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Tumor Necrosis Factor-Induced Neutrophil Adhesion Occurs Via Sphingosine Kinase-1-Dependent Activation of Endothelial $\alpha_5\beta_1$ Integrin

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Leukocyte recruitment plays a major role in the immune response to infectious pathogens, as well as during inflammatory and autoimmune disorders. The process of leukocyte extravasation from the blood requires a complex cascade of adhesive events between the leukocytes and the endothelium, including initial leukocyte rolling, adhesion, and finally transendothelial migration. Current research in this area aims to identify the key leukocyte subsets that initiate a given disease and to identify the trafficking molecule(s) that will most specifically inhibit those cells. Herein we demonstrate that tumor necrosis factor (TNF)α activates the integrin $\alpha_5\beta_1$ without altering total expression levels of β_1 integrin on human umbilical vein endothelial cells. Moreover, our studies suggest that TNFα-induced β_1 activation is dependent on sphingosine kinase-1, but independent of the sphingosine-1-phosphate family of G protein-coupled receptors. We also show, using a parallel plate flow chamber assay, that neutrophil adhesion to TNFα-activated endothelium can be attenuated by blocking $\alpha_5\beta_1$ or its ligand angiopoietin-2. These observations add new complexities that broaden the accepted concept of cellular trafficking with neutrophil adhesion to TNFα activated endothelial cells being sphingosine kinase-1, $\alpha_5\beta_1$, and angiopoietin-2 dependent. Moreover, this work supports the notion that sphingosine kinase-1 may be the single target required for an effective broad spectrum approach to combat inflammation and immune disorders. ***(Am J Pathol 2010, 177:436–446; DOI: 10.2353/ajpath.2010.091016)***

To fulfill their surveillance function, leukocytes continuously patrol the human body, shuttling back and forth between the blood stream, the lymphatic fluid, secondary lymphoid organs, and peripheral tissues.[1] Leukocyte recruitment to sites of inflammation is critical for the development and maintenance of the immune response. During injury and pathogen invasion, inflammatory cytokines, such as tumor necrosis factor (TNF)α, are released to recruit leukocytes. However, excessive and remaining cytokines at these sites often result in prolonged inflammation, tissue damage, and disease. When leukocytes leave the blood stream, they undergo a sequential adhesion cascade to overcome both the high shear forces within the blood vessel and the tight seal of endothelial cells that line these vessels. The classical paradigm for leukocyte recruitment states that the selectin-family (ie, P-selectin, E-selectin, and L-selectin) uses transient interactions with carbohydrates to initiate tethering and rolling (reviewed in 2). Leukocyte arrest during rolling is rapidly triggered by chemoattractants (eg, chemokines) and is mediated by the binding of leukocyte integrins to immunoglobulin superfamily members, such as vascular and cellular adhesion molecule (VCAM)−1 and intercellular adhesion molecule (ICAM)−1, expressed by endothelial cells (ECs). This stabilization of the rolling leukocytes to the endothelium enables their emigration from the microvasculature. Undoubtedly, the diversity in selectivity and extent of leukocyte recruitment are regulated by the intrinsic complexity of pro-adhesive signaling networks expressed by the vasculature.

The family of integrins are significant contributors to leukocyte adhesion, with their qualitative and quantitative variations of expression and activation states. In the past

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decade, new insights have been gained in understanding the combination and activation of the 18α and 8β integrin subunit family members, which associate in pairs to form at least 24 $\alpha\beta$ receptors (reviewed in 3, 4). Moreover, modulation of integrin ligand affinity is now widely recognized as a crucial step in agonist-induced leukocyte arrest under flow.[5] Indeed, specific integrin blocking molecules are effective therapeutic strategies in multiple sclerosis and psoriasis as they modulate leukocyte trafficking.[6,7] However, their inability to provide absolute protection suggests that the precise mechanisms underpinning cellular recruitment remain incompletely understood.[8]

TNFα is one of the most pleiotropic cytokines involved in systemic inflammation and has been implicated in a multitude of pathologies including autoimmune disease, insulin resistance, and cancer (reviewed in 9). A major site for TNFα action is the vascular endothelium where it binds to membrane receptors and instigates a cascade of intracellular signaling events for EC production of cytokines and induction of adhesion molecule expression. TNFα also stimulates the activation of sphingomyelinase and sphingosine kinase (SK)−1, yielding sphingosine-1-phosphate (S1P) (reviewed in 10). Although most cells can synthesize S1P, large amounts are present in platelets,[11] and recent reports have identified erythrocytes as well as vascular endothelium as major contributors of S1P in circulation.[12–14] S1P can act extracellularly through the G protein coupled S1P receptors ($S1P_{1\text{-}5}$). Mature ECs express S1P receptors $S1P_{1\text{-}3}$ and these ligand/receptor interactions promote EC survival, migration, proliferation, adherens junction assembly, increased revascularization, and wound healing both *in vitro* and *in vivo* (reviewed in 15). However, S1P can also act intracellularly, possibly through histone deacetylases[16] or other as yet unknown binding partners, where the ablation of receptor signaling through both chemical or genetic mechanisms does not abrogate S1P effects on cell proliferation, Ca^{2+} mobilization, EC survival, nor the differentiation of endothelial progenitor cells.[10,17] SK-1 has two functional states, an intrinsic or basal state and an agonist-induced activated state, which requires its phosphorylation and is responsible for its oncogenic properties.[18] More recently we observed that SK-1 activates $\alpha_v\beta_3$ integrin to mediate EC survival signaling pathway via formation of a heterotrimeric complex between SK-1, $\alpha_v\beta_3$ and CD31.[19] In ECs, TNFα-induced up-regulation of E-selectin, VCAM-1 and ICAM-1 expression is an SK-1-dependent process.[20,21] Together, it is tempting to speculate that SK-1 may also contribute to integrin-mediated leukocyte adhesion under shear stress, and as such, act as a master switch for adhesion molecules and thus become a single target for therapeutic intervention.

In this study, we have examined the role of SK-1 in direct leukocyte adhesion under physiologically relevant shear flow. We show that although TNFα stimulation of human umbilical vein endothelial cells (HUVECs) does not alter total β_1 protein levels it significantly increases the formation of $\alpha_5\beta_1$ (very late antigen−5) and promotes angiopoietin (Ang)−2 expression at the EC surface. Furthermore, we hypothesize that an active conformation of β_1 integrin exists on these cells as identified by a unique anti-β_1 integrin monoclonal antibody, QE.2E5. This antibody was originally developed by Faull et al[22] following immunization of mice with lipopolysaccharide-activated HUVECs and binds to a site remote from that of the ligand, as well as other function-modifying β_1 antibodies. Importantly, QE.2E5 is itself without effect on the adhesive, proliferative, and tube-forming capabilities of both untreated and phorbol-12-myristate-13-acetate-treated HUVECs.[23] This study now proposes a novel mechanism of neutrophil adhesion under shear flow involving TNFα-induced $\alpha_5\beta_1$ activation and Ang-2 expression, which is SK-1-dependent.

Materials and Methods

Reagents and Antibodies

Murine monoclonal antibodies directed against β_1 (QE.2E5, 61.2C4, and 58.7H2), VCAM-1 (51.10C9) and E-selectin (68.5H11) were used along with isotype control (23.1F11; gifts, Prof Jennifer Gamble, Centenary Institute, NSW, Australia); α_5 (P1D6), α_2 (P1E6), PECAM-1 (M-20), and Ang-2 (N-18) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); $\alpha_5\beta_1$ (BMC5 and MAB1999), $\alpha_v\beta_3$ (LM609), and actin were from Chemicon International Inc (Temecula, CA); VCAM (BBIG-V1) and ICAM (BBIG-I1) were purchased from R&D Systems, Inc. (MN); $\alpha_5\beta_1$ (BMC5), ERK (137F5), and P-ERK (197G2) were purchased from Cell Signaling Technologies (Danvers, MA). Secondary anti-goat Alex-555, anti-mouse Alexa-488 and -594 antibodies were purchased from Invitrogen (Carlsbad, CA), and anti-mouse-, goat-, and rabbit-HRP were purchased from Pierce (Rockford, IL). Human recombinant TNFα was purchased from R&D Systems. Small interfering (si)RNA against human Ang-2 was purchased from Invitrogen (Mount Waverley, Victoria, Australia).

Cells and Cell Culture

HUVECs were grown in M199 medium containing 20% fetal calf serum (JRH, Brooklyn, Victoria, Australia) and endothelial growth factor supplement (BD BioSciences, North Ryde, NSW, Australia) as previously described.[24] Cells were used at passage two or less.

Flow Cytometry

Expression of β_1, α_5, $\alpha_5\beta_1$, VCAM-1, and E-selectin were determined on HUVECs with one-color flow cytometry using 1 μg of antibodies described above added to the cells at 4°C for 30 minutes. The secondary antibody was anti-mouse Alexa 488 at 1:1000 dilution. The median fluorescence intensity was determined using a Beckman Coulter XL-MCL (Gladesville, NSW, Australia).

SK Activity Assay

SK activity was determined as previously described.[25] D-Erythro sphingosine (Biomol, Plymouth Meeting, PA) solubilized by 0.05% Triton X-100 and [γ^{32}P]ATP (Perkin Elmer, Victoria, Australia) were used as substrates to incubate with the whole cell lysates. The radioactively labeled phospholipid was resolved by two thin-layer chromatography (Sigma, St. Louis, MO) separations in the solvents containing butanol, ethanol, water, and acetic acid (8:2:2:1 ml). The radioactive spots were quantified by Phosphorimaging Typhoon 9410 (Fullerton, CA) and ImageQuant 5.2 program (GE Health care, Rydalmere, NSW, Australia).

Inhibition Studies Using Human SK-1 Adenovirus Transduction

Generation of wild-type human SK-1 wild-type (SK-1) and mutants possessing an aspartate at Glycine82 (SK-1-DN) have been made as previously described.[26] For infection with adenoviral constructs, HUVECs were exposed to one plaque forming unit/cell for 2 hours in M119 medium with 2% fetal calf serum and a further 72 hours with medium containing 20% fetal calf serum. Cells were infected with a dose of virus previously determined to lead to a five- to tenfold increase in SK-1 activity and the same dose of control EV adenovirus was used.[17]

Immunofluorescence Microscopy

Untreated or adenovirus infected EV, SK-1, and SK-1-DN HUVECs were replated at 5×10^4 cells/well in LabTek chamber slides (Nunc, NY) with fibronectin coated at 50 μg/ml. Confluent cells were fixed with 4% paraformaldehyde at room temperature for 15 minutes before blocking with 2% bovine serum albumin/PBS at room temperature for 30 minutes. Activated β_1 (QE.2E5), $\alpha_5\beta_1$ (BMC5), Ang-2, or control antibodies (1 μg) were added to cells at 4°C overnight followed by anti-mouse Alexa 488 or anti-goat Alexa 594 antibodies (1:1000) incubation at room temperature for 1 hour. The slides were mounted with anti-fading agents (Biomeda Corp, CA) before visualization by fluorescence microscopy (Olympus IX2-UCB, Olympus, Mt Waverley, Victoria, Australia). Five images, with four cells per image, were collected per sample and analyzed using Analysis LifeSciences software (Olympus).

S1P Receptor Activation and Inhibition Studies

S1P receptor activation and inhibition studies used S1P (1 μmol/L, Cayman Chemical Co., Ann Arbor, MI), JTE-013 (1 μmol/L, Cayman Chemical Co.), and VPC23019 (10 μmol/L, Avanti Polar Lipids Inc., Alabaster, AL), which were administered either alone (for S1P) or 30 minutes before TNFα stimulation of cells (5 ng/ml, 5 hours). All reagents were proven functionally effective in paralleled human umbilical vein EC studies.[17]

Parallel Plate Flow Chamber Assay

Confluent HUVECs were cultured on Corning petri dishes (Sigma) and treated with or without 5 ng/ml TNFα for 5 hours. Control, blocking antibodies (10 μg/ml), or SK inhibitor SKi (1 μmol/L, Cayman Chemical Co.) were added for 30 minutes before cell perfusion. Using published methods, heparinized whole blood was diluted 1:10 in Hank's balanced salt solution (Sigma)[27] or used to isolate neutrophils[28] and cells (diluted blood or 1×10^6 isolated neutrophils) were perfused across the substratum by a syringe pump (NE-1000, New Era Pump System, Inc, Wartagh, NY) at a constant rate of 2 dynes/cm^2 for 5 minutes followed by Hank's balanced salt solution wash. Interactions of unlabeled cells were visualized using $\times$10/0.3 NA objectives and phase-contrast microscopy on an inverted microscope and images were recorded using a digital camera (Olympus IX70 and SIS F-view, Olympus). Five random areas per dish were recorded for analysis using AnalySIS Life Sciences software (Olympus). Adherent cells were defined as those remaining stationary for at least 10 seconds. Dishes were stained using the using May-Grunwald Giemsa (Sigma) according to the product protocol to identify leukocyte subsets.

siRNA Transfection

Adapted from methods previously described,[29] Ang-2 siRNA (50 nmol/L) (HSS100480: 5′-AAUACUUCAGCACAGUCUCUGAAGC-3′;HSS100482:5′-UUCUCCUGAAGGGUUACCAAAUCCC-3′) were transfected into HUVECs using HiPerfect (Qiagen, Doncaster, Victoria, Aust.) in EGMII medium (Clonetics/Lonza, Basel, Switzerland) when cells were at 60% confluency. After 24 hours, culture media were changed to the aforementioned tissue culture media until cells were harvested at 48 hours post-transfection.

Immunoprecipitation and Immunoblotting

HUVECs were harvested and lysed in lysis buffer containing 1% NP40 and sonicated for immunoprecipitation. Following the manufacturers instructions, cell lysates were incubated with $\alpha_5\beta_1$ (MAB199) or Ang-2 (N-18) antibody and protein A magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) on ice for 30 minutes before selection by magnetic columns (Miltenyi Biotech). Whole lysates or immunoprecipitates were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to Hybond-P (Amersham Biosciences, NJ). Primary antibodies to phosphorylated ERK (197G2), total ERK (137F5), Ang-2 (N-18), or β_1 (58.7H2) and secondary antibodies were used to probe the membranes before visualization by enhanced chemiluminescence (GE Health Science, Piscataway, NJ) and a luminescent image analyzer (LAS4000, Fujiflim; Stamford, CT).

Statistical Analysis

Data are shown as mean $\pm$ SEM and statistically analyzed by Student's *t*-test, 1- or 2-way analysis of variance

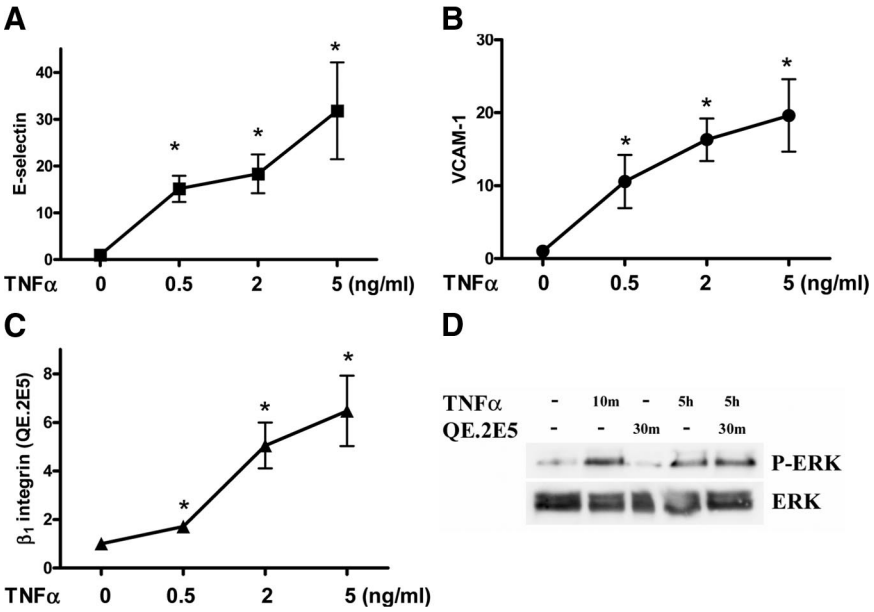

Figure 1. TNFα induced E-selectin, VCAM-1, and β_1 integrin expression on ECs. HUVECs were treated with TNFα (0, 0.5, 2, or 5 ng/ml, four hours) and examined for E-selectin (**A**), VCAM-1 (**B**), and activated β_1 integrin (**C**) using the QE.2E5 Ab using flow cytometric analysis. Results are normalized to untreated control and expressed as the mean ± SEM of seven experiments; $^*P < 0.05$ versus untreated controls (0). In **D**, HUVECs treated without and with TNFα for ten minutes or five hours without or with QE.2E5 (30 minutes at 4°C) before immunoblotting equal lysate amounts for P-ERK and total ERK. Results are representative of three experiments.

for multiple comparisons. $P < 0.05$ was considered as significant.

Results

TNFα Activates β_1 Integrin on ECs

With a similar profile to that observed for TNFα-induced E-selectin and VCAM-1 expression, Figure 1, A–C shows a dose-dependent increase in β_1 integrin on HUVECs. As these flow cytometric data were obtained using the β_1 integrin QE.2E5 antibody, we propose that this increase in β_1 integrin may in fact represent an increase in β_1 activation. The concept of QE.2E5 binding to activated β_1, without causing activation, stems from it originally being developed against activated HUVECs[22] and previous observations of it being without effect on the adhesive, proliferative, and tube-forming capabilities of both untreated and phorbol-12-myristate-13-acetate-treated HUVECs.[23] To confirm that QE.2E5 does not activate HUVECs, we examined phosphorylation of ERK, an integrin-activated downstream signaling molecule. As shown in Figure 1D, addition of QE.2E5 to HUVEC for 30 minutes at 4°C did not phosphorylate ERK. By contrast, ERK is phosphorylated by TNFα at both 10 minutes and 5 hours (Figure 1D).

TNFα-Induced Activation of β_1 Integrin Is Sphingosine Kinase-1-Dependent

To study the underlying mechanism of TNFα-induced β_1 integrin activation we investigated a role for SK-1. First, TNFα-induced activation of SK-1 in ECs was confirmed using an enzymatic assay. As shown in Figure 2, TNFα activates SK-1 in a biphasic pattern with significant increases observed at 10 minutes and 4 to 6 hours after TNFα treatment. We next examined a role for SK-1 in β_1 integrin activation using an adenoviral delivery system to either overexpress SK-1 or knock down SK-1 catalytic activity with the dominant negative SK-1 mutant (SK-1-DN).[26,30] Data shown as supplemental Figure S1 (at *http://ajp.amjpathol.org*) demonstrate that both SK-1 and SK-1-DN infected ECs exhibit a significant increase in SK-1 protein when compared with empty vector (EV) controls but that only SK-1 infected ECs express catalytically active SK-1 above control levels (see supplemental Figure S1, A and B, at *http://ajp.amjpathol.org*). Moreover, ECs overexpressing SK-1 exhibited a further increase in SK activity following TNFα stimulation for 4 hours. This was not observed in the SK-1-DN cells (see supplemental Figure S1B at *http://ajp.amjpathol.org*).

Flow cytometric analysis (using the QE.2E5 antibody) suggests that levels of activated β_1 integrin in untreated ECs do not differ between EV controls and SK-1 or SK-1-DN overexpressing cells (Figure 3B). Following TNFα activation, levels of active β_1 in EV-infected ECs increased approximately sevenfold. This increase was fur-

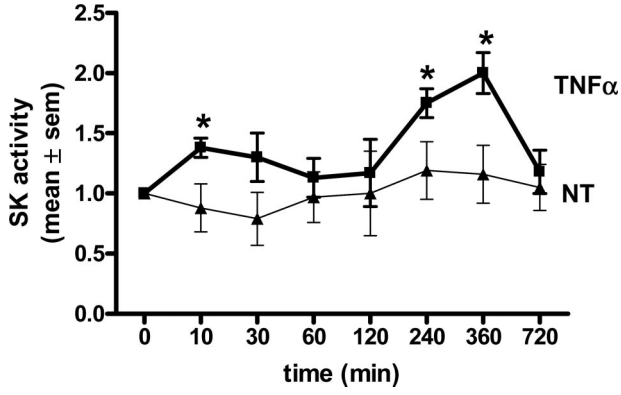

Figure 2. SK activity in ECs stimulated with TNFα. Equal lysate amounts from HUVECs treated without (triangle, NT) and with TNFα (square) (5 ng/ml: 10, 30, 60, 120, 240, 360, and 720 minutes) were assessed for SK activity by enzymatic assay. Results are the mean ± SEM of $n = 4$. $^*P < 0.05$ versus NT controls.

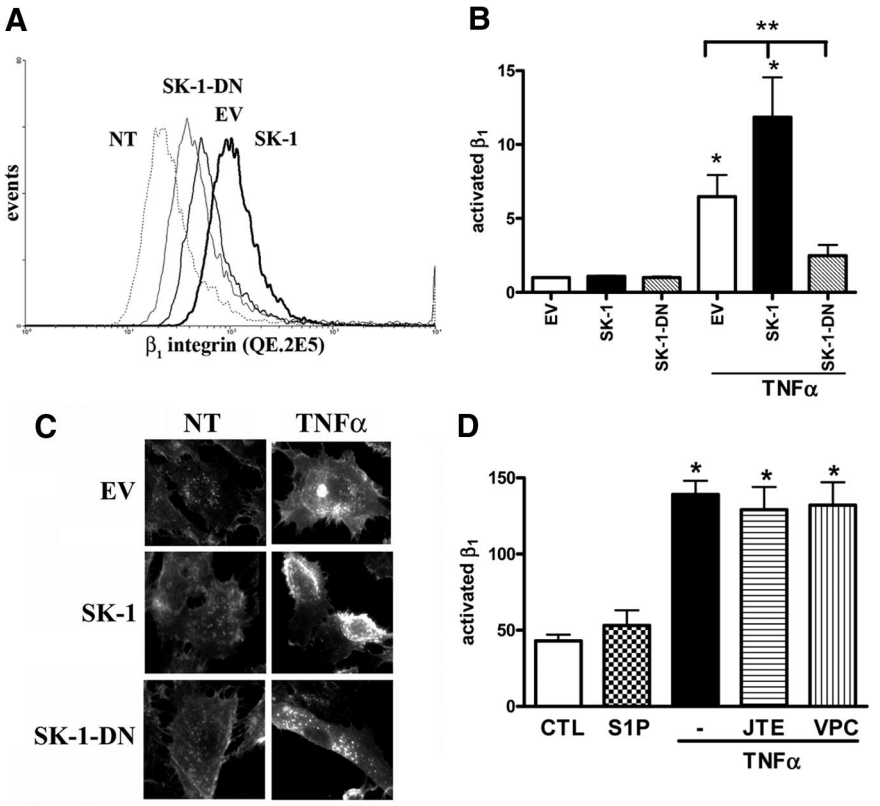

Figure 3. TNFα induces β_1 integrin activation in ECs overexpressing SK-1. HUVECs overexpressing SK-1, SK-1 dominant negative mutant (SK-1-DN), or EV control were stimulated without and with TNFα (5 ng/ml, 4 hours) and assessed for β_1 activation. In **A**, trace files show a representative experiment with untreated (NT) controls and TNFα treated EV, SK-1, and SK-1-DN. In **B**, flow cytometric analysis of QE.2E5 stained pooled experiments as the mean ± SEM, $n = 7$. $^*P < 0.05$ versus untreated control; $^{**}P < 0.05$ versus TNFα treated EV. In **C**, immunofluorescence and confocal microscopy using the QE.2E5 Ab in NT and TNFα treated EV, SK-1, and SK-1-DN cells. In **D**, HUVECs were cultured without or with JTE-013 (1 μmol/L) or VPC23019 (10 μmol/L) 30 minutes before S1P (1 μmol/L, 4 hours) or TNFα (5 ng/ml, 4 hours) stimulation. β_1 activation on ECs was assessed using immunofluorescence and mean fluorescence intensity quantified. Results are the mean ± SEM of four cells/view, five views per sample, and three experiments. $^*P < 0.05$ versus untreated control.

ther augmented in the ECs overexpressing SK-1 and not observed in the SK-1-DN ECs, which exhibited significantly less active β_1 integrin than both EV and SK-1 ECs (Figure 3, A and B). These results were supported by fluorescence microscopy, wherein increased active β_1 integrin was observed on the cell surface and edges of TNFα-treated EV control cells. This effect was augmented in TNFα-treated ECs overexpressing SK-1 and absent in the TNFα-treated SK-1-DN cells (Figure 3C).

We next investigated whether the family of S1P receptors were involved in activation of β_1 integrin, using two methods. First, ECs cultured with 1 μmol/L S1P, a concentration known to result in receptor-mediated signaling events,[20] exhibited no change in β_1 activation (Figure 3D). Second, chemical inhibition of the three S1P receptors identified on ECs (ie, $S1P_1$, $S1P_2$, and $S1P_3$)[31] using JTE-013 and VPC23019 previously shown to inhibit S1P-induced SK-1 activation in HUVECs[17] failed to inhibit TNFα-induced activation of β_1 integrin (Figure 3D).

Together, these results suggest that TNFα activates β_1 integrin at the cell surface, that SK-1 is integral to this process, and that this occurs independently of $S1P_{1\text{-}3}$.

Activation of β_1 Integrin by TNFα Does Not Correlate with Increased β_1 Integrin Protein

We next investigated whether TNFα-induced activation of β_1 integrin coincided with an increase in β_1 protein levels at the cell surface. Using flow cytometry and the 58.7H2 β_1 antibody, which does not specifically identify an activated conformation, we observed that the surface expression levels of β_1 integrin in EV, SK-1, and SK-1-DN were similar without and with TNFα treatment (Figure 4A). These observations were confirmed with a second antibody that recognizes a ligand binding site of β_1 integrin (61.2C4; Figure 4B). In addition to surface expressed β_1 integrin levels, we investigated total β_1 protein levels via immunoblotting. With a representative immunoblot shown in supplemental Figure S2 (at *http://ajp.amjpathol.org*) and pooled experimental results shown in Figure 4C; EV, SK-1, and SK-1-DN expressing ECs exhibit similar levels of β_1 integrin protein both without and with TNFα treatment. Together, these results suggest that neither TNFα treatment nor SK-1 overexpression altered total β_1 integrin protein levels either within or on the surface of ECs.

TNFα Activates $\alpha_5\beta_1$ Co-Association in ECs

To identify the α integrin subunit partnering β_1 following TNFα activation we performed immunoprecipitation assays using TNFα-treated EC lysates and the activated β_1 integrin antibody QE.2E5. As shown in Figure 5A, immunoblots identified a close association between β_1 and α_5 in untreated as well as TNFα-treated ECs. Notably, from five experiments we observed an average of 18 ± 5% increase in α_5 association with β_1 integrin following TNFα stimulation. Despite previous studies reporting α_2 subunit associating with β_1 in TNFα-treated ECs, we were unable to detect this association following activated β_1 (QE.2E5) antibody pull-down (Figure 5A). Flow cytometric analysis confirmed a significant increase in surface expression

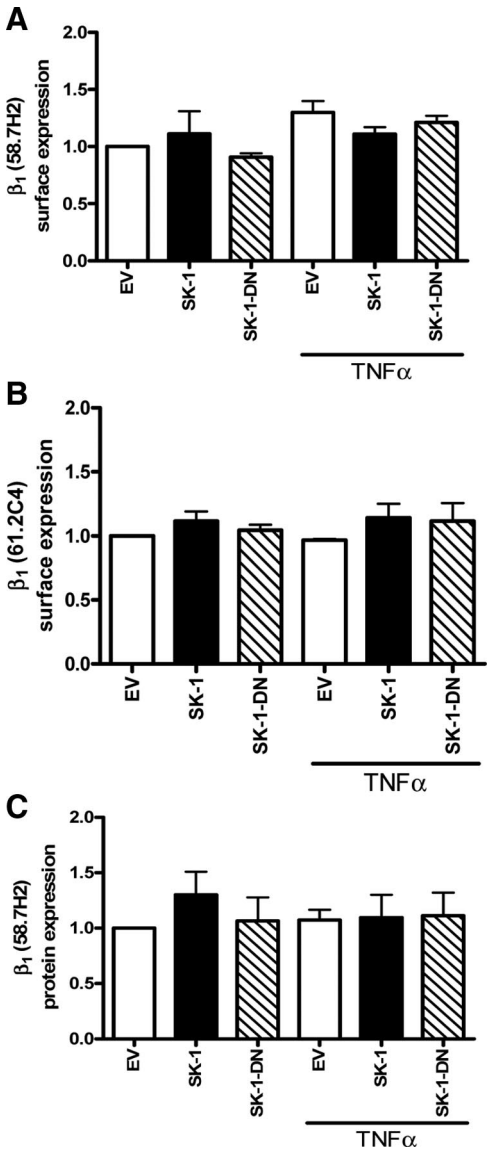

Figure 4. β_1 integrin surface expression and protein levels in ECs in response to TNFα treatment. HUVECs overexpressing SK-1, SK-1 dominant negative mutant (SK-1-DN), or EV control, were stimulated without and with TNFα (5 ng/ml, four hours) and assessed for β_1 expression by flow cytometric analysis using 58.7H2 and 61.2C4 Abs (**A** and **B**) and immunoblotting using the 58.7H2 Ab (**C**). Results are expressed as the mean ± SEM of three experiments (**A** and **B**) or four experiments (**C**).

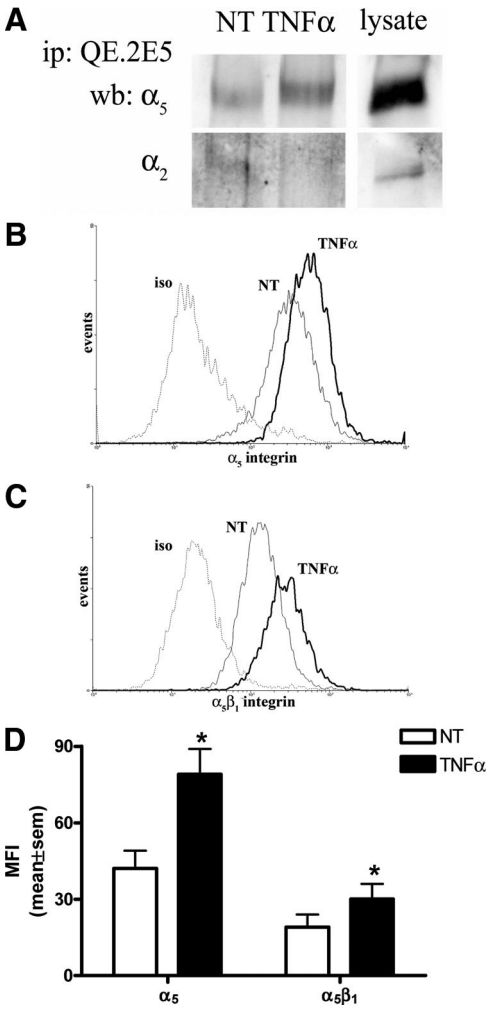

Figure 5. TNFα activated β_1 integrin is co-associated with α_5. HUVECs were treated without and with TNFα (5 ng/ml, four hours) before immunoprecipitation of equal lysate amounts with QE.2E5 to pull down activated β_1 integrin (**A**). Precipitates were examined for β_1 co-association with α_5 and α_2 integrin via immunoblotting. Gel lanes from the same experiment have been cut and re-orientated to better illustrate the whole HUVEC lysate positive controls (lysate). Results are representative of five experiments. In **B** and **C**, HUVECs were treated without (NT) and with TNFα (5 ng/ml, four hours) before flow cytometric analyses for α_5 and $\alpha_5\beta_1$ integrin surface expression, respectively, and are a representative of three experiments relative to isotype control (iso). In **D,** similar experiments were quantified for mean fluorescence intensity (MFI) of α_5 and $\alpha_5\beta_1$ surface expression. Results are mean ± SEM, $n = 3$; $^*P < 0.05$ versus NT.

of α_5, as well as $\alpha_5\beta_1$, on TNFα-treated HUVEC (Figure 5, B–D).

TNFα-Induced Activation of $\alpha_5\beta_1$ on HUVECs Mediates Leukocyte Adhesion

To investigate the function of TNFα-induced activation of β_1 integrin on ECs, we examined its role in leukocyte adhesion by a parallel plate flow chamber assay. As shown in Figure 6A (and supplemental Video S1, at *http://ajp.amjpathol.org*), when human blood was perfused over untreated HUVECs at a rate of 2 dynes/cm^2, negligible leukocyte adhesion was observed. By contrast, when stimulated with TNFα, a significant increase in leukocyte adhesion occurred. When TNFα stimulated ECs were exposed to a β_1 blocking antibody (61.2C4) 30 minutes before flow chamber assay, the number of adherent cells was significantly reduced. A similar profile of reduced leukocyte adhesion was also observed with an α_5 blocking antibody. No change was observed with either an isotype control or a blocking antibody to α_2 (confirmed to inhibit ECs attachment to collagen) (Figure 6A). A role for SK in TNFα-induced leukocyte adhesion was confirmed in experiments wherein an SK inhibitor, SKi, was added 30 minutes before flow chamber assay (Figure 6A). These results suggest that TNFα-induced leukocyte adhesion to ECs is, at least in part, mediated by the integrin $\alpha_5\beta_1$ and the SK/S1P pathway. The role for α_5 and β_1 combined in leukocyte adhesion was next examined us-

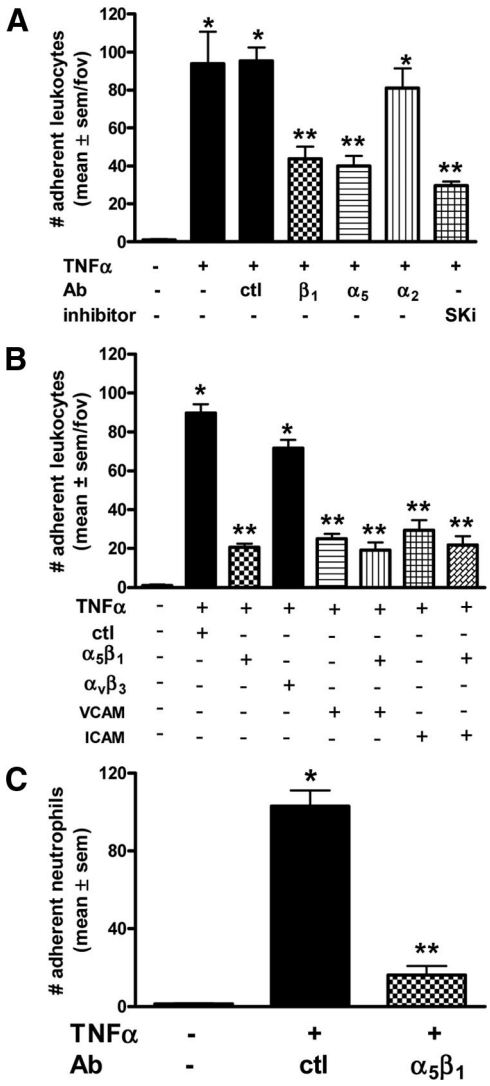

Figure 6. Leukocyte adhesion to TNFα-activated ECs under shear flow. Adhesion of leukocytes on HUVECs stimulated without and with TNFα (5 ng/ml, four to five hours) using the parallel plate flow chamber assay at a constant shear rate of 2 dynes/cm^2 and pre-incubation of ECs in **A** with control (ctl) or blocking Abs to β_1, α_5, α_2, or the SK inhibitor (SKi, 1 μmol/L), for 30 minutes prior. In **B**, blocking Abs to $\alpha_5\beta_1$, $\alpha_v\beta_3$, VCAM, and ICAM for 30 minutes prior. In **C**, isolated neutrophils are perfused (1×10^6/ml) as above and pre-incubation of ECs with control (ctl) or blocking Abs to $\alpha_5\beta_1$ for 30 minutes prior. Data are expressed as the mean ± SEM per field of view (fov) with four to five fov captured for three to four separate experiments. *$P < 0.05$ relative to untreated (−) controls; **$P < 0.05$ relative to TNFα stimulated adhesion.

ing the $\alpha_5\beta_1$ blocking antibody (BMC5). As shown in Figure 6B, administration of the $\alpha_5\beta_1$ antibody significantly reduced leukocyte adhesion to TNFα-treated HUVECs. This inhibition was not observed with the $\alpha_v\beta_3$ blocking antibody (LM609) (Figure 6B), an integrin also activated by SK-1.[19] As TNFα is also known to regulate VCAM-1 and ICAM-1 expression on HUVECs via SK-1,[20] we next investigated their contribution to $\alpha_5\beta_1$-mediated leukocyte adhesion. As shown in Figure 6B, antibodies to $\alpha_5\beta_1$, VCAM-1 and ICAM-1, alone, or together, demonstrated a similar level of inhibition.

May-Grunwald Giemsa staining of the dishes at completion of the aforementioned experiments identified neutrophils as the leukocyte subset targeted by $\alpha_5\beta_1$ for adhesion (data not shown). This was confirmed with isolated neutrophils in flow chamber assays. As shown in Figure 6C, when TNFα-stimulated ECs were exposed to the $\alpha_5\beta_1$ blocking antibody (BMC5) 30 minutes before flow chamber assay, the majority of neutrophils could no longer adhere.

Angiopoietin-2 Associates with $\alpha_5\beta_1$ to Mediate TNFα-Induced Leukocyte Adhesion

Previous studies have shown that Ang-2 production by ECs is significantly increased following TNFα stimulation.[32] More importantly for this study, Ang-2 is a known ligand for $\alpha_5\beta_1$ on ECs.[33] Increased Ang-2 expression by ECs following TNFα activation was first confirmed by fluorescence microscopy on HUVECs overexpressing SK-1, SK-1-DN, or EV control. As shown in supplemental Figure S3A (at *http://ajp.amjpathol.org*), untreated ECs exhibit an Ang-2 expression that is low and diffuse over the cell surface. This expression increases in a similar pattern following TNFα activation. Figure 7A shows quantitation of the immunofluorescence data and suggests that TNFα promotes Ang-2 surface presentation by approximately fourfold. When ECs overexpress SK-1, TNFα-mediated Ang-2 expression is further increased. This effect is not observed in the SK-1-DN ECs where TNFα-induced Ang-2 expression is significantly abrogated (Figure 7A).

As Ang-2 is a known ligand for $\alpha_5\beta_1$ on ECs[33] we next investigated their endogenous association in ECs without and with TNFα activation. Immunoprecipitations targeting Ang-2 identified its close association with β_1 integrin (Figure 7B) with repeated experiments suggesting an increase of approximately 10% following TNFα activation. Reciprocal immunoprecipitations targeting $\alpha_5\beta_1$ and immunoblotting against Ang-2 confirmed an increase in $\alpha_5\beta_1$:Ang-2 complex formation in ECs following TNFα activation. From repeated experiments, an average of 21 ± 12% increase in Ang-2 association with $\alpha_5\beta_1$ integrin occurred following TNFα activation.

We next investigated whether Ang-2 is involved in leukocyte adhesion under flow using Ang-2 specific siRNAs. Immunoblots first confirmed a significant reduction in Ang-2 protein levels when compared with ECs transfected with a negative control siRNA (see supplemental Figure S3B at *http://ajp.amjpathol.org*). Importantly, when ECs are depleted of Ang-2 by siRNA the number of adherent leukocytes under flow is significantly reduced when compared with the control siRNA-transfected ECs (Figure 7C and supplemental Video S2 at *http://ajp.amjpathol.org*). These results were confirmed using a second Ang-2 siRNA and Giemsa May-Grunwald staining of the dishes supported previous data of neutrophils being targeted by this process. Finally, Figure 7D shows immunofluorescence staining following flow chamber assay for $\alpha_5\beta_1$ (green) and Ang-2 (red), which indicate similar expression patterning on TNFα-treated HUVECs. The close

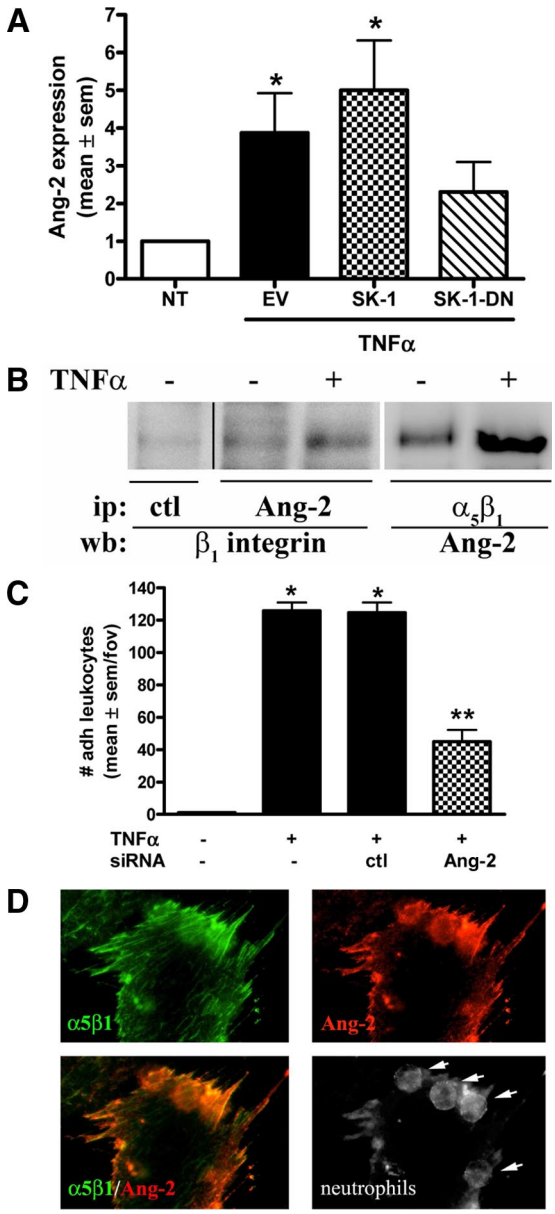

Figure 7. Ang-2 expression in TNFα-treated ECs and leukocyte adhesion under shear flow. In **A**, HUVECs overexpressing SK-1, SK-1 dominant negative mutant (SK-1-DN), or EV control were stimulated without (NT) and with TNFα (5 ng/ml, four hours) and quantified for Ang-2 surface expression by immunofluorescence. Results are the normalized mean ± SEM of approximately 100 cells from a total of five experiments; $^{*}P < 0.05$ versus untreated controls (NT). In **B**, equal lysate amounts from HUVECs treated without and with TNFα (5 ng/ml, four hours) were immunoprecipitated for Ang-2 or $\alpha_5\beta_1$. Precipitates were examined for $\alpha_5\beta_1$ and Ang-2 co-association via immunoblotting. Gel lanes from the same experiment have been cut and re-orientated to better illustrate the changes from isotype control Ab (ctl) ip. Results are representative of five experiments. In **C**, HUVECs containing siRNA to Ang-2 or ctl were stimulated without and with TNFα (5 ng/ml, four to five hours) and assessed for leukocyte adhesion by flow chamber assay at a constant shear rate of 2 dynes/cm^2. Data are expressed as the mean ± SEM per field of view (fov) with four to five fov captured for five separate experiments. $^{*}P < 0.05$ relative to untreated (−) controls; $^{**}P < 0.05$ relative to TNFα stimulated ctl siRNA cells. In **D**, $\alpha_5\beta_1$ (green) and Ang-2 (red) surface expression, as well as neutrophil localization (**arrows**) were viewed by immunofluorescence in HUVECs treated with TNFα (5 ng/ml, four hours) by confocal microscopy.

association of these two proteins is further supported by the merged image in Figure 7D ($\alpha_5\beta_1$/Ang-2) where $\alpha_5\beta_1$ and Ang-2 clearly overlay. Furthermore, a change in focal plane of this image identified the localization of neutrophils in close proximity to $\alpha_5\beta_1$ and Ang-2 at the cell junction (Figure 7D, neutrophils, arrows). Together these results support our hypothesis that TNFα-induced neutrophil adhesion to ECs under flow is mediated by a complex of active $\alpha_5\beta_1$ and Ang-2.

Discussion

Inappropriate activation or recruitment of leukocytes has been implicated in the pathogenesis of various inflammatory diseases (reviewed in 2). Herein we demonstrate that TNFα induces $\alpha_5\beta_1$ activation via an SK-1-dependent pathway in HUVECs. We show that SK-1 overexpression in ECs, to levels of activity equivalent to that seen after vascular endothelial growth factor or other growth factor stimulation, sensitizes the cells to TNFα and has a crucial role in $\alpha_5\beta_1$ integrin activation and neutrophil adhesion under shear flow. Evidence for this comes from firstly, the binding of the QE.2E5 antibody, previously shown to bind to activated β_1 integrin via a conformation sensitive epitope[22] when SK-1 is overexpressed. Notably, this TNFα-induced activation of β_1 does not coincide with increased protein levels but does correlate with increased expression of α_5 as well as formation of $\alpha_5\beta_1$, as demonstrated by immunoblotting and flow cytometric analysis of TNFα-treated ECs. TNFα-induced $\alpha_5\beta_1$ activation in ECs promotes neutrophil adhesion under physiologically relevant shear flow and this occurs in an SK-1-dependent, $S1P_{1\text{-}3}$-independent, manner. Finally, in response to TNFα activation, ECs exhibit an SK-1-dependent, increased surface expression of Ang-2, which is biologically important for neutrophil adhesion.

Herein, we propose that TNFα treated HUVEC exhibit increased activation of β_1 integrin. This concept is primarily derived from increased binding of the QE.2E5 monoclonal antibody, which was originally developed against lipopolysaccharide-activated HUVEC.[22] QE.2E5 is unique in that it binds to β_1 integrin at amino acid residues 426-587, a site 194 amino acids distal from the binding site of ligands and other function-modifying β_1 antibodies.[22] Our data support the notion that QE.2E5 identifies activated β_1 integrin on HUVECs with 1) TNFα-activated HUVECs expressing more $\alpha_5\beta_1$ complex at their cell surface, 2) no change in β_1 surface expression or protein using antibodies 58.7H2 and 61.2C4, and 3) increased binding of Ang-2 to $\alpha_5\beta_1$ on TNFα-treated HUVEC. With the assumption that β_1 integrin does not exist without an α partner at the cell surface, this implies that the TNFα-induced $\alpha_5\beta_1$ formation is a result of β_1 switching from another integrin heterodimer. The integrin from which this β_1 subunit is derived is yet to be determined with $\alpha_2\beta_1$ a likely candidate (Figure 5A). Interestingly, despite QE.2E5 previously shown to activate β_1 integrin function on the erythroleukemic cell line K562[22] and thymocytes[34] it remains without effect on the adhesive, proliferative, and tube-forming capabilities of both untreated and phorbol-12-myristate-13-acetate-treated HUVECs,[23] as well as ERK activation on TNFα-treated HUVECs (Figure 1). The physiological significance of such alternative conformational effects by QE.2E5 on

different cell types is uncertain. It is tempting to speculate that the lipopolysaccharide-activated HUVECs, first used to develop QE.2E5, underwent an inside-out signaling event, which altered the conformation of β_1 integrin at residues 426–587, and as such, identifies agonist induced β_1 activate conformation specific to HUVECs.

Despite our significant progress in the knowledge of the neutrophil adhesion cascade, there are still several caveats in our understanding. In particular, the relative importance of each adhesion receptors in response to specific stimuli for tissue- and vascular bed-specific inflammatory cell recruitment needs to be established. Some light may have been shed on this issue by previous studies, which demonstrated a role for $\alpha_5\beta_1$ in the migration of neutrophils,[35–37] as well as the adhesion of monocytes.[38] These studies clearly suggest that leukocyte interactions with the endothelium may rely on the assembly of complexes, which contain different adhesion molecules. Our observations that inhibiting β_1 abrogated, in part, neutrophil adhesion to TNFα-treated HUVEC under shear flow is in keeping with other studies demonstrating the contribution of TNFα-induced expression of VCAM-1 and ICAM-1 to leukocyte adhesion (reviewed in 39). Furthermore, when $\alpha_5\beta_1$ is blocked in addition to VCAM-1 and ICAM-1 a slight, but insignificant reduction in neutrophil adhesion was observed. The full relationship between $\alpha_5\beta_1$ and other TNFα-induced adhesion molecules is still to be fully elucidated, but our work suggests that an interrelationship between these molecules may exist. Given the wide distribution of integrins throughout the body, the need for stringent regulation of their interactions might be well anticipated. Indeed, studies to date suggest that a certain threshold level of integrin expression, as well as conversion to the active form, is required to engage ligands—a conversion that is cell type- and stimuli-specific. Such selectivity is exemplified by opioids that trigger $\alpha_5\beta_1$ integrin-mediated monocyte adhesion[38] and f-Met-Leu-Phe, but not leukotriene B4 activation of $\alpha_5\beta_1$ for neutrophil adhesion to fibronectin.[40] In further support, studies suggest that the activated form of $\alpha_5\beta_1$ is a transient property of activated ECs, and can therefore be used only within a confined time frame (reviewed in 3). Our data now extend this work by suggesting that TNFα activates the endothelium for $\alpha_5\beta_1$-dependent neutrophil adhesion under shear flow.

Ang-1 and Ang-2 are structurally related endothelial growth factors found on the extracellular surface of ECs with Tie-2 considered to be their principle cell surface receptor.[41] While Ang-1 mediates vessel maturation and maintains vessel integrity, Ang-2, in contrast, is classically considered as a functional antagonist of Ang-1 and binds to Tie-2 without inducing signal transduction.[42] Mice transgenically overexpressing *Ang-2* have an embryonic lethal phenotype that essentially phenocopies *Ang-1*-deficient and *Tie-2*-deficient phenotypes.[41–43] Interestingly, genetic ablation of Ang-2 is also lethal with postnatal mortality of newborn pups occurring by day 14. However, this lethality is entirely dependent on the genetic background of the mice with the 129/J and C57BL/6 strains being more and less susceptible, respectively.[44,45] Ang-2 acts by an autocrine mechanism and is stored in Weibel-Palade bodies from where it can be rapidly released on stimulation.[46] A role for Ang-2 in leukocyte recruitment is evidenced by Fiedler and colleagues who recently showed that *Angpt-2−/−* mice could not elicit an inflammatory response in thioglycollate-induced or *Staphylococcus aureus*-induced peritonitis, or in the dorsal skin fold chamber model.[45] Furthermore, intravital microscopy showed normal TNFα-induced leukocyte rolling but not adhesion in the vasculature of *Angpt-2−/−* mice. Cellular experiments also suggest that Ang-2 promotes cell adhesion by modulating TNFα-induced expression of the EC adhesion molecules ICAM and VCAM.[45] Finally, a close association between the angiopoietins and $\alpha_5\beta_1$ has previously been demonstrated in pull-down assays and is suggested to be Tie-2 independent.[47,48] Our study supports these observations with TNFα-induced neutrophil recruitment being both $\alpha_5\beta_1$- and Ang-2-dependent and that a relationship with VCAM-1 and ICAM-1, but not $\alpha_v\beta_3$, may exist.

SK-1 can exist in a basal, intrinsic state that we have shown inhibits EC permeability.[29] SK-1 also has an agonist-induced activated state, which occurs, at least in response to TNFα and phorbol ester, as a direct consequence of phosphorylation at serine 225 by ERK1/2.[30] The effects of this single phosphorylation are two-fold: it is required for agonist induced increases in the catalytic activity of SK-1, and it is necessary for translocation of this protein from the cytosol to the plasma membrane.[18] We have recently demonstrated a requirement of SK-1 phosphorylation at serine 225 for increased heterotrimeric complex formation between SK-1, $\alpha_v\beta_3$ and CD31 following factor deprivation where $\alpha_v\beta_3$ activation and subsequent EC survival signals include the Bcl-X and nuclear factor κB pathways.[19] Herein we demonstrate, for the first time, that SK-1 is integral for TNFα-induced $\alpha_5\beta_1$ activation, Ang-2 expression and neutrophil adhesion under shear flow. Whether phosphorylation of SK-1 is also involved in this process is still to be determined. Furthermore, we suggest that TNFα-induced activation of $\alpha_5\beta_1$ is S1P receptor independent as inhibition of $S1P_{1\text{-}3}$ function, as well as S1P administration (at a concentration specific for receptor engagement[49]) did not alter QE.2E5 antibody binding capabilities. The possibility that TNFα-induced adhesion molecule expression requires SK-1/S1P to act intracellularly supports the initial observations by Xia and co-workers.[50] Briefly, Xia et al[50] demonstrated that the effective concentrations of S1P to mediate adhesion molecule expression were in the micromolar range despite the K_d for $S1P_{1\text{-}3}$ on ECs being 20 to 60 nmol/L. Furthermore, neither pertussis toxin (a $G\alpha_i$ inhibitor) nor suramin (a nonspecific phospholipid receptor inhibitor) could effectively block S1P induction of adhesion molecules.[20] These observations are however in contrast to the suppressive effect of S1P on $\alpha_5\beta_1$-mediated monocyte adhesion to ECs.[51] Aoki and colleagues[51] recently demonstrated that S1P-induced activation of HUVECs inhibited U937 adhesion by shifting the localization of $\alpha_5\beta_1$ from the apical surface to the basal surface. This S1P-induced suppressive effect was $S1P_1$ and $S1P_3$ receptor-mediated, as demonstrated by inhibition studies, as well as specific inhibitors of $G\alpha_i$ protein, Src family proteins, phosphatidylinositol 3-kinase, and Rac1.[51] The

differences observed between this and our own observations may be explained by the use of a myeloid cell line and a static adhesion assay by Aoki et al[51] versus ours of whole blood or neutrophils and adhesion under conditions of physiologically relevant shear flow. The very recent identification of histone deacetylases as intracellular targets of S1P by Hait and colleagues[16] suggests a new paradigm of S1P signaling within the nucleus. Whether transcriptional regulation of EC adhesion molecules occurs via this mechanism is interesting to speculate and requires further investigation. Another aspect that requires further elucidation with respect to TNFα-induced, SK-1-dependent adhesion molecule expression is the timing of SK activity. We show here a biphasic response of SK activity in ECs following TNFα activation: the first occurring within 10 minutes and the second approximately 4 to 6 hours later. Whether TNFα-induced E-selectin, VCAM-1, ICAM-1, and now, $\alpha_5\beta_1$, expression are reliant on the first and/or second wave of SK activity is currently under investigation within our laboratory.

Neutrophils are the most abundant blood-borne leukocytes in healthy humans and they accumulate within hours at sites of acute inflammation. Moreover, they are essential for combating bacterial and fungal infections, but their activation also releases cytotoxic mediators, causing tissue damage. These studies support that the paradigm of neutrophil trafficking as a multistep cascade determined by a variety of adhesion receptors continues to serve as a useful model, but needs to be refined to accommodate Ang-2 and other non-selectin and cellular adhesion molecules. The findings presented here add new complexities that broaden the accepted concept of neutrophil trafficking, as we show that primary adhesive events of neutrophils, *in vitro*, are dependent on $\alpha_5\beta_1$ and Ang-2. The precise mechanisms underpinning $\alpha_5\beta_1$:Ang-2-mediated neutrophil adhesion require further investigation. We propose a sandwich type configuration between $\alpha_5\beta_1$ and Ang-2, where Ang-2 acts as a bridge to mediate $\alpha_5\beta_1$:$\alpha_5\beta_1$-dependent cellular events between neutrophils and TNFα-activated HUVECs. A similar system was recently observed with Th1 and Th2 lymphocytes, wherein hyaluronan mediates the CD44:CD44 dependent rolling and adhesion to the intestine of TNFα-treated mice.[27] Our observations now suggest that SK-1/S1P is integral to controlling all three families of adhesion molecules, namely, selectins, cellular adhesion molecules, and integrins. Taken together these results suggest that SK-1 may be the single target required for an effective broad spectrum therapeutic target to combat inflammatory and immune disorders.

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