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Monoclonal Antibody Blockade of L-Selectin Inhibits Mononuclear Leukocyte Recruitment to Inflammatory Sites *in Vivo*

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L-selectin interacting with inducible endothelial counterreceptors mediates in part the initial adhesive interactions, termed rolling, between circulating blood leukocytes and vascular endothelium. While blockade of L-selectin function in* in vivo *models of inflammation reduces both neutrophil and lymphocyte influx at early times, little is known concerning the role of L-selectin in leukocyte recruitment at later times (>24 hours). Using an* in vivo *murine model of experimentally induced inflammation of the peritoneum, the role of L-selectin in recruitment of mononuclear leukocytes to chronic sites of inflammation (48 hours) was investigated. Saturating levels of function blocking anti-L-selectin monoclonal antibody (MEL-14) or control rat IgG were maintained for 48 hours using surgically implanted miniosmotic pumps; this treatment did not alter the circulating leukocyte cell count or differential. In animals receiving MEL-14 monoclonal antibody (MAb), macrophage and lymphocyte accumulation in response to thioglycollate was reduced by 60% ($P \leq 0.0002$) and >90% ($P < 0.001$), respectively, at 48 hours as compared with animals implanted with pumps containing saline. Similarly, MEL-14 MAb dramatically inhibited granulocyte influx by 80% ($P < 0.03$) at 6 hours; recruitment at 24 and 48 hours was reduced by 50%. In contrast, the effects of purified rat IgG was not significantly different from saline. Our results suggest L-selectin, interacting with its inducible endothelial counterreceptor(s), plays an important role in circulating mononuclear leukocyte extravasation at sites of inflammation. (Am J Pathol 1994, 145:461–469)

L-selectin (murine lymphocyte homing receptor or MEL-14 antigen),[1–5] a member of the selectin gene family, is constitutively expressed on circulating neutrophils, eosinophils, basophils, monocytes, and most B and T lymphocytes (reviewed in ref. 5). The N-terminal region of L-selectin contains a C-type lectin binding domain that is involved in lymphocyte recirculation in peripheral blood lymph node tissues via attachment to histologically distinctive postcapillary venules, termed high endothelial venules (HEV) *in vivo* and *in vitro*[1,6] and in blood neutrophil, lymphocyte, and monocyte adhesion to cytokine-activated endothelium *in vitro*.[6–10] Recent studies have shown that L-selectin in part mediates the initial adhesive interaction, termed "rolling," between circulating blood neutrophils and monocytes and the "activated" peripheral vascular endothelium *in vivo* or under defined flow conditions *in vitro*.[7–9,11–13] Thus, L-selectin interacting with its endothelial counterreceptors(s)[7,10,14–16] may regulate initial leukocyte-endothelial interactions at sites of inflammation.

Inhibition of L-selectin function by function blocking monoclonal antibody (MAb) MEL-14[6,17,18] or by soluble chimeric receptor molecules[17] greatly reduces neutrophil recruitment into the inflamed peritoneum. However, these studies examined neutrophil influx at early time points of inflammation (2–4 hours) but not at later times. In addition, although L-selectin mediates monocyte rolling and adhesion to IL-4-activated endothelial monolayers under flow conditions *in vitro* (F. W. Luscinskas, G. S. Kansas, H. Ding, B. E. Schleiffenbaum, T. F. Tedder, and M. A. Gimbrone, J Cell Biol, in press), a detailed analysis of the role of L-selectin in recruitment of mononuclear leukocytes (lymphocytes and monocytes) to

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sites of inflammation at later time points of inflammation (≥24 hours) has yet to be undertaken. In the present study, an *in vivo* model of experimentally induced inflammation and a function blocking MAb to L-selectin were used to investigate the role of the L-selectin adhesion pathway in recruitment of circulating lymphocytes, monocytes, and granulocytes to sites of chronic (up to 48 hours) inflammation. The results suggest that L-selectin, interacting with its inducible endothelial counterreceptors, plays an important role in the influx of circulating leukocytes in chronic inflammation.

Materials and Methods

Animals

Eight- to 10-week-old C57BL/6J female mice were obtained from Jackson Laboratory, Bar Harbor, ME. Swiss nude mice were obtained from Taconic, Georgetown, NY. Mice were housed in a virus antibody-free facility. Animals used in these studies were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

Buffers and Materials

Thioglycollate solution (4% w/v; Difco, Detroit, MI) was prepared as detailed by the manufacturer. DMEM and RPMI 1640 for culture of hybridoma cells lines were purchased from Whittaker Bioproducts (Walkersville, MD). Lactated Ringer's solution for irrigation was purchased from Baxter Healthcare (Deerfield, IL). FCS was obtained from HyClone (Orem, UT). All other chemicals and reagents were of the highest grade available from Baker Chemicals.

Antibodies

MEL-14 (rat IgG2a; clone HB 132, American Type Culture Collection, Rockville, MD) is a function-blocking MAb directed to murine L-selectin.[1] MAb M18/2.a (rat IgG2a) is a non-function-blocking MAb that recognizes CD18 (ATCC clone TIB 218).[19] MAb RB6–8C5 (rat IgG2b) recognizes the murine granulocyte series of myeloid cells and was the gift of Dr. Coffman (DNAX, Palo Alto, CA).[20] MAb 29B (rat IgG1) is directed to mouse CD3.[21] MAb 6B2 (rat IgG1) recognizes murine CD45RO and is a marker of B lymphocytes.[22] Purified rat IgG was purchased from Caltag (S. San Francisco, CA). MEL-14 ascites was produced by injection of the hybridoma cell line into Swiss nude mice. MEL-14 MAb IgG was purified from ascites fluid by ammonium sulfate precipitation and immobilized protein G column chromatography (Pierce, Rockford, IL). $F(ab')_2$ fragments of MEL-14 MAb were prepared from pure IgG as previously detailed.[23] In brief, purified IgG was sequentially dialyzed against 100 mmol/L sodium formate buffer, pH 2.8, and 100 mmol/L sodium acetate buffer, pH 4.5. Dialyzed MEL-14 IgG was then digested with 1% (w/v) pepsin (Sigma Chemical Co., St. Louis, MO) in 0.1 mol/L sodium acetate buffer, pH 4.5, for 6 hours at 37 C. Purity (~90%) of IgG and $F(ab')_2$ was assessed on 10% reducing and nonreducing SDS-PAGE. For use in Alzet miniosmotic pumps, purified MEL-14 IgG or rat IgG were concentrated to 33 mg/ml by centrifugation on Centricon 30 membranes (Amicon Inc., Beverly, MA). Levels of endotoxin were ≤10 pg/ml as determined in a *Limulus* amoebocyte lysate kit (sensitivity, 0.5 EU/ml; Cape Cod Associates, Woods Hole, MA). MAb used in indirect immunofluorescence analyses (MAbs RB6–8C5, 29B, and 6B2) were precipitated from spent hybridoma culture supernatant or ascites (MEL-14 and M18/2) by ammonium sulfate and purified by ion-exchange chromatography on Bakerbond ABx beads (Baker Inc., Phillipsburg, NJ) according to manufacturer's instructions. MAb (purified IgG) were labeled with FITC[24] or biotin (Sigma).[25]

MAb Inhibition in Vivo

Alzet miniosmotic pumps (model 1003D; Alza Corp., Palo Alto, CA) were loaded with MAb or saline according to the manufacturer's instructions. The overall dimensions of assembled pumps were 1.7 × 0.6 cm. Pumps containing MAb or saline were implanted into a pocket created by blunt dissection in the subcutaneous space on the dorsal side of anesthetized mice. Surgery required 5 to 10 minutes. The pump capacity is 90 ± 4.0 μl and its nominal pumping rate is 1.1 ± 0.05 μl/h. Based on this flow rate and 3 mg of affinity-purified MEL-14 or purified rat IgG in 90 μl, the calculated release rate of MAb is 36 μg/h. To insure that adequate blood levels of MAb were achieved during the time period before the pumps were active, a single bolus (0.4 mg) of appropriate MAb was injected in the tail vein immediately after surgery. For MEL-14 MAb, this quantity saturated antigen (Ag) on leukocytes for at least 4 hours (see Results, Table 1).

Table 1. *MEL-14 MAb Binding to Circulating Blood Leukocytes*

Treatments*	Time (hours)	n	Mean Channel Fluorescence†		
			Lymphocytes	Monocytes	Granulocytes
Saline	4	3	37 ± 3	57 ± 2	35 ± 1
MEL-14 IgG	4	3	111 ± 4	139 ± 7	116 ± 7
MEL-14 IgG	6	2	39 ± 1	60 ± 2	56 ± 1
MEL-14 $F(ab')_2$	4	2	107 ± 2	129 ± 2	114 ± 7
Ex vivo FITC-MEL-14‡		2	106 ± 1	109 ± 23	117 ± 6

* Treatments were intravenous bolus injections of various preparations of purified MEL-14 MAb or saline as detailed in Materials and Methods.

† MEL-14 MAb binding was detected by incubation of whole blood samples with FITC-labeled goat anti-rat IgG as detailed in Materials and Methods.

‡ Whole blood from control mice (received no pumps or intraperitoneal injections) was treated with saturating levels of FITC-labeled MEL-14 MAb and the level of FITC fluorescence on 10,000 leukocytes determined. FACScan software used for flow cytometry data analysis was LYSIS II, version 2.2 (1992).

Measurement of Leukocyte Accumulation in Peritoneal Lavage

One ml of thioglycollate or lactated Ringer's irrigation buffer was injected intraperitoneally in conscious mice 30 minutes after pump implantation. Blood samples were taken from the tail vein of mice before sacrifice at 0, 6, 24, and 48 hours. Mice were euthanized and their peritoneal contents were obtained by injection of 5 ml of warm medium (RPMI 1640–2% FCS containing 2 mmol/L EDTA) followed by gentle massage of the peritoneum. Total leukocyte cell counts were determined with an electronic particle counter (Model Zf, Coulter Electronics, Hialeah, FL). Granulocytes, lymphocytes, and macrophages were identified by distinctive forward and side light scatter profiles and indirect immunofluorescence flow cytometry using leukocyte-specific MAb markers 29B (CD3, pan T-lymphocyte), 6B2 (B-lymphocyte), and RB6.8C5 (granulocyte marker) MAb as detailed below. Wright-Giemsa-stained cytospin preparations were used to verify the relative percentage of mononuclear *versus* polymorphonuclear leukocytes.

Indirect Immunofluorescence Assay

Immunofluorescence assays for surface Ag expression on leukocytes were performed using directly labeled (FITC or biotin) primary MAb. Aliquots of whole blood (50 μl) or peritoneal lavage fluid ($5–10 \times 10^5$ leukocytes) were incubated at 4 C with saturating concentrations of FITC- or biotin-labeled primary MAb in RPMI 1640 medium containing 5% heat-inactivated rabbit serum for 20 minutes on ice. Biotinylated MAb were detected by a 10 minutes incubation with FITC-conjugated avidin (Sigma) at 1:200 dilution. The relative binding of MEL-14 on circulating leukocytes was determined on whole blood samples incubated for 30 minutes at 4 C with FITC-labeled $F(ab')_2$ goat anti-rat IgG (1:100 dilution; Caltag). Erythrocytes were lysed using the Coulter Whole Blood Immunofluorescence kit as detailed by the manufacturer (Coulter). FITC fluorescence was determined on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) by counting the fluorescence of 10,000 leukocytes. The data are presented as single parameter histograms with the mean channel fluorescence (4 decade log scale, *x* axis) versus the relative cell number (*y* axis).

Statistics

Analysis of variance was used to analyze data and Student's paired *t*-test was used to determine statistical significance (Minitab statistical software, version 7.1). *P* values of ≤ 0.05 were considered significant.

Results

Saturation of MEL-14 Ag on Circulating Leukocytes

Previous studies have reported that MEL-14 MAb recognizes the lectin domain of L-selectin expressed on circulating neutrophils, lymphocytes, and monocytes[18,26] and that MEL-14 inhibits lymphocyte and neutrophil binding to peripheral lymph node HEV *in vivo*.[1] Preliminary experiments were carried out to determine whether intravenous injection of MEL-14 MAb (0.4 mg/animal) could saturate binding sites on circulating leukocytes for up to 24 hours (Table 1). Indirect flow cytometric analyses, using goat anti-rat IgG to detect MEL-14 bound to each leukocyte type, revealed that MEL-14 Ag on neutrophils, lymphocytes, and monocytes was fully saturated at 4 hours but not after 6 hours (Table 1). Similar results were observed after administration of $F(ab')_2$ fragments (0.4 mg/animal) of MEL-14 MAb, suggesting that the

short circulating half-life of MEL-14 was not due to clearance via Fc-mediated cross-linking.

We next examined whether surgically implanted Alzet miniosmotic pumps containing MEL-14 MAb, which have been used previously to deliver precalibrated volumes of MAb over sustained time periods,[27] could be used to saturate MEL-14 binding sites on circulating leukocytes for longer time periods. In these experiments, pumps containing MEL-14 MAb (3 mg/animal) were implanted and blood samples were taken at 0, 6, 24, and 48 hours. MEL-14 binding sites on circulating leukocytes were saturated at 6, 24, and 48 hours (Figure 1 and Table 2). Analysis of the data in Table 2 revealed that there was no statistical difference between the levels of MEL-14 MAb bound at 6, 24, or 48 hours for each leukocyte type. Figure 1 shows the relative binding of MEL-14 MAb to each leukocyte type over the time course of the experiments. For comparison, surface expression of MEL-14 Ag on peripheral blood from control animals is shown. This model was used, therefore, to examine the role of L-selectin in leukocyte recruitment to experimentally induced chronic inflamed peritoneum.

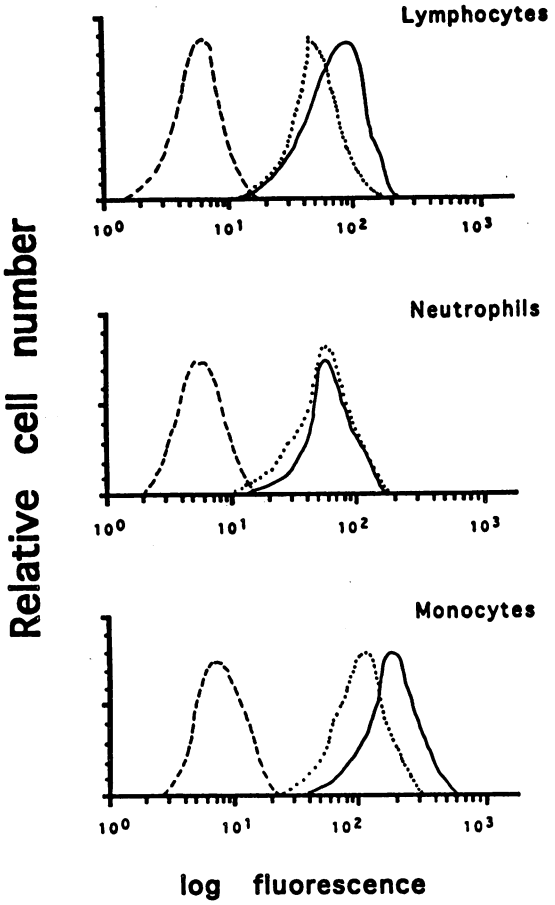

Figure 1. *MEL-14 MAb binding to circulating blood lymphocytes, neutrophils, and monocytes 48 hours after thioglycollate injection intraperitoneally. MEL-14 MAb binding to circulating leukocytes was assessed using FITC-labeled goat anti-rat IgG as detailed in Materials and Methods. Mice were implanted with Alzet miniosmotic pumps loaded with 3 mg of purified MEL-14 IgG MAb (·····) or saline (- - -), injected with 1 ml of thioglycollate intraperitoneally, and blood samples taken after 48 hours. For comparison, surface expression of MEL-14 Ag on peripheral blood leukocytes from normal control animals was determined by single step indirect immunofluorescence flow cytometry using FITC-labeled MEL-14 MAb (——).*

MEL-14 MAb Inhibits Neutrophil, Lymphocyte, and Monocyte Recruitment in Vivo

In mice implanted with saline-loaded pumps, the total number of macrophages (blood derived monocytes), neutrophils, and lymphocytes accumulated in peritoneal lavage at 0, 6, 24, 48, and 96 hours after thioglycollate injection is shown (Figure 2). Macrophage and lymphocyte accumulation did not increase significantly until 24 hours and was maximal by 48 hours, whereas neutrophil accumulation was maximal by 6 hours and declined at later times. Thus, we tested the effects of test or control antibodies between 0 and 48 hours.

Using the osmotic pump delivery system in this model of inflammation, the effect of MEL-14 MAb on leukocyte recruitment to the peritoneum in response to thioglycollate was determined (Figure 3). MEL-14 MAb significantly decreased the number of macrophages accumulated in response to thioglycollate at later times as compared with saline (Figure 3A). At 24 and 48 hours, macrophage accumulation was reduced by 95% ($P < 0.0002$) and 63% ($P < 0.003$), respectively. Lymphocyte accumulation was almost totally inhibited at 24 (88% inhibition, $P < 0.0025$) and 48 hours (100%, $P < 0.001$) (Figure 3B). Although lymphocyte and macrophage accumulation in the control rat IgG treatments were lower than saline treatments, these differences were not statistically significant. Neutrophil recruitment was greatly reduced (80% inhibition, $P < 0.0064$) at 6 hours with MEL-14 MAb as compared with saline (Figure 3C). However, at 24 and 48 hours, less inhibition was observed (24 hours, 48% inhibition, $P < 0.0086$; 48 hours, 64% $P \leq 0.0023$). The effects of control rat IgG treatment on neutrophil accumulation was not significantly different from saline at any time point.

The inhibitory effects of MEL-14 MAb were not due to a reduction in the total number of circulating leukocytes (ie, leukopenia). In mice receiving saline intraperitoneally, the circulating neutrophil and mononuclear leukocyte (lymphocyte + monocyte) counts at 4, 24, and 48 hours after implantation with pumps

Table 2. *Immunodetection of MEL-14 MAb Binding to Circulating Blood Leukocytes in Animals Implanted with Alzet Pumps Loaded with MEL-14 IgG MAb or Saline and Receiving Thioglycollate Intraperitoneally*

Treatment*	Time (hours)	n	Mean Channel Fluorescence†		
			Lymphocytes	Monocytes	Granulocytes
MEL-14	6	4	43 ± 19	107 ± 25	54 ± 13
Saline	6	4	4 ± 2	10 ± 4	5 ± 1
MEL-14	24	4	44 ± 19	99 ± 13	72 ± 11
Saline	24	4	4 ± 1	5 ± 2	5 ± 3
MEL-14	48	5	51 ± 21	107 ± 42	79 ± 25
Saline	48	5	5 ± 2	9 ± 2	9 ± 6
Ex vivo‡		2	53 ± 2	89 ± 1	93 ± 2

* Mice were implanted with Alzet pumps containing saline or MEL-14 MAb and injected intraperitoneally with thioglycollate as detailed in Materials and Methods.

† Whole blood was obtained from animals at various times and incubated with FITC-labeled goat anti-rat IgG. FITC fluorescence (linearized value) was determined as detailed in Materials and Methods. FACScan software used for data analysis was BDIS consort 30, version G (1989).

‡ Whole blood samples were taken from control animals that were not implanted with pumps and incubated *ex vivo* with FITC-MEL-14 MAb. The level of MEL-14 expression determined as in Table I legend. Data are expressed as means ± SD of n experiments.

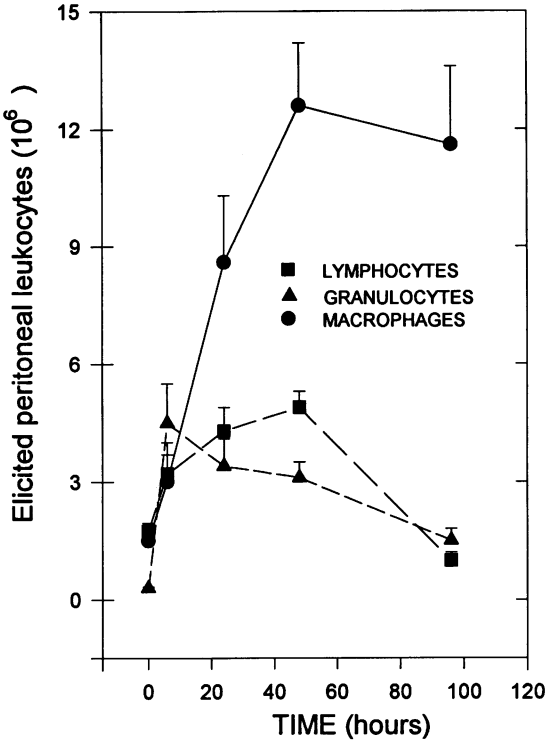

Figure 2. *Time course of circulating monocyte, lymphocyte, and granulocyte recruitment to the peritoneum cavity in response to thioglycollate. Elicited peritoneal leukocytes were harvested at 0, 6, 24, 48, and 96 hours after thioglycollate injection. Total leukocyte count was measured by Coulter counter and the leukocyte differential count was determined by distinctive forward and side light scatter profiles and indirect immunofluorescence flow cytometry using leukocyte-specific MAb. Data are represented as means ± SEM, with three or four animals in each group.*

containing MEL-14 or saline were not significantly different (Table 3).

Discussion

The findings in this report provide direct evidence that L-selectin is involved in mononuclear leukocyte accumulation at experimentally induced chronic inflammatory sites. In particular, recruitment of peripheral blood lymphocytes to the inflamed peritoneum after 24 hours was reduced by >85% and macrophage recruitment also was reduced by >60% during this time period. The current results with neutrophils confirm previous studies[6,17,18] that MEL-14 MAb blocks most of the neutrophil influx at 4 to 6 hours and provides new information that neutrophil accumulation also is reduced by this treatment at later times. In addition, the data presented (Tables 1–3 and Figure 1) indicate that saturating levels of MEL-14 MAb were achieved in our model throughout the 48 hours experimental time period and that MEL-14 MAb does not alter the leukocyte count or differential. These data suggest that L-selectin, interacting with its inducible counterreceptor(s), is an important adhesion pathway for both lymphocyte and monocyte, as well as neutrophil, accumulation at chronic inflammatory sites *in vivo*.

Current models of leukocyte-endothelial adhesive interactions suggest sequential involvement of multiple members of the selectin, integrin, and Ig gene families during the process of initial attachment (rolling), stable adhesion (arrest), spreading, and, ultimately, diapedesis.[12,28] The initial attachment of polymorphonuclear leukocytes is mediated in part by L-selectin, while subsequent arrest is mediated by β_2-integrins.[5,7,8,11,12] Our recent studies with blood monocytes under flow (ref. 10; F. W. Luscinskas, G. S. Kansas, H. Ding, P. Pizcueta, B. E. Schleiffenbaum, T. F. Tedder, and M. A. Gimbrone, J Cell Biol, in press) indicate that L-selectin mediates monocyte rolling and also facilitates $\alpha_4\beta_1$-dependent arrest, whereas β_2-integrins are necessary for spreading of firmly attached monocytes. Based on these above models, inhibition of L-selectin by MEL-14 MAb presumably

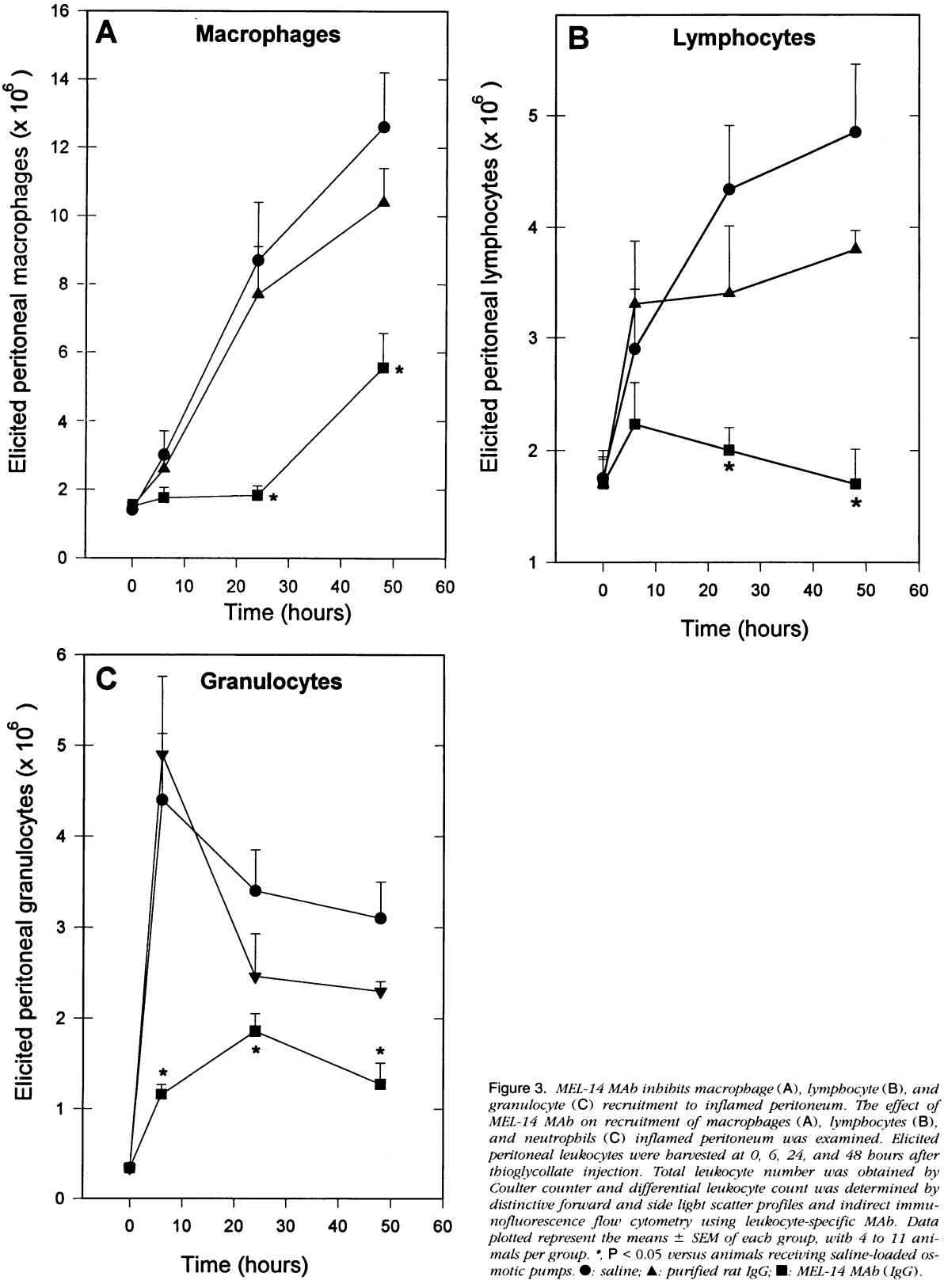

Figure 3. *MEL-14 MAb inhibits macrophage* (A), *lymphocyte* (B), *and granulocyte* (C) *recruitment to inflamed peritoneum. The effect of MEL-14 MAb on recruitment of macrophages* (A), *lymphocytes* (B), *and neutrophils* (C) *inflamed peritoneum was examined. Elicited peritoneal leukocytes were harvested at 0, 6, 24, and 48 hours after thioglycollate injection. Total leukocyte number was obtained by Coulter counter and differential leukocyte count was determined by distinctive forward and side light scatter profiles and indirect immunofluorescence flow cytometry using leukocyte-specific MAb. Data plotted represent the means ± SEM of each group, with 4 to 11 animals per group. *, P < 0.05 versus animals receiving saline-loaded osmotic pumps. ●: saline; ▲: purified rat IgG; ■: MEL-14 MAb (IgG).*

Table 3. *MEL-14 MAb IgG Does Not Alter Peripheral Blood Leukocyte Cell Count or Differential in Normal Mice*

Treatment*	Time (hours)	Mononuclear Leukocytes ($\times 10^6$/ml)†	Granulocytes ($\times 10^6$/ml)†
PBS	0	3.95 ± 0.79	0.70 ± 0.14
PBS	6	4.13 ± 1.51	0.86 ± 0.30
MEL-14	6	3.10 ± 0.19	0.92 ± 0.07
PBS	24	3.51 ± 1.40	0.70 ± 0.28
MEL-14	24	4.28 ± 2.41	0.88 ± 0.49
PBS	48	3.63 ± 0.38	0.72 ± 0.11
MEL-14	48	4.70 ± 1.43	0.64 ± 0.19

* Treatments were implantation of Alzet miniosmotic pumps containing MEL-14 MAb or saline in normal mice.

† The total leukocyte number was determined by Coulter counter. The percentages of mononuclear cells and granulocytes were determined by distinctive forward and side light scatter profiles and Wright's stained smears. The mononuclear leukocyte and granulocyte counts were determined by multiplying the differential times the total leukocyte count. Data are means ± SD from three separate experiments.

blocks the initial lymphocyte attachment (rolling interactions) to "inflamed" vascular endothelium, while subsequent steps, such as arrest and diapedesis, would require additional adhesion pathways. Other molecules have also been implicated in lymphocyte recruitment to immune or inflammatory reactions. Both $\alpha_4\beta_1$ (VLA-4) and $\alpha_L\beta_2$ (LFA-1) integrins are involved in lymphocyte adhesion to cultured human umbilical vein endothelium (HEC)[7,29,30] and to cultured rat microvascular endothelium[31–33] under static conditions. *In vivo*, the combination of function-blocking anti-$\alpha_4\beta_1$ and $\alpha_L\beta_2$ MAb maximally inhibit cutaneous delayed-type hypersensitivity reactions.[31] Taken together, these data suggest that at chronic sites of inflammation, L-selectin mediates lymphocyte rolling interactions with chronic "inflamed" endothelial cells, whereas $\alpha_4\beta_1$ and $\alpha_L\beta_2$ are involved in subsequent steps such as arrest and emigration.

Macrophage accumulation was also reduced by MEL-14 MAb, although the inhibitory effect was not as dramatic as with lymphocytes (compare Figure 3A to 3B). This result suggests other adhesion pathways are involved in the initial interactions of monocytes with endothelium. Inducible endothelial molecules implicated in such interaction include both P- and E-selectin (see ref. 34 for review). In preliminary experiments, both human E-selectin and P-selectin stably expressed on CHO cell and L-cell monolayers, respectively, supported human monocyte rolling and transient adhesion under laminar flow at 1.8 dynes/cm^2 (H. Ding and F. W. Luscinskas, manuscript in preparation). However, a major role for E-selectin in monocyte recruitment in the current model is unlikely since previous studies have demonstrated that its surface expression *in vivo* declines after 24 hours and is usually associated with neutrophil influx.[35–37] The role of P-selectin in leukocyte recruitment at chronic sites of inflammation has yet to be addressed although recent studies by Mayadas and coworkers,[38] using P-selectin-deficient mice, revealed defects in leukocyte recruitment. In particular, P-selectin-deficient mice exhibit a near total absence of rolling leukocytes in exteriorized mesenteric venules and a delayed neutrophil recruitment into the peritoneum 2 to 6 hours after thioglycollate injection.

Recent reports have identified endothelial cell molecules from lymphoid tissues that may function as ligands for L-selectin. Lasky, Rosen, and coworkers have identified glyCAM-1[14] and CD34,[15] which are mucin-like molecules, as ligands for a L-selectin chimera molecule, presumably through carbohydrate determinants. Salmi and Jalkanen[39] reported the immunopurification of a 90-kd ligand, VAP-1, from tonsillar extracts that supports lymphocyte adhesion. Michie and coworkers[16] have reported that carbohydrate determinants on the human peripheral lymph node vascular addressin, identified by MECA-79 MAb, also may participate in L-selectin dependent adhesion and recruitment at sites of chronic inflammation. In addition, MAdCAM-1, which contains Ig domains and a mucin-like domain and is expressed on HEV, has recently been shown to support L-selectin-dependent lymphoid cell line rolling interactions.[40] Further examination of the integrative function of these molecules will be necessary to ascertain their role in leukocyte influx at sites of inflammation.

In summary, we have used a model which allows examination of the role of the L-selectin adhesion pathway in chronic inflammatory leukocyte recruitment to sites of inflammation. Our results suggest L-selectin, interacting with its inducible endothelial counterreceptor(s), plays an important role in both neutrophil and mononuclear leukocyte extravasation at sites of inflammation.

Note Added in Proof

Arbonés, Tedder and colleagues (Immunity, in press), using *L*-selectin-deficient mice have reported that neutrophil recruitment into the inflamed peritoneum is significantly reduced at 4 hr; however, no data concerning mononuclear leukocyte recruitment into the peritoneum was presented.

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References

1. Gallatin WM, Weissman IL, Butcher EC: A cell-surface molecule involved in organ-specific homing of lymphocytes. Nature 1983, 304:30–34
2. Siegelman MH, van de Rijn M, Weissman IL: Mouse lymph node homing receptor cDNA clone encodes a glycoprotein revealing tandem interaction domains. Science 1989, 243:1165–1172
3. Lasky LA, Singer MS, Yednock TA, Dowbenko D, Fennie C, Rodriguez H, Nguyen T, Stachel S, Rosen SD: Cloning of a lymphocyte homing receptor reveals a lectin domain. Cell 1989, 56:1045
4. Tedder TF, Isaacs CM, Ernst TJ, Demetri GD, Adler DA, Disteche CM: Isolation and chromosomal localization of CDNAS encoding a novel human lymphocyte cell surface molecule, LAM-1. J Exp Med 1989, 170: 123–133
5. Tedder TF, Luscinskas W, Kansas GS: Regulation of leukocyte migration by L-selectin: mechanisms, domains and ligands. Behring Inst Mitt 1993, 92:165–177
6. Lewinsohn DM, Bargatze RF, Butcher EC: Leukocyte-endothelial cell recognition: evidence of a common molecular mechanisms shared by neutrophils, lymphocytes and other leukocytes. J Immunol 1987, 138: 4313–4321
7. Spertini O, Luscinskas FW, Munro JM, Kansas GS, Griffin JD, Gimbrone MA Jr, Tedder TF: Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial ligand to support leukocyte adhesion and transmigration. J Immunol 147:2565–2573
8. Smith CW, Kishimoto TK, Abbas O, Hughes B, Rothlein R, McIntire LV, Butcher E, Anderson DC: Chemotactic factors regulate lectin adhesion molecule (LECAM-1)-dependent neutrophil adhesion to cytokine-stimulated endothelial cell *in vitro*. J Clin Invest 87:609–618
9. Hallmann R, Jutila MA, Smith CW, Anderson DC, Kishimoto TK, Butcher EC: The peripheral lymph node homing receptor, LECAM-1, is involved in CD18-independent adhesion of human neutrophils to endothelium. Biochem Biophys Res Comm 1991, 174:236
10. Spertini O, Luscinskas FW, Gimbrone MA Jr, Tedder TF: Monocyte attachment to activated human vascular endothelium *in vitro* is mediated by leukocyte adhesion molecule-1 (L-selectin) under non-static conditions. J Exp Med 175:1789–1792
11. Ley K, Gaehtgens P, Fennie C, Singer MS, Laskey LA, Rosen SD: Lectin-like cell adhesion molecule 1 mediates leukocyte rolling in mesenteric venules *in vivo*. Blood 1991, 77:2553–2555
12. Von Andrian UH, Chambers JD, McEvoy LM, Bargatze RF, Arfors KE, Butcher EC: Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles of LECAM-1 and the leukocyte β_2-integrins *in vivo*. Proc Natl Acad Sci USA 1991, 88: 7538–7542
13. Ley K, Tedder TF, Kansas GS: L-selectin mediates leukocyte rolling in untreated mesenteric venules *in vivo* independent of E- or P-selectin. Blood 1993, 82:1632–1638
14. Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel WJ, Grimley C, Fennie C, Gillett N, Watson SR, Rosen SD: An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell 1992, 69:927–938
15. Baumhueter S, Singer MS, Henzel W, Hemmerich S, Renz M, Rosen SD, Lasky LA: Binding of L-selectin to the vascular sialomucin CD34. Science 1993, 262: 436–438
16. Michie SA, Streeter PR, Bolt PA, Butcher EC, Picker LJ: The human peripheral lymph node vascular addressin: an inducible endothelial ligand involved in lymphocyte homing. Am J Pathol 1993, 143:1688–1698
17. Watson SR, Fennie C, Lasky LA: Neutrophil influx into an inflammatory site inhibited by a soluble homing receptor-IgG chimera. Nature 1991, 349:164–167
18. Jutila MA, Rott L, Berg EL, Butcher EC: Function and regulation of the neutrophil MEL-14 antigen *in vivo*: comparison with LFA-1 and MAC-1. J Immunol 1989, 143:3318
19. Sanchez-Madrid F, Simon P, Thompson S, Springer TA: Mapping of antigenic and functional epitopes on the α- and β-subunits of two related mouse glycoproteins involved in cell interactions, LFA-1 and MAC-1. J Exp Med 1983, 158:586–602
20. Holmes KL, Langdon WA, Fredrickson TN, Coffman RL, Hoffman PM, Hartley JW, Morse HC: Analysis of neoplasms induced by CAS-BR-M MuLV tumor extracts. J Immunol 1986, 137:679–686
21. Portoles P, Rojo J, Golby A, Bonneville M, Gromkowski S, Geenbaum L, Janeway CA Jr, Murphy DB, Bottomly K: Monoclonal antibodies to murine CD3 epsilon define distinct epitopes, one of which may interact with CD4 during T cell activation. J Immunol 1989, 142: 4169–4175
22. Johnson P, Greenbaum L, Bottomly K, Trowbridge IS: Identification of the alternatively spliced exons of murine CD45 (T200) required for reactivity with B220 and other T200-restricted antibodies. J Exp Med 1989, 169:1179–1184
23. Rousseaux J, Rousseaux-Prevost R, Bazin H: Optimal conditions for the preparation of Fab and F(ab')$_2$ fragments from monoclonal IgG of different rat IgG subclasses. J Immunol Methods 1983, 64:141–146
24. Goding JW: Conjugation of antibodies with fluorochromes: modifications to the standard methods. J Immunol Methods 1976, 13:215–226
25. Harlow E, and Lane D: Antibodies: A Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor

Laboratory, 1988, pp 319–358
26. Bowen BR, Fennie C, Lasky LA: The MEL-14 antibody binds to the lectin domain of the murine peripheral lymph node homing receptor. J Cell Biol 1990, 110: 147–153
27. Herlyn DM, Steplewski Z, Herlyn MF, Koprowski H: Inhibition of growth of colorectal carcinoma in nude mice by monoclonal antibody. Cancer Res 1980, 40: 717–721
28. Butcher EC: Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell 1991, 67:1033–1036
29. Oppenheimer-Marks N, Davis LS, Bogue DT, Ramberg J, Lipsky PE: Differential utilization of ICAM-1 and VCAM-1 during the adhesion and transendothelial migration of human T lymphocytes. J Immunol 1991, 147:2913–2921
30. Carlos TM, Harlan JM: Membrane proteins involved in phagocyte adherence to endothelium. Immunol Rev 1992, 114:5–28
31. 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
32. Issekutz TB, Wykretowicz A: Effect of a new monoclonal antibody, TA-2, that inhibits lymphocyte adherence to cytokine-stimulated endothelium in the rat. J Immunol 1991, 147:109–116
33. Issekutz TB: Inhibition of lymphocyte endothelial adhesion and *in vivo* lymphocyte migration to cutaneousinflammation by TA-3, a new monoclonal antibody to rat LFA-1. J Immunol 1992, 149:3394–3402
34. Bevilacqua MP: Endothelial-leukocyte adhesion molecules. Annu Rev Immunol 1993, 11:767–804
35. Cotran RS, Gimbrone MA, Bevilacqua MP, Mendrick DL, Pober JS: Induction and detection of a human endothelial activation antigen *in vivo*. J Exp Med 1986, 164:6614–666
36. Munro JM, Pober JS, Cotran RS: Tumor necrosis factor and interferon-γ induce distinct patterns of endothelial activation and associated leukocyte accumulation in skin of papio anubis. Am J Pathol 1989, 135:121–133
37. Pober JS, Cotran RS: Cytokines and endothelial cell biology. Physiol Rev 1990, 70:427–451
38. Mayadas TN, Johnson RC, Rayburn H, Hynes RO, Wagner DD: Leukocyte rolling and extravasation are severely compromised in P-selectin deficient mice. Cell 1993, 74:541–554
39. Salmi M, and Jalkanen S: A 90-kilodalton endothelial cell molecule mediating lymphocyte binding in humans. Science 1992, 257:1407–1409
40. Berg EL, McEnvoy LM, Berlin C, Bargatze RF and Butcher EC: L-selectin-mediated lymphocyte rolling on MAdCAM-1. Nature 366:695–698