American Journal of Pathology, Vol. 151, No. 6, December 1997
Copyright © American Society for Investigative Pathology

Lymphocyte Recruitment and the Kinetics of Adhesion Receptor Expression during the Pulmonary Immune Response to Particulate Antigen

Frances M. Wolber,* Jeffrey L. Curtis,†‡ Alicja M. Milik,† Tina Fields,* Gerami D. Seitzman,† KyungMann Kim,§ Sucha Kim,† Joanne Sonstein,† and Lloyd M. Stoolman*§

From the Departments of Pathology and Internal Medicine† (Pulmonary and Critical Care Medicine Division), University of Michigan Medical Center, the Pulmonary and Critical Care Medicine Section, Department of Veterans Affairs Medical Center,‡ and the University of Michigan Comprehensive Cancer Center,§ Ann Arbor, Michigan*

The selectins and β2 integrins participate in the recruitment of neutrophils in acute pulmonary inflammation. However, the cell adhesion receptors that mediate lymphocyte trafficking into the lung have not been defined. This study examined the relationship between cell adhesion molecules on the pulmonary vasculature and on lymphocytes recovered from the lung during a pulmonary immune response to intratracheal (IT) sheep red blood cells (SRBCs) in sensitized C57BL/6J mice. Silver-enhanced immunogold staining and reverse transcriptase polymerase chain reaction of lung tissues revealed sustained induction of VCAM-1, E-selectin, and P-selectin on the pulmonary vasculature for up to 7 days after IT-SRBC challenge. Neither the MECA 79 nor MECA 367 antigens were induced on the pulmonary vasculature during this period. In the peripheral blood, both CD4 and CD8 T-cell subsets showed an initial increase in P-selectin ligand expression after IT-SRBC challenge. The number of P-selectin ligand-positive T cells in the peripheral blood fell as T cells with both P-selectin and, to a lesser extent, E-selectin ligands accumulated in the bronchoalveolar lavage fluid. We conclude that IT-SRBC challenge in sensitized mice elicits prolonged synthesis of P-selectin, E-selectin, and VCAM-1 by the lung vasculature as well as selectin ligand synthesis by responding T cells. Furthermore, the entry of selectin-ligand-positive T cells into the circulation and their accumulation in the bronchoalveolar lavage fluid indicates that these receptors may contribute to T cell recruitment. Finally, VCAM-1 on the vasculature may also participate; however, the vascular addressins, required for homing to peripheral and mucosal lymphoid organs, are not essential for T-cell entry into the lung following IT-SRBC challenge. ***(Am J Pathol 1997, 151:1715–1727)***

Leukocyte recruitment is central to such clinically important immunological lung diseases as asthma, sarcoidosis, transplant rejection, and pulmonary fibrosis.[1,2] In many organs, recruitment begins with interactions between complementary cell adhesion molecules (CAMs) on the circulating leukocytes and activated endothelia within target tissues.[3,4] A widely held paradigm states that the initial contact between the leukocyte and the endothelial cell is mediated by a subset of adhesion molecules including the selectins[5–7] and the α4-integrins.[8–10] These receptors mediate transient adhesive interactions, or rolling behavior, under shear. Locally produced chemokines trigger a rapid increase in the binding affinity of β1 and β2 integrins on the rolling leukocytes. These integrins then stabilize attachment by forming shear-resistant bonds to endothelial counter-receptors of the immunoglobulin supergene family, including vascular cell adhesion molecule-1 (VCAM-1) and the intercellular adhesion molecules (ICAMs).

Experiments in animal models confirm that CAMs regulate leukocyte recruitment and contribute to cell-mediated tissue damage *in vivo*. The selectins mediate granulocyte recruitment in acute inflammatory processes in the lung,[11–14] peritoneal cavity,[15,16] meninges,[17] and heart.[18] T-lymphocyte recruitment into delayed-type hypersensitivity lesions of the skin[19–22] and kidney[23] use the selectins as well. The α4 integrins contribute to T lymphocyte and monocyte recruitment during immune-mediated tissue damage in the brain,[24–26] pancreas,[27] kidney,[28] skin,[29] and synovium.[30] Finally, the vascular addressins, which initiate lymphocyte recirculation

Portions of these data were presented in abstract form at the Ninth International Congress of Immunology meeting, San Francisco, CA (July 1995), and FASEB, New Orleans, LA (June 1996).

Supported in part by NIH/NHLBI P50-HL46487 (J. L. Curtis and L. M. Stoolman), NIH HL31963 (L. M. Stoolman), and F32-HL08763 (S. Kim) and by research funds from the Department of Veterans Affairs. J. L. Curtis is a career investigator of the American Lung Association of Michigan.

Accepted for publication September 8, 1997.

Address reprint requests to Dr. Lloyd M. Stoolman, Department of Pathology, University of Michigan Medical Center, Ann Arbor, MI 48109-0602.

through lymphoid organs,[31–33] appear at sites of intense immunological activity in rheumatoid synovium[34] and in murine insulinitis.[35] Consequently, these receptors may augment recruitment into nonlymphoid organs as well.

Despite this extensive literature, the contribution of CAMs to T lymphocyte recruitment in the lung is not well defined. *In vivo* blocking studies provide some support for the involvement of α4/VCAM-1[36,37] and LFA-1/ICAM-1[38] interactions during ovalbumin and *Ascarus*-induced T lymphocyte recruitment in rodents. In humans, T lymphocytes in the bronchoalveolar lavage fluid (BAL) of patients with stable chronic inflammatory lung diseases, including asthma, do not express high levels of E-selectin ligands, implying that selectins may not participate in T cell recruitment into the lung.[39] However, the prospective analysis of CAM kinetics early in the development of an immunological response is difficult to conduct in humans and has not been reported in animal models. Furthermore, no information is available on the regulation of P-selectin and its ligands on the T cell during pulmonary immune responses.

Consequently, the current study evaluated CAM expression on pulmonary vascular endothelium and T lymphocytes during the response to intratracheal (IT) injection of sheep red blood cells (SRBCs) in sensitized mice. In this model,[40,41] mild edema and infiltrates of polymorphonuclear leukocytes develop within 1 day after intratracheal injection (post-IT). As the neutrophilic infiltrate resolves, large perivascular, peribronchial, and alveolar infiltrates of T cells, B cells, and monocytes develop. These infiltrates peak at 3 to 4 days post-IT, begin to decline by day 7, and gradually resolve over 4 to 6 weeks.

This well characterized model was chosen for study because T cell recruitment is both antigen specific and T cell dependent.[40,42] Specifically, the mononuclear infiltrates develop only after intraperitoneal sensitization to SRBCs. This response cannot be elicited by nonspecific irritants or by erythrocytes other than those used for priming. In addition, blockade of the CD4 epitope before IT challenge suppresses development of the mononuclear infiltrates without depleting CD4-positive T cells from the host. Thus, SRBC-specific, CD4-dependent immunological responses are required for development of the mononuclear infiltrates in this model. The experiments described herein demonstrate that P-selectin, E-selectin, and VCAM-1 are potential mediators of T cell recruitment during the pulmonary immune response to SRBCs. In contrast, no evidence for participation of the peripheral lymph node or mucosal vascular addressins in lung lymphocyte recruitment is found.

Materials and Methods

Animals

All experiments were performed on specific-pathogen-free female C57BL/6 mice, 10 to 16 weeks of age. Mice were obtained from Jackson Laboratory (Bar Harbor, ME) and housed in specific-pathogen-free animal rooms at the Veterans Administration Medical Center Animal Care Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were given routine animal chow (Rodent Lab Chow 5001, Purina, St. Louis, MO) and chlorinated tap water *ad libitum*. All procedures were performed according to a protocol approved by the Animal Care Committees of the VA Medical Center and the University of Michigan Medical Center. This study complied with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (DHEW publication (NIH) 80-23, revised 1978, Office of Science and Health Reports, DRR/NIH, Bethesda, MD).

Animal Model and Experimental Design

The secondary pulmonary immune response was induced in previously primed mice by IT challenge of particulate antigen using SRBCs (Colorado Serum Co., Boulder, CO; sheep 4158). Briefly, mice were primed by an intraperitoneal injection of 1×10^8 saline-washed SRBCs, and 14 to 21 days later the animals were lightly sedated with intraperitoneal pentobarbital (0.05 mg/g body weight; Nembutal, Abbott Laboratories, North Chicago, IL) and restrained in the supine position. The trachea was exposed via a vertical neck skin incision and sharp dissection of the neck muscles, taking care to avoid lacerating the great veins. SRBCs (5×10^8 cells in 50 μl of physiological saline) were injected directly into the trachea using a tuberculin syringe and a 30-gauge needle. Successful antigen administration was confirmed by the immediate increase in respiratory rate. The restraint board was immediately elevated to favor antigen retention in the distal airspace. The incision was closed using stainless steel skin clips, and the mice were allowed to recover under a warming lamp. The lungs of animals sacrificed immediately after challenge show a nonuniform distribution of SRBCs in bronchi and alveoli in multiple lobes bilaterally. The distribution varies from one animal to the next, but previous studies showed that a reproducible, statistically significant, CD4-dependent recruitment of mononuclear leukocytes occurs in all sensitized animals.[40,42,43]

At various times after initiating the pulmonary response, lungs and control lymphoid tissue were harvested for use in various assays. Three types of experiments were performed on separate groups of mice. To define the temporal and spatial distribution of CAMs, tissues from mice at various stages in the SRBC-induced inflammatory response were cryopreserved and snap-frozen for immunohistochemistry studies. To demonstrate transcriptional regulation of endothelial selectin expression, semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) was performed on RNA extracted from lung tissue of mice. As a positive control, tissues were harvested from unchallenged mice 4 hours after intraperitoneal injection with 50 μg of lipopolysaccharide (LPS; Sigma Chemical Co., St. Louis, MO), which is known to induce expression of E-selectin, P-selectin, and VCAM-1 in murine lungs.[44–46] To measure P- and

Table 1. Sources and Concentrations of Antibodies

Antigen	Clone	Source	Isotype	Concentration
CD4-FITC	RM4-5	PharMingen	Rat IgG2a	5 μg/ml
CD8-FITC	54-6.7	PharMingen	Rat IgG2a	5 μg/ml
CD90.2-FITC	53-2.1	PharMingen	Rat IgG2a	5 μg/ml
CD45-FITC	30F11.1	PharMingen	Rat IgG2a	5 μg/ml
E-selectin chimera	N/A	J. Lowe	Human IgM	1:2 (culture supernatant)
P-selectin chimera	N/A	J. Lowe	Human IgM	1:2 (culture supernatant)
VCAM-1	429	Pharmingen	Rat IgG2a	0.1 μg/ml
E-selectin	10E9/6	D. Vestweber	Rat IgG2a	1:10 (culture supernatant)
P-selectin	polyclonal	D. Vestweber	Rabbit IgG	2 μg/ml
HEV antigen	MECA 325	E. Butcher	Rat IgG1	10 μg/ml
Peripheral node addressin	MECA 79	E. Butcher	Rat IgM	10 μg/ml
CD31 (PECAM-1)	MEC 13.3	Pharmingen	Rat IgG2a	5 μg/ml
Mucosal addressin (MAdCAM)	MECA 367	E. Butcher	Rat IgG2a	5 μg/ml

E-selectin ligand expression, leukocytes were collected from lung and peripheral blood of normal and inflamed mice and assayed by two-color flow cytometry using leukocyte subset-specific antibodies and selectin chimeric proteins.

Tissue Collection and Processing for Immunohistochemistry

Mice were deeply anesthetized with pentobarbital (80 mg/kg) and exsanguinated. The trachea was cannulated with plastic tubing (PE-50, Clay-Adams, Parsippany, NJ), and the lungs were fixed *in situ* by instilling freshly made 2% formaldehyde (Tousimis, Rockville, MD) containing 0.01 mol/L sodium metaperiodate (Sigma) in a 3:1 solution of 100 mmol/L L-lysine hydrochloride (Sigma)/50 mmol/L phosphate buffer, pH 7.4. The lungs were then removed, sliced parasagittally into four sections, and infused with the above fixative for 1 hour followed by three 5-minute washes in phosphate-buffered saline (PBS; Gibco, Gaithersburg, MD) containing 5% sucrose (Sigma) at 5°C. The tissues were then incubated with agitation at 5°C in sequential solutions of 7.5, 10, 12.5, and 15% sucrose in PBS for 30 minutes. After a final 30-minute immersion in 14% sucrose/33% OCT compound (Miles Laboratories, Elkhart, IN) at room temperature, lung blocks were embedded in OCT, snap-frozen in liquid-nitrogen-cooled 2-methylbutane, and stored at −70°C until use.

Preparation of Tissue Sections for Immunohistochemistry

Standard 5-μm-thick sections were cut from lung blocks using a refrigerated microtome (Frigocut 2800, Cambridge Instruments Inc., Buffalo, NY) and placed on saline-treated glass slides (Vectabond, Zymed, South San Francisco, CA). Tissue sections were hydrated in PBS and pretreated for 10 minutes with a blocking solution containing equal amounts of casein (Cas-Block, Zymed) and 10% normal goat serum (Zymed) in PBS to block nonspecific binding. All incubations were carried out in a humidity chamber at room temperature. After draining the excess blocker, the primary antibodies were diluted in the above blocking solution and placed on the tissues. The characteristics and sources of the primary antibodies used are listed in Table 1. To determine optimal staining concentrations, all antibodies and conjugates were titered on sections of spleen, lymph node, or lung tissue from LPS-stimulated mice. Isotype-matched antibodies of irrelevant specificity (The Binding Site, San Diego, CA) were used as controls in every experiment.

Gold Staining

After a 1-hour incubation with primary antibody, sections were rinsed three times in PBS and incubated for 30 minutes with a secondary antibody conjugated to 4-nm-diameter gold particles (Accurate, Westbury, NY). After three washes in PBS, the staining was enhanced with silver (SilvEnhance, Zymed), and the sections were washed three times in distilled water, counterstained with hematoxylin, dehydrated in ethanol and xylene, and coverslipped using Histomount (Zymed).

Fluorescent Staining

After a 1-hour incubation with primary antibody, sections were rinsed three times in PBS and then incubated with a secondary antibody conjugated to phycoerythrin (Southern Biotechnology, Birmingham, AL) at room temperature in a humidity chamber. The slides were then briefly rinsed twice in PBS and fixed with 2% paraformaldehyde for 20 minutes at 5°C. Excess paraformaldehyde was shaken off, and the sections were mounted under Aquamount (Lerner Laboratories, Pittsburgh, PA).

Quantitation of Stained Tissues

Immunostained slides were coded and analyzed microscopically in a blinded and randomized fashion at ×400 (VCAM-1), ×200 (P-selectin), and ×1000 (E-selectin). The lung sections included the peripheral, central, and hilar lung fields to minimize bias. The density and extent of the silver-enhanced gold staining observed on the lumens of each was graded on a scale of 0 (no-stain) to 4+ (dense stain covering the entire lumen) using an Olympus light microscope. In each tissue section, non-

overlapping microscopic fields covering most of the section were examined. Sixty to eighty individual vessels in six to seven lung sections taken from three mice were evaluated for each adhesion receptor and time point. The mean percentages of stained vessels and the mean intensity of vascular staining in areas with and without leukocytic infiltrates were determined for each treatment condition. Nonparametric and regression analyses (see below) were performed to evaluate the significance of the observed differences.

The many steps involved in the silver-enhanced immunogold technique made this approach unwieldy for the semiquantitative studies. Therefore, a two-step immunofluorescence stain (described above) was also developed and used to detect VCAM-1 expression. Sections were read on an Olympus epifluorescence microscope with a mercury-vapor lamp. Phycoerythrin-conjugated secondary antibodies were used as the molar quantum yield of phycoerythrin is ~10-fold greater than other fluorochromes, ensuring the highest level of sensitivity. Furthermore, tissue autofluorescence is lower in the near-red (phycoerythrin emission) than green (fluorescein isothiocyanate (FITC) emission) region of the spectrum. The criterion used for positive vessels and staining intensity were the same as with immunogold except that fluorescence intensity, rather than silver density, was evaluated by the observer.

Pre-Embedding and Thin Section Immunohistochemistry

Thirty-micron-thick sections were cut from lung blocks using a refrigerated microtome and applied to gelatin-coated plastic slides (Miles Laboratories). The staining procedure was the same as described for the 5-μm-thick sections, except that incubation times were increased to 3 hours with the blocking solution and to >12 hours with the primary and secondary antibodies. The incubations were conducted in a humidity chamber at 5°C with three 1-hour washes in PBS between each step. The slides were soaked in distilled water (1 hour), fixed in 1.5% glutaraldehyde (Tousimis) in 0.15 mol/L cacodylate buffer (1 hour), rinsed twice in cacodylate buffer, and infused with 4% OsO_4 (Sigma) in cacodylate-buffer (1 hour). The tissues were then washed twice in cacodylate buffer, dehydrated in ethanol, partially dried, and embedded by inverting a capsule filled with epoxy (Epon, Polysciences, Warrington, PA) over the tissue section. The capsules were removed from the slides and labeled after an overnight incubation at 60°C.

One-micron-thick sections were cut from the Epon-embedded tissue using a glass knife and microtome. The sections were collected in a water-filled boat, retrieved using a wooden pick, and placed in a drop of sterile water on a glass slide. The sections were dried on a heated plate (10 minutes), stained with 1% toluidine blue (5 minutes), rinsed exhaustively with sterile water, redried (30 minutes), dehydrated with ethanol, infused with xylene, and coverslipped under Histomount for light microscopy.

Polymerase Chain Reaction (PCR)

Tissues were homogenized in RNA STAT-60 (TEL-TEST, Friendswood, TX) homogenization buffer, followed by chloroform/isopropanol extraction and ethanol precipitation. RNA pellets were briefly air dried and resuspended in diethylpyrocarbonate (DEPC)-treated RNAse-free water, and the OD_{260} was measured (Beckman Spectrophotometer 480). Reverse transcription (RT) reactions for all samples were performed simultaneously using Moloney murine leukemia virus RT (Gibco/BRL), deoxynucleotide triphosphates (dNTPs; Boehringer Mannheim, Indianapolis, IN) and dithiothreitol. Control DNA was isolated from plasmids containing full-length cDNA inserts of murine L-, E-, or P-selectin (gift of John Lowe, University of Michigan).

First-strand cDNA concentrations in each sample were equalized to control for differences in starting mRNA concentrations and for possible differences in RT efficiency between samples. Serial 1:5 dilutions were amplified using primers for the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and each sample was adjusted to give equivalent GAPDH product. The accuracy of this equalization was confirmed by amplifying aliquots of each adjusted sample using primers for cyclophilin. The equalized cDNA samples were amplified with primers for P- and E-selectin for 33 and 30 cycles, respectively, which resulted in amplification in the exponential part of the curve. Five micrograms of first-strand cDNA were amplified in 25 μl of DEPC water containing the sense and antisense primers (0.5 μl, 200 nmol/L), dNTPs (2.5 μl, 10 mmol/L), 0.5 U of *Taq* polymerase (Gibco/BRL) and 5 μl of PCR buffer A (the PCR optimizer kit; Invitrogen Corp., San Diego, CA). PCR was performed in a DNA Thermocycler 480 (Perkin-Elmer, Norwalk, CT) under the following amplification conditions: 60 seconds of denaturation at 95°C, 120 seconds of annealing at 55°C, and 60 seconds of extension at 72°C. A final extension of 7 minutes at 72°C was performed after the last cycle only. In all experiments, control reactions lacking cDNA yielded no reaction products.

PCR was performed using the following specific primer pairs (sense and antisense, respectively): cyclophilin, 5′ACCTAAAGTCACAGTCAAGG-3′ and 5′-TGGTGTC-TTTGCCTGCATTG-3′ (gift of Nick Lukacs and Steven Kunkel, University of Michigan); GADPH, 5′-CAGTCT-TCTGAGTGGCAGTG-3′ and 5′-CTGGTGCTGAGTAT-GTCGTG-3′; P-selectin, 5′-AGACGGATCAGAGAGGA-CAT-3′ and 5′-GACGTCATT-GAGGTGAGCGA-3′; E-selectin, 5′-GATCCAACGCCAGAACAACA-3′ and 5′-TGCACTGGCTTCATCATACG-3′; and L-selectin, 5′-GAT TGGCTCTGGAGATGACC-3′ and 5′-TCTGCTACACAG CCTCTTGC-3′. Selectin primers were designed using ALIGN Plus (version 2) software (Scientific and Educational Software, State Line, PA), based on sequences obtained from GenBank, and synthesized by the University of Michigan DNA Synthesis Core Facility.

PCR reaction products were analyzed by electrophoresis on agarose gels containing ethidium bromide, visualized by ultraviolet transillumination, and immedi-

ately photographed. One microgram of 100-bp ladder marker (Gibco/BRL) was run in parallel for molecular size marker comparison, and the specificity of the amplified target sequences was validated by their predicted size. The relative concentrations of final reaction products were determined using the gel macros in NIH Image version 1.60 (NIH Image is available on the Internet at http//rsb.info.nih.gov/nih.image/). Gels shown are representative of three to four separate amplification reactions.

Lymphocyte Collection and Purification

Mice were deeply anesthetized as above and exsanguinated via direct heart puncture with a heparinized syringe. The peripheral blood was subjected to hypotonic lysis to remove erythrocytes, washed, and resuspended in cold PBS. Lungs were perfused via the right ventricle with approximately 5 ml of saline until the tissue blanched completely. The trachea was then cannulated with plastic tubing and the lungs lavaged with 10 successive 1-ml washes of PBS containing 0.5 mmol/L EDTA (Sigma) as described elsewhere.[42] The BAL cells were washed, resuspended in PBS, and kept on ice until use. Total leukocyte counts were determined by hemocytometer counts of peripheral blood and BAL after hypotonic lysis of erythrocytes.

Lymphocyte Expression of Selectin Ligands

Leukocytes from BAL and peripheral blood were resuspended in Dulbecco's minimal essential medium (DMEM) staining buffer containing 0.1% sodium azide and 0.1% bovine serum albumin (Sigma) at 5×10^6 cells/ml. The entire assay was carried out at 5°C, and 100-μl aliquots of the cell suspensions were placed in a 96-well round-bottomed plate (Corning, Corning, NY). After washing and centrifuging once, the cells were vortexed and resuspended in 50 μl of a cell culture supernatant containing the murine P- or E-selectin-human IgM chimeric protein (gift of John Lowe, University of Michigan) as described previously.[16] EDTA (10 mmol/L) was added to all solutions in some wells as a control for nonspecific binding (physiologically relevant selectin-chimera binding is Ca^{+2} dependent). After 30 minutes of incubation at 5°C, 150 μl of staining buffer was added and the cells washed once. Then, 50 μl of biotinylated goat anti-human IgM secondary antibody (Zymed) was added and the cells incubated as above. After washing once as above, 50 μl of a streptavidin/phycoerythrin conjugate (Pharmingen, diluted 1:200 in staining buffer) was added, together with 5 μg/ml FITC-labeled anti-mouse CD4 or CD8 (Pharmingen, San Diego, CA). After a final wash to remove unbound reagents, the cells were resuspended in fixative (2% paraformaldehyde in PBS containing 1 g/L $CaCl_2$ and $MgCl_2$) and stored at 5°C. Samples were read within 36 hours by two-color flow cytometry (FACScan, Becton Dickinson, Mountain View, CA) and analyzed using the WinList analysis program (Verity Software House, Topsham, ME).

Statistical Analysis

All data are expressed as mean ± SEM. The immunohistochemical studies were analyzed initially using analysis of variance (ANOVA) followed by Student-Neuman-Keuls (SNK) *t*-test or unpaired *t*-test as indicated in the figure legends. In addition, the random-effects binary (probit) and ordinal regression (cumulative probit) models of Hedeker and Gibbons,[47,48] respectively, were also used to analyze the selectin expression studies. These analyses assumed a binomial distribution for the estimates of positivity, a multinomial distribution for the estimates of expression intensity, and an underlying, or latent, normal distribution for both data sets. The models accounted for the fact that observations in individual animals showed less variance than observations made on different animals within a treatment group. In general, regression analysis revealed higher degrees of significance between treated and untreated animals than the nonparametric analysis. The flow cytometry data were analyzed by ANOVA, followed by Student-Neuman-Keuls (SNK) *t*-test for multiple samples or by Student's paired or unpaired *t*-test for two samples. $P \leq 0.05$ was considered significant in all data analyses.

Results

Selectin Kinetics and Distribution

Minimal E-selectin staining was seen in the normal mouse lung. After IT-SRBC challenge, E-selectin expression peaked at 24 hours with greater than 90% of the vessels showing at least some degree of positive staining. Both the percentage of stained vessels (Figure 1A) and the intensity of staining (Figure 1B) for E-selectin were significantly increased in the challenged lungs. The staining reaction was specific as little reactivity was seen with an isotype-matched control antibody (Figure 2, A and B). Initially, E-selectin staining was significantly elevated on vessels in areas both with and without leukocytic infiltrates (Figure 1). In areas without infiltrates, the percentage of positive vessels and the intensity of staining were elevated on days 1 and 2 and then fell to near baseline on days 3 to 7. In areas with infiltrates, staining for E-selectin remained above baseline for 4 to 7 days.

RT-PCR demonstrated that E-selectin mRNA levels in whole-lung extracts roughly paralleled the level of protein expression on the vasculature. As shown in Figure 3, normal lung tissue contained very little detectable message for E-selectin. Upon initiation of the pulmonary lesion, E-selectin mRNA rose rapidly to a level nearly eight times that seen in unchallenged lung. The message level remained elevated during the first 2 days post-IT and then diminished rapidly, returning to baseline by day 4 post-IT.

In contrast to E-selectin, weak P-selectin staining was observed on >95% of the vessels in normal mouse lung. Whether this low reactivity reflects constitutive cell surface expression, staining of cytoplasmic storage pools, or staining of activated platelets cannot be determined.

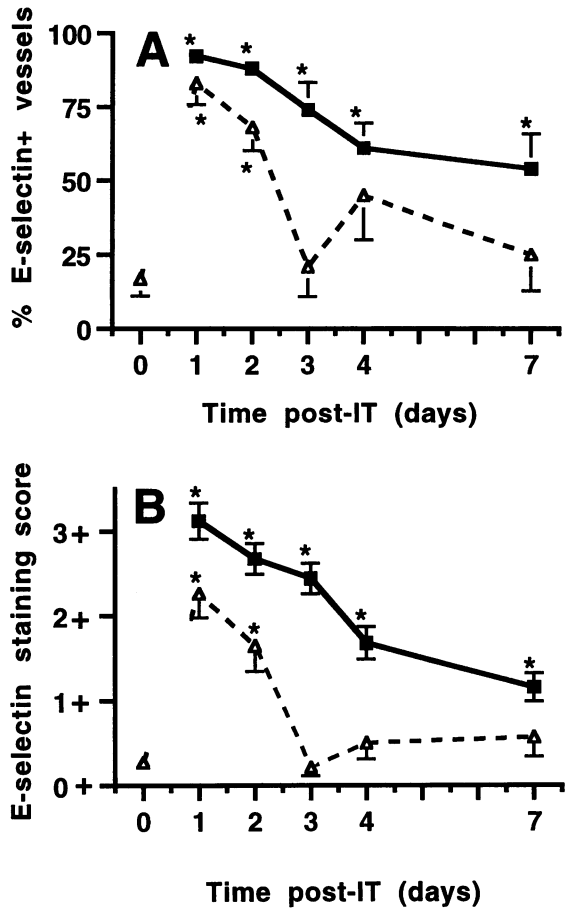

Figure 1. E-selectin expression on pulmonary endothelium before and after IT-SRBC challenge. SRBC-sensitized animals were sacrificed at the indicated times after IT challenge with 5×10^8 SRBCs. E-selectin expression was quantified on silver-enhanced, immunogold-stained sections as described in Materials and Methods for vessels in areas with (■) and without (△) leukocytic infiltrates. **A:** Mean percentage of blood vessels positive for E-selectin staining to any degree. **B:** Mean intensity of E-selectin staining on blood vessels, graded on a scale of 0 (no staining) to 4 (100% of vessel surface strongly stained). *$P < 0.05$ by ANOVA and <0.025–0.001 by random-effects binary (probit) and ordinal regression (cumulative probit) analysis compared with day 0 (see Materials and Methods).

However IT-SRBC challenge significantly increased the staining intensity on vessels within 24 hours (Figure 4). The receptor was associated with arteries, veins, and venules. As with E-selectin, staining intensities were initially elevated throughout the lung vasculature (Figure 4). In areas without infiltrates, the levels fell to baseline by day 4. In areas with infiltrates, staining for P-selectin remained elevated throughout the 7-day observation period. No staining was seen with an isotype-matched control antibody (Figure 2, C and D).

P-selectin is sequestered in cytoplasmic storage granules (eg, Weibal-Palade bodies in humans) before mobilization to the cell surface. As only expression on the vascular lumen is physiologically significant, pre-embedding immunocytochemistry was used to examine the distribution of the receptor in the inflamed lung. Thirty-micron slabs of cryopreserved frozen tissue were stained for P-selectin and embedded in epoxy resin before sectioning. Tissue processed in this manner can be sectioned at 0.5 to 1 μm rather than the 4 to 8 μm typical for routine frozen sections. The thin sections clearly showed reactivity for the P-selectin protein on the endothelial surface but not in the cytoplasm. High density staining was evident in areas of mononuclear leukocyte infiltration as late as day 7 post-IT (Figure 2E). There was no endothelial staining with an irrelevant control antibody (Figure 2F).

P-selectin mRNA, presumably of endothelial origin, was also induced in the lung during the immune response. As shown in Figure 5, message for P-selectin increased dramatically within 24 hours of initiating the pulmonary lesion, reaching a level nearly 20-fold higher than that seen in unchallenged lung. P-selectin mRNA levels remained elevated longer than E-selectin levels, falling to baseline only at day 7. Thus, both P-selectin mRNA and vascular protein levels were elevated throughout the period of peak lymphocyte recruitment.

VCAM-1 and Vascular Addressins: Kinetics and Distribution

VCAM-1 was readily detected at low levels on most pulmonary vessels in untreated, specific-pathogen-free mice when visualized by either immunogold or immunofluorescent staining. In contrast, staining was not observed on alveolar capillaries, alveolar macrophages, or mononuclear cells in the interstitial space of untreated animals. As shown in Figure 6, IT-SRBC challenge up-regulated vascular VCAM-1 expression within 1 day ($P < 0.025$). Levels remained elevated during the period of peak lymphocyte recruitment, falling toward baseline at the 7-day time point (Figure 2, G and H, and Figure 6). Up-regulation was observed on pulmonary arteries, veins, and venules in areas with and without mononuclear infiltrates. In addition, prominent receptor expression was noted within the mononuclear infiltrates on days 3 to 7 (not shown). The resolution of routine tissue sections was not sufficient to identify the stained structures; however, both large mononuclear cells and interstitial capillaries appeared to be stained.

In contrast to the inducible endothelial CAMs, no expression of the peripheral lymph node addressin (MECA 79), the mucosal vascular addressin (MECA 367), or the nonselective high endothelial venule (HEV) marker MECA 325 was detected on the pulmonary vasculature at any time point (data not shown). Special attention was paid to discern staining within the small perivascular vessels, which previous studies identified as the sites of earliest mononuclear cell entry into the peribronchovascular bundles.[41] Paired sections from lymph node and Peyer's patch showed strong luminal staining of the HEV with the appropriate antibodies.

Selectin Ligands: Expression and Kinetics

Expression of the carbohydrate ligands for P- and E-selectin on T lymphocytes was assessed by their ability to bind selectin-IgM chimeric proteins (Figure 7). The histograms show representative two-color plots of selectin-IgM binding *versus* CD4 expression on cells from periph-

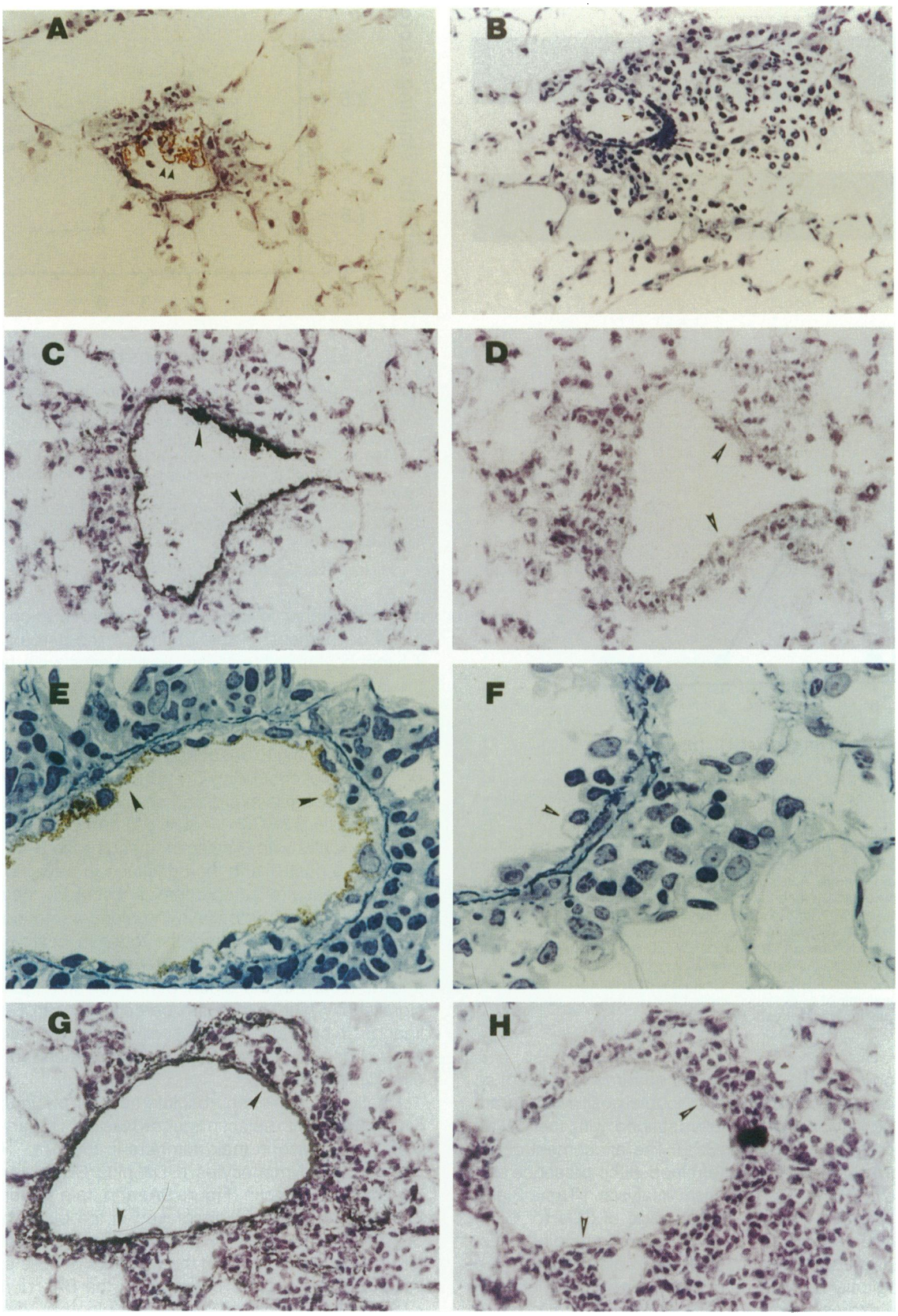

Figure 2. Examples of staining for E-selectin, P-selectin, and VCAM-1 (**arrows**) after IT-SRBC challenge. **A** and **B**: E-selectin (**A**) and isotype-matched control IgG (**B**) staining at day 2. **C** and **D**: P-selectin (**C**) and species-match control IgG (**D**) staining at day 3. **E** and **F**: Pre-embedding immunocytochemistry showing P-selectin (**E**) and species-matched control IgG (**F**) staining at day 7. **G** and **H**: VCAM-1 (**G**) and isotype-matched control IgG (**H**) staining at day 7. Magnification, ×400 (**C**, **D**, **G**, and **H**; 5-μm sections) and ×1000 (**A**, **B**, **E**, and **F**; 1-μm sections).

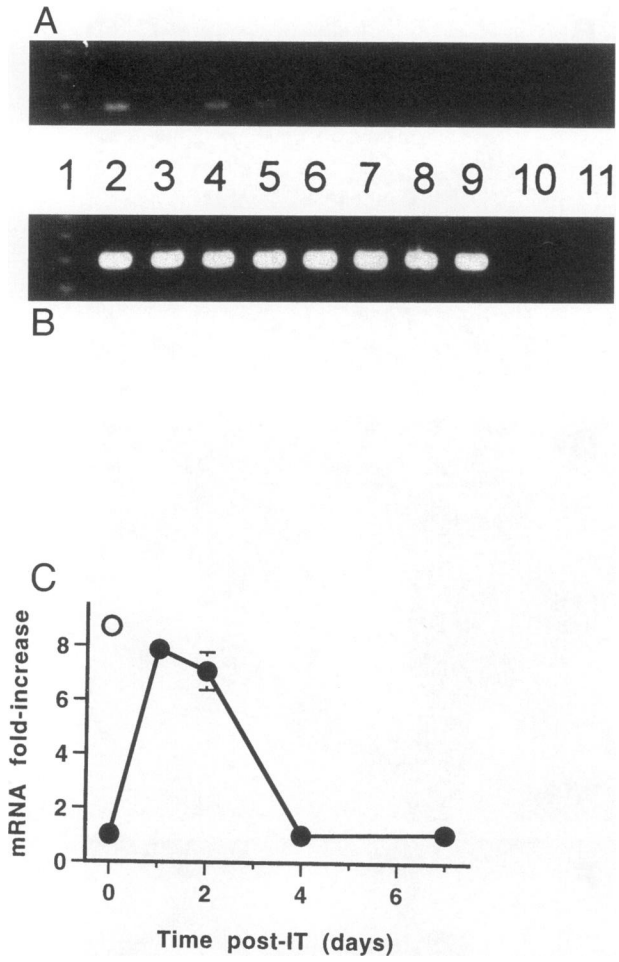

Figure 3. RT-PCR for E-selectin mRNA levels in lung extracts before and after IT-SRBC challenge. SRBC-sensitized animals were sacrificed at the indicated times after IT challenge with 5×10^8 SRBCs. Mice stimulated with a single intraperitoneal dose of LPS 4 hours before sacrifice were used as a positive control. mRNA extracts from whole lungs were prepared, reverse transcribed, equilibrated (for levels of GAPDH), and amplified by PCR as detailed in Materials and Methods. **A:** Representative gel of E-selectin mRNA expression. **Lane 1**, 100-kb ladder; **lane 2**, LPS-stimulated (positive control); **lanes 3** to **9**, days 0, 1, 2, 4, 7, 9, and 14 after IT; **lanes 10** and **11**, no cDNA (negative control). **B:** cDNA samples in **A** amplified for GAPDH. **C:** Densities of the ethidium-bromide-stained PCR products on gels from IT-SRBC-treated (●) and LPS-treated (○) animals *versus* untreated animals. The data are the mean ratios (+SEM) of the E-selectin bands amplified from lung extracts of treated and untreated animal as described above (three to four PCR amplifications and densitometry scans for each time point).

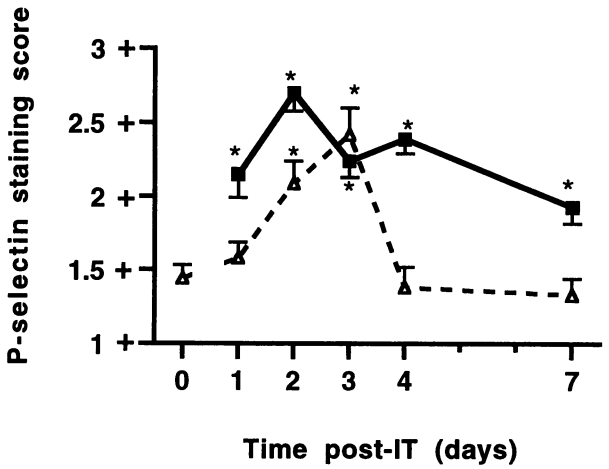

Figure 4. P-selectin expression on pulmonary endothelium before and after IT-SRBC challenge. See Figure 1 for a description of the experiment. P-selectin expression was quantitated on silver-enhanced, immunogold-stained sections as described in Materials and Methods for vessels in areas with (■) and without (△) leukocytic infiltrates. Low-level staining of all vessels was observed in untreated animals; therefore, only changes in staining intensity were estimated (as described in Figure 1B). $^*P < 0.05$ by ANOVA/SNK and <0.001 by random-effects binary (probit) and ordinal regression (cumulative probit) analysis compared with day 0 (see Materials and Methods).

eral blood and BAL. The analysis quadrants were set so that no events appeared in the double-positive quadrant (upper right) with control cells (stained with the CD45-IgM chimera and pooled IgG2a). The attachment of selectin-IgM chimeras was determined in the presence and absence of EDTA. The statistical analysis in Table 2 and Figures 8 and 9 used the percentage of CD4 (or CD8) lymphocytes with EDTA-sensitive selectin-binding sites. Only these Ca^{+2}-dependent selectin-binding sites are physiologically relevant. The histograms show that such sites are expressed on a small percentage of circulating CD4 lymphocytes and a much larger percentage of CD4 lymphocytes in the lung exudate. Statistical analysis of paired samples confirmed that the percentage of both the CD4 and CD8 subsets with selectin ligands was markedly higher in BAL than in peripheral blood at 2.5 and 4 days post-IT (Table 2). In fact, the percentages of T lymphocytes with selectin-binding sites were higher in the BAL than in the blood at all time points examined.

The percentage of circulating T lymphocytes with selectin-binding sites changed during the course of the pulmonary immune response (Figure 8). Only a small percentage of circulating T lymphocytes in either naive or sensitized animals expressed selectin ligands. However, the proportions of CD4 (Figure 8A) and CD8 (Figure 8B) T lymphocytes with P-selectin ligands rose 6- to 10-fold by day 2.5 post-IT and then declined to baseline by day 4. The increase reflected a transient surge in the number of P-selectin-positive cells in the blood as no significant changes occurred in either the total leukocyte or differential counts during this period. At day 2.5, the percentage of T cells in the blood with P-selectin ligands was significantly higher than the percentage with E-selectin ligands. In contrast, no statistically significant differences were observed in the number of ligand-positive cells at other time points.

Finally, the fall in the absolute number of circulating T lymphocytes with selectin ligands was accompanied by a marked increase in their numbers in the BAL. The total number of T lymphocytes (CD4 plus CD8) expressing ligands for P-selectin (Figure 9A) and, to a lessor extent, E-selectin (Figure 9B) decreased in the blood between days 2.5 and 4 post-IT. In contrast, an approximately four- to sixfold increase in the numbers of cells expressing these ligands was observed in the BAL during this interval. As noted in the peripheral blood on day 2.5, a greater percentage of the T cells in BAL expressed ligands for P-selectin than for E-selectin. The difference was less than in the peripheral blood and varied from one experiment to the next (Table 2; Figures 7 to 9) but was

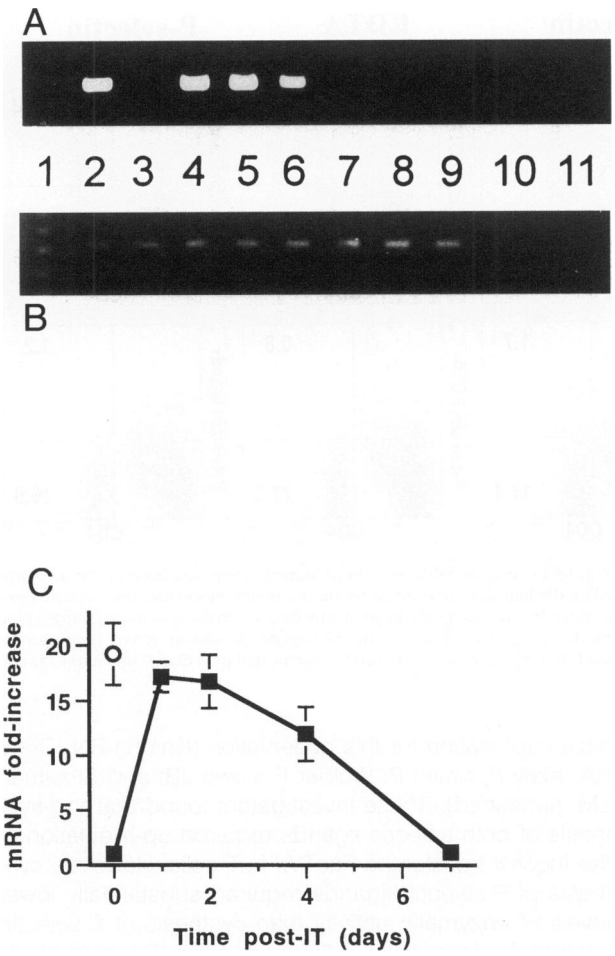

Figure 5. RT-PCR for P-selectin mRNA levels in lung extracts before and after IT-SRBC challenge. See Figure 3 for description of experiment. **A:** Representative gel of P-selectin mRNA expression in mouse lung. **Lane 1**, 100-kb ladder; **lane 2**, LPS-stimulated (positive control); **lanes 3** to **9**, days 0, 1, 2, 4, 7, 9, and 14 after IT; **lanes 10** and **11**, no cDNA (negative control). **B:** cDNA samples in **A** amplified for HPRT. **C:** Densities of the ethidium-bromide-stained PCR products on gels from IT-SRBC-treated (●) and LPS-treated (○) animals relative to untreated animals. The data are the mean ratios (+SEM) of the P-selectin-specific bands amplified from lung extracts of treated and untreated animal as described above (three to four PCR amplifications and densitometry scans for each time point).

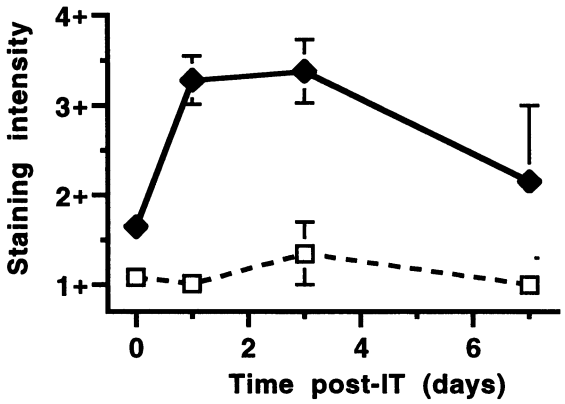

Figure 6. VCAM-1 expression on pulmonary endothelium before and after IT-SRBC challenge. See Figure 1 for a description of the experiment. VCAM-1 staining was quantitated on immunofluorescently stained frozen tissue sections as described in Materials and Methods. Low-level staining of all vessels was observed in untreated animals; therefore, only changes in staining intensity were estimated (as described in Figure 1B). Results from VCAM-1 ◆) and isotype-matched control IgG (□) are shown.

present throughout the period of peak recruitment (days 2 to 4 post-IT).

Discussion

This study resulted in several major findings. The mRNA and vascular-associated protein levels for both E-selectin and P-selectin were induced for days in sensitized mice by challenge with SRBCs. In addition, the peripheral blood showed a transient surge in the absolute number of T lymphocytes carrying P-selectin ligands beginning several days after IT-SRBC challenge. These cells rapidly disappeared from the peripheral blood in parallel with the appearance of cells with a similar phenotype in the inflamed lung. In fact, T lymphocytes recovered from BAL were enriched for expression of selectin ligands at all time points after IT challenge. VCAM-1 was also up-regulated during peak lymphocyte recruitment in this model. In contrast, neither the peripheral nor the mucosal vascular addressins were detected on the pulmonary vasculature at any point.

The protein and mRNA levels for both vascular selectins remained elevated throughout the periods of maximal granulocyte, monocyte and lymphocyte recruitment. These kinetics differ from those reported in selectin-dependent models of acute lung injury where transient elevations in one or both receptors last for less than 24 hours.[11,45,49] Pre-embedding immunocytochemistry confirmed that P-selectin was expressed on the vascular lumen after SRBC challenge and that it was generally not associated with intact platelets. Although a contribution from platelet fragments cannot be ruled out, the marked induction of P-selectin mRNA indicates that new receptor synthesis by endothelial cells is a significant factor as well.

The time course for induction of selectin-specific mRNA and vascular protein implied that SRBC challenge up-regulated synthesis at the pretranslational level. A variety of pro-inflammatory cytokines, including interleukin (IL)-1,[50] tumor necrosis factor (TNF)-α,[45,51,52] IL-3,[53] and IL-4,[54] are known to act at this level. Furthermore, the shift in selectin expression from all vessels to those associated with infiltrates of mononuclear leukocytes suggests a change in the distribution/mix of soluble mediators as the reaction progressed. For example, the release of TNF-α and IL-1 from interstitial or alveolar macrophages may induce the widespread expression of the selectins (and VCAM-1) initially. The reaction of SRBC-specific CD4 memory cells (recruited early) with antigen-presenting cells in peribronchial/perivascular areas might trigger the local release of T-cell cytokines (eg, IL-3 and IL-4) and maintain the output of monokines such as TNF-α.[55] These mediators could then control the makeup and distribution of the late infiltrates through actions on local endothelial CAMs and chemokine synthesis. These hypotheses are currently under investigation.

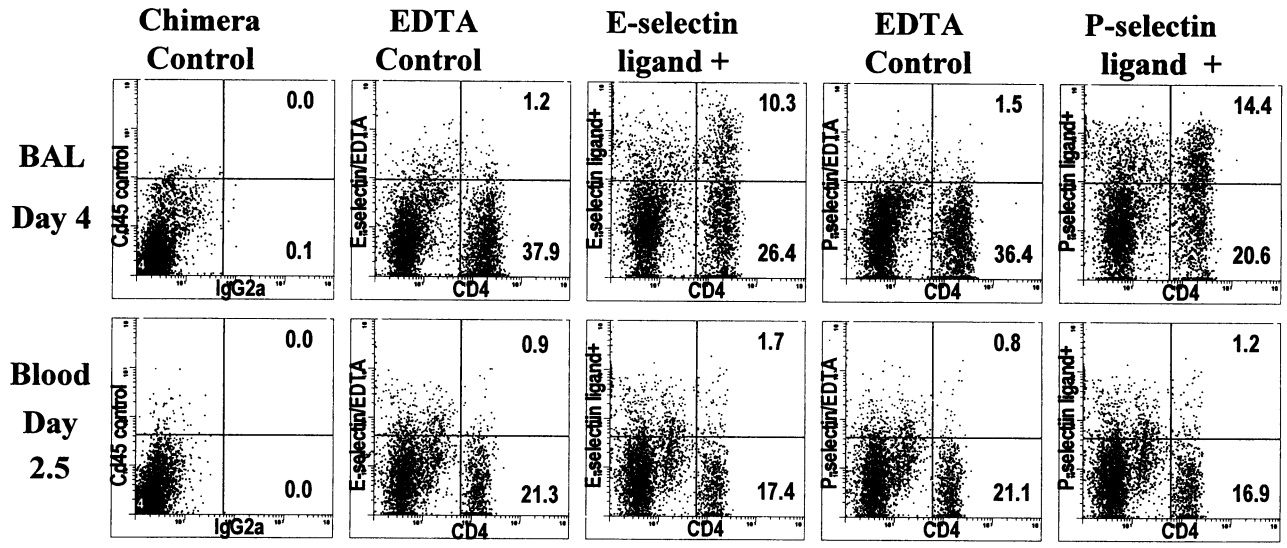

Figure 7. Representative histograms of lymphocytes stained with anti-CD4 and selectin-IgM chimeras. SRBC-sensitized animals were sacrificed at the indicated times after IT challenge with 5×10^8 SRBCs. Two-color flow cytometry was performed on the lymphocyte populations in peripheral blood and BAL. The analysis quadrants were set to exclude nonspecifically stained cells as described in Results. The numbers give the percentage of lymphocytes in the specimen falling within the quadrant. **Top panels:** BAL from day 4 post-IT mouse. **Bottom panels:** Blood from day 2.5 post-IT mouse. From left to right are shown (*x* axis *versus y* axis) IgG2a *versus* CD45-IgM (control), anti-CD4 and E-selectin-IgM plus EDTA, anti-CD4 and E-selectin-IgM, anti-CD4 and P-selectin-IgM plus EDTA, and anti-CD4 and P-selectin-IgM.

Longitudinal studies showed that challenge with SRBCs induced new synthesis of ligands for P-selectin and, to a lessor extent, E-selectin on T lymphocytes. The CD4 and CD8 epitopes were used to identify T-cell subsets. In mice, the CD4 antigen is not expressed to a significant degree on monocytes.[43] Murine lymphocytes carrying the CD8 antigen do not lyse the YAC-1 cell line and, thus, are functionally distinct from true natural killer (NK) cells.[56] The small subset of murine cells that co-express the CD8 or CD4 epitopes and the NK antigen NK 1.1 carry a Vα14+ α/β T-cell receptor as well.[57] The physiological activity of this unusual T-cell subset is not defined. Therefore, the current study did not separate its selectin ligand expression from that of other CD4- and CD8-positive cells.

The ratio of P:E ligand-positive T cells was significantly greater in peripheral blood on days 1 to 2.5 post-IT than at later time points in either BAL or peripheral blood. Recent findings by Kibbs and Stoolman provide a plausible explanation for this observation (Knibbs RN, Craig RA, Màly P, Smith P, Wolber F, Lowe JB, and Stoolman LM, submitted). These investigators found that the synthesis of both selectin ligands required up-regulation of the fucosyl transferase FucTVII in T cells. However, synthesis of P-selectin ligands required substantially lower levels of enzymatic activity than synthesis of E-selectin ligands. Consequently, P-ligand-positive, E-ligand-negative T cells in the blood at early time points may be recently activated cells with relatively low levels of the FucTVII enzyme. Sustained or elevated activity of the FucTVII enzyme in some cells may then increase production of E-selectin ligands at late time points in both BAL and peripheral blood. Whether synthesis occurred primarily in hilar lymph nodes, a major site of immunological activity in this model,[60] or in the lung parenchyma cannot be determined from the data. Nonetheless, the findings show clearly that pulmonary antigenic challenge leads to selectin ligand synthesis on a significant percentage of the responding T cells.

Table 2. Percentages of Selectin Ligand-Positive T Cells in Blood and BAL after IT-SRBC

	Day 2.5		Day 4	
	Blood	BAL	Blood	BAL
CD4: ESL^+	3.1 ± 0.9	21.4 ± 3.4*	1.8 ± 1.3	31.8 ± 2.8†
CD4: PSL^+	11.2 ± 4.7	36.2 ± 5.6*	4.8 ± 1.0	47.2 ± 3.0†
CD8: ESL^+	0.0 ± 0.0	14.0 ± 3.0*	1.2 ± 0.7	17.2 ± 2.5†
CD8: PSL^+	3.5 ± 2.4	19.0 ± 4.5*	0.0 ± 0.0	24.9 ± 1.8†

Peripheral blood and BAL specimens were collected from SRBC-sensitized mice at the indicated times after IT challenge with 5×10^8 SRBCs. The percentage of E-selectin ligand-positive (ESL^+) or P-selectin ligand-positive (PSL^+) CD4 and CD8 T cells was determined by two-color flow cytometry as described in Figure 8. Data are shown as the mean percentage of positive cells (±SEM) from three to seven individual samples (each sample includes blood from one to two mice). *$P \leq 0.043$ and †$P \leq 0.007$ for the differences between blood and BAL, by Student's paired *t*-test.

The loss of P-selectin-ligand-positive cells from the blood correlated with their accumulation in the lung BAL. Specifically, between days 2.5 and 4 post-IT, the absolute number of P-selectin ligand-positive CD4 and CD8 T cells fell ninefold in the bloodstream while increasing sixfold in the BAL. The proliferation of resident selectin-positive cells cannot account for this increase as 18 to 24 hours is required for a single round of cell division. In addition, bromodeoxyuridine studies indicate that fewer than 5 to 10% of the T cells recovered from the lung in this model are cycling (G. D. Seitzman, J. Sonstein, W. Chow, and J. L. Curtis, manuscript submitted). Furthermore, induction of selectin ligand synthesis on negative cells *in vitro* requires several days before a substantial increase in the percentage of positive cells is observed.[58] Consequently, the most likely explanation for the accumulation

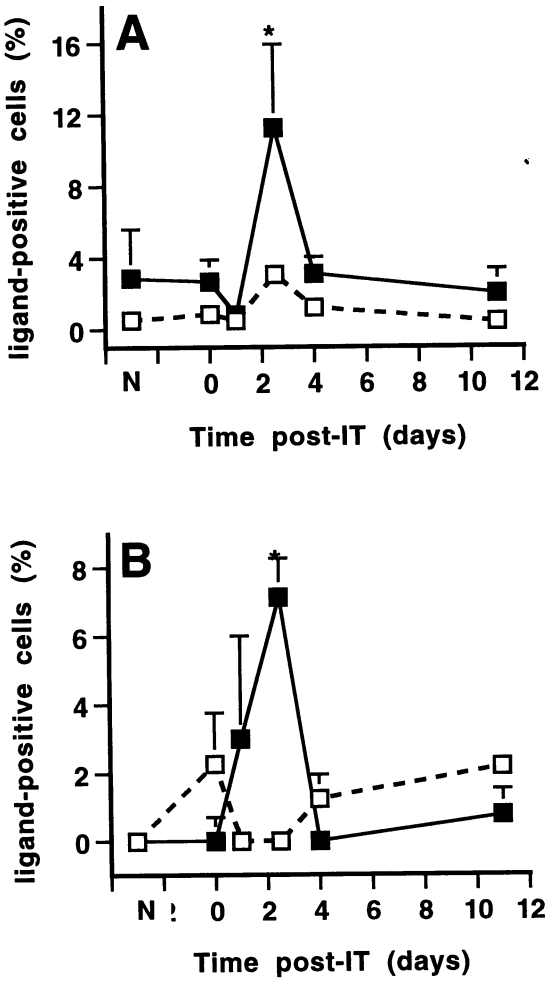

Figure 8. Expression of selectin ligands on T cells in the peripheral blood before and after IT-SRBC. Peripheral blood was collected from untreated and IT-SRBC-challenged animals at multiple time points. The $CD4^+$ and $CD8^+$ T lymphocytes were analyzed for expression of ligands for P-selectin (—■—) and E-selectin (--□--) by two-color flow cytometry as shown in Figure 7. The percentage of selectin-ligand-positive cells was calculated by dividing the percentage of selectin-ligand-positive cells (upper right quadrants in two-color histograms; see Figure 7) by the percentage of the T-cell subset in the specimen (sum of upper right and lower right quadrants). **A:** Percentage of CD4 T cells expressing selectin ligands. **B:** Percentage of CD8 T cells expressing selectin ligands. On the *x* axes, N indicates untreated animals, and the numbers indicate the number of days after IT SRBC challenge of sensitized mice. On the *y* axes are the mean percentages of selectin-ligand-positive lymphocytes (±SEM) in three to six independent samples (each sample pooled from one to three mice). $^*P < 0.02$ by ANOVA/SNK, compared with day 0 after IT.

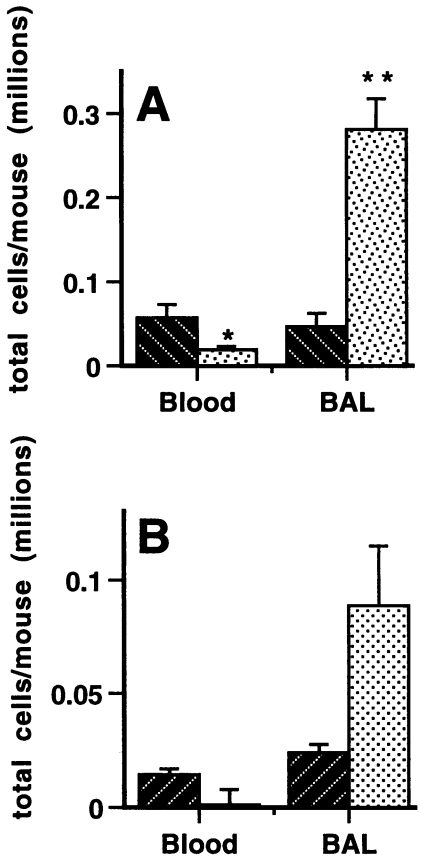

Figure 9. Total number of selectin-ligand-positive T cells in blood and BAL after IT-SRBC challenge. Peripheral blood and BAL specimens were collected from SRBC-sensitized mice at the indicated times after IT challenge with 5×10^8 SRBCs. The absolute counts were calculated by multiplying the percentage of selectin-ligand-positive T lymphocytes (Figure 7) times the total lymphocyte count (lymphocyte differential (%) × leukocyte concentration × specimen volume). **A:** Number of P-selectin-ligand-positive T cells. **B:** Number of E-selectin-ligand-positive T cells. *x* axes, peripheral blood and BAL at 2.5 (**striped bars**) and 4 (**stippled bars**) days after IT; *y* axes, mean number of selectin-ligand-positive T lymphocytes per mouse in each compartment (±SEM) calculated from three to six independent, paired samples at each time point. $^*P < 0.05$ and $^{**}P \leq 0.004$ between the two time points, by Student's *t*-test.

of selectin-ligand-positive cells in the lung is their enhanced recruitment from the circulation.

Of course, such a correlation cannot establish a causal role for P- or E-selectin in lymphocyte recruitment into lung. Short-term trafficking experiments with labeled lymphocyte populations are traditionally used for this purpose. However, existing techniques are inadequate for the study of lung inflammation due to the high levels of nonspecific retention observed in uninflamed lung.[21] Improved techniques are currently under development but are beyond the scope of this report. Nevertheless, the findings reported here support the hypothesis that selectin-ligand-positive T cells synthesized in response to SRBC challenges enter the peripheral blood and then traffic into the lung through the selectin-positive vasculature.

The absence of staining with MECA 79 and MECA 367 indicates that the receptors involved in recirculation through peripheral lymph nodes[32] and gut-associated lymphoid tissues[31] are not involved in leukocyte recruitment in this system. Previous morphological studies in this model did not detect classical, well developed HEV,[41] and the current study found no expression of the nonspecific HEV marker MECA 325[61] on vessels at sites of lymphocytic infiltration in the lung. Thus, the pathways used by lymphocytes to migrate into lung differ from those required for recirculation through organized lymphoid tissues. It remains possible, however, that the lymph node homing receptor (L-selectin) on lymphocytes augments recruitment through interactions with immobilized neutrophils as has been postulated for L-selectin on neutrophils themselves.[7]

Induction of endothelial VCAM-1, like the selectins, peaked at 24 hours and persisted on the pulmonary

vasculature during the period of mononuclear recruitment. Several recent studies demonstrated that α4 integrin/VCAM-1 interactions can both tether cells to the endothelium in the absence of selectins[8–10,62] and strengthen adhesion initiated by the selectins.[63] Consequently, VCAM-1 on the pulmonary vasculature may contribute to both selectin-dependent and -independent recruitment of T cells from the circulation.

In conclusion, previous studies concluded that selectins are essential for T cell recruitment into cellular immune responses in the skin. The current findings expand this paradigm by documenting the sustained synthesis of the endothelial selectins and their counter-receptors on T lymphocytes during the pulmonary immune response to SRBCs. VCAM-1 may also contribute to T cell recruitment, but neither the peripheral nor mucosal vascular addressins are essential.

Acknowledgments

We thank Lang Li for his effort in performing the regression analyses. Rabbit polyclonal antibody to murine P-selectin, rabbit polyclonal control IgG, and rat monoclonal antibody to murine E-selectin were the generous gifts of Dr. Dietmar Vestweber, Hans-Spemann Laboratory, Max-Planck-Institute fur Immunbiologie, Germany. MECA 325, MECA 79, and MECA 367 were the generous gifts of Dr. Eugene Butcher, Stanford University, Palo Alto, CA. John Lowe and Peter Smith, University of Michigan, kindly provided the selectin-IgM chimeric proteins.

References

1. Stoolman LM: Adhesion molecules involved in leukocyte recruitment and lymphocyte recirculation. Chest 1993, 103:79S–86S
2. Lipscomb MF, Bice DE, Lyons CR, Schuyler MR, Wilkes D: The regulation of pulmonary immunity. Adv Immunol 1995, 59:369–455
3. Butcher EC, Picker LJ: Lymphocyte homing and homeostasis. Science 1996, 272:60–66
4. Springer TA: Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. Annu Rev Physiol 1995, 57:827–872
5. Lawrence MB, Springer TA: Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell 1991, 65:1–20
6. von Andrian UH, Berger EM, Ramezani L, Chambers JD, Ochs HD, Harkin JM, Paulson JC, Etzioni A, Arfors KE: In vivo behavior of neutrophils from two patients with distinct inherited leukocyte adhesion deficiency syndromes. J Clin Invest 1993, 91:2893–2897
7. Bargatze RF, Kurk S, Butcher EC, Jutila MA: Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells. J Exp Med 1994, 180:1785–1792
8. Wolber F, Craig R, Abassi O, Ballew J, Lobb R, Stoolman L: VLA-4 mediates lymphocyte binding to endothelium under shear. FASEB J 1993, 7(3):A639–3704
9. Alon R, Kassner PD, Carr MW, Finger EB, Hemler ME, Springer TA: The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. J Cell Biol 1995, 128:1243–1253
10. Berlin C, Bargatze RF, Campbell JJ, von Andrian UH, Szabo MC, Hasslen RD, Nelson RD, Berg EL, Erlandsen SL, Butcher EC: Alpha-4 integrins mediate lymphocyte attachment and rolling under physiologic flow. Cell 1995, 80(3):413–422
11. Mulligan MS, Polley MJ, Bayer RJ, Nunn MF, Paulson JC, Ward PA: Neutrophil-dependent acute lung injury: requirement for P-selectin (GMP-140). J Clin Invest 1992; 90:1600–1607
12. Ridings PC, Windsor AC, Jutila MA, Blocher CR, Fisher BJ, Sholley MM, Sugerman HJ, Fowler AA: A dual-binding antibody to E- and L-selectin attenuates sepsis-induced lung injury. Am J Respir Crit Care Med 1995, 152:247–253
13. Lo SK, Bevilacqua B, Malik AB: E-selectin ligands mediate tumor necrosis factor-induced neutrophil sequestration and pulmonary edema in guinea pig lungs. Circ Res 1994, 75:955–960
14. Henriques GM, Miotla JM, Cordeiro SB, Wolitzky BA, Woolley ST, Hellewell PG: Selectins mediate eosinophil recruitment in vivo: a comparison with their role in neutrophil influx. Blood 1996, 87:5297–5304
15. Bullard DC, Kunkel EJ, Kubo H, Hicks MJ, Lorenzo I, Doyle NA, Doerschuk CM, Ley K, Beaudet AL: Infectious susceptibility and severe deficiency of leukocyte rolling and recruitment in E-selectin and P-selectin double mutant mice. J Exp Med 1996, 183:2329–2336
16. Maly P, Thall A, Petryniak B, Rogers CE, Smith PL, Marks RM, Kelly RJ, Gersten KM, Cheng G, Saunders TL, Camper SA, Camphausen RT, Sullivan FX, Isogai Y, Hindsgaul O, von Andrian UH, Lowe JB: The alpha(1,3)fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin ligand biosynthesis. Cell 1996, 86:643–653
17. Tang T, Frenette PS, Hynes RO, Wagner DD, Mayadas TN: Cytokine-induced meningitis is dramatically attenuated in mice deficient in endothelial selectins. J Clin Invest 1996, 97:2485–2490
18. Pinsky DJ, Naka Y, Liao H, Oz MC, Wagner DD, Mayadas TN, Johnson RC, Hynes RO, Heath M, Lawson CA, Stern DM: Hypoxia-induced exocytosis of endothelial cell Weibel-Palade bodies: a mechanism for rapid neutrophil recruitment after cardiac preservation. J Clin Invest 1996, 97:493–500
19. Picker LJ, Kishimoto TK, Smith CW, Warnock RA, Butcher EC: ELAM-1 is an adhesion molecule for skin-homing T cells. Nature 1991, 349:796–799
20. Subramaniam M, Saffaripour S, Watson SR, Mayadas TN, Hynes RO, Wagner DD: Reduced recruitment of inflammatory cells in a contact hypersensitivity response in P-selectin-deficient mice. J Exp Med 1995, 181:2277–2282
21. Austrup F, Vestweber D, Borges E, Lohning M, Brauer R, Herz U, Renz H, Hallmann R, Scheffold A, Radbruch A, Hamann AP: P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. Nature 1997, 385:81–83
22. Staite ND, Justen JM, Sly LM, Beaudet AL, Bullard DC: Inhibition of delayed-type contact hypersensitivity in mice deficient in both E-selectin and P-selectin. Blood 1996, 88:2973–2979
23. Tipping PG, Huang XR, Berndt MC, Holdsworth SR: P-selectin directs T lymphocyte-mediated injury in delayed-type hypersensitivity responses: studies in glomerulonephritis and cutaneous delayed-type hypersensitivity. Eur J Immunol 1996, 26:454–460
24. Yednock TA, Cannon C, Fritz LC, Sanchez-Madrid F, Steinman L, Karin N: Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. Nature 1992, 356: 63–66
25. Christensen JP, Andersson EC, Scheynius A, Marker O, Thomsen AR: Alpha 4 integrin directs virus-activated $CD8^+$ T cells to sites of infection. J Immunol 1995, 154:5293–5301.
26. Baron JL, Madri JA, Ruddle NH, Hashim G, Janeway CA Jr: Surface expression of alpha 4 integrin by CD4 T cells is required for their entry into brain parenchyma. J Exp Med 1993, 177:57–68
27. Baron JL, Reich EP, Visintin I, Janeway CA Jr: The pathogenesis of adoptive murine autoimmune diabetes requires an interaction between alpha 4-integrins and vascular cell adhesion molecule-1. J Clin Invest 1994, 93:1700–1708
28. Molina A, Sanchez-Madrid F, Bricio T, Martin A, Barat A, Alvarez V, Mampaso F: Prevention of mercuric chloride-induced nephritis in the brown Norway rat by treatment with antibodies against the alpha 4 integrin. J Immunol 1994, 153:2313–2320
29. Issekutz TB: Dual inhibition of VLA-4 and LFA-1 maximally inhibits cutaneous delayed-type hypersensitivity-induced inflammation. Am J Pathol 1993, 143:1286–1293
30. Issekutz AC, Issekutz TB: Monocyte migration to arthritis in the rat utilizes both CD11/CD18 and very late activation antigen 4 integrin mechanisms. J Exp Med 1995, 181:1197–1203
31. Streeter PR, Berg EL, Rouse BT, Bargatze RF, Butcher EC: A tissue-specific endothelial cell molecule involved in lymphocyte homing. Nature 1988, 331:41–46
32. Streeter PR, Tien B, Rouse N, Butcher EC: Immunohistologic and

functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. J Cell Biol 1988, 107: 1853–1862
33. Kikuta A, Rosen SD: Localization of ligands for L-selectin in mouse peripheral lymph node high endothelial cells by colloidal gold conjugates. Blood 1994, 84:3766–3775
34. Michie SA, Streeter PR, Bolt PA, Butcher EC, Picker LJ: The human peripheral lymph node vascular addressin: an inducible endothelial antigen involved in lymphocyte homing. Am J Pathol 1993, 143:1688–1698
35. Onrust SV, Hartl PM, Rosen SD, Hanahan D: Modulation of L-selectin ligand expression during an immune response accompanying tumorigenesis in transgenic mice. J Clin Invest 1996, 97:54–64
36. Nakajima H, Sano H, Nishimura T, Yoshida S, Iwamoto I: Role of vascular cell adhesion molecule 1/very late activation antigen 4 and intercellular adhesion molecule 1/lymphocyte function-associated antigen I interactions in antigen-induced eosinophil and T cell recruitment into the tissue. J Exp Med 1994, 179:1145–1154
37. Pretolani M, Ruffie C, Lapa JD, Lobb RR, Vargaftig BB: Antibody to very late activation antigen 4 prevents antigen-induced bronchial hyperreactivity and cellular infiltration in the guinea pig airways. J Exp Med 1994, 180:795–805
38. Rabb HA, Olivenstein R, Issekutz TB, Renzi PM, Martin JG: The role of the leukocyte adhesion molecules VLA-4, LFA-1, and Mac-1 in allergic airway responses in the rat. Am J Respir Crit Care Med 1994, 149:1186–1191
39. Picker LJ, Martin RJ, Trumble A, Newman LS, Collins PA, Bergstresser PR, Leung DY: Differential expression of lymphocyte homing receptors by human memory/effector T cells in pulmonary versus cutaneous immune effector sites. Eur J Immunol 1994, 24:1269–1277
40. Curtis JL, Kaltreider HB: Characterization of bronchoalveolar lymphocytes during a specific antibody-forming cell response in the lungs of mice. Am Rev Respir Dis 1989, 139:393–400
41. Curtis JL, Warnock ML, Arraj SM, Kaltreider HB: Histologic analysis of an immune response in the lung parenchyma of mice: angiopathy accompanies inflammatory cell influx. Am J Pathol 1990, 137:689–699
42. Curtis JL, Byrd PK, Warnock ML, Kaltreider HB: Requirement of CD4-positive T cells for cellular recruitment to the lungs of mice in response to a particulate intratracheal antigen. J Clin Invest 1991, 88:1244–1254
43. Crocker PR, Jefferies WA, Clark SJ, Chung LP, Gordon S: Species heterogeneity in macrophage expression of the CD4 antigen. J Exp Med 1987, 166:613–618
44. Sanders WE, Wilson RW, Ballantye CM, Beaudet AL: Molecular cloning and analysis of in vivo expression of murine P-selectin. Blood 1992, 80:795–800
45. Weller A, Isenmann S, Vestweber D: Cloning of the mouse endothelial selectins: expression of both E- and P-selectin is inducible by tumor necrosis factor alpha. J Biol Chem 1992, 267:15176–15183
46. Hahne M, Lenter M, Jager U, Vestweber D: A novel soluble form of mouse VCAM-1 is generated from a glycolipid-anchored splicing variant. Eur J Immunol 1994, 24:421–428
47. Hedeker D, Gibbons RD: A random-effects ordinal regression model for multilevel analysis. Biometrics 1994, 50:933–944
48. Hedeker D, Gibbons RD: MIXREG: a computer program for mixed-effects regression analysis with autocorrelated errors. Comput Methods Programs Biomed 1996, 49:229–252
49. Mulligan MS, Varani J, Dame MK, Lane CL, Smith CW, Anderson DC, Ward PA: Role of endothelial-leukocyte adhesion molecule 1 (ELAM-1) in neutrophil-mediated lung injury in rats. J Clin Invest 1991, 88:1396–1406
50. Bevilacqua MP, Stengelin S, Gimbrone MA Jr, Seed B: Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science 1989, 243:1160–1164
51. Neumann B, Machleidt T, Likfa A, Pfeffer K, Vestweber D, Mak TW, Holzmann B, Kronke M: Crucial role of 55-kilodalton TNF receptor in TNF-induced adhesion molecule expression and leukocyte organ infiltration. J Immunol 1996, 156:1587–1593
52. Hahne M, Jager U, Isenmann S, Hallmann R, Vestweber D: Five tumor necrosis factor-inducible cell adhesion mechanisms on the surface of mouse endothelioma cells mediate the binding of leukocytes. J Cell Biol 1993, 121:655–664
53. Khew-Goodall Y, Butcher CM, Litwin MS, Newlands S, Korpelainen EI, Noack LM, Berndt MC, Lopez AF, Gamble JR, Vadas MA: Chronic expression of P-selectin on endothelial cells stimulated by the T-cell cytokine, interleukin-3. Blood 1996, 87:1432–1438
54. Yao L, Pan J, Setiadi H, Patel KD, McEver RP: Interleukin 4 or oncostatin M induces a prolonged increase in P-selectin mRNA and protein in human endothelial cells. J Exp Med 1996, 184:81–92
55. Yamamoto N, Zou JP, Li XF, Takenaka H, Noda S, Fujii T, Ono S, Kobayashi Y, Mukaida N, Matsushima K: Regulatory mechanisms for production of IFN-γ and TNF by antitumor T cells or macrophages in the tumor-bearing state. J Immunol 1995, 154:2281–2290
56. Chadwick BS, Brady G, Miller RG: Characterization of murine lymphokine-activated killer cell cultures separated according to cell size. Cell Immunol 1993, 146:1–10
57. Davodeau F, Peyrat MA, Necker A, Dominici R, Blanchard F, Leget C, Gaschet J, Costa P, Jacques Y, Godard A, Vie H, Poggi A, Romagne F, Bonneville M: Close phenotypic and functional similarities between human and murine $\alpha\beta$ T cells expressing invariant TCR α-chains. J Immunol 1997, 158:5603–5611
58. Knibbs RN, Craig RA, Natsuka S, Chang A, Cameron M, Lowe JB, Stoolman LM: The fucosyltransferase FucT-VII regulates E-selectin ligand synthesis in human T cells. J Cell Biol 1996, 133:911–920
59. Kaltreider HB, Curtis JL, Arraj SM: The mechanism of appearance of specific antibody-forming cells in lungs of inbred mice after immunization with sheep erythrocytes intratracheally. II. Dose-dependence and kinetics of appearance of antibody-forming cells in hilar lymph nodes and lungs of unprimed and primed mice. Am Rev Respir Dis 1987, 135:87–92
60. Bishop DK, Jutila MA, Sedmak DD, Beattie MS, Orosz CG: Lymphocyte entry into inflammatory tissues in vivo: qualitative differences of high endothelial venule-like vessels in sponge matrix allografts vs isografts. J Immunol 1989, 142:4219–4224
61. Gerszten RE, Luscinskas FW, Ding HT, Dichek DA, Stoolman LM, Gimbrone MA Jr, Rosenzweig A: Adhesion of memory lymphocytes to vascular cell adhesion molecule-1-transduced human vascular endothelial cells under simulated physiological flow conditions in vitro. Circ Res 1996, 79:1205–1215
62. Luscinskas FW, Ding H, Lichtman AH: P-selectin and vascular cell adhesion molecule 1 mediate rolling and arrest, respectively, of $CD4^+$ T-lymphocytes on tumor necrosis factor alpha-activated vascular endothelium under flow. J Exp Med 1995, 181:1179–1186