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FUNCTION AND REGULATION OF THE NEUTROPHIL MEL-14 ANTIGEN IN VIVO: COMPARISON WITH LFA-1 AND MAC-1[1]

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The CD11/18 (LFA-1, Mac-1) molecules participate in neutrophil adhesion to cultured endothelium in vitro and are critical for effective neutrophil localization into inflamed tissues in vivo. More recently, the MEL-14 Ag, which was first defined as a lymphocyte homing receptor, has also been implicated in inflammatory neutrophil extravasation. Here we compare the regulation and function of these adhesion molecules on neutrophils during the in vivo inflammatory response. The MEL-14 Ag is expressed at high levels on bone marrow and peripheral blood neutrophils, but is lost on neutrophils isolated from the thioglycollate-inflamed peritoneal cavity. In contrast, Mac-1 is up-regulated on inflammatory neutrophils and little change is seen in the level of LFA-1 expression. In vitro activation of bone marrow neutrophils with PMA or leukotriene B_4 results in a dose dependent increase in Mac-1 and decrease in MEL-14 Ag expression within 1 h after treatment, thus reflecting what is found during inflammation in vivo. Neutrophils activated in vitro or in vivo (MEL-14^{Low}, Mac-1^{Hi}) do not home to inflammatory sites in vivo, correlating with the loss of the MEL-14 Ag and the increased Mac-1 expression. Anti-LFA-1, anti-Mac-1, or MEL-14 antibody given i.v. suppress neutrophil accumulation within the inflamed peritoneum (38%, 30%, and 37% of medium control, respectively) without affecting the levels of circulating neutrophils. However, when FITC-labeled cells are precoated with the mAb and injected i.v., only MEL-14 inhibits extravasation into the inflamed peritoneum (25% of medium control). Finally, in ex vivo adhesion assays of neutrophil binding to high endothelial venules in inflamed-lymph node frozen sections MEL-14 inhibits >90%, anti-LFA-1 20 to 30% and anti-Mac-1 <10% of the binding of bone marrow neutrophils to inflamed-lymph node high endothelial venules. These results confirm that both the MEL-14 antigen and Mac-1/LFA-1 are important in neutrophil localization to inflamed sites in vivo, but suggest that their roles in endothelial cell interactions are distinct.

Extravasation of neutrophils from the blood into an inflammatory site requires neutrophil adherence to the vascular endothelium (1). Two classes of neutrophil surface Ag have been shown to be involved in this interaction—the LFA-1/Mac-1/p150,95 (CD11a-c/CD18) complex and the MEL-14 Ag. mAb specific for CD11/18 components block neutrophil adhesion to cultured endothelial cells in vitro (2–5) and when given i.v. inhibit neutrophil localization to sites of inflammation in vivo (6–9). The importance of the CD11/18 complex is dramatically illustrated in human patients lacking expression of these molecules on the surface of their leukocytes. These patients have recurrent bacterial infections and exhibit inflammatory lesions that are devoid of neutrophils (10, 11). We have recently shown that another adhesion molecule, defined in the mouse by mAb MEL-14 (12–14), can also participate in neutrophil-endothelial cell interactions in vivo. MEL-14 blocks neutrophil interactions with HEV[4] in lymphoid tissues in vitro, and preliminary studies studies suggest that it is important for neutrophil localization to nonlymphoid sites of acute inflammation in vivo (15).

The mouse offers the only system in which antibodies are available to both classes of adhesion molecules. Here we have compared the effects anti-LFA-1, anti-Mac-1, and MEL-14 antibodies on murine neutrophil localization to inflammatory sites *in vivo* and binding to lymphoid tissue HEV ex vivo (adhesion to endothelium taken directly from the animal). In addition, we have studied the regulation of these molecules during inflammation. Our results show that Mac-1 is specifically up-regulated and the MEL-14 Ag down-regulated on the surface of the neutrophil during inflammation in vivo or activation in vitro. Antibody blocking experiments suggest that, although both of these adhesion molecules are important to neutrophil extravasation in vivo, the MEL-14 Ag may be principally involved in the initial interaction between the neutrophil and the vascular endothelium, whereas Mac-1 may have a broader role in subsequent adhesion and diapedesis.

MATERIALS AND METHODS

Animals. Twelve-week-old BALB/c mice were obtained from Simonsen N. I. H. colony, Gilroy, CA.

Reagents. The following antibodies were used in this study: MEL-14, rat IgG2a anti-gp90-110 homing receptor (12); FD445.1, rat IgG2b anti-LFA-1 α-chain (16); M1/70, rat IgG2b anti-Mac-1 α-chain (17); 30G12, rat IgG2a, anti-T200 common leukocyte Ag (18); RB6-8C5, rat IgG2b anti-mouse neutrophil (15, 19); and Hermes-1, rat

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[4] Abbreviations used in this paper: HEV, high endothelial venule; LTB_4, leukotriene B_4; FMF, flow microfluorimetry; PE, phycoerythrin; SER, specific extravasation ratio.

IgG2a anti-human Hermes/CD44 (20). All antibodies were precipitated from serum-free culture supernatants with ammonium sulfate. Purity of the preparations was evaluated by SDS-PAGE and all preparations were at least 50% antibody. PMA (Sigma, St. Louis, MO) was dissolved in DMSO and stored at a 1 mg/ml solution at −30°C. LTB_4 (Sigma) was stored in ethanol at −70°C. Thioglycollate broth (BBL Microbiological Systems, Cockeysville, MD) was made up per manufacturer's instruction, autoclaved, and allowed to "age" at room temperature for 1 wk before use as an inflammatory agent.

Cell suspensions and in vitro activation. Peritoneal cell suspensions from thioglycollate-inflamed (1 to 2 ml injected i.p. 3 h earlier) or control animals were obtained by washing out the peritoneal cavity with 10 ml of pyrogen-free HBSS (Applied Scientific, San Francisco, CA). Bone marrow cells were collected from mouse femurs into HBSS. In all cases the cell suspensions were washed in HBSS and resuspended at 2×10^7 cell/ml for immunofluorescence staining or at other cell concentrations for in vitro activation or HEV binding studies (see below).

For in vitro activation, bone marrow neutrophils were resuspended at 1×10^7 cells/ml in RPMI (Applied Scientific) plus 5% FCS and 0.1 ml/well placed into 96-well plates. The cells were incubated with different concentrations of PMA or LTB_4 for 1 hour, washed and then stained for FMF analysis. Optimal neutrophil activating concentrations of LTB_4 and PMA were determined by increased expression of a neutrophil specific cell surface activation antigen SK2-105 (E. L. Berg, M. A. Jutila, L. Rott, and E. C. Butcher, in preparation) and were found to be 1×10^{-8} M and 50 ng/ml, respectively.

Immunofluorescence staining and FMF analysis. Immunofluorescence staining of cells was carried out in microtiter plates or 4-ml tubes. Briefly, 1×10^6 cells were initially incubated in 5% rabbit serum for 10 min on ice to block FcR. The cells were washed and then incubated with primary antibody at 50 to 100 μg/ml for 20 min on ice. After washing, bound antibodies were revealed by incubation with PE conjugated $F(ab)'_2$ goat anti-rat Ig (Tago, Burlingame, CA) at a 1/80 dilution in 5% FCS in DMEM. FMF analysis was performed on a FACS STAR (Becton Dickinson, Mountain View, CA) as described elsewhere (21). Neutrophils present in bone marrow, peritoneal exudate, and peripheral blood preparations were identified by distinctive forward and side light scatter profiles, the accuracy of which were confirmed by bright staining with the neutrophil antibody RB6-8C5 (15, 19, 21). Data were collected from 10,000 to 50,000 cells, and are presented as either percent positive cells, mode fluorescence, or as histograms.

Standard frozen section HEV-binding assay. The standard ex vivo lymph node HEV-binding assay was performed as described elsewhere (15, 22–24), with the following modifications. Isolated bone marrow neutrophils were resuspended at 1×10^7/ml in RPMI (2.5% FCS, 20 mM HEPES, pH 7.1), and 0.1 ml was applied to each frozen section of lymph nodes. After incubation (15 min, 6°C with agitation), the sections were fixed in 1.5% glutaraldehyde for at least 1 h, washed gently in a 10 mM bicarbonate solution, then viewed under dark-field microscopy. Greater than 90% of bone marrow cells that adhere to HEV are neutrophils (15). The number of cells/HEV was determined from at least 200 HEV/experiment: the mean and standard deviation of data from at least three experiments is presented. In antibody-blocking experiments, saturating levels of the above antibodies (normally 1 to 10 μg antibody/1 to 3×10^6 cells) were incubated with cells for 20 min at 4°C before the HEV-binding assay. In all blocking experiments the antibodies were left with the cell preparations throughout the assay. Assays were counted single blind. At 7°C nonspecific antibody-induced cell clumping did not occur.

Antibody blocking of neutrophil homing to sites of inflammation in vivo. Two approaches for studying the effect of antibodies on inflammatory neutrophil homing in vivo were used. The first was a modification of the method of Rosen and Gordon (25). Animals were injected i.v. with 500 μg of the various antibodies or saline alone 1 h before the induction of inflammation in the peritoneal cavity with 1 ml of thioglycollate broth. Three hours later, the peritoneal cavities of the mice were washed out with 10 ml of HBSS and the number of newly arrived peritoneal neutrophils was evaluated for each animal. Peripheral blood was also collected from each animal, RBC lysed, and the effect of the antibody treatment on circulating neutrophils quantitated. The percentage of neutrophils in the peritoneum and peripheral blood of each animal was determined by FMF analysis after staining with the neutrophil antibody RB6-8C5 and by Wright's stain differentials. Antibody blocking data are presented as percent of medium control.

The second approach was the method used by Lewinsohn et al. (15). Bone marrow neutrophils were labeled with FITC (Sigma), as previously described (24), and then 2 to 5×10^7 cells were injected i.v. into mice which received 1 to 2 ml of thioglycollate broth i.p. 3 h earlier. The FITC-labeled bone marrow neutrophils localize effectively to sites of inflammation in vivo (15). The cells that accumulated in the inflamed peritoneal cavity were revealed by FMF analysis of 50,000 cells. Two color staining with RB6-8C5 (anti-neutrophil antibody) followed by PE-conjugated goat anti-rat Ig second stage, indicated that all of the FITC-bone marrow cells that migrated into the inflamed peritoneum were neutrophils (RB6-8C5 bright, data not shown). Data were recorded as the percentage of FITC-labeled donor neutrophils vs unlabeled host neutrophils in the inflamed peritoneal cavity. The unlabeled host neutrophils serve as a standard for the level of inflammation in a given animal. Routinely the percentage of FITC-labeled neutrophils that accumulated in the inflamed peritoneum ranged from 2 to 8%. For blocking studies, FITC-labeled neutrophils were precoated with the antibodies at saturating concentrations for 20 to 30 min on ice. The cells were washed and then injected into animals that had received thioglycollate 3 h earlier. FMF analysis of 50,000 cells isolated from the peritoneal cavity of each mouse was performed. Clearance of antibody-coated cells was evaluated by examining peripheral blood levels from each test animal. The percentage of FITC-labeled neutrophils vs unlabeled host neutrophils in the peritoneum and the blood of each animal was determined. The data after antibody treatment are presented as a percentage of medium control. The specificity of antibody blocking was determined by calculating a SER for each animal by dividing the percent FITC-labeled neutrophils in the peritoneum by the percent FITC-labeled neutrophils in the peripheral blood. (SER = (FITC neutrophils/host neutrophils)$_{peritoneum}$/(FITC neutrophils/host neutrophils)$_{blood}$. If neutrophil localization at the inflammatory site is blocked due to clearance of the antibody coated cells from the circulation, this results in SER values similar to saline control.

In vivo homing of MEL-14^{Hi} control vs activated MEL-14^{Low} neutrophils into the thioglycollate-inflamed peritoneal cavity. MEL-14^{Low}, Mac-1^{Hi} cells were obtained by treatment of bone marrow neutrophils with 50 ng/ml PMA or by injecting 5×10^7 FITC-labeled bone marrow neutrophils into the thioglycollate-inflamed peritoneal cavity, waiting 1 hour and then isolating the cells by peritoneal lavage. MEL-14^{Hi}, Mac-1^{Hi} neutrophils were obtained by injecting 5×10^7 FITC-labeled cells into the uninflamed peritoneal cavity, followed 1 hour later by peritoneal lavage. The FITC-labeled cells isolated from the peritoneum of inflamed or uninflamed animals routinely contained less than 20% contamination with unlabeled host cells. Control unactivated cells, which were MEL-14^{Hi}, Mac-1^{Moderate}, were also collected. Each of the cell populations were adjusted to 2 to 4×10^7 FITC-labeled neutrophils/ml in HBSS and 0.5 ml was injected into animals which received thioglycollate i.p. 3 h earlier. After one hour the peritoneal cavity of each mouse was lavaged and peripheral blood samples collected for FMF analysis. The percent localization and percent peripheral blood neutrophil levels are presented.

RESULTS

Expression of LFA-1, Mac-1, and the MEL-14 Ag on bone marrow, peripheral blood, and inflammatory neutrophils. The levels of LFA-1, Mac-1, and the MEL-14 Ag on neutrophils isolated from the bone marrow, peripheral blood, and the 3-h thioglycollate-inflamed peritoneum were compared by flow cytometry. Inflammatory neutrophils expressed far less MEL-14 Ag in comparison with bone marrow or peripheral blood neutrophils (Table I and Fig. 1). In contrast, inflammatory neutrophils expressed substantially higher levels of Mac-1. The expression of the T200 Ag and of LFA-1 was similar on all three populations (Table I). The staining patterns of the bone marrow and inflammatory neutrophils were essentially unimodal (Fig. 1).

To determine if migration across the endothelium was essential to the loss or down-regulation of cell surface MEL-14 Ag, FITC-labeled neutrophils were given i.v. and allowed to localize to the inflamed peritoneum in vivo or were injected directly i.p. into the inflamed peritoneal cavity. The expression of Mac-1, LFA-1, and the MEL-14 Ag on the FITC-labeled neutrophils was determined. A decrease in MEL-14 Ag expression, as well as an increase in Mac-1, was seen on FITC neutrophils, whether they arrived in the peritoneum via extravasation or by direct

TABLE I

Expression of LFA-1, Mac-1, and the MEL-14 Ag on neutrophils isolated from the bone marrow, peripheral blood and the thioglycollate-inflamed peritoneal cavity

Antibody	Ag	Mode Fluorescence[a]		
		Bone marrow[b]	Peripheral blood[b]	Peritoneal exudate[b]
MEL-14	gp90-110	200	220	22
FD445.1	LFA-1	84	136	130
M1/70	Mac-1	225	500	1356
30G12	T200	350	380	430

[a] Cell surface staining by FMF analysis. The modal fluorescence of neutrophils stained with the indicated antibodies after subtraction of the fluorescence of cells stained with the control antibody Hermes-1. Values represent the mean of two experiments with similar results.

[b] Bone marrow, peripheral blood, and thioglycollate-elicited peritoneal exudate neutrophils were collected as described in the *Materials and Methods*.

injection (Table II). The Mac-1 and the MEL-14 Ag levels on transferred cells were equivalent to those seen on the unlabeled host neutrophils that accumulated in the inflamed peritoneum.

Interestingly, only a small decrease in MEL-14 Ag expression (mode fluorescence = 90) was detected on FITC-labeled neutrophils injected into the control uninflamed peritoneal cavity of mice. In contrast, Mac-1 expression increased (mode fluorescence = 900) to levels only slightly lower than those reached in the inflamed peritoneal cavity. This suggests that inflammation was necessary for effective suppression of the MEL-14 Ag, and that perhaps increased Mac-1 expression can be dissociated from MEL-14 Ag loss.

Expression of LFA-1, Mac-1, and the MEL-14 Ag on bone marrow neutrophils after in vitro activation with PMA and LTB_4. Isolated bone marrow neutrophils were treated for 1 h at 37°C in vitro with optimal activating doses of PMA and LTB_4 (see *Materials and Methods*), and then stained with anti-LFA-1, anti-Mac-1, MEL-14, and anti-T200 antibodies. Treatment with both PMA and LTB_4 resulted in increased Mac-1 and decreased MEL-14 Ag expression. LFA-1 and T200 expressions were not appreciably altered (Table III). Interestingly, the fraction of neutrophils losing MEL-14 Ag was dependent on the dose of activating factor, but the loss of the Ag appeared to be a discrete event for each cell. For example, as shown in Figure 2 (and as occurred with optimal doses of PMA and LTB_4 used for Table III) all neutrophils become MEL-14 low after treatment with 1×10^{-8} M LTB_4 (Fig. 2*F*), but staining was bimodal after treatment with the intermediate concentrations, 1×10^{-9} M and 2×10^{-9} M LTB_4, respectively (Fig. 2, *D* and *E*). Staining was unaffected after treatment with 1×10^{-10} M LTB_4 (Fig. 2*C*). The results suggest that LTB_4 triggers a discrete activation event associated with MEL-14 Ag loss on neutrophils, and that neutrophils as a population are heterogeneous with respect to their sensitivity to LTB_4. A more extensive study of the mechanism of the in vitro regulation of the neutrophil MEL-14 Ag is given in a separate report (26).

Capacity of activated MEL-14^{Low} neutrophils to home to inflammatory sites in vivo. We asked whether the loss of the MEL-14 Ag after cell activation was associated with the loss of the ability of neutrophils to home to inflammatory sites in vivo. MEL-14^{Low}, Mac-1^{Hi} bone marrow neutrophils were obtained by activation with PMA in vitro (Table III) or by injecting the cells into the inflamed peritoneal cavity and then collecting the cells by peritoneal lavage 1 h later (Table II). MEL-14^{Hi}, Mac-1^{Hi} neutrophils were obtained by injecting cells into the uninflamed peritoneum and then collecting the cells one hour later. The ability of the treated neutrophils to home to the inflamed peritoneum was compared with untreated control neutrophils as described in the Materials and Methods. As shown in Table IV, the in vitro and in vivo activated cells (MEL-14^{Low}, Mac-1^{Hi}) did not localize to the inflamed site, whereas the cells incubated in the uninflamed peritoneum (MEL-14^{Hi}, Mac-1^{Hi}) and control unactivated neutrophils (MEL-14^{Hi}, Mac-1^{Low}) localized well.

Clearance of the MEL-14^{Low}, Mac-1^{Hi} neutrophils from the peripheral blood did not account for these results. Circulating FITC-labeled neutrophils were present in all test animals. Although the blood levels of Mac-1^{Hi} neutrophils were generally lower than those of control Mac-$1^{Moderate}$ cells (Table IV), the circulating levels of activated cells (MEL-14^{Low}, Mac-1^{Hi}) were equal to or higher than the levels of the cells that were incubated in the uninflamed peritoneum (MEL-14^{Hi}, Mac-1^{Hi}), yet only the latter cells homed effectively to the inflamed peritoneum.

Antibody blocking of neutrophil localization to sites of inflammation in vivo. We used an approach similar to that of Rosen and Gordon (25) to compare the effects of anti-LFA-1, anti-Mac-1 and MEL-14 on inflammatory neutrophil localization in vivo. Mice were injected with 500 μg of each antibody, or of medium alone, and then one hour later were given 1 ml of thioglycollate broth i.p. The effects of the antibody treatments were evaluated 3 to 4 h later by FMF analysis of the cells that accumulated in the inflamed peritoneum. MEL-14, anti-LFA-1 and anti-Mac-1 treatment blocked neutrophil localization, to 37%, 38%, and 30% of medium control, respectively (Table V). Control antibody Hermes-1, which does not recognize mouse neutrophils, had no effect on localization, but anti-T200 antibody blocked approximately 50% of the accumulation of neutrophils. However, the anti-T200 antibody treatment resulted in significant clearance of neutrophils from the peripheral blood (to 50% of medium control), whereas anti-LFA-1, anti-Mac-1, and MEL-14

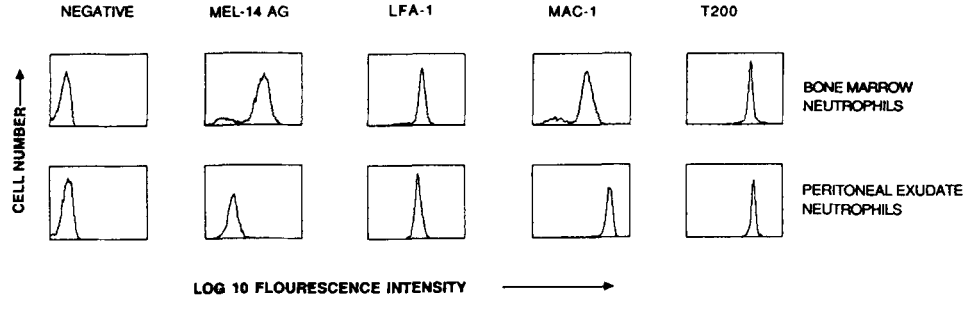

Figure 1. Expression of T200, LFA-1, Mac-1, and the MEL-14 Ag on neutrophils isolated from the bone marrow or from the thioglycollate-inflamed peritoneum.

TABLE II

Expression of LFA-1, Mac-1, and the MEL-14 Ag on FITC-labeled neutrophils injected directly into the thioglycollate-inflamed peritoneum and on FITC-labeled neutrophils that were allowed to migrate from the blood to the inflamed peritoneum after i.v. injection

Ag	Mode Fluorescence[a]			
	BM PMN[b]	PE-unlabeled PMN[c]	PE FITC-Labeled PMN[d]	
			i.v.	i.p.
MEL-14	185 ± 43	13 ± 3	17 ± 8	9 ± 3
LFA-1	92 ± 28	116 ± 12	92 ± 11	98 ± 22
Mac-1	249 ± 23	1200 ± 180	1564 ± 240	1350 ± 350
T200	390 ± 2.3	496 ± 70	450 ± 90	450 ± 50

[a] Cell surface staining by FMF analysis. The modal fluorescence of neutrophils stained with the indicated antibodies after subtraction of the fluorescence of cells stained with the control antibody Hermes-1. Values represent the mean ± SE of three separate experiments.
[b] Bone marrow polymorphonuclear neutrophils.
[c] Unlabeled peritoneal exudate polymorphonuclear neutrophils.
[d] Peritoneal exudate FITC-labeled polymorphonuclear neutrophils after i.v. or i.p. injection.

TABLE III

Expression of LFA-1, Mac-1, and the MEL-14 Ag on bone marrow neutrophils after in vitro activation with PMA and LTB_4

Antibody	Ag	Mode Fluorescence[a]		
		No treatment	PMA	LTB_4
MEL-14	gp90-110	290	5	12
FD445.1	LFA-1	146	136	141
M1/70	Mac-1	136	1145	360
30G12	T200	431	402	416

[a] Neutrophils were treated with 50 ng/ml PMA or 1×10^{-8} M LTB_4 for 1 h at 37°C and cell surface staining determined by FMF analysis. The modal fluorescence of neutrophils stained with the indicated antibodies after subtraction of the fluorescence of cells stained with the control antibody Hermes-1 are presented. Values are from a representative experiment.

had little or no effect (132%, 88%, and 130% of medium control, respectively). These results suggest that the MEL-14, anti-LFA-1, and anti-Mac-1 blocking was specific; whereas, the blocking seen with anti-T200 most likely was due to nonspecific clearance of neutrophils from the blood or prevention of their release from the bone marrow into the circulation.

We also used a second approach to evaluate the effects of these antibodies on neutrophil homing in vivo (15). In this method FITC-labeled bone marrow neutrophils were precoated with anti-LFA-1, anti-Mac-1 or MEL-14, washed, and injected i.v. into mice which received thioglycollate i.p. 3 h earlier. Cells which localized to the peritoneum were harvested and then evaluated by FMF. All antibodies, except MEL-14, resulted in both reduced accumulation of FITC-labeled neutrophils in the inflamed peritoneum and clearance of FITC-labeled neutrophils from the circulation. Anti-Mac-1, anti-LFA-1, and anti-T200 reduced localization into the inflamed peritoneum, 41%, 76%, and 60% of medium control, respectively, and lowered circulating levels of FITC-labeled neutrophils to 59%, 86%, and 76% of medium control, respectively (Table VI). In marked contrast, MEL-14 treatment reduced accumulation of neutrophils in the inflamed peritoneum to 41% of medium control, while at the same time consistently increased circulating levels of FITC-labeled neutrophils (146% of medium control) (Table VI).

To reflect the specificity of antibody blocking, a SER was calculated (see *Materials and Methods*). MEL-14, but not the other antibodies, showed significant inhibition of neutrophil localization ($p < 0.001$) independent from the clearance of the antibody coated cells from the peripheral blood (Table VI). Thus pretreatment of neutrophils in vitro with MEL-14, and not with anti-Mac-1, anti-LFA-1 or anti-T200 antibodies, blocks extravasation to the inflamed peritoneum in vivo.

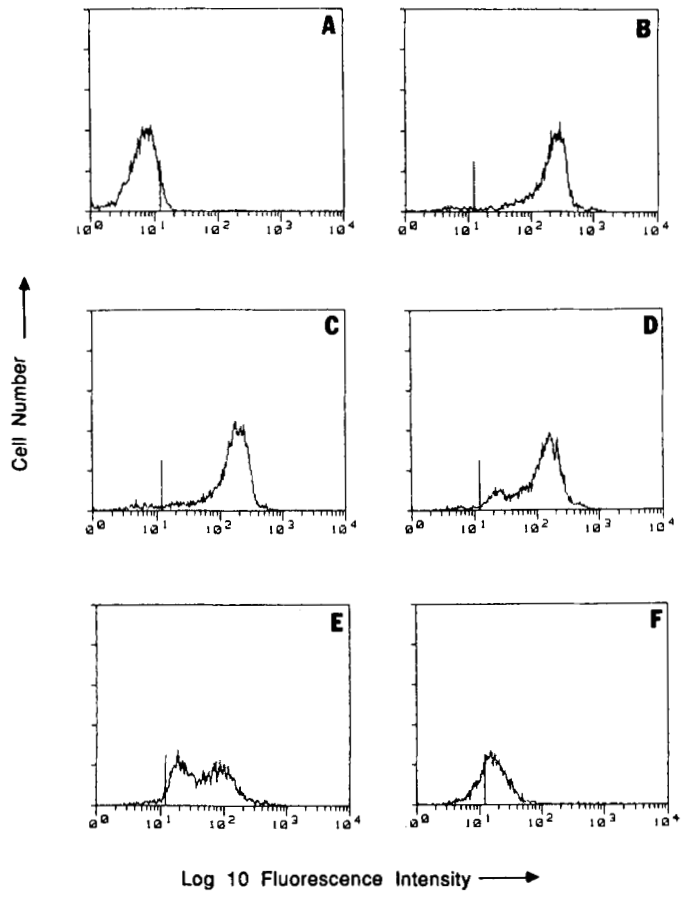

Figure 2. Expression of the MEL-14 Ag on bone marrow neutrophils treated with different concentrations of LTB_4 for one hour in vitro. *Panel A* represents background staining with the isotype control antibody Hermes-1. No changes were noted in background staining after treatment with LTB_4 (data not shown). MEL-14 staining of unactivated cells (*B*) and of cells treated with 1×10^{-10} M LTB_4 (*C*); 1×10^{-9} M LTB_4 (*D*); 2×10^{-9} M LTB_4 (*E*); or 1×10^{-8} M LTB_4 (*F*) is shown.

TABLE IV

Capacity of activated (MEL-14^{Low}) vs control (MEL-14^{High}) neutrophils to localize to the thioglycollate-inflamed peritoneal cavity

Treatment[a]	MEL-14, Mac-1 Levels	FITC-Labeled Cells as % of Polymorphonuclear Neutrophils in	
		Peritonium[b]	Blood[c]
None	MEL-14^{Hi}Mac-$1^{Moderate}$	1.45	8.0
Uninflamed Peritoneum	MEL-14^{Hi}Mac-1^{Hi}	1.7	2.9
Inflamed Peritoneum	MEL-14^{Low}Mac-1^{Hi}	<.01	4.3
PMA In Vitro	MEL-14^{Low}Mac-1^{Hi}	<.01	2.6

[a] Neutrophils isolated from the bone marrow were treated by either being injected into the uninflamed peritoneal cavity, injected into the thioglycollate-inflamed peritoneal cavity or activated with PMA in vitro. One hour later the cells were harvested, labeled with FITC, and injected i.v. into recipients which had received thioglycollate i.p. 3 h before. Values are from a representative experiment.
[b] The percentage of FITC-labeled neutrophils in the inflamed peritoneum after 2×10^7 cells were injected i.v. 1 h earlier.
[c] The percentage of FITC-labeled neutrophils in the peripheral blood after 2×10^7 cells were injected i.v. 1 h earlier.

Antibody blocking of neutrophil adhesion to HEV ex vivo. Neutrophils show specific binding to HEV within frozen thin sections of uninflamed lymph nodes. Lewinsohn et al. (15) showed that the MEL-14 antibody will

TABLE V

Blocking of neutrophil accumulation into the thioglycollate-inflamed peritoneal cavity after i.v. administration of MEL-14, anti-LFA-1, or anti-Mac-1 antibodies

Antibody	Ag	Percent of Medium Control[a]	
		Peritoneal exudate PMN	Peripheral blood PMN
MEL-14	gp90-110	37 ± 10.3	130 ± 24
FD445.1	LFA-1	38 ± 12.6	132 ± 19
M1/70	Mac-1	30 ± 10.0	88 ± 14
30G12	T200	50 ± 3.5	49 ± 8
Hermes-1	Control	92 ± 21.3	92 ± 13

[a] Numbers of polymorphonuclear neutrophils (PMN) in the peritoneal cavity and peripheral blood determined by FMF analysis and Wright's stained differentials. Values expressed as percentage of medium control and represent mean ± SE of three separate experiments.

TABLE VI

Blocking of FITC-labeled polymorphonuclear neutrophil (PMN) localization into the thioglycollate-inflamed peritoneal cavity after i.v. transfer of antibody coated cells

Antibody	Ag	Percent of Medium Control[a]		SER[b]
		Peritoneal FITC-PMN	Peripheral blood FITC-PMN	
MEL-14	gp90-110	41 ± 9.3	146 ± 25	4.0 ± 1.1
FD445.1	LFA-1	76 ± 22	84 ± 20	14.2 ± 3.4
M1/70	Mac-1	41 ± 3.0	59 ± 9.8	11.2 ± 3.6
30G12	T200	58 ± 15	76 ± 9.4	12.3 ± 3.1
Medium	NA[c]	NA	NA	14.8 ± 2.3

[a] FITC-labeled neutrophils were coated with the indicated antibody, injected i.v. into mice which received thioglycollate i.p. 3 h earlier, and 1 h later the percentage of FITC-labeled neutrophils in the peritoneum and peripheral blood was quantitated by FMF. Values are presented as percentage of control and represent mean ± SE of three separate experiments.

[b] SER-specific extravasation ratio = [(number of FITC neutrophils/number unlabeled neutrophils)$_{peritoneum}$/(number of FITC neutrophils/number unlabeled neutrophils)$_{blood}$]. Values represent the mean ± SE of three separate experiments.

[c] NA = not applicable.

TABLE VII

Antibody blocking of bone marrow neutrophil adhesion to CFA-inflamed lymphoid tissue HEV ex vivo

Antibody	Ag	Percent of Medium Control[a]
MEL-14	gp90-110	9 ± 6.8
FD445.1	LFA-1	76 ± 3.5
M1/70	Mac-1	92 ± 16
30G12	T200	122 ± 33

[a] Mean ± SE of three separate experiments.

block this interaction. We found that MEL-14 also blocked adhesion of neutrophils to HEV from CFA-inflamed lymph nodes (90.8 ± 6.8%). In contrast, anti-LFA-1 inhibited 24.3 ± 3.5% and anti-Mac-1 8.2 ± 16% of neutrophil binding. Anti-T200 had little effect on neutrophil binding to HEV (Table VII).

DISCUSSION

At least two classes of neutrophil surface membrane glycoproteins appear to be involved in neutrophil-endothelial cell interactions: the CD11a-c/CD18 (CD11/18) complex and the MEL-14 defined "homing" receptor. The current experiments were designed to characterize further the role of the neutrophil MEL-14 Ag and compare its function and regulation with LFA-1 and Mac-1. We show that during inflammation there is an inverse regulation of the cell surface expression of Mac-1 and the MEL-14 Ag. Mac-1 expression greatly increases upon neutrophil activation in vivo or in vitro (shown here in Tables II, III, IV, and in Refs. 3, 27–30). This is due to translocation of preformed molecules contained in intracellular granules to the cell surface, which is rapid (occurring within minutes) and can be triggered by a variety of stimuli (3, 27, 28, 31).

In direct contrast to the up-regulation of Mac-1, inflammation in vivo or activation in vitro causes a rapid loss of the MEL-14 antigen from the surface of the neutrophil. Other experiments suggest that loss of the MEL-14 antigen occurs within minutes upon stimulation (26) and we show here that it is dependent upon the dose of the agent used to activate the neutrophil (Fig. 1). Importantly, the down-regulation of surface expression is specific for the MEL-14 Ag: little change in surface LFA-1 and T200 Ag expression (Tables II to IV), or of four additional Ag studied in other experiments (M. A. Jutila, unpublished observations), was observed upon activation.

The MEL-14 Ag on lymphocytes, and most likely on monocytes as well, is also down-regulated upon activation. Treatment with Con A in vitro causes the loss of the MEL-14 Ag from the surface of lymphocytes. This is due to MEL-14 positive cells becoming MEL-14 negative and not simply the mitogen induced expansion of MEL-14 negative lymphocytes (32, 33). Though inflammatory monocytes express the MEL-14 molecule, the level of expression is much lower than found on peripheral blood monocytes (M. A. Jutila, unpublished observations). These results, in addition to the studies presented here, show that a direct consequence of activation of MEL-14-positive leukocytes is down-regulation of surface expression of the MEL-14 Ag. It will be interesting to determine if the MEL-14 Ag is internalized, has simply lost the MEL-14 epitope or is actually released from the cell surface like the FcRIII receptor on human neutrophils (34).

The loss of the MEL-14 Ag on activated neutrophils suggests that its role in neutrophil function may be limited to the early stages of migration from the blood. This is in contrast to Mac-1, which is important not only in the early events of extravasation, but also for many functions once the neutrophil is within the inflamed tissues, such as complement-mediated phagocytosis and chemotaxis (3, 31, 35). We demonstrate here that the loss of the MEL-14 Ag on the neutrophil after activation is associated with the loss of the capacity to home to inflammatory sites in vivo. This is similar to the inability of lymphocytes to home to peripheral lymph nodes in vivo after mitogen stimulation in vitro (32, 33), which also correlates with the loss of MEL-14 antigen expression. The lack of homing of activated neutrophils could also be explained by the deactivation of the Mac-1 molecule. Wright and Meyer (36) showed that treatment of neutrophils with PMA for 1 h results in the inability of these cells to bind C3bi-coated E, a function mediated by Mac-1. Even though it is not known whether the C3bi binding capacity of the Mac-1 molecule is important in extravasation, these results suggest that other explanations, in addition to the loss of the MEL-14 Ag, can be given for the inability of activated neutrophils to home to inflammatory sites in vivo. However, this observation in combination with in vitro and in vivo antibody blocking experiments, and the regulation studies (Tables V to VII and Ref. 15, discussed below) is consistent with the MEL-14 Ag being important in neutrophil adhesion to the vascular endothelium.

Unlike other experimental animal models, the mouse

system allows a direct comparison of the function of the MEL-14 Ag and the CD11/18 complex in vivo and in vitro, since antibodies to both classes of molecules are available. Here we show that anti-Mac-1, anti-LFA-1, and MEL-14 antibodies are equally effective in blocking the accumulation of neutrophils into the thioglycollate-inflamed peritoneum when given i.v. 1 h before induction of inflammation. However, when FITC-labeled neutrophils were precoated with the antibodies in vitro and then injected i.v., only MEL-14 inhibited accumulation into the inflamed peritoneum. In the latter experiment, the lack of affect of anti-Mac-1 is likely due to the rapid expression or translocation of new Mac-1 to the cell surface. This suggests that the Mac-1 present initially on unactivated neutrophils may not be required for the initial interaction with the vascular endothelium. However, other explanations can also be given to account for these results. For instance, the lack of blocking by precoating with anti-Mac-1 and anti-LFA-1 could be due to conformational changes in Mac-1 or LFA-1 in vivo resulting in the loss of the monoclonal antibody from the surface of the neutrophil. It is also possible that the anti-Mac-1 and anti-LFA-1 antibodies used in this study do not recognize relevant adhesion epitopes. Indeed, multiple functional domains of Mac-1 have been shown (25, 35, 37). Some anti-Mac-1 antibodies can block C3bi binding, but have no effect on adhesion to or spreading on plastic. Finally, it could be that anti-Mac-1 or anti-LFA-1 mediate blocking of neutrophil extravasation by affecting other cells, such as endothelial cells, as well as the neutrophil.

In vitro studies suggest that, under appropriate conditions, the MEL-14 antigen and CD11/18 may each be able to play a dominant role in neutrophil-endothelial cell interactions. Anti-CD11/18 antibodies block neutrophil binding to cultured endothelial cells when binding is studied over a 30- to 60-min period of time, under little or no shear force and at 37°C. The CD11/18-mediated adhesion in this setting requires either activation of the neutrophil, resulting in increased expression of Mac-1 and transition of surface Mac-1 to an "active" form (3, 21–30), or activation of the endothelium (38–40). In contrast, the in vitro neutrophil-endothelial cell adhesion event shown to be blocked by MEL-14 is measured in an ex vivo assay in which neutrophils adhere to HEV in frozen sections of lymphoid tissues under shear forces (agitation) and at 7°C. Activation of the neutrophil is not required for adhesion in this ex vivo assay and we show here that anti-LFA-1 and anti-Mac-1 have little effect at blocking this interaction. The low temperature of the HEV-binding assay (4 to 7°C) most likely contributes to these results, since CD11/18 mediated events are thought to be temperature sensitive (41). (In addition, the cells used in adhesion to HEV are unstimulated bone marrow neutrophils that express low levels of Mac-1 (Tables I and II) which may be in the functionally "inactive" state.) Thus the relative contribution of MEL-14 antigen and CD11/18 in neutrophil-endothelial cell interactions depends on the experimental setting. In this context, it is likely that the relative importance of CD11/18 and MEL-14 Ag-mediated adhesion may vary between particular situations in vivo as well. Furthermore, it is likely that additional adhesion mechanisms of importance await to be discovered.

However, taken together our results lead us to a working hypothesis that in the experimental conditions studied here the MEL-14 Ag mediates an early interaction between neutrophils and the vascular endothelium during inflammation, which is not dependent on activation of the neutrophil, but most likely is dependent upon activation of the endothelium. This interaction would not involve CD11/18. Then upon activation by factors released at the inflammatory site, a rapid transition of the neutrophil CD11/18 complex to an "active" form plus increased surface expression of the molecules occurs, which "cements" the neutrophil adhesion to the endothelium through distinct mechanisms. After this point, the MEL-14 Ag may be lost and the cell eventually responds to chemotactic signals and migrates into the underlying tissue. Interestingly, it has been recently shown that CD11/18-deficient neutrophils bind activated endothelial cells in vitro, and that activation of the neutrophil greatly reduces this binding. Thus, an integrin-independent neutrophil-endothelial cell adhesion interaction has been demonstrated in the endothelial cell culture system, which is down-regulated upon activation of the neutrophil (C. W. Smith, personal communication). It is possible that the MEL-14 antigen mediates this novel neutrophil-activated endothelial cell interaction. It will be interesting to determine if inducible endothelial cell adhesion ligands, such as ELAM-1 (42), ICAM-1 (43) or other as yet uncharacterized surface molecules, are involved in the proposed MEL-14 Ag-mediated events.

The leukocyte molecules described to date do not appear sufficient to account for the leukocyte and inflammation specificity of neutrophil extravasation. Although, little difference is evident in the expression of CD11/18 and the MEL-14 Ag on neutrophils and monocytes, these cells display clearly distinct homing behaviors. For example, they show different kinetics in localization into inflammatory sites as well as different capacities to localize in response to various stimuli. This is even more dramatically illustrated by the behavior of eosinophils, which are also CD11/18 and MEL-14 positive, but which extravasation only during allergic or parasitic inflammation. Therefore, it is likely that other leukocyte and inflammation specific interactions, and/or leukocyte specific chemotactic factors, await our discovery.

In conclusion, the down-regulation of the MEL-14 Ag during inflammation, combined with the antibody blocking experiments, provide substantial support for a role of the MEL-14 Ag in the earliest interaction of the neutrophil with the vascular endothelium. MEL-14 Ag and CD11/18 most likely have distinct roles, but apparently are equally important in controlling neutrophil extravasation. Other interactions may also contribute. Understanding the mechanisms controlling inflammation and leukocyte specific extravasation may lead to better treatments of diverse chronic and acute inflammatory diseases. For example, the MEL-14 Ag may provide a target for specific intervention of neutrophil endothelial cell interactions without affecting other neutrophil functions many of which also involve CD11/18.

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