to demonstrate the effects of IL-21 on endothelial cells and compared it to the effects of IL-2. Our results demonstrate that IL-21 mediates angiostatic properties, as determined by *in vitro* and *in vivo* assays. This contributes to anti-tumor activity of IL-21 *in vivo*. These results suggest that IL-21 is a very powerful

anti-tumor compound, having the ability to induce an effective anti-tumor immune response and simultaneously inhibit tumor angiogenesis. This is the first demonstration of the anti-angiogenic property of IL-21, which may have considerable implications for the use of IL-21 in future clinical trials.

Material and Methods

Cell culture and reagents

The murine EC cell line SVEC4-10 and TME were purchased from ATCC and cultured in DMEM (Gibco Invitrogen, Breda, The Netherlands) supplemented with 10% FCS (Hyclone Perbio Science, Erembodegem-Aalst, Belgium), 2mM HEPES and 2mM L-glutamine (Gibco) in 0.2% gelatine (Sigma, Zwijndrecht, the Netherlands) coated tissue culture flasks (Costar Corp., Cambridge, MA). The cells were subcultured 1:10. Human umbilical vein derived endothelial cells (HUVEC) were cultured as described previously³¹. Angiogenic stimulation of the ECs was induced by 10ng/ml bFGF and/or 40ng/ml VEGF (Peprotech, London, United Kingdom). Primary mouse endothelial cells were isolated as described earlier³⁸. Normal heart tissues derived from C57Bl/6 mice were mechanically and enzymatically digested. The obtained single cell suspensions were allowed to adhere to gelatin coated tissue culture flasks (Costar Corp.). The remaining adherent cell population was stained for the CD31 marker and separated from other cells by sorting (FACS Aria; BD Biosciences, Alphen a/d Rijn, The Netherlands). Cells were cultured in DMEM containing 20% FCS.

Primary human tumor and normal endothelial cells were isolated from fresh tissues as described previously³⁹. Briefly, colorectal carcinoma tissues and distant normal colon tissue of the same patients were obtained directly after surgery, minced and digested to create a single-cell suspension. ECs were immunolabeled with a combination of anti-CD31 (clones JC/70A, DAKO, Carpinteria, CA, and EN4, Monosan, Sanbio, Uden, The Netherlands) and anti-CD34 (clone Qbend10, Novocastra, New Castle, United Kingdom) antibodies, subsequently captured on goat anti-mouse IgG-coated paramagnetic beads (Dyna, Oslo, Norway), and used directly for RNA isolation.

EG7 thymoma cells transfected with OVA were cultured in RPMI (Gibco) supplemented with 10% FCS (Perbio Science), 25mM Hepes buffer, 2mM L-glutamine, 100U/ml Penicillin, 100µg/ml Streptomycin, 1mM sodium pyruvate, 1x non-essential amino acids and 50µM 2-mercaptoethanol (all Gibco). Cells were maintained with addition of 40µg/ml G418. Before injection in mice, G418 concentration was increased to 400µg/ml for two rounds of expansion.

Recombinant mouse IL-21 protein was obtained as previously described⁴⁰. Recombinant mouse IL-2 was obtained from R&D systems (Minneapolis, MN). Anginex was

kindly provided by Dr. K. Mayo (Department of Biochemistry, Minnesota, Minneapolis, MN).

In vitro and ex vivo sprouting assay

In vitro sprouting and tube formation was studied using Cytodex-3 beads overgrown with SVEC4-10 endothelial cells in a three-dimensional collagen gel (PureCol, INAMED biomaterials, Fremont, CA) containing 10 ng/ml bFGF as described previously⁴¹. Sprout formation was stimulated with medium containing 10 ng/ml bFGF and 40ng/ml VEGF. Recombinant IL-21, IL-2 or anginex were added in the medium on top of the gel. After 72 hours the length of the sprouts was analyzed.

Ex vivo sprouting was investigated by using rings of the mouse thoracic aorta as was described earlier³⁴. 1-mm-thick cross-sectional rings were placed in the wells of a 96-well tissue culture plate in the previous described collagen gel. Medium (DMEM, 20% FCS, 2 mM L-glutamine, 2 mM sodiumpyruvate, 20 mM HEPES, 1% nonessential amino acids, 1.5% MEM vitamins, antibiotics, 1 IU/100 ml heparin (Gibco Invitrogen, Breda, The Netherlands) containing recombinant IL-21, IL-2 or anginex was put on top of the gels. Vascular sprouting from each ring was examined using an inverted microscope. The width of the tube formation area was measured at three different predefined places of the aortic ring. Different culture conditions were tested at least in triplicate.

Flow cytometry

Endothelial cells were fixed with 1% PFA for 30min at room temperature. IL-21 receptor α (IL21-R α) expression was detected biotinylated mAb to IL-21R α ¹⁰. Subsequently the cell suspensions were incubated with phycoerithrine labeled strepdavidine (DAKO, Glostrup, Denmark). Stained cells were analyzed on a FACSScan flow cytometer using Cell Quest software (Becton Dickinson, Sunnyvale, CA).

Proliferation and apoptosis measurement

Endothelial cell proliferation was measured using a [³H]thymidine (Amersham Life Science, Roosendaal, The Netherlands) incorporation assay as described previously⁴¹. Angiogenically stimulated SVEC4-10 were exposed for 3 days to recombinant mouse IL-21, IL-2 or anginex before proliferation was assessed. At least three independent experiments were performed. In each experiment measurements were done in triplicate.

Apoptosis was measured by propidium iodide (PI) (Brunschwig Chemie, Amsterdam, The Netherlands) staining as described previously⁴¹. bFGF (10 ng/ml) stimulated cells were cultured for 3 days in the presence or absence of IL-21, IL-2 or anginex. After this period, the cells were harvested, fixed in 70% ethanol, centrifuged, resuspended in DNA extraction buffer and incubated for 20' at 37°C. After incubation, PI was added at a final concentration of 20 µg/ml, and the DNA profile was directly analyzed with the FACScalibur.

Migration measurement

Migration of murine endothelial cells was measured using the wound assay³⁴. In brief, SVEC4-10 cells were seeded in gelatin-coated cell culture plates (Costar Corp., Cambridge, MA) and grown to confluence. Using a blunt pipette tip, a cross-shaped wound was made in the well. Cells were washed with PBS and fresh medium containing 10 ng/ml bFGF with or without IL-21, IL-2 or anginex. Wound width was measured at four predefined locations at start and at 2, 4, 6, 8, and 24 hours after wounding.

Chorioallantoic membrane assay (CAM)

The chorioallantoic membrane (CAM) assay was performed in fertilized White Leghorn eggs as described previously⁴¹. In brief, CAMs were treated by daily addition of sterile PBS, recombinant mouse IL-21 or IL-2 from days 10 to 13. On day 14, the CAMs were photographed. Quantification of vascularization was done by enumeration of intersections with five concentric rings that were superimposed on the photographs.

Mouse tumor model

C57Bl/6 mice were injected subcutaneously at the base of the spine with 5×10^6 mouse EG7 tumor cells on day 0. On day 10, 12, 14, 16 and 18 after tumor inoculation, mice were treated with intraperitoneal injections of either PBS, 2000 I.U. IL-2 or 20 µg IL-21 in a volume of 500 µL. At day 20, tumors were excised and snap frozen in liquid nitrogen.

Immunohistochemistry

Immunohistochemical staining was performed on frozen sections (5 µm), fixed in acetone on ice and air dried. Immersing the slides in 0.3% hydrogen peroxidase in PBS for 30min blocked endogenous peroxidase. After washing three times with PBS, aspecific binding was blocked by PBS containing 20% FCS and 0.1% Tween 20 for 15 min. The sections were incubated with rat anti mouse CD31 (Pharmingen BD Company, Woerden, The

Netherlands) primary antibody for 1hour, followed by incubation with peroxidase labeled goat anti-rat IgG antibody (Immunotech, Marseille, France). The peroxidase activity was detected using diaminobenzidine (DAB; Sigma, Zwijndrecht, The Netherlands).

Quantitative real time RT-PCR (qRT-PCR)

Total RNA isolation from cultured cells, cDNA synthesis and quantitative real-time RT-PCR were performed essentially as described previously⁴² using iQ SYBR Green PCR Supermix (Bio-Rad, Veenendaal, The Netherlands). The expression of each target gene was normalized to the expression of the control gene cyclophilin A by calculation of dCt values (Ct target – Ct reference gene). Primer sequences are shown in supplementary table 1.

Western Blot analysis

10 ng/ml bFGF stimulated SVEC4-10 cells or primary mouse endothelial cells were incubated with IL-2, IL-21 or anginex for 5 or 30 minutes. Cells were harvested and permeabilized in NP40 lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% NP40, 1 mM Na₃VO₄, 5 mM NaF, 1 mM AEBSF, 0.8 mM Aprotinin, 21 mM Leupeptin, 36 mM Bestatin, 15 mM Pepstatin A, and 14 mM E-64). Whole cell lysates (about 10 ug/sample) were fractionated on 4-12% polyacrylamide gels (Invitrogen), western blotted. Blots were probed with antibodies to phosphorylated Stat1 (Y701), Stat3 (Y705), Stat5 (Y694 for Stat5a and Y699 for Stat5b)(Cell Signalling), and then re-probed with antibodies to Stat1, Stat3, Stat5a, Stat5b (Santa Cruz).

Statistic analysis

All data are expressed with standard error of the mean (SEM) and were statistically analyzed using Mann-Whitney U tests (using SPSS-10 software). Probabilities below 0.05 were considered statistically significant.

Results

IL-21 inhibits sprout formation by EC

The sprouting of primary aortic EC into a 3-dimensional semi-natural collagen matrix was inhibited by approximately 60% in the presence of IL-21 (200 ng/ml, Figure 1A,B). In the present study we compared the effects of IL-21 with those of IL-2, a member of the same cytokine family and previously suggested to inhibit blood vessel formation. Interestingly, treatment with recombinant mouse IL-2 in this assay, did not lead to significantly inhibited sprout formation by mouse aortic EC (Figure 1B).

A similar *in vitro* assay using the SVEC4-10 cell line was used to demonstrate a direct effect on EC. This murine cell line is an excellent tool for angiogenesis research since it is inducible by angiogenic growth factors³⁴. Addition of IL-21 inhibited bFGF-induced sprouting by 20-30% (Figure 1C, D). Similar as in the aortic ring assay, IL-2 did not inhibit the sprouting of SVEC cells. The positive control anginex reduced the sprout formation by approximately 50% (Figure 1C).

The IL-21 receptor is expressed by EC

Flow cytometry identified the IL-21 receptor to be expressed by SVEC. Similar results were found for primary human endothelial cells (HUVEC, Figure 2A and 2B). These data were confirmed for various endothelial cell lines at the transcriptional level by quantitative real-time RT-PCR (qRT-PCR). The expression level of the IL-21 receptor was low in the endothelial cells compared to the positive control spleen (average dCt values of 17 for EC samples vs 10 for spleen) but within the boundaries of our assay. Analysis of the PCR products on agarose gel showed unambiguously the presence of the IL-21 receptor α -chain in primary human and mouse endothelial cells and endothelial cell lines (Figure 2C). In addition, we tested the mRNA expression of the IL-21 receptor on freshly isolated human tumor endothelial cells and normal cells³⁹. Although expression was low (average dCt=11), a clear expression of IL-21 receptor could be detected when we analyzed the qRT-PCR on agarose gel (Figure 2C).

The expression of the IL-21 receptor α -chain on endothelial cells suggests that the inhibitory effects by IL-21 on sprout formation as described above occur via a direct effect on the endothelial cells. Similarly, the presence of the IL-21 receptor on human tumor endothelial cells suggests that IL-21 has a direct effect on endothelial cells in tumors.

Analysis of the IL-2 receptor α -chain by qRT-PCR demonstrated that this receptor is also present on various types of endothelial cells (Figure 2D). These data together demonstrate that the tested endothelial cells can directly respond to IL-21 and IL-2 treatment via their specific receptor on the cell membrane.

IL-21 and IL-2 inhibit growth factor induced proliferation but not migration of endothelial cells

To further investigate the mechanism by which IL-21 caused inhibition of EC sprouting and thus angiogenesis *in vitro*, the anti-proliferative activity of IL-21 was tested in a ³H-thymidine incorporation assay using SVEC4-10 cells. Cells were stimulated with bFGF and cultured for three days in the presence of IL-21, IL-2 or anginex. A slight but significant reduction of EC proliferation by approximately 20% was observed after treatment with IL-21. Comparable results were found for IL-2 (Figure 3A). The inhibition of proliferation by IL-21 and IL-2 was not associated with induction of cell death, as flow cytometric analysis of DNA fragmentation showed, in contrast to the positive control anginex³⁴, no increase of apoptotic or necrotic cells after IL-21 or IL-2 treatment (data not shown).

Next to proliferation, migration of endothelial cells is a key process in the formation of new blood vessels during angiogenesis. EC migration was investigated using the wound assay. While anginex inhibited the wound closure efficiently, IL-21 as well as IL-2 did not have an effect. It is concluded that IL-21 does not affect migration of EC (Figure 3B).

IL-21 and IL-2 affect in vivo angiogenesis in the chick embryo chorioallantoic membrane (CAM) assay

In vivo angiogenesis was first investigated in the chorioallantoic membrane (CAM) of the chicken embryo, an assay that represents developmental angiogenesis. Although treatment of the CAMs with IL-21 did not reduce vessel density (Figure 4B), a disturbed, tortuous and irregular growth of vessels was detected as compared to control CAMs (Figure 4A). Recombinant IL-2 showed a similarly disturbed vessel outgrowth. In addition, IL-2 did reduce vessel density in the CAMs by 25% (Figure 4A, B). This response to IL-2 confirms results presented in earlier reports³⁶.

IL-21 inhibits tumor angiogenesis in vivo

Effects of IL-21 and IL-2 on tumor angiogenesis *in vivo* were determined by treatment of EG7 tumor bearing mice with IL-21, IL-2 or PBS (control) at concentrations that have previously been shown to be effective (Figure 4C) ⁴⁰. Immunohistochemical staining of tumor sections of mice treated with recombinant mouse IL-21 using CD31 antibody showed a significantly decreased microvessel density, as compared to PBS treated mice (Figure 4C, D). Treatment of mice with IL-2 showed a decreased vessel formation in tumors as well, but this inhibition was not found to be statistically significant (Figure 4C, D).

IL-21 and IL-2 regulate expression of angiogenesis-related genes in activated EC

In order to investigate the feature of the angiostatic response of EC after exposure to IL-21 and IL-2, we performed qRT-PCR on samples of *in vitro* cultured endothelial cells and the above presented mouse tumor tissues. Absolute Ct-values can be found in the supplemental table 2. In cultured endothelial cells, the major angiogenesis related growth factor vascular endothelial growth factor A (VEGFA) and both of its receptors VEGFR1 and -2 are significantly decreased by exposure to IL-21, as compared to bFGF treatment alone (Figure 5A). The IL-21 induced downregulation of VEGF-A and VEGFR2 is shared with the angiogenesis inhibitor anginex (Figure 5A). The expression of angiopoietins, mediators of vessel sprouting, maturation and remodeling, are also affected by IL-21 treatment *in vitro*. Angiopoietin-1 and -3 (ANG1, -3) show a lower expression after IL-21 treatment (Figure 5B), while neither IL-2 nor anginex treatment show this effect in ECs. The expression of the tyrosine kinase receptor-1 (TIE1), one of both receptors of the angiopoietin family, is diminished after IL-21 treatment, an effect that is shared with IL2 and anginex in endothelial cells (Figure 5C). These results support the observation of anti-angiogenesis activity by IL-21 and IL-2. In tumor tissues of mice treated with IL-21 we also observed a significant decrease of VEGFA and both receptors VEGFR1, and -2, as compared to PBS treatment (Figure 5D). The mRNA expression of angiopoietins is also reduced upon IL-21 treatment (Figure 5E), though only significantly for angiopoietin-2. A significant decline of approximately 75% is determined for TIE1 and TIE2 expression in tumor tissues of IL-21 treated mice (Figure 5F). In experiments treatment with IL-2 did never induce significant changes of expression.

Regulation of Stat phosphorylation in EC after IL-21 treatment

In leukocytes, IL-21 signals through the IL21-R and the JAK/Stat pathway. JAK1 and/or JAK3 can activate Stat1, Stat3 and to a lesser extent Stat5 by phosphorylation after

induction by IL-21. In order to investigate if similar signaling can occur in endothelial cells we performed western blot analysis on protein lysates of SVEC4-10 (Figure 6A) and primary mouse EC (Figure 6B).

Western blot analysis of bFGF stimulated SVEC4-10 showed presence of activated (phosphorylated) Stat3 (P-Stat3) and a slight activation of Stat1 (P-Stat1, Figure 6A lane 1). Interestingly, incubation of SVEC4-10 for 30 minutes with IL-21 induced a dramatic and significant decrease in the expression of phosphorylated Stat3, a feature shared with the angiogenesis inhibitor anginex. Similar effects were observed for IL-2. This regulation was not visible after 5 minutes of exposure to either IL-21, IL-2 or anginex.

Primary mouse endothelial cells showed expression of P-Stat3 after bFGF stimulation, as well (Figure 6B, lane 1). Also in these primary endothelial cells, phosphorylation of Stat3 was diminished after 30 minutes of exposure to the compounds (Figure 6B, lane 2-6). No differences could be detected for the other Stat proteins tested. Since Stat3 activation has been implicated in angiogenesis before ^{21,22}, the diminishing effects of IL-21 and IL-2 on Stat3 phosphorylation reinforces the angiostatic activities of both IL-21 and IL-2. In addition, it implicates the role of diminished STAT3 activation in the observed anti-angiogenesis effects.

Discussion

IL-21 is a pleiotropic cytokine with immune-regulatory properties and immune-therapeutic capacity. In this study we have identified a novel function for IL-21. We demonstrate that the IL-21 receptor is present on normal and tumor endothelial cells and can mediate angiostatic properties by several *in vitro* assays. While IL-21 moderately inhibits the proliferation of ECs, it markedly inhibited sprouting of angiogenically stimulated endothelial cells. In addition, it also reduces vessel formation and disturbs vessel architecture *in vivo*. IL-21 was found to reduce the expression of VEGFR2 and TIE1 in endothelial cells, contributing to the lower angiogenic profile. Also, exposure of endothelial cells to IL-21 led to a decrease in expression of VEGF-A, angiopoietin-1 and -3. Similar results on gene expression were found when testing samples of intact tumor tissue, indicating that the effects are shared by other cells, including the tumor cells, as well. The results of the current report are in line with an earlier report on disruption of the vascular network as found in tumors of mice treated with IL-21⁴³. Furthermore, the decrease of Stat3 phosphorylation in activated endothelial cells after treatment with IL-21, an effect that is shared with the angiogenesis inhibitor anginex, suggests that angiostatic signaling by IL-21 in EC is mediated through Stat3.

The current report also confirms the earlier described angiostatic power of IL-2. This type-I cytokine reduced proliferation of angiogenically stimulated endothelial cells *in vitro* as well as vessel formation *in vivo*. In contrast to IL-21, IL-2 did not reduce sprouting neither of aortic rings nor of endothelial cells. This difference, and the fact that IL-21 seems to be a more powerful angiogenesis inhibitor than IL-2, could be explained by differential expression of ANG1, ANG3 and TIE1 in endothelial cells induced by IL-21 and IL-2 treatment. Angiopoietins and their receptors are essential for vessel sprouting, remodeling and maturation⁴⁴. A decrease of their expression might interfere with these processes during angiogenesis. This is also in line with our observations in the CAM assay that showed a disturbed and tortuous character of the vessels after IL-21 treatment. Interestingly, IL-21 also decreases the expression of VEGFR1 in EC. This is also consistent with the observed inhibition of sprouting and disturbed vessel morphology because a defect in VEGFR1 can lead to the assembly of endothelial cells into abnormal vascular channels⁴⁵. However we could detect irregular vessel morphology in the CAMs after IL-2 treatment as well, indicating that other factors, e.g. nitric oxide³⁶, might be involved in the disturbed vessel outgrowth in CAMs as well. These data suggest that IL-2 is likely to be involved in survival rather than in differentiation mechanisms, which IL-21 seems to be. The regulation of VEGFA, VEGFR1 and -2 gene expression in tumor tissues is similar for both IL-21 and IL-2, although the effects

of IL-21 are stronger. In addition to these VEGF related genes, IL-21 decreases the expression of the angiopoietin family as well. This differential regulation of gene expression can explain the predominant effects of IL-21 over IL-2, on tumor angiogenesis. Since IL-2 and IL-21 both affect hematopoietic cells differently^{2,40}, these cells, when present e.g. in the ex vivo sprouting assay and the tumor growth experiment, could mediate the effects of the cytokines on the endothelium. The clear effects in the cultured EC experiments, favors the view of different effects directly on the endothelium.

Here we show that, γ_c -dependent cytokines can have different effects on endothelium. For IL-15 it has been shown that it can induce angiogenesis^{46,47}. Our results demonstrate that both IL-2 and IL-21 can inhibit angiogenesis but in a different way.

As mentioned above, IL-21 can have different effects on leukocytes and thereby affect their pro-angiogenic function. This can have implications on tumor angiogenesis since IL-21 might reduce the pro-angiogenic factors secreted by leukocytes. However, the observed expression of the IL-21 receptor on tumor endothelial cells supports the idea that microvessel density is decreased by a direct negative effect of IL-21 on tumor endothelial cells.

In leukocytes IL-21 signaling has been shown to occur via the Janus family tyrosine kinases, JAK1 and JAK3. These kinases then mediate activation of Stat1, Stat3 and to a lesser extent Stat5a and Stat5b². The activation of a specific Stat-molecule by IL-21 depends on the leukocyte subgroup^{2,19}. We reasoned that similar signaling may occur in endothelial cells and therefore tested whether phosphorylated Stat3 is detectable in EC. To our surprise, phosphorylated Stat3 is present in activated endothelial cells. In addition, Stat3 phosphorylation is decreased in endothelial cells after 30 minutes of incubation with either IL-21 or IL-2, an effect that is shared with the angiostatic compound anginex. Interestingly, phosphorylated Stat3 in EC has recently been linked to the induction of angiogenesis^{21,22}. Thus, angiogenesis inhibitors including IL21, IL2 and anginex may exert their effect, at least in part, by reducing Stat3 phosphorylation. Inactivation of Stat3 in tumor cells has already been implied to be a promising strategy for therapy in various cancer types by inhibiting tumor derived VEGF action and angiogenesis⁴⁸⁻⁵⁰. Our data suggest that inhibition of Stat3 could also inhibit tumor angiogenesis by a direct effect on endothelial cells.

In conclusion, the ability of IL-21 to inhibit angiogenesis *in vitro* and *in vivo*, in combination with its previously reported beneficial effect on survival of tumor bearing mice⁴⁰, provide further impetus for the use of IL-21 in therapeutic approaches for cancer. In addition, further elucidation of the role of Stat3 phosphorylation in tumor angiogenesis may provide additional

clues for the development of novel and more potent angiogenesis inhibitors and their mechanism of action.

Authors contributions

Karolien Castermans: designed research, performed experiments, collected data, analyzed and interpreted data, performed statistical analysis, and wrote paper.

Sebastien P. Tabruyn: designed research, performed experiments, interpreted data, and wrote paper.

Rong Zeng: performed experiments

Judy van Beijnum: performed experiments and wrote paper.

Cheryl Eppolito: performed experiments

Warren J. Leonard: wrote paper

Protul A. Shrikant: wrote paper

Arjan W. Griffioen: designed research, analyzed and interpreted data, and wrote paper

References

1. Brandt K, Singh PB, Bulfone-Paus S, Ruckert R. Interleukin-21: A new modulator of immunity, infection, and cancer. *Cytokine Growth Factor Rev.* 2007;18:223-232.
2. Leonard WJ, Spolski R. Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. *Nat Rev Immunol.* 2005;5:688-698.
3. Parrish-Novak J, Dillon SR, Nelson A, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature.* 2000;408:57-63.
4. Parrish-Novak J, Foster DC, Holly RD, Clegg CH. Interleukin-21 and the IL-21 receptor: novel effectors of NK and T cell responses. *J Leukoc Biol.* 2002;72:856-863.
5. Sivakumar PV, Foster DC, Clegg CH. Interleukin-21 is a T-helper cytokine that regulates humoral immunity and cell-mediated anti-tumour responses. *Immunology.* 2004;112:177-182.
6. Brady J, Hayakawa Y, Smyth MJ, Nutt SL. IL-21 induces the functional maturation of murine NK cells. *J Immunol.* 2004;172:2048-2058.
7. Kasaian MT, Whitters MJ, Carter LL, et al. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. *Immunity.* 2002;16:559-569.
8. Wang G, Tschoi M, Spolski R, et al. In vivo antitumor activity of interleukin 21 mediated by natural killer cells. *Cancer Res.* 2003;63:9016-9022.
9. Zeng R, Spolski R, Finkelstein SE, et al. Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. *J Exp Med.* 2005;201:139-148.
10. Jin H, Carrio R, Yu A, Malek TR. Distinct activation signals determine whether IL-21 induces B cell costimulation, growth arrest, or Bim-dependent apoptosis. *J Immunol.* 2004;173:657-665.
11. Mehta DS, Wurster AL, Whitters MJ, Young DA, Collins M, Grusby MJ. IL-21 induces the apoptosis of resting and activated primary B cells. *J Immunol.* 2003;170:4111-4118.
12. Ozaki K, Spolski R, Ettinger R, et al. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol.* 2004;173:5361-5371.
13. Ozaki K, Spolski R, Feng CG, et al. A critical role for IL-21 in regulating immunoglobulin production. *Science.* 2002;298:1630-1634.
14. Asao H, Okuyama C, Kumaki S, et al. Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. *J Immunol.* 2001;167:1-5.
15. Mehta DS, Wurster AL, Grusby MJ. Biology of IL-21 and the IL-21 receptor. *Immunol Rev.* 2004;202:84-95.
16. Vosshenrich CA, Di Santo JP. Cytokines: IL-21 joins the gamma(c)-dependent network? *Curr Biol.* 2001;11:R175-177.
17. Habib T, Senadheera S, Weinberg K, Kaushansky K. The common gamma chain (gamma c) is a required signaling component of the IL-21 receptor and supports IL-21-induced cell proliferation via JAK3. *Biochemistry.* 2002;41:8725-8731.
18. Suto A, Wurster AL, Reiner SL, Grusby MJ. IL-21 inhibits IFN-gamma production in developing Th1 cells through the repression of Eomesodermin expression. *J Immunol.* 2006;177:3721-3727.
19. Zeng R, Spolski R, Casas E, Zhu W, Levy DE, Leonard WJ. The molecular basis of IL-21-mediated proliferation. *Blood.* 2007.

20. Spolski R, Leonard WJ. Interleukin-21: Basic Biology and Implications for Cancer and Autoimmunity. *Annu Rev Immunol.* 2007.
21. Hilfiker-Kleiner D, Limbourg A, Drexler H. STAT3-mediated activation of myocardial capillary growth. *Trends Cardiovasc Med.* 2005;15:152-157.
22. Valdembri D, Serini G, Vacca A, Ribatti D, Bussolino F. In vivo activation of JAK2/STAT-3 pathway during angiogenesis induced by GM-CSF. *Faseb J.* 2002;16:225-227.
23. di Carlo E, de Toter D, Piazza T, Fabbi M, Ferrini S. Role of IL-21 in immune-regulation and tumor immunotherapy. *Cancer Immunol Immunother.* 2007;56:1323-1334.
24. Sondergaard H, Frederiksen KS, Thygesen P, et al. Interleukin 21 therapy increases the density of tumor infiltrating CD8(+)T cells and inhibits the growth of syngeneic tumors. *Cancer Immunol Immunother.* 2007.
25. Davis ID, Skrumsager BK, Cebon J, et al. An open-label, two-arm, phase I trial of recombinant human interleukin-21 in patients with metastatic melanoma. *Clin Cancer Res.* 2007;13:3630-3636.
26. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med.* 1971;285:1182-1186.
27. Molema G, Griffioen AW. Rocking the foundations of solid tumor growth by attacking the tumor's blood supply. *Immunol Today.* 1998;19:392-394.
28. Ferrara N, Hillan KJ, Novotny W. Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. *Biochem Biophys Res Commun.* 2005;333:328-335.
29. Laird AD, Vajkoczy P, Shawver LK, et al. SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. *Cancer Res.* 2000;60:4152-4160.
30. Mendel DB, Laird AD, Xin X, et al. In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. *Clin Cancer Res.* 2003;9:327-337.
31. Griffioen AW, van der Schaft DW, Barendsz-Janson AF, et al. Anginex, a designed peptide that inhibits angiogenesis. *Biochem J.* 2001;354:233-242.
32. Kusaka M, Sudo K, Fujita T, et al. Potent anti-angiogenic action of AGM-1470: comparison to the fumagillin parent. *Biochem Biophys Res Commun.* 1991;174:1070-1076.
33. O'Reilly MS, Boehm T, Shing Y, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell.* 1997;88:277-285.
34. van der Schaft DW, Dings RP, de Lussanet QG, et al. The designer anti-angiogenic peptide anginex targets tumor endothelial cells and inhibits tumor growth in animal models. *Faseb J.* 2002;16:1991-1993.
35. Orucevic A, Lala PK. Role of nitric oxide in IL-2 therapy-induced capillary leak syndrome. *Cancer Metastasis Rev.* 1998;17:127-142.
36. Sakkoula E, Pipili-Synetos E, Maragoudakis ME. Involvement of nitric oxide in the inhibition of angiogenesis by interleukin-2. *Br J Pharmacol.* 1997;122:793-795.
37. Volpert OV, Fong T, Koch AE, et al. Inhibition of angiogenesis by interleukin 4. *J Exp Med.* 1998;188:1039-1046.
38. Dirkx AE, Oude Egbrink MG, Kuijpers MJ, et al. Tumor angiogenesis modulates leukocyte-vessel wall interactions in vivo by reducing endothelial adhesion molecule expression. *Cancer Res.* 2003;63:2322-2329.
39. van Beijnum JR, Dings RP, van der Linden E, et al. Gene expression of tumor angiogenesis dissected: specific targeting of colon cancer angiogenic vasculature. *Blood.* 2006;108:2339-2348.

40. Moroz A, Eppolito C, Li Q, Tao J, Clegg CH, Shrikant PA. IL-21 enhances and sustains CD8+ T cell responses to achieve durable tumor immunity: comparative evaluation of IL-2, IL-15, and IL-21. *J Immunol.* 2004;173:900-909.
41. van der Schaft DW, Toebes EA, Haseman JR, Mayo KH, Griffioen AW. Bactericidal/permeability-increasing protein (BPI) inhibits angiogenesis via induction of apoptosis in vascular endothelial cells. *Blood.* 2000;96:176-181.
42. Thijssen VL, Brandwijk RJ, Dings RP, Griffioen AW. Angiogenesis gene expression profiling in xenograft models to study cellular interactions. *Exp Cell Res.* 2004;299:286-293.
43. Di Carlo E, Comes A, Orengo AM, et al. IL-21 induces tumor rejection by specific CTL and IFN-gamma-dependent CXC chemokines in syngeneic mice. *J Immunol.* 2004;172:1540-1547.
44. Eklund L, Olsen BR. Tie receptors and their angiopoietin ligands are context-dependent regulators of vascular remodeling. *Exp Cell Res.* 2006;312:630-641.
45. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature.* 1995;376:66-70.
46. Kuniyasu H, Ohmori H, Sasaki T, et al. Production of interleukin 15 by human colon cancer cells is associated with induction of mucosal hyperplasia, angiogenesis, and metastasis. *Clin Cancer Res.* 2003;9:4802-4810.
47. Rodella L, Zamai L, Rezzani R, et al. Interleukin 2 and interleukin 15 differentially predispose natural killer cells to apoptosis mediated by endothelial and tumour cells. *Br J Haematol.* 2001;115:442-450.
48. Masuda M, Ruan HY, Ito A, et al. Signal transducers and activators of transcription 3 up-regulates vascular endothelial growth factor production and tumor angiogenesis in head and neck squamous cell carcinoma. *Oral Oncol.* 2006.
49. Weerasinghe P, Garcia GE, Zhu Q, et al. Inhibition of Stat3 activation and tumor growth suppression of non-small cell lung cancer by G-quartet oligonucleotides. *Int J Oncol.* 2007;31:129-136.
50. Xu Q, Briggs J, Park S, et al. Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways. *Oncogene.* 2005;24:5552-5560.

Figure legends

Figure 1. *IL-21 inhibits angiogenic sprouting of endothelial cells*

A) A mouse aortic ring treated with bFGF (10ng/ml) and bFGF and IL-21 200ng/ml. **B)** Quantification of sprouting of primary mouse endothelial cells from isolated aortic rings in a 3D collagen matrix. **C)** Sprouting of bFGF (10ng/ml) stimulated SVEC4-10 cells (mouse endothelial cell line) grown on beads embedded in a 3D collagen matrix. **D)** An example of sprouting of SVEC4-10 cells treated with bFGF (10ng/ml, upper panel) and bFGF and IL-21 (lower panel). Data are represented as relative mean values of at least 3 experiments. (**p< 0.0005, *p< 0.05 compared to control).

Figure 2. *IL-21 receptor α -chain is expressed by endothelial cells*

A) FACS analysis of human umbilical vein derived endothelial cells (HUVEC) stained for IL-21-R α -chain (white), conjugate control is represented by grey histogram. **B)** SVEC4-10 stained for IL-21-R α . **C)** mRNA expression of IL-21-R α mouse endothelial cell lines SVEC and TME in primary (pr) human (h) and mouse (m) endothelial cells, and freshly isolated human tumor (TEC) and normal endothelial cells (NEC)³⁹. **D)** mRNA expression of IL-2-R α in primary human, primary mouse and freshly isolated human tumor and normal endothelial cells. Positive control is spleen tissue, negative control is no template in the PCR reaction (H₂O).

Figure 3. *Proliferation but not migration is inhibited by IL-21 or IL-2 treatment*

A) Proliferation of angiogenically stimulated (bFGF 10ng/ml) SVEC4-10 is inhibited by 3 days of treatment with IL21, IL-2 or anginex. **B)** Wound assay of SVEC4-10 cells. Migration of EC is not effected by treatment with IL-21 nor IL-2. The angiogenesis inhibitor anginex inhibits migration of SVEC4-10 by approximately 70%. Data are represented as relative mean values of at least 4 experiments. (**p< 0.0005, *p<0.005, p< 0.05 compared to control).

Figure 4. *IL-21 and IL-2 inhibit angiogenesis in vivo*

A-B) Treatment of CAMs with 200ng/ml IL-2 reduces vessel formation in CAMs whereas IL-21 does not effect the number of vessels in CAMs (n=7 for each group). Morphological examination shows that vessels in CAMs treated with 20ng/ml or 200ng/ml IL-21 are tortuous and irregular compared to control CAMs (**B**). **C)** Images of CD31 staining of EF7 tumor sections of mice treated with PBS (control), 20 μ g IL-21 or 2000 I.U. IL-2. **D)** IL-2 (n=4) and

IL-21 (n=5) treatment reduce microvessel density in tumor tissues compared to control (PBS, n=5). Results are presented as relative mean values (**p<0.005, *p<0.05 compared to control).

Figure 5. *IL-21 and IL-2 regulate expression of angiogenesis-related genes in activated EC and tumor tissues*

A-C) Regulation of mRNA expression levels of angiogenesis factors in bFGF (10ng/ml) stimulated SVEC4-10 after three days of IL-21, IL-2 or anginex treatment. Concentrations of the compounds are as indicated in previous figures, first bar 20ng/ml, second bar 200ng/ml and anginex 25 μ M. Results are represented as relative means of at least 3 experiments. (*p<0.05, #p<0.005 compared to bFGF). **D-E)** Regulation of mRNA expression levels of angiogenesis factors in tumor tissues of EG7 tumors after PBS (n = 4), IL21 (20 μ g, n = 4) or IL-2 (I.U. 2000, n = 3) treatment. Results are represented as relative means (*p<0.05).

Figure 6. *Decreased Stat3 activation is correlated with angiostatic activity.*

A) Western blot analysis (n = 2) of cell lysates of 10 ng/ml bFGF stimulated SVEC4-10 after 5 or 30 minutes incubation with IL-2, IL-21 or anginex. After 30 minutes, IL-2, IL-21 and anginex treated cells showed decreased Stat3 phosphorylation (P-Stat3). **B)** Western blot analysis (n=1) of cell lysates of bFGF stimulated primary mouse endothelial cells after 30 minutes with IL-21, IL-2 or anginex.

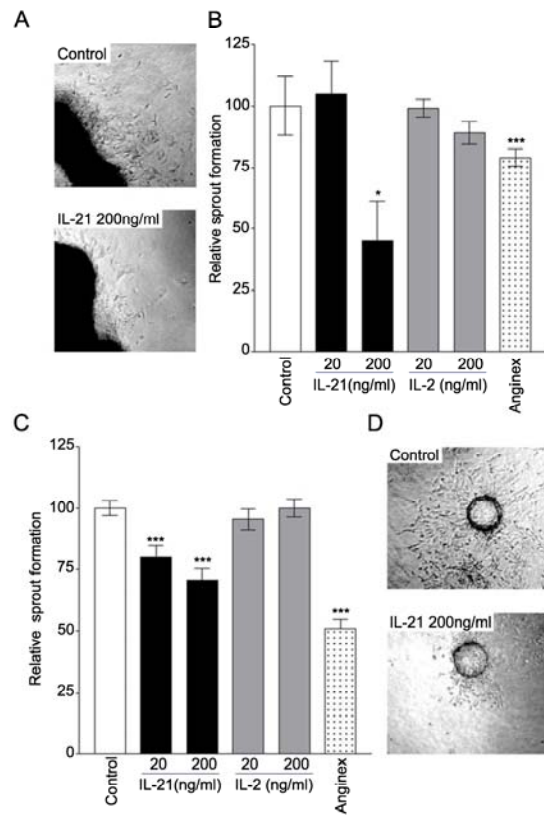
Supplemental table 1. Real-time RT-PCR primers (5' - 3')

		Forward	Reverse	Product length (bp)
Mouse	Cyclophilin A	ATTTCTTTTGACTTGCGGGC	AGCTAGACTTGAAGGGGAATG	156
Human	Cyclophilin A	CTCGAATAAGTTTGACTTGTG TTT	CTAGGCATGGGAGGGAACA	165
Mouse	IL21R	CTCCCCCTGAACGTGACT	TTGCCCTCAGCACGTAGTT	97
Human	IL21R	CGTGGGAGTCAGCATGCC	TGTCGTCGGCCATGAAGTG	290
Mouse	IL2R	CACAGTCTATGCACCAAGAG AA	GTAGTGAACACTCTGTCCTTCCA	113
Human	IL2R	GGTCACTCTATATGCTCT	GTGCACTTGTTTCGTTGTGTT	105

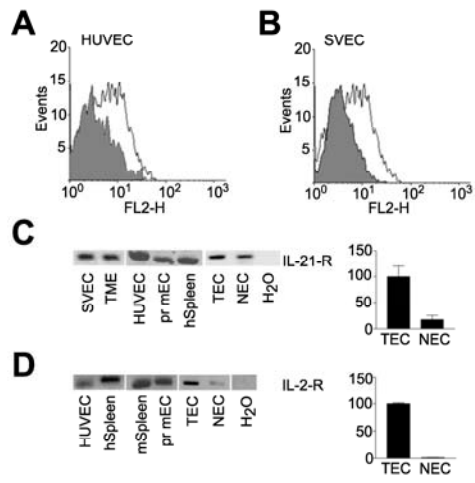
Supplemental table 2. Absolute Ct values of qRT-PCR on mouse endothelial cells and tumor tissues.

	SVEC4-10		Tumor tissue	
	Mean Ct	H₂O Ct	Mean Ct	H₂O Ct
CycloA	15,8 ± 0,9	N/A	17,5 ± 1,1	N/A
VEGFA	20,0 ± 0,9	37,4	24,0 ± 1,24	33,9
VEGFR1	25,6 ± 0,7	32,9	28,5 ± 1,0	33,1
VEGFR2	30,7 ± 1,2	36,5	29,4 ± 1,1	38,5
ANG1	28,0 ± 1,2	N/A	31,6 ± 2,5	N/A
ANG2	30,6 ± 0,7	35,9	27,9 ± 1,3	34,7
ANG3	30,5 ± 0,8	33,4	31,2 ± 1,9	32,8
TIE1	33,5 ± 1,2	37,9	33,8 ± 1,8	36,1
TIE2	27,8 ± 1,1	34,19	29,6 ± 1,6	35,9

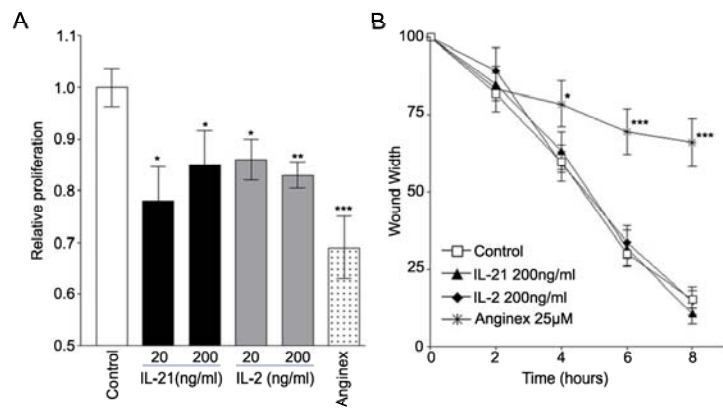
Data are represented as mean values of at least 4 experiments ± standard deviation.



Castermans et al. Figure 1 ^top

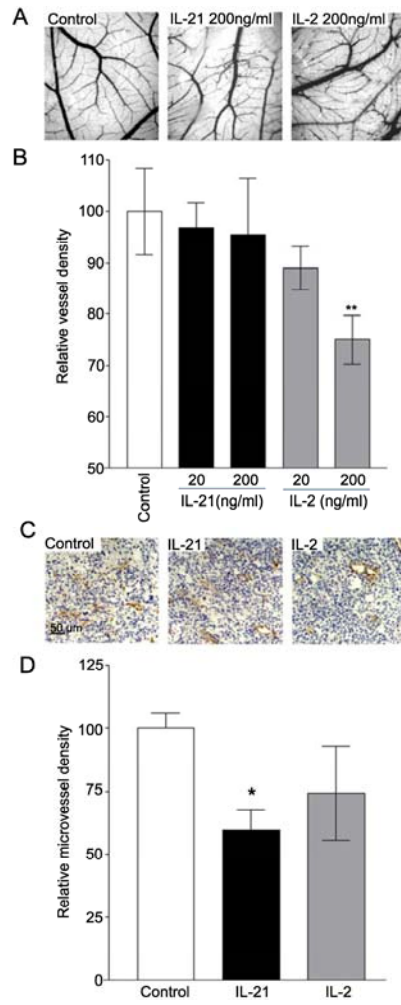


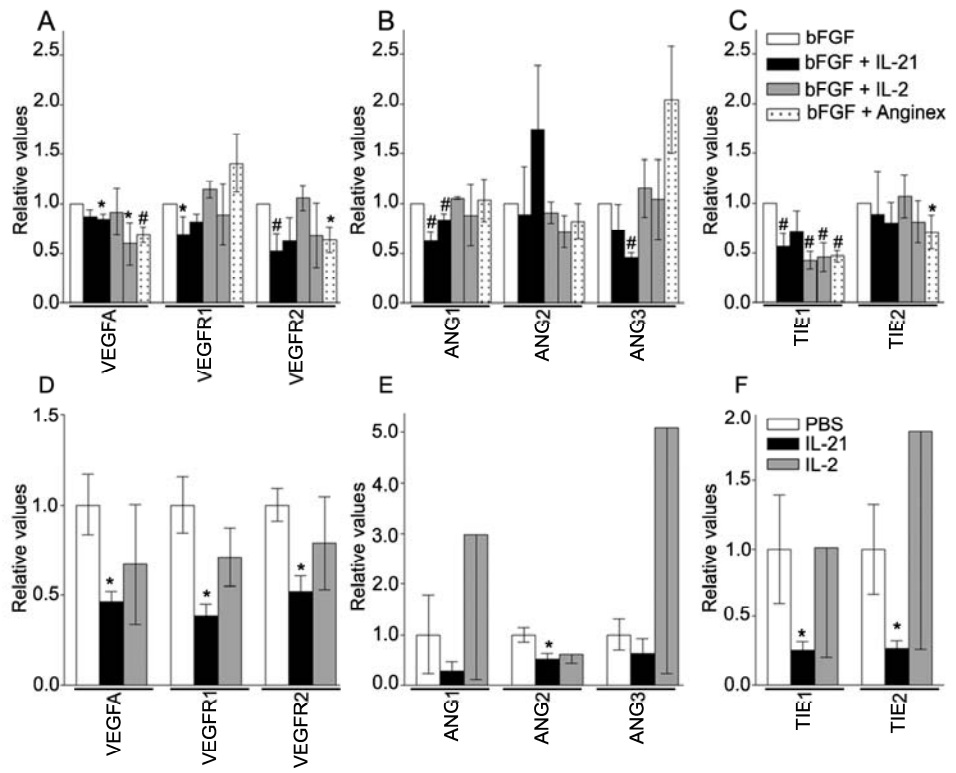
Castermans et al. figure 2 ^top



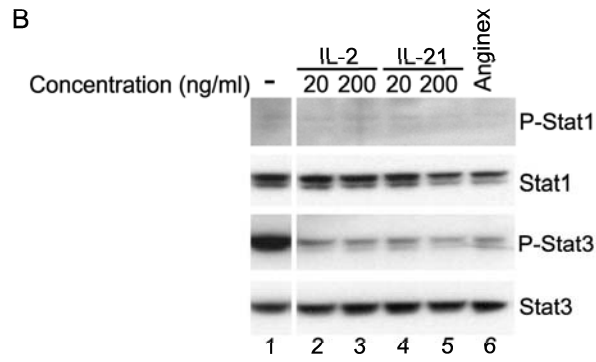
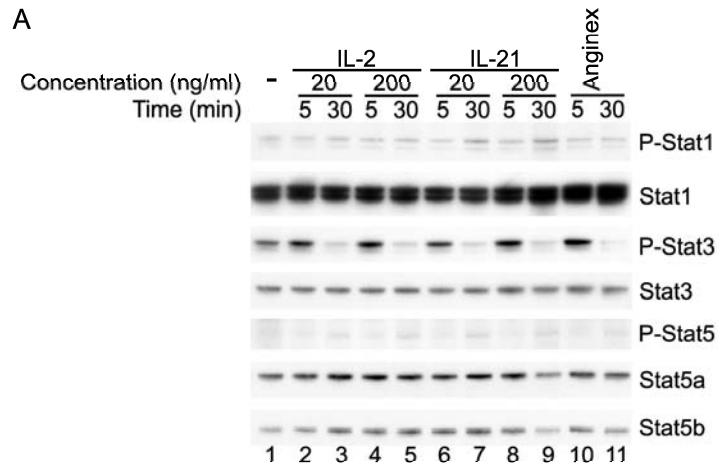
Castermans et al. Figure 3 ^top

Castermans et al. Figure 4 ^top





Castermans et al. Figuur 5 ^top



Castermans et al. Figuur 6 ^{top}