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Laminar flow attenuates interferon-induced inflammatory responses in endothelial cells

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Abstract

Objective: Atherosclerosis is a chronic disease that involves inflammation, in which cytokines, including interferon-gamma (IFNγ), participate. Endothelial cells (ECs) exposed to IFNγ increase the expression of CXC chemokines. ECs subjected to laminar flow (LF) are atheroprotective, despite an unclear mechanism. This study was conducted to analyze whether ECs under LF were protected from IFNγ-induced responses.

Methods: IFNγ-treated human umbilical cord ECs were subjected to LF in a well-defined flow chamber system. IFNγ-induced STAT1 activation and downstream target genes were examined.

Results: ECs exposed to IFNγ triggered STAT1 activation via the phosphorylation of Tyr701 and Ser727 in STAT1. ECs exposed to LF alone did not activate STAT1. LF exposure of IFNγ-treated ECs significantly attenuated IFNγ-induced Tyr701 phosphorylation in a shear-force- and time-dependent manner, whereas Ser727 phosphorylation was unaffected. Consistently, LF inhibited IFNγ-induced STAT1 binding to DNA. ECs treated with IFNγ induced the expression of three T-cell-specific CXC chemokines (CXCL9, CXCL10 and CXCL11) as well as CIITA, a transcriptional regulator of major histocompatibility complex class II (MHCII). Consistently, LF exposure of IFNγ-treated ECs reduced the expression of CXC chemokines and CIITA.

Conclusions: LF attenuates IFNγ-induced responses via the suppression of STAT1 activation. Inhibition by LF of the interferon-induced ECs' response may explain some aspects of LF's atheroprotective effects on the endothelium.

Keywords: Endothelial cells; Laminar flow; Interferon-gamma; STAT1; CXC chemokines

1. Introduction

Endothelial cells (ECs) that are subjected to steady laminar flow (LF) align themselves in the direction of the flow and show a decreased turnover rate, two essential characteristics for maintaining vessel integrity [1,2]. LF triggers biomechanical signaling mechanisms that modulate endothelial functions, including the regulation of vascular tone, endothelial growth, and leukocyte/EC interaction. Atherosclerotic lesions often develop in areas where ECs are subjected to nonlaminar flow, or disturbed flow, associated with low shear stress [3–5]. However, the detailed mechanism of the atheroprotective effects of LF on ECs remains elusive. It has been shown that ECs exposed to LF, but not to disturbed flow, upregulate the level of endothelial nitric oxide synthase (eNOS) [6]. In addition, LF alters the expression of redox-related enzymes by increasing superoxide dismutase and heme oxygenase-1 and by decreasing thioredoxin-interacting protein, all of which contribute to atheroprotection [7–9]. All these protective effects of LF

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on vessel walls are transcriptional events. Recent studies indicated that LF protected ECs from oxidative stress- or TNFα-induced responses, a process involving a LF-induced post-transcriptional event or signaling modulation [10,11]. We earlier demonstrated that LF attenuated serum- or IL6-induced STAT3 activation in ECs in a shear-force-dependent manner [12,13]. This LF-induced inhibitory effect is an acute response and is mediated via signaling modulation. However, the detailed signaling pathways and downstream effects in ECs that contribute to LF-induced atheroprotection remain to be further defined.

Immunological and/or inflammatory responses that are mediated by cytokines and chemokines play a crucial role during atherogenesis. Patients with atherosclerosis were shown to have an elevated level of cytokines in serum [14,15] and accumulated T cells in atheromas [16], implying that cytokines and chemokines involved in activation and/or recruitment of T cells are tightly associated with this vascular dysfunction. Interferon-gamma (IFNγ), a cytokine predominantly secreted by activated $CD4^+$ T cells and NK cells, is well known for its involvement in atherosclerosis [17]. Hypercholesterolemia-induced T cell adhesion to ECs was diminished in IFNγ knockout mice [18]. In addition, IFNγ aggravated atherosclerosis in apoE-deficient mice [19]. These observations confirm that IFNγ is a major cytokine that contributes to atherogenesis. IFNγ induces the JAK/STAT1 signaling pathway in cells [20]. IFNγ binds to its receptor, followed by activation of JAK1/2. Subsequently, cytosolic STAT1 is recruited to the receptor complex and is phosphorylated at Tyr701 and Ser727. Upon phosphorylation, STAT1 forms a homodimer and translocates to the nucleus, where it binds conserved DNA sequences in the promoter region of its downstream target genes and activates their transcription. Those target genes include IFNγ-inducible CXC chemokines and major histocompatibility complex class II (MHCII) [21]. The IFNγ-inducible CXC chemokines CXCL9 (Mig), CXCL10 (IP-10) and CXCL11 (I-TAC) share a certain degree of structural homology and bind to the same receptor, CXCR3 [22]. Analysis of human atherosclerotic lesions showed that these CXC chemokines were highly expressed in ECs associated with atheroma [16]. These chemokines may serve as chemotactic factors for the recruitment of T cells to sites of inflammation [16]. Chronically activated T cells are frequently detected in atherosclerotic lesions [23], indicating that T cells participate in this inflammatory process. Activation of T cells requires the presence of the MHCII molecule on the surface of the antigen-presenting cell. T cells recognize antigens presented by endothelial MHCII that is induced by interferon [24,25]. Furthermore, the STAT1-regulated gene product CIITA is a transcriptional regulator of MHCII expression. CIITA induction thus plays a key role in the regulation of immune response during vascular disorders, including graft atherosclerosis [26].

This study aimed to elucidate the protective mechanisms of LF to ECs under cytokine IFNγ treatment. Our results clearly show that ECs subjected to LF suppress IFNγ-induced STAT1 activation and this suppression seems to be shear force dependent. This suppression reduces the expression of CXC chemokines and CIITA.The suppression of IFNγ-induced responses in ECs by LF provides new insights into the atheroresistant nature of the straight segments of the arterial tree.

2. Methods

2.1. Materials and cell culture

Recombinant human IFNγ was purchased from PeproTech EC Ltd (London, UK). Antibodies to phospho-Tyr701 of STAT1, STAT1 and tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-Ser727 of STAT1 and phospho-ERK were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies to Akt, phospho-Ser473 of Akt, STAT3 and ERK1 were obtained from BD Transduction Laboratories (Lexington, KY). ECs were isolated from human umbilical cords as previously described [27]; the investigation conforms with the principles outlined in the Declaration of Helsinki for use of human tissue or subjects. Isolated ECs were cultured in M199 supplemented with 20% fetal bovine serum (FBS) for two days and then subcultured onto glass slides until confluent. The culture medium was then changed to M199 containing 2% FBS overnight prior to experiments. All shear stress experiments were carried out in medium containing 2% FBS.

2.2. Shear flow system

ECs were subjected to LF in a parallel flow chamber system as previously described [28]. ECs were subjected to continuous flow with culture medium circulating in a closed loop. All shear stress experiments were carried out with M199 medium containing 2% FBS. For most studies, ECs were subjected to a constant shear stress at 25 dyne/cm^2. For shear force studies, ECs were exposed to various shear stresses as indicated.

2.3. Electrophoretic mobility shift assay (EMSA)

We prepared nuclear extracts from ECs as previously described [13]. The nuclear extracts were incubated with ^{32}P-labeled hSIE (5′-GTGCATTTCCCGTAAATCTTGTC-TACA-3′; Santa Cruz Biotechnology) in binding buffer at room temperature for 30 min. For supershift assays, the antibody to STAT1 or STAT3 was incubated with the nuclear extracts for 30 min prior to the addition of a radiolabeled hSIE probe. DNA-protein complexes were resolved on a nondenaturing 5% polyacrylamide gel, followed by autoradiography.

2.4. RNA isolation and quantitative RT-PCR

RNA extraction was performed using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. The quantitative analysis

of mRNA was done using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The TaqMan™ fluorescent probes, forward and reverse primers for human CXCL9, CXCL10, CXCL11, CIITA and GAPDH were designed and synthesized by Proligo LLC (Boulder, CO). The TaqMan™ probes were labeled with the fluorescent reporter dye 6-FAM at the 5′ end and with the quencher dye BHQ-1 at the 3′ end. The sequences of the primers and the probes for each chemokine and CIITA were as follows: CXCL9 (Accession No. NM_002416), 5′-TCTTG CTGGTTCTGATTGGAGTG-3′ (forward), 5′-GATAGTCCCTTGGTTGGTGCTG-3′ (reverse), 5′-CAGGAACAGCGACCCTTTCTCACTACTGG-3′ (probe); CXCL10 (Accession No. NM_001565), 5′-GCTTCCAAGGATGGACCACAC-3′ (forward), 5′-GCAAACTAAGAACAATTATGGCTTGAC-3′ (reverse), 5′-CTGCCTCTCCCATCACTTCCCTACAT-3′ (probe); CXCL11 (Accession No. NM_005409), 5′-AAGAGGACGCTGTCTTTGC-3′ (forward), 5′-C AGTTGTTACTTGGGTACATTATGG-3′ (reverse), 5′-CTTTCTCAATATCTGCCACTTTCACTGCT-3′ (probe); CIITA (Accession No. NM_000246), 5′-CCTGTGCCTCTACCACTT CTATG-3′ (forward), 5′-GTGTCTGTGTCGGGTTCTGAG-3′ (reverse), 5′-CTCAATCTCTTCTTCTCCAGCCAGGTCCA-3′ (probe); GAPDH (Accession No. NM_002046), 5′-GGACCTGACCTGCCGTCTAG-3′ (forward), 5′-AGCCCAGGATGCCCTTGAG-3′ (reverse), 5′-TCCGACGCCTGCTTCACCACCTTCTTG-3′ (probe). One-step RT-PCR was performed by using the TaqMan™ One-Step PCR Master Mix Reagents Kit. Briefly, we used 20 μl of reaction mixture [100 ng of total RNA, 1x Master Mix buffer, 200 μM each of dATP, dGTP and dCTP, 600 μM dUTP, 0.2 μM forward and reverse primers, 0.1 μM TaqMan probe, 0.5 U of MultiScribe (reverse transcriptase) and 0.5 U of AmpliTaq Gold (DNA polymerase)]. One-step RT-PCR was achieved as following: 30 min at 48 °C, 10 min at 95 °C, and then 40 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C. Real-time quantitative RT-PCR reactions were performed triplicately for each sample. In a parallel experiment, human CIITA and GAPDH gene expressions were performed using one-step RT-PCR System SuperScript™III (Invitrogen, San Diego, CA). Thermal cycling conditions were shown as following: reverse transcription at 55 °C for 30 min; denaturation at 94 °C for 2 min; 25 cycles of denaturation at 94 °C for 15 s, annealing at 56 °C for 30 s, elongation at 68 °C for 1 min; an additional elongation step of 5 min at 68 °C. Results were analyzed by electrophoresis in 1% agarose gels.

2.5. Western Blot analysis

Western blot was performed as previously described [27]. Equal amounts of protein were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blotted with a specific antibody followed by detection with horseradish peroxidase-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG. Results were analyzed using an enhanced chemiluminescence detection system (Pierce, Rockford, IL).

2.6. Small interfering RNA (siRNA) transfection

ECs were cultured in M199 complete medium containing 20% FBS and 25% EGM-2MV (Clonetics Corp., San Diego, CA). For STAT1 knockdown experiments, STAT1-specific siRNA or nonspecific control siRNA (50 nM; Dharmacon, Chicago, IL) was transfected into ECs by electroporation using Nucleofector™ Kit (Amaxa Biosystems, Gaithersburg, MD). ECs were cultured for one day in M199 complete medium followed by culturing in M199 containing 2% FBS overnight. ECs were treated with IFNγ for 4 hours, and cell lysates were obtained for western blotting. Extracted RNA was harvested to analyze the expression of CXC chemokines and CIITA.

2.7. ELISA

Secreted CXCL10 in the culture supernatant, collected from ECs immediately after LF experiment, was determined

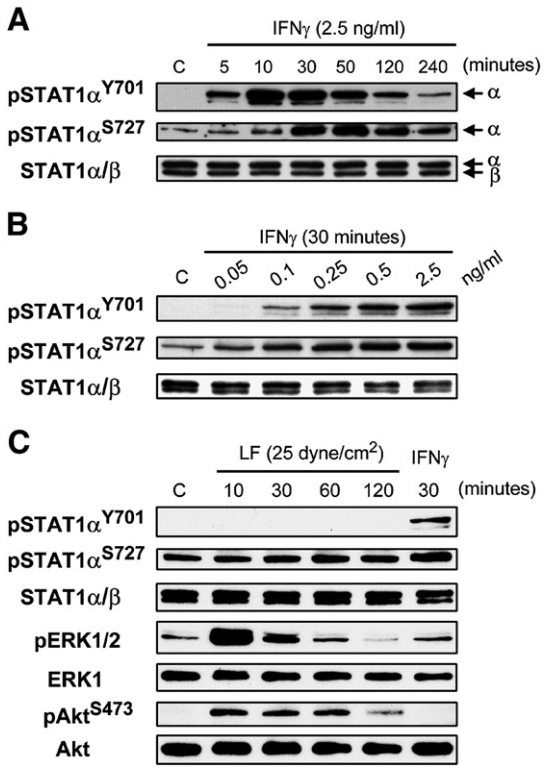

Fig. 1. IFNγ, but not LF, stimulates phosphorylation of Tyr701 and Ser727 in STAT1. (A) IFNγ induces phosphorylation in STAT1 in a time-dependent manner. ECs were treated with IFNγ at the indicated time intervals. (B) IFNγ induced the phosphorylation in STAT1 in a concentration-dependent manner. ECs were treated with IFNγ for 30 min. (C) ECs were maintained under static conditions or were exposed to LF (25 dyne/cm^2) at the indicated time intervals. ECs treated with IFNγ (2.5 ng/ml) for 30 min were used as positive controls. They were then lysed and subjected to western blotting using various antibodies as indicated. Arrows indicate STAT1α and STAT1β. Results are representative of three independent experiments.

using a sandwich ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.8. Statistical analysis

All results are expressed as means±S.E. Statistical analysis was performed using one-way ANOVA. Statistically significant was defined as $P<0.05$.

3. Results

3.1. IFNγ, but not LF, induces STAT1 activity in ECs

Activated STAT1 is phosphorylated at Tyr701 and Ser727, facilitating STAT1 dimerization and nuclear translocation, and binds to conserved DNA sequences in the promoter region of target genes. ECs that were stimulated with IFNγ showed a time- and dose-dependent increase in the phosphorylation of Tyr701 and Ser727 in STAT1 (Fig. 1A and B). Phosphorylation at Tyr701 reached a peak around 10 to 30 min after IFNγ treatment and remained at an elevated level for up to 4 hours (Fig. 1A). The phosphorylation at Ser727, however, was delayed and reached its peak around 50 min after treatment. This increase was shown to be sustained up to 4 hours after IFNγ treatment (Fig. 1A). ECs subjected to LF (25 dyne/cm^2) did not induce Ser727 phosphorylation, as the phosphorylation at Ser727 remained at an elevated level similar to that of unsheared controls (Fig. 1C). Thus, LF alone does not induce STAT1 activation in ECs under basal conditions. In contrast, ERK1/2 and Akt were phosphorylated immediately after LF was initiated (Fig. 1C), a phenomenon consistent with previous reports [12,29].

3.2. LF attenuates IFNγ-induced Tyr701 phosphorylation in STAT1 in a time- and shear-force-dependent manner

LF protects ECs from IL6-induced Tyr705 phosphorylation in STAT3 [12,13]. In the present study, we examined the atheroprotective effect of LF on ECs under IFNγ stimulation. ECs were exposed to IFNγ (2.5 ng/ml) for 10 min, followed by LF (25 dyne/cm^2) treatment with the same medium containing IFNγ for various intervals. ECs were then collected and analyzed for the phosphorylation in STAT1. ECs pretreated with IFNγ for 10 min followed by LF for an additional 10 or 20 min significantly inhibited IFNγ-induced Tyr701 phosphorylation by about 40% or 80%, respectively, compared with those IFNγ-treated ECs under static conditions (Fig. 2A). Moreover, the inhibition of IFNγ-induced STAT1 Tyr701 phosphorylation was shear-

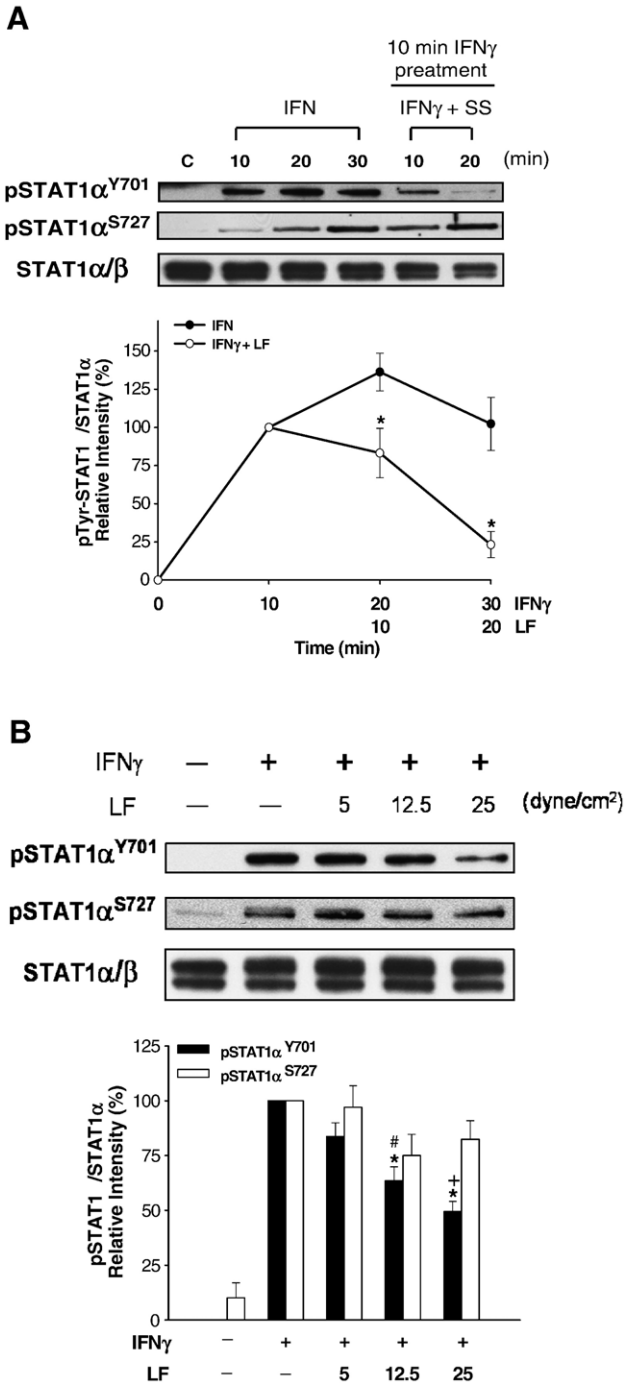

Fig. 2. Laminar flow suppresses IFNγ-induced phosphorylation of Tyr701 in STAT1 in a time- and force-dependent manner. (A) ECs pretreated with IFNγ (2.5 ng/ml) for 10 min were either maintained under static conditions or exposed to LF (25 dyne/cm^2) containing IFNγ for an additional 10 or 20 min. Cell lysates were subjected to western blotting. The intensity of Tyr701 phosphorylation was normalized to the level of STAT1α and is presented as the mean±S.E. of three independent experiments. The intensity of Ser727 phosphorylation was also shown. *$P<0.05$ vs. respective IFNγ-treated ECs under static condition. (B) ECs were treated with IFNγ for 30 min under static conditions or were pretreated with IFNγ for 10 min under static conditions followed by LF for 20 min in an IFNγ-containing medium with increasing shear forces as indicated. Phosphorylation of STAT1 was normalized to the level of STAT1α and is presented as the mean±S.E. from three independent experiments. *$P<0.05$ vs. ECs treated with IFNγ for 30 min under static conditions. # $P<0.05$ vs. ECs under shear stress of 5 dyne/cm^2. + $P<0.05$ vs. ECs under shear stress of 12.5 dyne/cm^2.

force dependent i.e., the higher the shear stress that was applied to the ECs, the greater was the suppression of Tyr701 phosphorylation that we observed (Fig. 2B). In contrast, IFNγ-induced Ser727 phosphorylation was not affected by LF (Fig. 2B), indicating the specificity of this shear effect on Tyr701. The suppression of Tyr701 phosphorylation in STAT1 decreased STAT1/DNA binding activity, as shown by an electrophoretic mobility shift assay (EMSA) using the oligonucleotide corresponding to the STAT1 binding sequences (hSIE). The increased STAT1/DNA binding after IFNγ treatment was greatly suppressed in ECs exposed to LF (Fig. 3). This STAT1 binding to DNA was specific, because preincubation of the nuclear protein extract with the antibody to STAT1, but not STAT3, largely eliminated the STAT1/STAT1 homodimer band. These results indicate that LF to ECs specifically suppresses IFNγ-induced Tyr701 phosphorylation in STAT1 and consequently inhibits STAT1-mediated transcriptional activity.

3.3. LF suppresses IFNγ-induced expression of CXC chemokines and CIITA

IFNγ triggers JAK/STAT1 signaling and subsequently upregulates the IFNγ-inducible CXC chemokines, CXCL10, CXCL9 and CXCL11 in ECs [30]. In human atherosclerotic lesions, these chemokines are highly expressed in ECs overlying the plaque [16]. Because of the focal nature of atherosclerosis, the expression of CXC chemokines was analyzed in cytokine-treated ECs under the influence of LF. As shown, IFNγ treatment of ECs greatly induced gene expression of CXC chemokines and CIITA which reached their plateau 4 hours after IFNγ exposure (Fig. 4A). To clarify whether those genes encoding CXC chemokines and CIITA are downstream targets of STAT1, a specific siRNA was used to knock down STAT1 in ECs. ECs transfected with siRNA to STAT1 greatly reduced the level of STAT1 but not of STAT3 (Fig. 4B). Consequently, the expression of the IFNγ-inducible CXC chemokines and CIITA were significantly suppressed compared with those ECs transfected only with nonspecific siRNA (Fig. 4B). These results

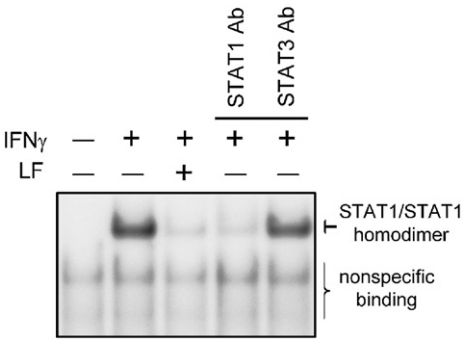

Fig. 3. Laminar flow inhibits IFNγ-induced STAT1 binding to DNA. ECs were maintained under static conditions (lane 1) or treated with IFNγ (2.5 ng/ml) for 4 hours and 10 min (lane 2). ECs pretreated with IFNγ for 10 min were subjected to LF with a medium containing IFNγ for an additional 4 hours (lane 3). Nuclear extracts were analyzed by EMSA using a ^{32}P-labeled hSIE oligonucleotide probe. The specificity of the supershifted complex (STAT1/STAT1 homodimer) was assessed by preincubating the nuclear extracts with antibody to STAT1 (lane 4) or STAT3 (lane 5). Results are representative of three independent experiments.

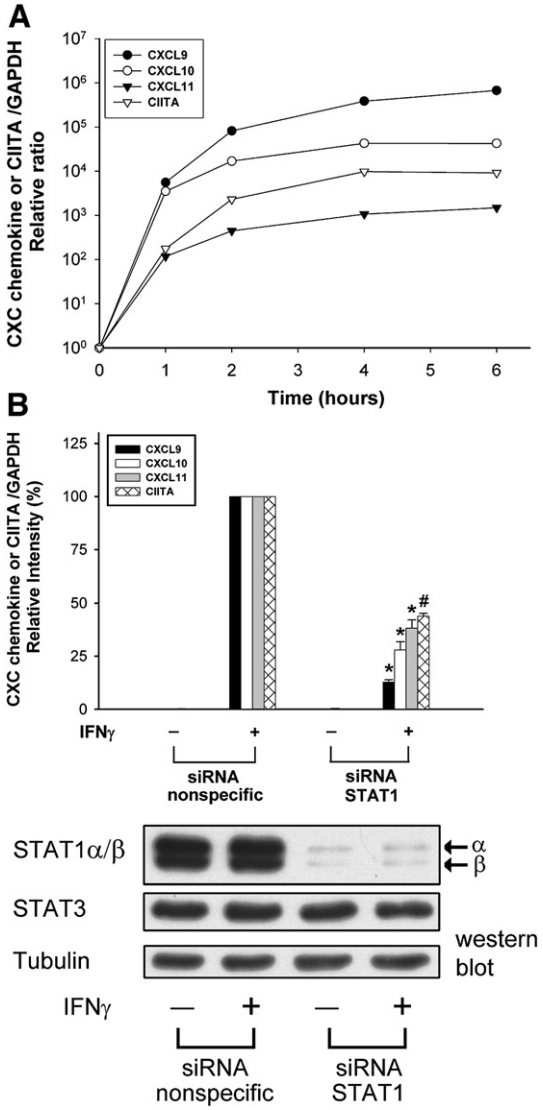

Fig. 4. IFNγ induces expression of CXC chemokines via STAT1 activation. (A) ECs were maintained under basal conditions or treated with IFNγ (2.5 ng/ml) at the indicated time intervals; the expression of each chemokine or CIITA was determined by real-time quantitative RT-PCR. (B) STAT1 knockdown reduced IFNγ-inducible CXC chemokines and CIITA. ECs transfected with either STAT1-specific or nonspecific siRNA were treated with or without IFNγ (2.5 ng/ml) for 4 hours, and the expression of chemokine or CIITA was analyzed using real-time quantitative RT-PCR. Chemokine expression was normalized to GAPDH and was presented as the mean ± S.E. from three independent experiments. *$P<0.01$, #$P<0.05$ vs. respective gene expression in IFNγ-treated ECs transfected with nonspecific siRNA. In a parallel experiment, expressions of STAT1, STAT3 and tubulin in ECs transfected with STAT1-specific or nonspecific siRNA were analyzed by western blotting.

indicate that the IFNγ-induced expression of CXC chemokines is mainly mediated via STAT1. To examine the atheroprotective effects of LF on ECs, IFNγ-treated ECs were subjected to LF for 4 hours. In contrast to the IFNγ-treated ECs, ECs subjected to LF only did not induce CXC chemokines (Fig. 5A). When ECs were pretreated with IFNγ for 10 min followed by LF for 4 hours in the presence of IFNγ, the expression of IFNγ-inducible CXC chemokines was significantly attenuated as compared with those ECs

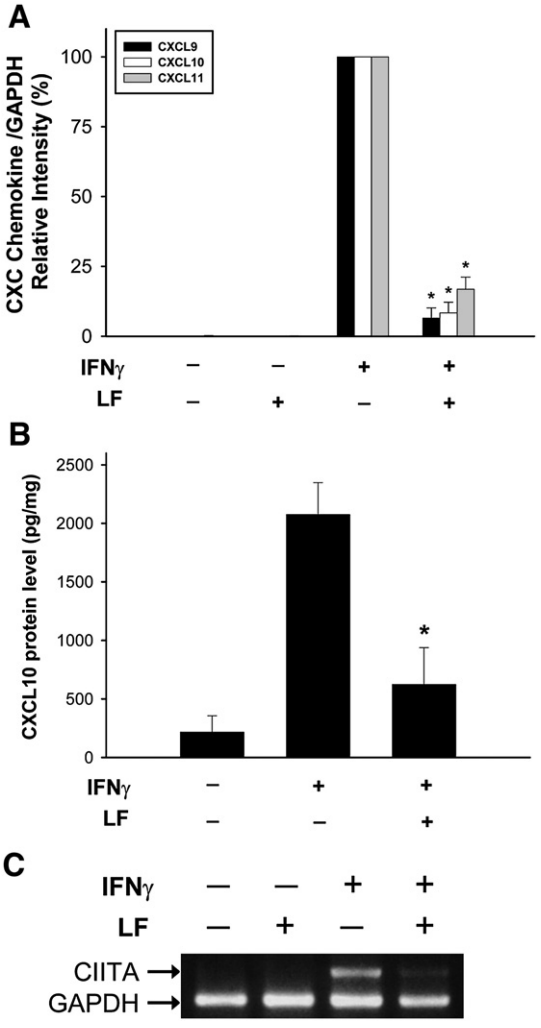

Fig. 5. Laminar flow suppresses IFNγ-inducible CXC chemokines and CIITA. (A) ECs were maintained under static conditions (lane 1) or exposed to LF (25 dyne/cm^2) for 4 hours and 10 min (lane 2); ECs were treated with IFNγ for 4 hours and 10 min (lane 3) or exposed to IFNγ for 10 min followed by LF for 4 hours in a medium containing IFNγ (lane 4). CXCL9, CXCL10 and CXCL11 mRNA levels were determined by real-time quantitative RT-PCR. Chemokine expression was normalized to GAPDH. (B) LF suppresses IFNγ-induced CXCL10 protein production. ECs were treated as in lanes 1, 3 and 4 of A. Conditioned medium was collected for ELISA analysis. CXCL10 protein is expressed as pg/mg of total protein. (C) LF attenuated IFNγ-induced CIITA expression in ECs pretreated with IFNγ. The expression of CIITA was analyzed using RT-PCR. Results are presented as the mean±S.E. from three independent experiments. *P<0.05 vs. IFNγ-treated ECs under static condition.

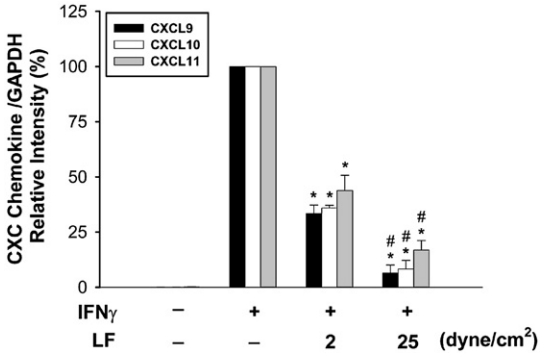

Fig. 6. Laminar flow suppresses IFNγ-inducible CXC chemokines in a shear-force-dependent manner. ECs were maintained under static condition, treated with IFNγ for 4 hours and 10 min, or pretreated with IFNγ (2.5 ng/ml) for 10 min and then subjected to LF in a medium containing IFNγ for 4 hours under shear forces as indicated. CXCL9, CXCL10 and CXCL11 mRNA levels were determined using real-time quantitative RT-PCR. Chemokine expression was normalized to GAPDH and was presented as the mean±S.E. from three independent experiments. *P<0.05 vs. respective chemokine expression of IFNγ-treated ECs under static condition. #P<0.05 vs. respective chemokine expression of IFNγ-treated ECs subjected to LF with lower shear stress (2 dyne/cm^2).

treated with IFNγ under static conditions (Fig. 5A). Consistently, LF greatly suppressed CXCL10 secretion into a cultured medium from IFNγ-treated ECs (Fig. 5B). In addition to CXC chemokines, the IFNγ-induced expression of CIITA, a crucial transcriptional regulator of MHCII expression, was also attenuated by LF (Fig. 5C). Taken together, LF attenuated IFNγ-induced STAT1 activation and subsequently suppressed the expression of CXC chemokines and CIITA in ECs.

3.4. Suppression of IFNγ-induced CXC chemokines and CIITA expression by LF is shear-force dependent

To examine the protective effects of LF on ECs further, we examined IFNγ-induced STAT1 downstream target genes, CXC chemokines and CIITA in ECs under LF with different shear stress. IFNγ treatment of ECs greatly induced the expression of CXCL9, CXCL10 and CXCL11 (Fig. 6). Those ECs under LF with a higher shear stress (25 dyne/cm^2) significantly suppressed the expression of CXCL9, CXCL10 and CXCL11 by more than 75%. In contrast, those ECs under LF with a lower shear stress (2 dyne/cm^2) had a minimal effect on the expression of CXC chemokines. Thus, LF shear force dependently exerts its protective effect by suppressing IFNγ-induced CXC chemokines. These results completely agree with the above-noted shear-force-dependent inhibition of IFNγ-induced STAT1 activation in ECs (Fig. 2B).

4. Discussion

At the vessel bifurcation of an arterial tree, disturbed flow accompanied by nonunidirectional and low shear flow

predisposes ECs to inflammation in which cytokines are involved, whereas steady LF exerts atheroprotective effects [31–33]. In this study, we have shown that LF suppresses IFNγ-induced endothelial responses. IFNγ is known to participate in many immunological and inflammatory responses and contributes to many pathological cellular events, such as apoptosis, cell cycle and innate/adaptive immunity [34,35]. IFNγ acts in concert with other cytokines, such as TNFα, IL-1β and IL6, to augment inflammatory responses. ECs lining the vessel wall directly encounter various cytokines released during local or systematic inflammation. We demonstrate that ECs under LF protect themselves from IFNγ-induced inflammatory responses. Several lines of evidence support this notion. First, LF suppresses IFNγ-induced phosphorylation of Tyr701 in STAT1, a requirement for STAT1 nuclear translocation [36]. Ser727 phosphorylation is required for the maximal transcriptional activation of STAT1 [36]. However, IFNγ-induced phosphorylation of Ser727 was not affected by LF, indicating the specificity of this inhibitory effect. Second, this inhibition by LF of Tyr701 phosphorylation is shear force dependent, i.e., higher shear forces lead to greater inhibition. Third, LF inhibits IFNγ-induced STAT1-DNA binding, which decreases STAT1-mediated transcriptional activity. Fourth, this inhibition is LF dependent, as shown by the decreases of CXC chemokines, CIITA expression, and CXCL10 protein secretion from ECs after LF treatment. Fifth, LF suppression of IFNγ-induced CXC chemokines appears to be shear force dependent. Furthermore, the suppressive effect of LF on IFNγ-induced CXCL10 expression was also observed in those ECs presheared for 30 min followed by IFNγ exposure during the subsequent 4 hours of shear flow (data not shown). This further substantiates the protective effect of LF on ECs exposed to cytokines. All these results support the notion that LF exerts its atheroprotective effects by suppressing cytokine-induced responses.

Our previous studies have indicated that LF attenuates serum- or IL-6-induced STAT3 activation in ECs [12,13]. Here, we further demonstrate that LF suppresses IFNγ-induced STAT1 activity in ECs. Interestingly, this suppression is shear force dependent. LF with lower shear stress, as compared to LF with higher shear stress, decreased its inhibitory effect on the phosphorylation of Tyr701 of STAT1. This result is consistent with the finding that ECs under lower shear stress show less atheroprotective effect than those ECs subjected to higher shear force [37–39].

LF, in contrast to disturbed flow, can attenuate IFNγ-induced STAT1 activation. The detailed protection mechanisms remain elusive. It is known that nitric oxide (NO) released as a result of eNOS activity is important in protecting ECs from inflammation. LF to ECs constantly activates eNOS via the PI3K/Akt pathway [6]. NO has been shown to impair IFNγ-induced STAT1 activation in macrophages [40]. IFNγ-induced JAK/STAT1 activity and expression of CXC chemokines and MHCII were all suppressed by treating ECs with NO [16,41]. The immediate release of NO upon eNOS activation by LF may be involved in this inhibitory effect of LF on IFNγ-induced STAT1 activity. Because IFNγ-induced tyrosine phosphorylation of STAT1 was significantly attenuated after the onset of LF, the protein tyrosine phosphatase (PTP) is likely involved. Suppression of IFNγ-induced STAT1 activity by LF was alleviated in the presence of sodium orthovanadate, a comprehensive phosphatase inhibitor (data not shown), supporting the participation of PTP in this protection effect by LF. LF also activates glutathione reductase and protects against oxidative stress [11]. Whether the change of redox status triggered by LF affects IFNγ-induced STAT1 activity remains to be determined and warrants further investigation.

LF to ECs suppresses the IFNγ-induced STAT1 target genes CXCL9, CXCL10 and CXCL11. These CXC chemokines bind to CXCR3 receptor on activated T cells and NK cells. Thus, LF exerts atheroprotective effects by suppressing these chemokines and may ultimately reduce T cell recruitment. IFNγ-induced CIITA expression was also suppressed by LF. CIITA is a master regulator for the expression of MHCII, which presents antigens to $CD4^+$ T cells, leading to T cell activation and differentiation. Atherosclerotic lesions contain activated T cells [16,23]. A decrease in CIITA may help to suppress T cell activation. Taken together, this study's findings demonstrate the key role of STAT1 in regulating endothelial responses to cytokine. LF exerts its atheroprotective effects by suppressing IFNγ-induced STAT1 activity, which decreases the expression of IFNγ-inducible CXC chemokines and of CIITA, possibly helping to suppress atherogenesis.

In addition to inflammation, recent evidence indicates that both innate and adaptive immune mechanisms are involved in atherogenesis [31,42,43], during which IFNγ plays an important role. Atherosclerotic lesions are frequently located on the outer walls of vascular bifurcations, where disturbed flow is located [3]. In contrast to the protective effect of LF, disturbed flow induces ECs to express higher levels of toll-like receptor 2 (TLR-2) and to be more sensitive to TLR-2 ligands [31]. Shear flow, with various flow patterns or forces to ECs, thus distinctly affects the signaling process and proteins' expression. The differential signaling mechanisms under LF may contribute to this protective effect. Our results clearly show a protective effect of LF on ECs exposed to IFNγ. Elucidation of these detailed protection mechanisms by flow may provide a basis for the development of new therapeutic treatments for atherosclerosis.

Acknowledgments

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