Interleukin 15 Induces Endothelial Hyaluronan Expression In Vitro and Promotes Activated T Cell Extravasation through a CD44-dependent Pathway In Vivo

By Pila Estess, Animesh Nandi, Mansour Mohamadzadeh, and Mark H. Siegelman

From the Laboratory of Molecular Pathology, Department of Pathology, The University of Texas Southwestern Medical Center, Dallas, Texas 75235-9072

Summary

T cell recruitment to extralymphoid tissues is fundamental to the initiation and perpetuation of the inflammatory state during immune and autoimmune responses. Interleukin (IL)-15 is a proinflammatory cytokine whose described functions largely overlap with those of IL-2. The latter is attributable in large part to its binding of the heterotrimeric receptor that contains the β and γ chains of the IL-2R in combination with an unique IL-15Rα chain. However, unlike IL-2, IL-15 and its receptor have a wide tissue and cell type distribution, including endothelial cells. Here, we examine the effect of IL-15 on hyaluronan expression by endothelial cells, and investigate its role in vivo in promoting the extravasation of antigen-activated T cells through a CD44-dependent pathway. The expression of hyaluronan on primary endothelial cells and microvascular endothelial cell lines is induced by IL-15, whereas IL-2 has no such activity. Moreover, intraperitoneal administration of IL-15 or TNF-α in the absence of other exogenous proinflammatory stimuli allows the extravasation of superantigen-stimulated T cells into this site in vivo in a CD44-dependent manner. T cell recruitment induced by IL-15 requires expression of an intact IL-2Rβ chain, indicating that IL-15 operates in this context through the traditional IL-15R. The results suggest that IL-15 can regulate endothelial cell function and thereby enables a CD44-initiated adhesion pathway that facilitates entry of activated T lymphocytes into inflammatory sites. They further demonstrate a novel role for IL-15 (distinct from any of IL-2) in regulating microvascular endothelial cell adhesive function, help to understand the role of IL-15R expression on endothelium, and further support a central position for this cytokine in orchestrating multiple sequential aspects of T cell effector function and therefore chronic inflammatory processes.

Key words: CD44 • endothelial cell • hyaluronate • interleukin 15 • lymphocyte adhesion

The specificity and regulation of leukocyte extravasation results in part from the differential expression of various types of adhesion receptors on endothelium within tissues and on leukocyte subsets, and partially from the fact that these receptors can act in sequential fashion. Our laboratory has been engaged in the description and characterization of a novel primary adhesion (rolling) interaction between the activated form of the cartilage link proteoglycan family member CD44 on lymphocytes and its major ligand, the glycosaminoglycan hyaluronan (HA)[1] on endothelial cells (ECs) (1). We have shown in a mouse model that this interaction can function in an extravasation pathway that results in the egress of antigen-activated lymphocytes in an inflamed vascular bed (2, 3). We have further demonstrated that HA expression is increased on microvascular primary EC cultures and EC lines in response to proinflammatory stimuli such as TNF-α, IL-1β, and LPS in vitro. This elevated expression results in increased interactions of activated lymphocytes with endothelium under shear forces (4). These observations have lead us to propose that the activated form of CD44 is induced on lymphocytes as a result of antigen stimulation, followed by release of activated cells from secondary lymphoid sites into the peripheral circulation. Activated CD44 would then function to initiate lymphocyte extravasation at sites of antigen localization or inflammation, where HA has been upregulated on the endothelium. In support of this model, circulating lymphocytes bearing

[1]*Abbreviations used in this paper:* bPG, bovine proteoglycan; CFDA-SE, 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester; EC, endothelial cell; HA, hyaluronan; HUVEC, human umbilical vein endothelial cell; ICAM, intracellular adhesion molecule; MLN, mesenteric lymph node; RT-PCR, reverse transcription polymerase chain reaction; SA-PE, phycoerythrin-labeled Streptavidin; SEB, Staphylococcal enterotoxin B; VCAM, vascular cell adhesion molecule.

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activated CD44 have been shown to be elevated during autoimmune exacerbations in both arthritis and systemic lupus erythematosus in humans (5). This finding suggests that such cells represent a pathogenically important subset of activated T cells and may serve as a reliable marker for autoimmune or other chronic inflammatory disease activity.

IL-15 is a cytokine originally identified as a T cell growth factor and described to have biological activities similar to IL-2 (6, 7). These properties include induction of T cell proliferation; potent T cell chemotaxis; costimulation of B cell growth and isotype switching; and promotion of natural killer cell growth, cytotoxicity, and cytokine production (8). IL-15 has also been reported to inhibit apoptosis and to enhance the survival of activated T cells in vivo (9, 10). However, unlike IL-2, IL-15 is expressed in diverse types of tissues, including skeletal muscle and kidney, as well as in a variety of cell types such as activated monocytes, fibroblasts, and keratinocytes. IL-15 generally mediates its activity through a heterotrimeric receptor consisting of a unique IL-15Rα chain in combination with the IL-2Rβ and γ chains (8). Like IL-15, the unique IL-15Rα chain has a broad tissue distribution, suggesting potential functions for this cytokine outside of the hematolymphoid system. IL-15 thus has been shown recently to have anabolic effects on skeletal muscle (11) and may serve as an angiogenic factor (12).

IL-15 has been suggested to play a role in the setting of the chronic autoimmune disease rheumatoid arthritis, particularly in the recruitment and activation of synovial T cells. This cytokine has been reported to be elevated within the synovial fluid of affected patients, and administration of IL-15 into the hind footpad of mice resulted in an enhanced mononuclear cell infiltrate, predominantly T cells, at the site of injection, although the mechanism for this was not elucidated (8, 13). Recently, in a mouse model of collagen-induced arthritis, a soluble form of the IL-15Rα chain was shown to be effective in ameliorating disease (14). This cytokine also has been shown to have clear effects on chemoattraction and migration of lymphocytes (8, 15). However, a pivotal element of inflammatory cell recruitment not previously examined for this cytokine is extravasation from the blood. Because we have defined a CD44-dependent adhesion pathway pertinent to activated T cells, it was of interest to investigate whether IL-15 has a role in the extravasation of T cells as well.

In the study presented here, we demonstrate that ECs directly respond to IL-15 in vitro with increased cell surface expression of HA and that, as with other proinflammatory stimuli (16), this responsiveness appears largely restricted to small venular endothelial sources. Moreover, in an in vivo model of peritoneal inflammation, IL-15 is sufficient to promote the extravasation of superantigen-activated T cells, and this is dependent on CD44 interactions with HA. Neither the in vitro nor the in vivo activity is induced by IL-2, suggesting this is a unique function for IL-15, although the shared receptor chain β appears to be required. These results place a function for IL-15 at the interface between the blood and vasculature, a vital position in the sequence of T cell effector processes, through regulation of CD44/HA interactions.

Materials and Methods

Chemicals and Reagents. Biotinylated bovine proteoglycan (bPG) extracted from bovine nasal cartilage (17) was provided by C. Underhill (Georgetown University School of Medicine, Washington, DC). Rooster comb HA and Staphylococcal enterotoxin B (SEB) were purchased from Sigma Chemical Co. 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE), was purchased from Molecular Probes, Inc. KM81, a rat anti–mouse CD44 that blocks HA binding (18) and anti–mouse intracellular adhesion molecule (ICAM)-1 (clone YN1/1.7.4; reference 19) were obtained from the American Type Culture Collection and anti–mouse vascular cell adhesion molecule (VCAM)-1 (clone M/K-2.7; reference 20) was provided by Dr. P. Thorpe (University of Texas Southwestern Medical Center, Dallas, TX). Antibody was purified from tissue culture supernatants by Protein A–Sepharose column chromatography. Rat anti–mouse H-2 (clone M1/42) was provided by K. Fischer-Lindahl (Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX). Anti–human IL-15 was obtained from R&D Systems, Inc. Rat anti–mouse TNF-α and biotinylated anti–mouse CD62P and anti-CD62E were obtained from PharMingen.

Murine TNF-α (5×10^7 U/mg), murine IL-1β (2.2×10^7 U/mg), and human IFN-γ (10^7 U/mg) were obtained from Genzyme, Inc. Human IL-15 was obtained from R&D Systems (ED_{50} in proliferation assays 0.5–2 ng/ml) and recombinant human IL-2 (10^7 U/ml) was obtained from the Biological Response Modifiers Program (Frederick, MD).

EC Culture and Stimulation. SVEC4-10 is an SV40 transformed murine LN EC line provided by K.A. O'Connell (Johns Hopkins University School of Medicine, Baltimore, MD) (21). The TME-3H3 endothelial line was similarly derived (22) and was provided by A. Hamann (Medizinischen Klinick, Hamburg, Germany) and J. Lesley (Salk Institute, San Diego, CA). LEII is a murine lung capillary EC line derived by A. Curtis (University of Glasgow, Glasgow, UK) and provided by P. Thorpe (University of Texas Southwestern Medical Center, Dallas, TX). Cells were maintained in RPMI 1640–high glucose, 15% FCS plus 1 mM pyruvate, 2 mM glutamine, and 50 μm β-mercaptoethanol. EC lines were taken from frozen storage and used up to a maximum of five passages. Human umbilical vein ECs (HUVECs) were obtained from S.W. Caughman (Skin Center at Emory University, Atlanta, GA) and were used upon reaching confluence or after their first or second passages. Primary LN EC (1°LNEC) cultures were made by pooling cervical and axial nodes from three animals, as described (23, 24). Organs were minced, rinsed to remove lymphocytes, treated with collagenase for 30 min at 37°C, and plated on 35-mm culture dishes in supplemented IMDM (20% FCS). These cultures at confluence showed the morphologic and phenotypic characteristics indicating >95% EC content, as previously described (1). After reaching initial confluence, cells were passaged and used directly or after one additional passage to fresh plates.

For stimulation of ECs, cells were passaged 24–48 h before addition of stimuli, when cultures were subconfluent. Stimuli were added to 1 ml of culture medium as follows, except as noted in dose–response experiments: TNF-α and IFN-γ to a final concentration of 10 ng/ml; IL-2 and IL-15 to a final concentration of 50 ng/ml. Cells were maintained at 37°C in a 5% CO_2 atmosphere for 4 h, unless otherwise noted. Control cultures without exogenous stimuli were incubated in parallel in all experiments. Cells were harvested for FACS® analysis and RNA isolation by gentle pipetting after incubation with Versene (GIBCO BRL) for 5 min at 37°C. Anti–IL-15 or anti–TNF-α was included in some

EC cultures at a final concentration of 100 ng/ml. For rolling assays, ECs were grown in 35-mm Corning tissue culture dishes.

FACS® Analysis. 5×10^5 cells were stained with bPG-biotin or primary antibody in 100 μl PBS/2% FCS for 30 min and then washed with 500 μl of PBS/2% FCS. PE-labeled Streptavidin (SA-PE; Caltag Labs.) or goat anti–rat immunoglobulin-FITC (Caltag Labs.) was added for an additional 30 min. Cells were washed and analyzed using a FACScan™ instrument and CellQuest™ software (Becton Dickinson).

Reverse Transcription PCR. Total RNA was isolated according to the manufacturer's instructions using RNAzolB (Biotecx). Reverse transcription (RT)-PCR amplification was performed as previously described (25). The following primer pairs were used: IL-2Rα, 5′ CCTACAAGAACGGCACCATCCTA/5′ CACCCCGTTTTC-CCACACTTC; IL-2Rβ, 5′ CTCCGTGGACCTCCTTGACA-TAAATGTGG/5′ TGTTTCGTTGAGCTTTGACCCTCACC-TGG; IL-2Rγ, 5′ ATGTCCAGTGCGAATGAAGA/5′ CTC-CGAACCCGAAATGTGTA; IL-15Rα, 5′ CTCCAGGCTGA-CACC/5′ CCATGGTTTCCACCTCAACACGGC. Cycling conditions were 95°C for 60 s, 55°C for 90 s, and 72°C for 120 s. PCR reactions were performed semi-quantitatively and compared with β-actin PCR amplifications run in parallel. For liquid hybridization of RT-PCR products, oligonucleotide probe was end-labeled with [^{32}P]dCTP and T4 polynucleotide kinase. 5% (5 μl) of PCR product from the IL-2Rα or γ reactions were hybridized in liquid phase (240 mM NaCl, 2.4 mM Tris/1 mM EDTA) with 10 μl of ^{32}P–end-labeled internal probe (IL-2Rγ chain: 5′ AGCTGAGATGGAAAAGCAGACA; IL-2Rα chain: 5′ GCTCCCTGCAGTGACCTGTAA-GGTTCTCTT) and analyzed by electrophoresis on a 4% acrylamide gel. Gels were vacuum dried and exposed to Kodak XAR-5 film. CTLL cells (American Type Culture Collection) maintained in the presence of IL-2 were used as a source of control RNA for the IL-2Rα and γ chains in liquid hybridization experiments.

Adhesion Assay under Flow Conditions. Physiological flow conditions were produced using a parallel plate flow chamber as previously described (1, 26). In brief, flow occurs over a 35-mm tissue culture dish containing an adherent cell monolayer of ECs. The culture dish and an opposing Plexiglas chamber are held 1.27×10^{-2} cm apart by a silicon gasket cut to form two flow chambers, each 0.6 cm wide. Experiments were carried out at a wall shear stress of 2.0 dynes/cm^2 unless otherwise indicated. Murine LN cells which had been cultured in vitro in the presence of SEB (50 μg/ml) for 48 h, resulting in 12–30% of cells bearing activated CD44 (2), were used. After equilibration of flow with medium alone, SEB-activated LN cells were resuspended at a concentration of 3×10^6 cells/ml in RPMI 1640 equilibrated to 37°C and pulled continuously across the flow chamber. For blocking studies, antibody was added at saturating concentrations to the cell suspension before flow. Interaction of lymphoid cells with the EC monolayer after equilibration of flow was monitored for 10–15 min with an inverted phase contrast microscope connected to a video camera and recorder. Only primary (rolling) adhesions were analyzed, and rolling cells were scored visually. Data is reported as the average number of rolling cells/mm^2 per min, based on an actual field of view of 0.6 mm $\times$ 0.8 mm.

Short-term Homing Assay. Short-term homing of fluorescently labeled cells was performed as previously described (3), with the following modifications. Donor BALB/c mice were injected intraperitoneally with 500 μl of sterile PBS or with 50 μg of the superantigen SEB in 500 μl of PBS to provide a source of in vivo–activated T cells. 20 h later, mesenteric LNs (MLNs) were removed. MLN cells were fluorochrome labeled by resuspending at a concentration of 10^7 cells/ml in HBSS plus 2 μm CFDA-SE, incubating them at room temperature for 20 min, and then washing the cells twice in HBSS. Recipient mice that had been injected intraperitoneally 20 hours previously with SEB (50 μg), IL-15 (350 ng), IL-2 (200 ng), TNF-α (200 ng), IFN-γ (200 ng), or IL-1β (200 ng) were then injected intravenously with 10^7 fluorescent donor cells/mouse in 500 μl HBSS. Some cytokine-injected recipients were also given 200 μg blocking KM81 anti-CD44 or control antibody at the time of donor cell injection. 2 h after the infusion of labeled cells, recipient mice were killed and cells in the peritoneal cavity were collected by lavage with 5 ml of 37°C RPMI and analyzed for the presence of CFDA-labeled donor cells. Some animals also received 200 ng/500 μl of anti–IL-15 or anti–TNF-α at the time of cytokine injection.

Depletion of Vβ8 T cells was performed using anti-Vβ8-biotin (PharMingen) and Streptavidin-conjugated magnetic beads (Dynal). Depletion of Vβ14 T cells was done using anti-Vβ14 (PharMingen) and goat anti–rat immunoglobulin-conjugated beads (Dynal). 5×10^6 cells were incubated for 20 min with 10^7 beads in 1 ml RPMI 1640/5% FCS at 4°C on a rocking platform. Vβ8$^+$ or Vβ14$^+$ T cells were removed by magnetic separation. After depletion, $<$1% of the remaining cells were shown to be Vβ8 or Vβ14 cells by FACS® analysis. Samples were pooled and cell numbers were readjusted to 10^7/500 μl before injection.

IL-2Rβ–deficient mice were obtained by breeding heterozygous animals purchased from The Jackson Laboratory (27). Animals were maintained at our facility under specific pathogen-free conditions. Offspring were tested by PCR analysis of genomic tail DNA and homozygous deficient and homozygous wild-type animals were used at 4–5 wk of age.

Results

IL-15, But Not IL-2, Induces Increased HA Expression on Cultured ECs. IL-15 has not been reported previously to induce increased levels of adhesion molecule expression. To examine the effect of IL-15 on the level of surface HA expression, the LN-derived EC line SVEC4-10 was incubated for 4 h in the presence of IL-15, TNF-α, IL-2, or IFN-γ. Using a biotinylated form of the HA-binding proteoglycan bPG to detect cell surface HA, we found that IL-15 treatment resulted in a marked increase in the amount of surface HA expression, which was comparable to levels seen with TNF-α treatment (reference 4 and Fig. 1 A). Moreover, HA surface expression appeared selectively induced, as other endothelial adhesion molecules showed no substantial alterations in levels of expression after IL-15 treatment (Fig. 1 B), although VCAM-1 and P-selectin were both increased on this cell line after LPS treatment (data not shown). Kinetic studies further demonstrated that surface HA expression was maximal at 4 h, consistent with the time course previously demonstrated with other proinflammatory cytokines (4). In contrast to IL-15, IL-2 had no effect on the level of bPG binding on these ECs, although these two cytokines have overlapping functions in many biological systems (15). IFN-γ (Fig. 1 A) and IL-12 (4) had no effect on HA expression, as previously reported. Also, bPG staining of TNF-α–treated SVEC4-10 cells was blocked by preincubating cells with soluble HA before staining with bPG (1), indicating specificity of this reagent (17). Thus, the proin-

flammatory cytokine IL-15 acts as a potent and selective regulator of surface HA expression.

To compare the effects of IL-15 and contrast them with IL-2 on a variety of EC sources, we used several other EC lines, including another SV40-transformed murine LN line (TME-3H3), early passages of primary murine LN ECs (1°LNEC), a murine lung capillary EC line (LEII), and HUVECs. Results are shown in Fig. 1 C, where it can be seen that IL-15 increases HA expression on the other two LN-derived ECs, also derived from microvascular venular endothelium. In contrast, IL-15 had no effect on expression of HA by the lung capillary line LEII or by HUVECs. This is consistent with our prior results, which indicate that ECs derived from microvenular sources, where leukocyte trafficking predominantly occurs, selectively exhibit cytokine inducible HA expression (4). In contrast to the effects of IL-15, treatment with IL-2 had no effect on HA expression in any of the ECs tested.

To determine the dose responsiveness of SVEC cells to IL-15 and whether IL-2 has any influence at higher concentrations, dose–response curves were performed. Evaluation of the response to IL-2 over a range of concentrations indicated that this cytokine had no effect on HA expression levels even at the highest concentrations used (Fig. 1 D). The maximal response to human IL-15 was attained at 50 ng/ml. The dose–response curves suggest that IL-15 is effective in a concentration range comparable with those seen in other cytokines (4). In contrast, murine IL-2 failed to have any effect at concentrations as high as 400 ng/ml (data not shown). Thus, unlike many of its other described functions, IL-15 behaves in a manner completely distinct from IL-2 with regard to endothelial HA induction.

ECs Produce the IL-2Rβ and γ Chains and IL-15Rα Chain, But Not the IL-2Rα Chain. We next addressed the expression of IL-15R and IL-2R chains in these ECs. RNA was prepared from SVEC4-10, TME-3H3, LEII, or 1°LNEC to examine these for the expression of IL-2Rβ and γ chain message, the IL-2Rα chain, and IL-15Rα chain. As seen in Fig. 2 A, agarose gel analysis of RT-PCR products revealed significant expression in unstimulated ECs of both the IL-2Rβ and the IL-15Rα chains. Since ECs did not make sufficient levels of either the IL-2Rα or γ chains for direct detection by ethidium staining, the PCR products for these reactions were further analyzed by hybridization to specific radiolabeled internal oligonucleotide probes. When the IL-2Rγ chain RT-PCR product was hybridized in liquid phase to a ^{32}P-labeled γ chain probe, a band of the appropriate molecular weight (573 bp) was observed (Fig. 2 B). However, similar analysis of the IL-2Rα chain RT-PCR product gave no signal in any of the EC-derived RNAs (Fig. 2 C), although the control IL-2–stimulated CTLL cells did show an appropriate positive signal. Thus, ECs express all three chains of the IL-15 cytokine receptor, but lack the unique α chain specific to the IL-2R, consistent with the relative pattern of induction of HA by these two cytokines. It is notable that, similar to HUVECs, LEII expresses mRNA for all IL-15R chains, yet these two EC lines are not induced to express increased HA (Fig. 1 C). This sug-

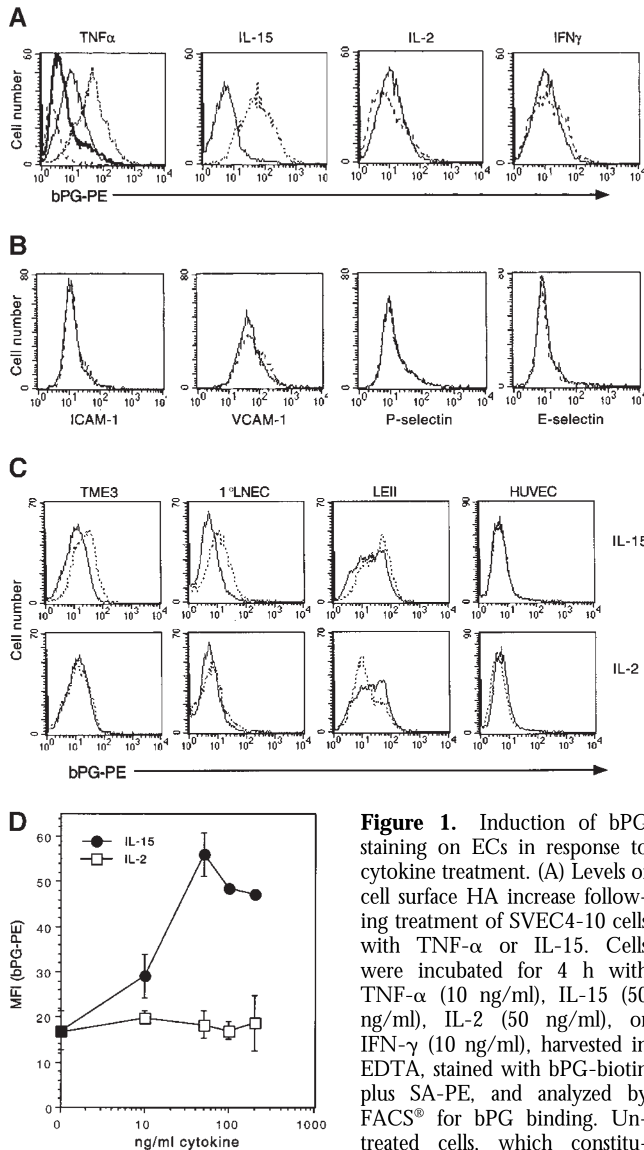

Figure 1. Induction of bPG staining on ECs in response to cytokine treatment. (A) Levels of cell surface HA increase following treatment of SVEC4-10 cells with TNF-α or IL-15. Cells were incubated for 4 h with TNF-α (10 ng/ml), IL-15 (50 ng/ml), IL-2 (50 ng/ml), or IFN-γ (10 ng/ml), harvested in EDTA, stained with bPG-biotin plus SA-PE, and analyzed by FACS® for bPG binding. Untreated cells, which constitutively express some HA, bind background levels of bPG (solid line). After treatment with TNF-α or IL-15, cells show even greater levels of staining (stippled line), whereas IL-2 has no effect. As previously shown, binding to bPG was not increased by incubation with IFN-γ (4). Baseline staining of SVEC4-10 cells with SA-PE alone is indicated by the dashed line in the TNF-α panel. Preincubating TNF-α–treated cells with soluble HA reduced bPG staining to near background levels (heavy line). (B) Expression of ICAM-1, VCAM, P-selectin, and E-selectin by SVEC4-10 cells before (stippled line) and after (solid line) IL-15 treatment. (C) TME-3H3, 1°LNEC, LEII, and HUVEC cells were treated with human IL-15 (top) or IL-2 (bottom), as in A. TME-3H3 and 1°LNEC respond to IL-15 treatment by expressing increased levels of HA, whereas LEII and HUVEC cells are not affected. In contrast to IL-15, IL-2 had no effect on any of the EC lines or primary ECs. Solid lines, untreated cells; stippled lines, treated cells. (D) Dose dependence of increased HA levels in response to IL-15 treatment. SVEC4-10 cells were incubated for 4 h with IL-15 or IL-2 at varying concentrations, as shown. Results are reported as the mean fluorescence intensity (MFI) of cells after staining with bPG-biotin plus SA-PE, as determined by FACS® analysis. IL-2 had no effect on HA expression by SVEC4-10 cells, even at 200 ng/ml, whereas IL-15 had a dose-dependent effect on levels of surface HA, with changes seen at 10 ng/ml. Data shown are the mean ± SD from three separate experiments.

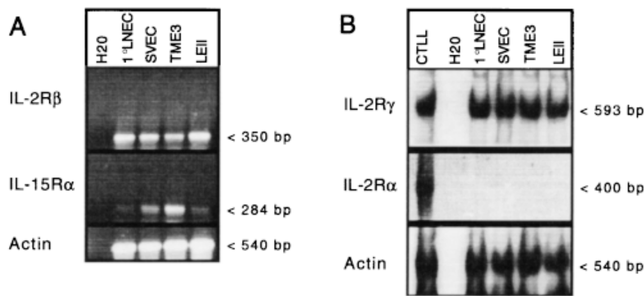

Figure 2. Endothelial cells express RNA for the IL-15R but not for IL-2R. (A) RT-PCR amplification of RNA from untreated 1°LNEC, SVEC4-10, TME-3H3, and LEII cells. Amplification was done with primers specific for the murine IL-2Rβ chain and the IL-15Rα chain as shown. For comparison, results of amplification of β-actin RNA run in parallel with the IL-15Rα chain are shown in the bottom panel. RT-PCR amplification indicates that the primary ECs and the three EC lines make detectable levels of RNA for each of the chains. (B) Autoradiogram of the RT-PCR product resulting from amplification with primers specific for the mouse IL-2Rγ or IL-2Rα chains. RT-PCR product was hybridized in liquid phase with IL-2Rγ– or α–specific ^{32}P-labeled oligonucleotide probes internal to the primer pairs used for amplification; hybridized sample was separated from radiolabeled probe by acrylamide gel electrophoresis and visualized by autoradiography. IL-2Rγ–specific product is clearly visible in all cells tested, whereas IL-2α–specific product was seen only in the IL-$2R^+$ control cell line CTLL.

gests that differences either in receptor protein expression or downstream signaling events in these cells account for their IL-15 unresponsiveness. Thus, IL15Rα expression appears not to be sufficient for responsiveness to IL-15.

Induction of Elevated Endothelial Surface HA Expression Results in Increased Rolling Interactions of SEB-activated T Cells under Shear Stress Conditions. We have previously demonstrated that the increases in HA induced by proinflammatory stimuli are sufficient to markedly enhance rolling interactions of the clonal T cell line, BW5147, under laminar flow analysis (4). In addition, we have shown that TCR signaling through conventional antigen or the superantigen SEB results in the conversion of CD44 to its activated form. With SEB stimulation, this activation of CD44 occurs selectively on the SEB-stimulated (Vβ8) subset of T cells (2). To determine whether the levels of HA induced on ECs by IL-15 and TNF-α had sufficient physiologic consequences to significantly alter CD44-dependent rolling interactions, normal peripheral LN T cells were stimulated in vitro with SEB and subjected to laminar flow on unstimulated ECs and ECs stimulated with IL-15 or TNF-α. Both SVEC4-10 and TME-3 cells were used in a parallel plate adhesion assay after 24 h treatment with either cytokine, when complete confluence and maximal HA expression of the monolayer was assured. Fig. 3 shows that on both EC lines induction with either IL-15 or TNF-α increased the number of rolling cells four- to fivefold over that seen on unstimulated ECs, although the cell density and viability of the monolayers were equivalent. Furthermore, blocking anti-CD44 antibody specifically inhibited rolling by >80%, suggesting VCAM-1/very late antigen (VLA)-4 or other interactions do not substantially contribute to rolling under these conditions. Unactivated T cells exhibited minimal rolling on treated ECs. Thus, the changes in HA levels measured by flow cytometry are sufficient for altering the EC capacity to support CD44-dependent tethering and rolling interactions under shear stress for normal activated T cells.

Intraperitoneal Administration of IL-15 Results in CD44-dependent Recruitment of Activated T Cells into the Peritoneal Cavity. Previous analysis has demonstrated that SEB, which specifically activates the well-represented Vβ8-bearing T cells, induces peritoneal inflammation that results in CD44-mediated recruitment of superantigen-activated T cells into the inflamed site (3). To directly assess the ability of IL-15 to generate an inflamed site similarly receptive to the CD44/HA extravasation pathway, short-term homing assays were performed using cytokines alone as the inflammatory stimulus. Lymphocytes expressing activated CD44 were generated in vivo by intraperitoneally injecting donor mice with SEB (3). 20 h later, MLN cells were isolated, washed, and labeled with CFDA-SE. The fluorescent cells were then injected intravenously into recipient mice that had received an intraperitoneal injection of cytokine(s) or SEB (positive control) 20 h previously. 2 h after intravenous injection of

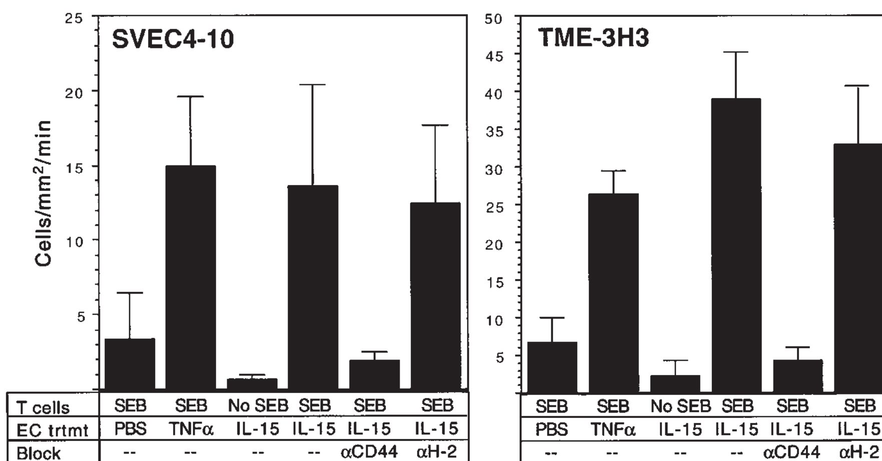

Figure 3. Rolling of SEB-activated LN T cells on TNF-α– or IL-15–treated SVEC4-10 and TME-3H3 monolayers. Cells were applied to feed solution already equilibrated under flow at a wall shear stress of 2.0 dynes/cm² and perfused over EC monolayers in the parallel plate flow assay. Rolling was analyzed as described above and the number of cells/mm² per min rolling across each monolayer was determined. For blocking, KM81 anti-CD44 or anti–H-2 was added to the T cell suspension before their introduction in the flow system. Rolling of SEB-activated T cells on TNF-α and IL-15–treated ECs compared with untreated ECs was significant for both SVEC ($P < 0.005$) and TME3 ($P < 0.001$), and anti-CD44 significantly reduced rolling numbers compared with those on IL-15 treated ECs ($P < 0.001$).

donor cells, peritoneal exudate was collected from recipient mice by peritoneal lavage and samples were analyzed cytofluorometrically for CFDA (green) fluorescence to identify cells that had trafficked to the site. As seen in Fig. 4 A, when SEB-activated MLN cells were given to IL-15– or TNF-α–treated recipients, significant extravasation of cells into the peritoneum occurred. This was comparable to the levels of emigration obtained in SEB-treated recipients. The migration of donor cells into IL-15– or TNF-α–treated sites was dependent upon activated CD44, since coadministration of an HA-blocking anti-CD44 antibody at the time of donor cell transfer inhibited the transit of cells from blood into the peritoneal cavity. Isotype control–matched anti–H-2 antibody did not inhibit cell migration. Moreover, magnetic bead depletion of Vβ8-bearing cells, the predominant lymphocyte population activated by SEB, completely ablated emigration to the site, whereas depletion of the equally well-represented non-SEB-responsive Vβ14 population did not. This indicated that, as with SEB (3), it is the activated T cells that emigrate. In contrast, when SEB-activated donor cells were transferred to mice that had received intraperitoneal IL-2, IFN-γ, or IL-1β, no significant emigration was detected. Injection of unactivated donor cells from PBS-treated animals also did not result in extravasation into the peritoneal cavities of IL-15–treated recipient mice. Giving optimal amounts of IL-15 and TNF-α together did not increase migration, indicating that maximal recruitment is achieved with either cytokine alone. This further suggests that optimal HA expression is attained with either of these treatments independently. Thus, TNF-α and IL-15, but not IL-2 or IFN-γ appear to alter the local vascular bed such that it is receptive to CD44-mediated extravasation. These results correlate well with our findings of HA regulation in vitro using the same cytokines, and suggest that the in vivo observations result from local changes in endothelial HA induced by the administered cytokine. Representative two-color immunofluorescence plots of cells recovered from the peritoneal cavity of an IL-15–treated mouse are shown in Fig. 4 B. 100,000 events were collected for each sample, and those cells with green (CFDA) fluorescence in the sec-

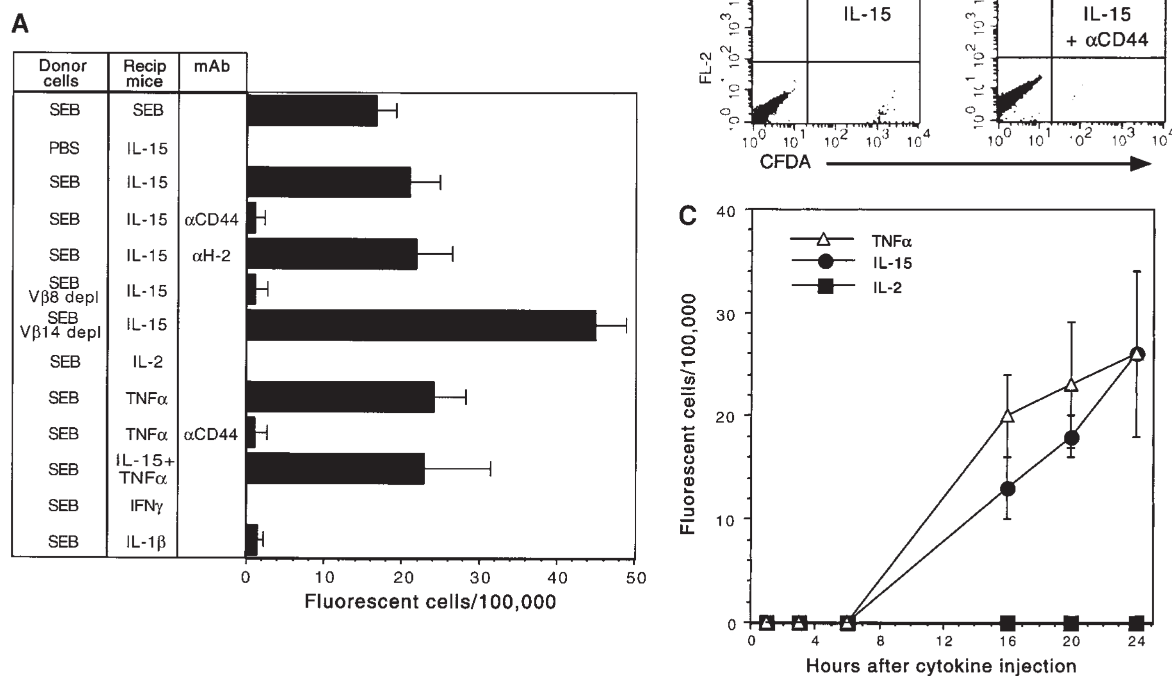

Figure 4. Intraperitoneal cytokine treatment allows CD44-dependent short-term homing of SEB-activated T cells into inflamed mouse peritoneal cavities. (A) CFDA-labeled MLN cells from SEB- or PBS-treated mice were injected intravenously into recipient mice that had received an intraperitoneal injection of SEB or cytokine(s) 20 h earlier. Cells from donor mice were injected without antibody, with HA-blocking anti-CD44 mAb (KM81), or with isotype-matched control anti–H-2 mAb. 2 h after injection, peritoneal cavities of recipient mice were lavaged and recovered cells were analyzed by FACS® for CFDA fluorescence. Data are shown as the number of fluorescent cells/100,000 total cells analyzed and represent the mean ± SEM from three separate experiments (three mice/group per experiment). (B) Representative analysis of cells recovered from the peritoneal cavity of an IL-15 recipient mouse. CFDA$^+$ cells exhibit green fluorescence and are visible in the lower right hand quadrant. As shown in the right hand panel, anti-CD44 treatment of the recipient animal blocks the emigration of CFDA$^+$ cells into the peritoneal cavity. 100,000 total events were collected. (C) Time course of induction of inflamed peritoneal cavities after cytokine treatment for short-term homing of SEB-activated T cells. CFDA-labeled mesenteric LN cells from SEB-treated mice were injected intravenously into either TNF-α–, IL-15–, or IL-2–treated recipients at varying time points after cytokine administration. Cells were recovered and analyzed as in A. No peritoneal accumulation of fluorescent cells is seen when injected at early time points after TNF-α or IL-15 administration (≤6 h); however, significant numbers of fluorescent cells are seen when transferred ≥16 h after cytokine treatment. No peritoneal accumulation of fluorescent cells is seen at any time point in IL-2 injected mice. Data are shown as the number of fluorescent cells/100,000 cells analyzed and represent the mean values ± SEM from at least three experiments (three mice/group per experiment).

ond to third decade are scored as positive (donor derived). KM81 anti-CD44 blocks the traffic of CFDA-labeled cells into the recipient peritoneal cavity (Fig. 4 B, right).

Although IL-15 and TNF-α both induce HA and result in trafficking of cells bearing activated CD44 into the treated site, it was possible that the kinetics of their in vivo effects differed. In addition, results with IL-2 could potentially have been different at time points other than 20 h after treatment if, for example, the timing of HA induction was altered. To address these issues, we conducted short-term homing experiments injecting donor cells at varying time points after cytokine administration to recipient animals. With both IL-15 and TNF-α, no significant migration of cells into the peritoneal cavity was seen until 16 h after cytokine injection (Fig. 4 C), and both cytokines resulted in similar kinetics. Moreover, no significant recruitment into the peritoneal cavities of IL-2–injected animals was seen at any time point.

IL-15 Induced Short-term Homing Is Dependent on the Traditional Heterotrimeric IL-15R. An alternate IL-15R unrelated to IL-2R chains is used by mast cells for their IL-15 responsiveness (28). To determine whether the conventional IL-15R is required for CD44-dependent activated T cell extravasation in vivo, IL-2Rβ–deficient mice (β−/−) were examined for their ability to support IL-15–induced trafficking to the peritoneum in the short-term homing model. When SEB-activated normal MLN donor cells were transferred intravenously into β−/− recipients treated intraperitoneally 20 h previously with IL-15, there was no subsequent accumulation of CFDA-labeled cells in the recipient peritoneal cavities (Fig. 5). However, when cells were transferred into β−/− recipients treated intraperitoneally with SEB or TNF-α, homing into the peritoneal cavity occurred at levels similar to those seen in wild-type recipients. Thus, the requirement for IL-2Rβ expression in the recipient tissues indicates that the IL-15 effect is dependent on expression of an intact traditional IL-15R. In addition, the inflammatory recruitment induced by SEB or TNF-α administration can occur in the absence of IL-15R interactions. These data suggest that the endothelial change(s) induced by TNF-α are intact in these animals and are independent of IL-15 pathways.

IL-15 and TNF-α Induce HA Expression Independently. It has been reported that TNF-α production in rheumatoid arthritis is mediated at least in part by IL-15 (29). In addition, some ECs are known to produce IL-15 at basal levels (30). We therefore investigated the possibility that TNF-α or IL-15 induction of endothelial HA occurred indirectly through the alternate cytokine and the resulting autocrine feedback mechanism occurred through the respective receptor. Cells were cultured as described above with IL-15 or TNF-α, either with or without the inclusion of anticytokine antibody. As illustrated in Fig. 6 A, the inclusion of anti–IL-15 antibody in TNF-α–stimulated EC cultures had no effect on the induction of HA. Likewise, the inclusion of anti–TNF-α antibody in IL-15–stimulated EC cultures had no effect on its induction of HA. Each antibody effectively blocked HA stimulation in control cultures (i.e., IL-15/anti–IL-15; TNF-α/anti–TNF-α). Together, the data suggest that IL-15 does not cause increased HA expression by increasing levels of TNF-α, nor does TNF-α result in increased HA expression by affecting levels of IL-15. Thus, at least two independent cell surface receptor pathways can result in changes in HA expression.

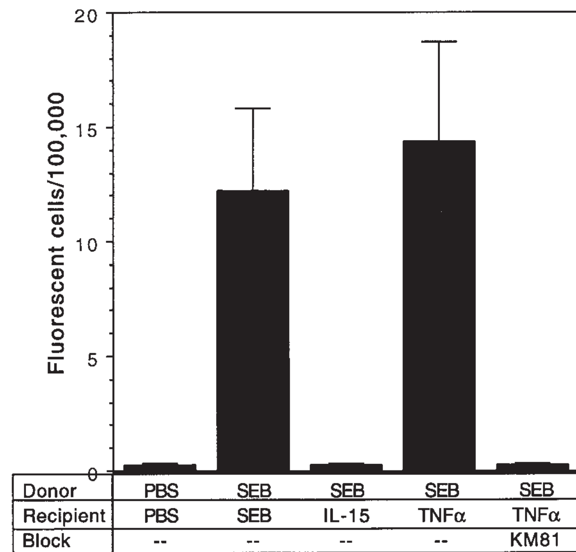

Figure 5. SEB-activated T cells do not enter the peritoneal cavities of IL-15–injected IL-2Rβ chain–deficient mice. IL-2Rβ knockout mice were injected intraperitoneally with PBS, SEB, IL-15, or TNF-α, as indicated. 20 h later, lymphocytes from MLN of SEB- or control-treated BALB/c donor mice were isolated, CFDA labeled, and injected intravenously into treated recipients. Cells were recovered and analyzed as in Fig. 4. Peritoneal accumulation of fluorescent cells is seen in recipient knockout mice injected intraperitoneally with either SEB or TNF-α; however, no fluorescent donor cells are found in recipient mice when treated intraperitoneally with IL-15. KM81 mAb blocks entry of activated donor cells into peritoneal cavities of TNF-α–treated recipients, indicating CD44 dependence. Data are shown as the number of fluorescent cells/100,000 cells analyzed and represent the mean ± SEM from at least three experiments (three mice/group per experiment).

Although IL-15 and TNF-α effects were clearly independent when using isolated ECs in vitro, it was of further interest to examine this issue in vivo using the short-term homing assay, where cytokines may derive from additional hematolymphoid or stromal cells within the peritoneum. Therefore, IL-15 or TNF-α was introduced intraperitoneally as above but in the presence or absence of blocking anticytokine antibody. As seen in Fig. 6 B, anti–TNF-α antibody did not inhibit the IL-15–induced recruitment to the peritoneum. Anti–IL-15 did effectively prevent IL-15–induced lymphocyte traffic, and anti–TNF-α likewise inhibited TNF-α–induced migration, demonstrating functional blocking by these antibodies. Thus, in this in vivo model, IL-15 appears to alter CD44-mediated homing patterns independent of TNF-α, consistent with our in vitro results.

Discussion

A variety of glycoprotein adhesion receptors primarily belonging to the immunoglobulin or selectin gene families are regulated on endothelium by cytokines or other inflammatory stimuli: E-selectin expression is induced on a variety

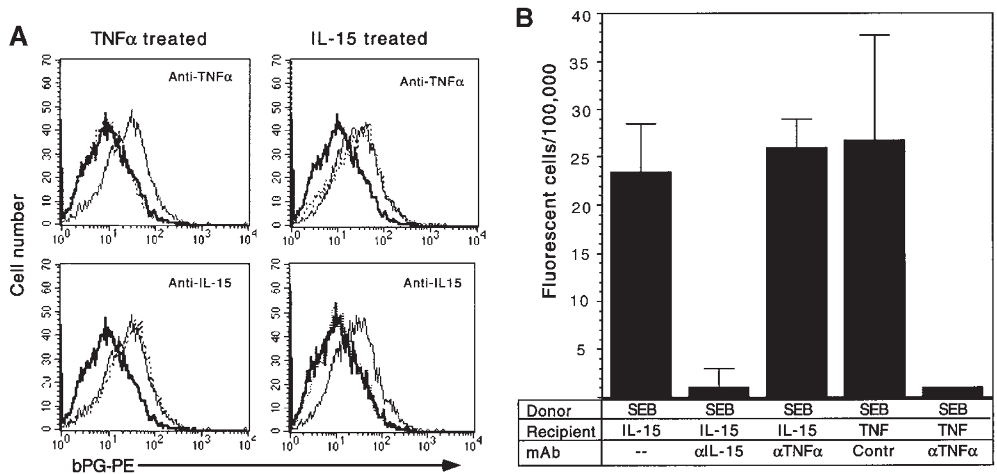

Figure 6. IL-15 and TNF-α induce increased HA expression via independent receptor pathways. (A) SVEC4-10 cells were stimulated for 4 h with IL-15 or TNF-α with or without the addition of either anti–IL-15 or anti–TNF-α antibody, as indicated. Inclusion of anti–TNF-α in IL-15–stimulated cultures had no effect on the increase of HA expression (upper right), nor did the inclusion of anti–IL-15 in TNF-α–stimulated cultures (lower left). Each antibody effectively blocked stimulation by the cytokine for which it was specific, as shown (upper left, lower right). Unstimulated cells, bold line; stimulated cells, solid line; stimulated cells plus antibody, stippled line. (B) In vivo administration of anti–TNF-α does not alter the short-term homing of activated T cells in response to IL-15.

of ECs stimulated with inflammatory agents such as TNF-α, IL-1, or bacterial LPS; VCAM-1 can be induced by IL-1, TNF-α, and IL-4; and levels of ICAM-1 have also been shown to be influenced by cytokines on a variety of ECs (31–33). We have recently added another class of molecule, the glycosaminoglycan hyaluronan, to the list of endothelial adhesion receptors that can be regulated in vitro, and have demonstrated that such elevated HA expression results in enhanced CD44-dependent primary adhesion interactions with lymphocytes (4). Thus, proinflammatory stimuli exert some of their effects by regulating the adhesion and thus recruitment of leukocytes to inflamed sites. IL-15, to our knowledge, has not been demonstrated previously to alter endothelial adhesion molecule expression. These studies show that, although adhesion glycoproteins generally do not appear to be induced by IL-15, the expression level of HA is increased to levels equivalent to that induced by other proinflammatory stimuli (Fig. 1). Given the evidence suggesting a role for the CD44/HA interaction in activated T cell recruitment during immune and autoimmune responses (1–3, 5), the selective upregulation of HA suggested a discrete function for IL-15 in the recruitment of activated T cells.

The role of cytokines in influencing T cell recruitment directly in vivo via the CD44/HA adhesion pathway had not been examined previously. Intraperitoneal SEB superantigen challenge has been used to create an inflamed site and to demonstrate the CD44 and HA dependence of T cell extravasation into such an inflamed site in vivo (3). The studies presented here demonstrate in a similar model that IL-15 as well as TNF-α treatment have the same effect on CD44-dependent T cell recruitment in the absence of further exogenous inflammatory stimuli. Other cytokines such as IFN-γ and IL-12, which did not result in the upregulation of EC surface HA expression in vitro (Fig. 1 A, reference 4), also had no effect in vivo (Fig. 4 A and data not shown). The concordance of these results suggests that IL-15 and TNF-α may be acting directly in vivo on the vascular endothelium of the peritoneum. However, it should also be noted that IL-1β, which could upregulate HA on ECs in vitro (4), had no appreciable effect in this in vivo model. Like these other cytokine receptors, IL-1βRs are expressed broadly, indicating that regulation does not occur purely at the level of receptor expression. Although IL-1 is known to be a potent inducer of endothelial adhesion molecule expression (34), its failure to induce lymphocyte recruitment in this model may be due to a lack of responsiveness of the type of endothelium in the peritoneum, the production in vivo of "decoy" IL-1R (type II) (35), or simply differences between in vivo and in vitro models. The anatomic details of extravasation into the peritoneum is complex and incompletely understood, but can potentially occur directly through the parietal peritoneum and/or through the serosal surfaces of any of the visceral organs contained within the peritoneum. Regardless, the high degree of specificity of the responsiveness supports the likelihood that IL-15 and TNF-α have distinct roles, at least in part, in the recruitment of activated T cells through a CD44-mediated pathway.

The functional activities ascribed to IL-15 have been described as broadly analogous to those of IL-2 (for review see reference 15). However, IL-15, but not IL-2, has been shown to have a protective role against apoptosis in nonlymphoid and lymphoid cells (10), and IL-15 has an effect on mast cells through an apparently entirely unique receptor unrelated to IL-2R components (28). In addition, it has recently been described that IL-15 induces the production by macrophages of another key proinflammatory cytokine, TNF-α, although IL-2 was also demonstrated to have a lesser effect (29). The role we have described for IL-15 on ECs adds a further distinct bioactivity that appears to be completely independent of IL-2 and also independent of TNF-α. Analysis of mRNA expression shows no detectable levels of IL-2Rα message in any of the murine ECs

tested (Fig. 2). This is in agreement with previous reports for human endothelium (12, 16), and is consistent with the lack of an effect of IL-2 on endothelial HA expression (Fig. 1). In addition, IL-2 had no effect on T cell recruitment in vivo (Fig. 4), and therefore also does not appear to operate through secondary cell types and/or cytokines that would support T cell recruitment in this model. These observations bring a functional relevance to the expression of IL-15Rα on ECs, and do so in such a way as to further accentuate its position in pathways of T cell function.

It is of interest that although all endothelial sources examined express IL-15Rα message as well as the coreceptor chains IL-2Rβ and IL-2Rγ (Fig. 2), only a subset of these responded to IL-15 with increased HA expression (Fig. 1). Although it is possible that the level of message expression does not reflect the protein products for these chains, the evidence is also consistent with the possibility that IL-15R expression alone is not sufficient for this response, and that downstream signaling mechanisms determine the responsiveness of particular ECs to HA production. These observations will require further investigation.

The studies presented here add the induction of EC adhesion receptors governing the initial phase of T cell adhesion to the list of effector functions coordinated by IL-15, and thus extend this cytokine's influence to the endothelial lumenal surface. Recently, IL-15 has been shown to enhance the transmigration of T cells through endothelium, although the mechanism was not clarified (30). Thus, it is attractive to suggest that under inflammatory conditions perivascular tissues and endothelium produce an IL-15 chemotactic gradient that induces endothelial HA production, thereby promoting adhesion and subsequent coordinated migration through the endothelium. Since IL-15 clearly has been shown to be a potent chemoattractant for T cells (6, 36, 37), this may further result in the migration of T cells towards the origin of the stimulus within the tissues proper. IL-15 has been shown to protect activated T cells from apoptosis (9, 38), and in particular has antiapoptotic effects on Fas-mediated death of activated T and B cells, as well as additional cell types in vivo (10). This suggests that IL-15 may prolong the survival of activated T cells under inflammatory states, favoring the execution of their effector functions. Recent observations that IL-15 selectively stimulates a CD44^{hi} CD8 T cell population in vivo and in vitro further supports a role for this cytokine in memory/effector T cell function (39) and is consistent with the evidence presented here. Therefore, IL-15 has the capacity to support multiple events in the life cycle of an effector T cell, beginning with its initial attachment to endothelium during extravasation, as demonstrated here, followed by migration through the vascular barrier and within tissues, and finally enhancement of survival within appropriate sites of antigenic challenge.

Although IL-15 and its various activities have been implicated in the pathogenesis of rheumatoid arthritis (13, 29) and may also play a prominent role in other chronic autoimmune diseases (40, 41), another cytokine thought to be key in the pathogenesis of rheumatoid arthritis is TNF-α. Approaches directed at inhibiting the effects of TNF-α have in fact shown significant therapeutic promise (42, 43). A significant relationship between IL-15 and TNF-α has been demonstrated, in that IL-15 was observed to induce TNF-α production from T cells as well as secondarily from macrophages in a contact-dependent fashion, suggesting that IL-15 acts upstream of TNF-α during rheumatoid arthritis (29). Since both TNF-α and IL-15 induce endothelial HA expression, and both can be produced by ECs, it was of interest to examine whether these cytokines acted coordinately or independently to achieve increased HA levels. The presence of antibody to either cytokine in culture had no effect on the ability of the other to induce HA expression, suggesting that these cytokines act independently at the receptor level and can each have their effects directly on ECs (Fig. 6 A). Moreover, administration of anti–TNF-α antibody in vivo had no effect on the ability of IL-15 to support T cell recruitment into the inflamed peritoneum (Fig. 6 B). This further suggests that even in the presence of additional resident peritoneal cells, such as macrophages, which may be induced by IL-15 to secrete TNF-α, it is not required for the CD44-mediated recruitment of T cells in response to IL-15. Thus, although TNF-α may frequently be a final mediator of inflammatory responses whose production is influenced by IL-15, the evidence presented here suggests that IL-15 has a more direct effect on endothelium itself in T cell extravasation.

In summary, these studies establish a unique function for IL-15 in affecting pathways of activated T cell recruitment to an inflamed site by directly inducing endothelial HA expression, which allows activated T cells to bind via activated CD44. The results reinforce the key role that IL-15 plays throughout multiple stages of T cell effector function, and helps to tie the expression of IL-15 and its receptor on endothelium to the central theme of IL-15 in T cell–mediated immune and autoimmune responses.

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Address correspondence to Mark Siegelman, Department of Pathology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9072. Phone: 214-648-4121; Fax: 214-648-4070; E-mail: siegelman@utsw.swmed.edu

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