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Mac-1 (CD11b/CD18) is the predominant β_2 (CD18) integrin mediating human neutrophil migration through synovial and dermal fibroblast barriers

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SUMMARY

Recently we reported that polymorphonuclear leucocyte (PMNL) migration *in vitro* through a barrier of human synovial fibroblasts (HSF) involves both β_2 (CD18) and β_1 (CD29) integrins on the PMNL. Here we investigated the role of the β_2 integrin family members, lymphocyte function-associated (LFA)-1 ($\alpha_L\beta_2$ or CD11a/CD18) and Mac-1 ($\alpha_M\beta_2$ or CD11b/CD18), in PMNL migration through HSF and human dermal fibroblast (HDF) monolayers. Treatment of PMNL with monoclonal antibody (mAb) to LFA-1 (anti-α_L) did not inhibit PMNL migration through either monolayer in response to C5a. In contrast, mAb to Mac-1 (CD11b) inhibited (by 30–40%) PMNL migration, and by virtually the same extent as mAb to the β_2 integrin chain (CD18) (40% inhibition). Addition of mAb to LFA-1 to mAb to Mac-1 did not result in greater inhibition. This was in contrast to PMNL migration through human umbilical vein endothelium (HUVE) monolayers, where mAb to LFA-1 or to Mac-1 each partially inhibited PMNL transendothelial migration, and when these mAbs were combined, synergistic inhibition of migration was observed, reaching 90–95% inhibition. Intercellular adhesion molecule 1 (ICAM-1) was not required for Mac-1 mediated migration through HSF or HDF, because treatment of the fibroblasts with mAb R6.5 ($F(ab)_2$) to ICAM-1, which blocks the Mac-1 binding site on ICAM-1, did not inhibit PMNL migration. An LFA-1–ICAM-1 mechanism of PMNL migration through HSF and HDF monolayers could be detected after treatment (4 hr) of the monolayers with TNF-α plus IFN-γ, which upregulated ICAM-1 on the fibroblasts. The results demonstrate the β_2 integrin dependent PMNL migration in connective tissue may involve primarily Mac-1, with little involvement of LFA-1 or ICAM-1, a situation in marked contrast to PMNL migration across endothelium. However, in inflammatory conditions in which TNF-α and/or IFN-γ may be generated, a role for LFA-1–ICAM-1 may be induced.

INTRODUCTION

The accumulation of leucocytes including polymorphonuclear leucocytes (PMNL) in connective tissues is a feature of acute and some forms of chronic inflammation in many diseases[1] and most notably in rheumatoid arthritis where PMNL accumulate in large numbers in joint fluid.[2,3] The process of PMNL infiltration requires initial migration across vascular endothelium and vascular basement membrane and then migration through connective tissue barriers. The initial steps in adhesion and migration through endothelium have been quite well characterized and involve a complex interplay between adhesion molecules on leucocytes, endothelium and leucocyte chemotactic factors.[4,5] Much less is known about mechanisms by which PMNL migrate in the extravascular space. However, it is likely that interactions between adhesion molecules on PMNL and on connective tissue cells, such as fibroblasts, as well as with extracellular matrix (ECM) proteins, is also important for PMNL to adhere and/or to migrate in connective tissue in response to chemotactic factors generated in inflamed tissues. In this context, the β_2 (CD18) and β_1 (CD29) integrin family members on PMNL have been shown to mediate adhesion of PMNL to ECM proteins such as laminin, fibronectin, collagen as well as to denatured proteins.[6–10] Recently, we have observed that PMNL migration, in response to chemotactic factors, through a confluent barrier of fibroblasts requires the function of both β_2 and β_1 integrins on the PMNL.[11,12]

The β_1 integrins comprise individual α chains and a common β_1 chain (CD29) and have at least nine heterodimer members.[13–15] Some of them are also known as very late activation antigen (VLA)-1 to VLA-6 ($CD49_{a-f}$). These molecules function as receptors for a variety of ECM proteins

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such as fibronectin, laminin and collagen, mediating cell–ECM interaction in development and inflammation.[13–15] On PMNL a receptor for fibronectin has been identified as VLA-5 ($\alpha_5\beta_1$),[6] and for laminin as VLA-6 ($\alpha_6\beta_1$).[6,16] We have observed that both of these integrins have a role in migration of PMNL through synovial or dermal fibroblast barriers, in addition to the β_2 (CD11/CD18) integrins.[11,12]

In response to chemotactic factors such as C5a and interleukin (IL)-8, PMNL migration through synovial or dermal fibroblasts is partly CD11/CD18, i.e. β_2 integrin dependent.[11,12] The β_2 (CD18) leucocyte integrins comprise three forms of heterodimeric glycoproteins, i.e. lymphocyte function-associated (LFA)-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18). These molecules share a common β_2 (CD18) chain, but have individual α (CD11) chains.[4,5,14,17] Both LFA-1 and Mac-1 recognize, as a ligand, intercellular adhesion molecule-1 (ICAM-1). However, LFA-1 and Mac-1 may have differential roles in leucocyte–endothelium or leucocyte–fibroblast and –ECM interaction, as Mac-1 also can bind to additional ligands including fibrinogen, C3bi, Arg–Gly–Asp sequences, factor X, denatured proteins, heparin-like glycosaminoglycans and some forms of collagen.[4,5,7,18–21] In the present study, we investigated the role of LFA-1 and Mac-1 in PMNL migration through synovial and dermal fibroblast monolayers, as a model of connective tissue, in parallel with PMNL migration through human umbilical vein endothelium, a model of a vascular barrier. The results show that Mac-1, but not LFA-1 mediates β_2 integrin dependent migration of PMNL through the fibroblast barriers, while both LFA-1 and Mac-1 are involved in transendothelial migration across endothelium.

MATERIALS AND METHODS

Monoclonal antibodies and reagents

The following murine monoclonal antibodies (mAbs) against human antigens were used as purified immunoglobulin G (IgG): 60.3 (anti-β_2 (CD18) integrin IgG_{2a}, provided by Bristol-Myers Squibb, Seattle, WA),[22,23] R15.7 (anti-β_2, IgG_1, provided by Dr R. Rothlein, Boehringer Ingelheim, Ridgefield, CT),[24] 3S3 (anti-β_1 integrin, IgG, from Dr J. Wilkins, Winnipeg, Manitoba, Canada),[11] and R6.5 (ICAM-1, $IgG_1[F(ab)_2]$, from Dr C. W. Smith)[24] and G25.2 (anti-LFA-1, IgG_{2a}; Becton Dickinson, San Jose, CA). The following mAbs were used as ascites: 2LPM19c (anti-Mac-1 I domain, IgG_{2a}, from Dr K. Pulford, Oxford, UK)[25] and mAb 543 (anti-CR1, IgG_1), 3C10 (anti-CD14, IgG_1) and TS1/22 (anti-LFA-1, IgG_1), were from the American Type Culture Collection (Bethesda, MD). Recombinant human tumour necrosis factor (TNF-α) (specific activity of 5×10^7 U/mg) and inteferon-γ (IFN-γ) (specific activity of 1×10^7 U/mg) were gifts from Genentech Inc. (South San Francisco, CA). Recombinant human C5a was a gift from CIBA-Geigy Pharmaceuticals (Summit, NJ).

Isolation and growth of human synovial and dermal fibroblasts

Human synovial fibroblasts (HSF) were aseptically isolated from synovium obtained at surgery or arthroscopy of knee or hip joints of patients with RA or osteoarthritis (provided by Dr J. Hanly, Division of Rheumatology, Victoria General Hosptial, Halifax, Nova Scotia, Canada). Human dermal fibroblasts (HDF) were isolated from normal skin resected during minor plastic surgery (kindly provided by Dr K. Wilson, Izaak Walton Killam Children's Hospital, Halifax, Nova Scotia, Canada). The tissues were minced in α-minimal essential medium (αMEM; Sigma Chemical Co., St. Louis, MO) and digested with 2 mg/ml collagenase type IV (512 U/mg; Sigma) in αMEM supplemented with 10% heat inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) and penicillin G/streptomycin (Gibco, Grand Island, NY), by incubation in a shaker (250 r.p.m.) at 37° for about 4 hr, as described previously.[11,12] After digestion, the single cells were recovered by centrifugation, washed and cultured in αMEM supplemented with 10% FBS, 50 μM 2-mercaptoethanol (2-ME) and penicillin G/streptomycin until cells grew confluent. The cells were passaged until the cultures became homogeneous and third to the 12th passage were used. They were harvested with 0·05% trypsin/0·02% EDTA (Flow Laboratories, Mississauga, Ontario, Canada) and seeded onto polycarbonate filters bearing 5 μm pores in Transwell culture plate inserts (6·5 mm diameter, Transwell 3421; Costar, Cambridge, MA), which were precoated with 0·01% gelatin overnight as described.[11,12] Seeding density was 2×10^4 of HDF or 3×10^4 HSF in 0·1 ml–10% FBS, 2-ME and antibiotics above the filter and 0·6 ml of this medium was added to the lower compartment beneath the filter. After 6–7 days culture, tight monolayers had formed on the filter, which allowed the diffusion of < 5% of ^{125}I-HSA (human serum albumin) in 45 min. In comparison, diffusion across bare filters was 25–30%.

Isolation and culture of endothelial cells

Human umbilical vein endothelial cells (HUVE) were isolated and cultured as described by Jaffe *et al.*[26] and HUVE monolayers on filters were grown as described previously.[27,28] Briefly, endothelial cells isolated from umbilical cords by collagenase treatment were grown in the following complete medium: RPMI-1640 (Sigma Chemical Co.) containing 2 mM L-glutamine, 2-ME, sodium pyruvate, penicillin G/streptomycin and supplemented with 20% FBS (Hyclone, Logan, UT), 25 μg/ml endothelial cell growth factor (Collaborative Research, Lexington, MA) and heparin (45 μg/ml) (Sigma Chemical Co.) in gelatin-coated flasks (Nunc, Gibco, Grand Island, NY). The HUVE were detached by 0·025% trypsin/0·01% Versene (MA Bioproducts, Walkerville, MD) and cultured on Transwell filters described above. For HUVE on filters, filters were prepared by coating with 0·01% gelatin (37° overnight) followed by application of 3 μg of human fibronectin (Collaborative Research) in 50 μl water at 37° for 2 hr. The HUVE (2×10^4 cells in 0·1 ml complete medium), from first or second passage, were then added above the filter and 0·6 ml medium was added to the lower compartment beneath filter. The HUVE grew confluent and formed a tight monolayer in 5–6 days, with permeability < 1·5% tested by ^{125}I-HSA diffusion, as described previously.[27,28]

Isolation of human polymorphonuclear leucocytes

PMNL were isolated as described previously.[27] Briefly, peripheral venous blood from healthy donors was collected into heparin (5 U/ml blood) and acid citrate dextrose (1·6 ml/10 ml blood; ACD formula A, Travenol, Malton, Ontario, Canada) anticoagulant. The red blood cells were sedimented with 6% dextran saline (1 part to 5 parts blood) (Travenol,

Malton, Ontario, Canada). The leucocyte rich plasma (LRP) was harvested and centrifuged at 150 *g* for 10 min. The pellet containing leucocytes and residual red blood cells was resuspended in Ca^{2+}, Mg^{2+}-free Tyrode's solution with 5% autologous platelet-poor plasma (PPP) and labelled with ^{51}Cr sodium chromate (40 μCi/ml; Amersham Corp. Oakville, Ontario, Canada) by incubation for 30 min at 37°. The labelled PMNL were separated on discontinuous 10% PPP–Percoll (Pharmacia Fine Chemicals, Dorval, Quebec, Canada) gradients (58%/73%) by centrifugation (30 min at 300 *g*). The PMNL were washed and suspended in RPMI-1640 medium containing 0·5% HSA (pyrogen free, Connaught Laboratories, Toronto, Ontario, Canada) (RHSA) and 10 mM HEPES, pH 7·4 to a final concentration of 2×10^6/ml. This method yielded >95% pure PMNL with >98% viability with essentially no red blood cell contamination.

Measurement of PMNL migration across fibroblast and endothelium monolayers

The PMNL migration assay was performed as previously.[27,28] Briefly, HSF, HDF or HUVE monolayers on the filters and the lower compartment were washed with RPMI-1640 and incubated for 4 hr in fresh RPMI-1640 with 10% FBS. After incubation, the filters were washed on the upper and lower surfaces with RMPI-1640 and transferred to a new, clean well (lower compartment). To this well, 0·6 ml RPMI-1640–0·5% HSA was added containing C5a as a chemotactic stimulus. Before immersion of the filter unit, 0·1 ml of medium containing 2×10^5 ^{51}Cr-labelled PMNL was added above the monolayer filter. After incubation, migration was stopped by washing above the filter twice with 150 μl RPMI-1640 to remove non-adherent PMNL. The undersurface of the filter was then swabbed with a cotton swab soaked in ice-cold phosphate-buffered saline (PBS)/0·2% EDTA and this was combined with the contents of the lower compartment. The cells which migrated into lower compartment were lysed by addition of 0·5% Triton X-100 and the medium in this compartment, plus the swab were analysed for ^{51}Cr and considered to be the migrated PMNL. The results are expressed as percentage of the total ^{51}Cr-labelled PMNL added above the HSF, HDF or HUVE monolayers, which migrated across the HSF, HDF or HUVE/filter unit. During the assay, >98% of the ^{51}Cr remained cell associated on PMNL. All the treatment conditions were performed in triplicate.

Antibody treatment

In some experiments, ^{51}Cr-labelled PMNL were treated for 20 min at room temperature with saturating concentrations of mAb (30–50 μg/ml or 1 : 100 dilution of ascites) before being added for migration. The antibodies were present throughout the migration assay except where otherwise indicated, when cells were washed after mAb treatment. In some cases, HSF or HDF monolayers were pretreated with mAb (40 min at 37°) before PMNL were added to the monolayers.

Statistical analysis

One-way analysis of variance (ANOVA), Student's *t*-test or paired *t*-test were used for analysis of the data. *P* values <0.05 were considered to be significant.

RESULTS

Mac-1 is required for β_2-integrin dependent PMNL migration through synovial fibroblast monolayers

A minor percentage, i.e. 5–10% of PMNL spontaneously migrated through HSF monolayers. This migration markedly increased when a chemotactic factor, such as C5a, was added into the lower compartment beneath the HSF/filter units (Fig. 1). Treatment of PMNL with mAbs to β_2 integrin (CD18) (60.3 or R15.7), which are known to block the adhesion/migration function,[22,24] inhibited the PMNL migration induced by C5a by about 35%. We investigated whether the LFA-1 (CD11a/CD18) and Mac-1 CD11b/CD18) members of the β_2 integrins are involved in PMNL migration through HSF monolayers. As shown, treatment of PMNL with two mAbs specific for LFA-1 (α_L or CD11a) (TS1/22 or G25.2), which block the adhesion function of LFA-1, and PMNL migration across vascular endothelium,[29,30] had no significant effect on PMNL migration through HSF monolayers induced by C5a (pooled results). However, treatment of PMNL with the mAb (2LPM19c) to Mac-1 (α_M or CD11b specific), which blocks Mac-mediated adhesion to at least four of its ligands,[31] significantly reduced PMNL migration, although slightly less inhibition was observed when compared to treatment with mAb to the β_2 chain (60.3). The difference between the inhibition by mAb to Mac-1 and by mAb to β_2 was not statistically significant. As also shown in Fig. 1, treatment with a combination of mAb to LFA-1 (TS1/22) and to Mac-1 (2LPM19c) had no additive effect on inhibition of migration, compared to treatment with mAb to Mac-1 (2LPM19c) alone. In two experiments, PMNL migration induced by IL-8 (50 ng/ml) was also examined. These results showed partial

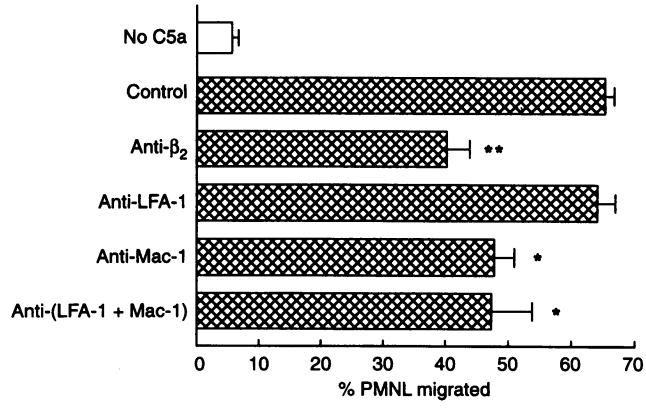

Figure 1. Effect of antibody to β_2 (CD18) integrins on C5a-induced PMNL migration across human synovial fibroblasts (HSF). The ^{51}Cr-labelled PMNL were treated (20 min, 22°) with mAb to the β_2 chain (60.3, 30 μg/ml), or with mAb to LFA-1 (TS1/22 or Becton Dickinson clone G25.2, 30 μg/ml) (pooled results), or to Mac-1 (2LPM19c) or with control mAb (543) to CR1, or by a combination of mAbs to LFA-1 (TS1/22) plus to Mac-1. All of the mAbs were present during the migration. The PMNL migration was induced by C5a (3×10^{-9} M) placed in the compartment beneath the HSF/filter unit. The migration time was 90 min. Results are expressed as a percentage of added PMNL which migrated through the HSF/filter unit. Values are mean ± SEM of three to six experiments performed with triplicates. $^*P < 0.05$; $^{**}P < 0.01$ compared to PMNL treated with control mAb.

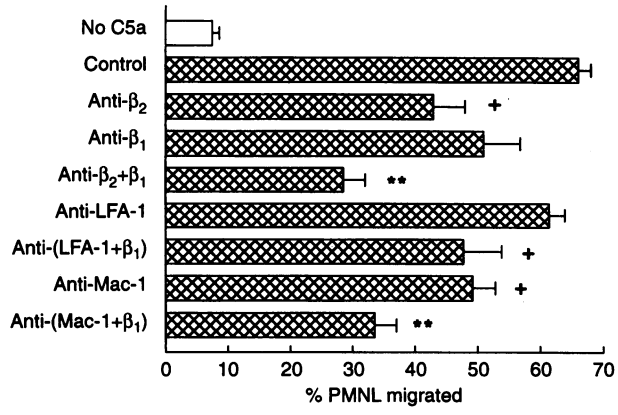

Figure 2. The role of LFA-1, Mac-1, and β_1 integrin in migration of PMNL through HSF monolayers. The ^{51}Cr-labelled PMNL were treated 22°, 20 min) with mAb to LFA-1 (TS1/22), Mac-1 (2LPM19c), β_1 (3S3), CR1 (543) as control or in combination as indicated. The migration was induced by C5a (3×10^{-9} M) added beneath HSF/filter unit. The migration time was 90 min. Values are mean ± SEM of three to seven experiments performed with triplicates. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$, as compared to PMNL treated with control mAb. $P < 0{\cdot}05$ when anti-(Mac-1 + β_1) compared to anti-Mac-1 alone.

and comparable inhibition by mAb to CD18 (60.3) and by mAb to Mac-1 (2LPM19c) (control = 35·9 ± 1% PMNL migrated; anti-β_2 = 18·9 ± 1·8%; anti-Mac-1 treated = 18·5 ± 1·1%).

We reported previously that β_1 integrins also can mediate PMNL migration through HSF monolayers, but this was only detected when the function of β_2 integrins was blocked by mAb.[11] Therefore, we considered that the role of LFA-1 might be obscured by the other integrin-mediated migration mechanisms. To examine this, we determined the effect on migration of blocking LFA-1 or Mac-1 when the β_1 integrin pathway was also blocked. As shown in Fig. 2, mAb (3S3) to the β_1 (CD29) integrin chain only slightly inhibited PMNL migration through HSF monolayers in response to C5a, but this was not statistically significant ($P > 0{\cdot}05$). This slight inhibition was markedly potentiated when PMNL were treated with a combination of mAb to β_2 (60.3) plus mAb (3S3) to β_1 integrins. Treatment of PMNL with mAb to LFA-1 (TS1/22) plus mAb (3S3) to β_1 integrins, inhibited PMNL migration slightly but not significantly more than mAb to β_1 integrins alone. In contrast, mAb to Mac-1 (2LPM19c), in combination with mAb to β_1 integrin, had a significant ($P < 0{\cdot}05$) additive inhibitory effect (47% inhibition) when compared to treatment with mAb to Mac-1 (25% inhibition) or β_1 integrin (20% inhibition) alone. The results in Figs 1 and 2 suggest that Mac-1, but not LFA-1 mediates PMNL migration through HSF monolayers in response to C5a.

The role of ICAM-1 in PMNL migration through synovial fibroblast monolayers

An important ligand for both LFA-1 and Mac-1 is ICAM-1 and this adhesion molecule is expressed on HSF[32–35] and expression on the HSF utilized here was confirmed by enzyme-linked immunosorbent assay (ELISA) on viable HSF (see below) and by immunofluorescence flow cytometry (not shown). Therefore, we investigated whether ICAM-1 may play a role in Mac-1 mediated migration. HSF monolayers were pretreated (40 min, 37°) with mAb R6.5 F(ab)$_2$ to ICAM-1, which blocks the binding of Mac-1 as well as LFA-1 to ICAM-1.[24,31] This treatment had no effect on PMNL migration through HSF monolayers (control mAb = 58·1 ± 5%; mAb R6.5 = 64·2 ± 4·7%, $n = 4$). In contrast, mAb R6.5 inhibited PMNL migration across IL-1 activated HUVE by 54% (not shown) as expected from previous reports.[29] These results suggest that ICAM-1 is not an important ligand in the Mac-1 mediated PMNL migration through HSF monolayers.

The role of LFA-1 and Mac-1 in PMNL migration through dermal fibroblast monolayers

In order to determine whether Mac-1 mediated PMNL migration through synovial fibroblasts was tissue-specific, we compared the role of LFA-1 and Mac-1 in PMNL migration through human dermal fibroblast (HDF) monolayers. As shown in Fig. 3, PMNL migration through HDF monolayers was partly β_2 integrin dependent, because mAb to β_2 inhibited migration by 43%, and partly β_1 (CD29) integrin dependent, because combination of the mAb (3S3) to β_1 integrins with mAb to β_2 inhibited PMNL migration by up to 70%. Further experiments showed that treatment of PMNL with mAb to LFA-1 (TS1/22) alone, or in combination with mAb to β_1 integrin, did not inhibit PMNL migration, as was observed also with HSF (compare with Fig. 2). In contrast, treatment with anti-Mac-1 (2LPM19c) significantly inhibited migration of PMNL through HDF monolayers, and further inhibition was observed when PMNL were treated with a combination of mAb to Mac-1 (2LPM19c) and to β_1 integrin (3S3). Addition of the mAb to LFA-1 (TS1/22) to this combination did not cause

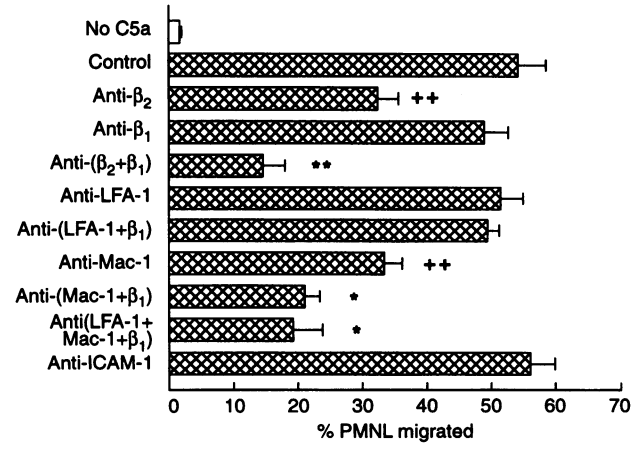

Figure 3. The effect of mAb to LFA-1, Mac-1 or ICAM-1 on PMNL migration across human dermal fibroblast (HDF) monolayers. The ^{51}Cr-labelled PMNL were treated (22°, 20 min) with mAb to β_2 integrin chain (60.3), LFA-1 (TS1/22), Mac-1 (2LPM19c), β_1 integrins (3S3), or the HDF were treated (37°, 40 min) with ICAM-1 (R6.5 F(ab)$_2$; 30 μg/ml) alone or in combination, as in Figs 1 and 2. C5a (3×10^{-9} M) was added beneath the HDF/filter unit to induce migration. Values are mean ± SEM of three to seven experiments performed with triplicates. $++P < 0{\cdot}01$, compared to PMNL treated with control mAb; $*P < 0{\cdot}05$, compared to PMNL treated with mAb to Mac-1 alone; $**P < 0{\cdot}01$, compared to PMNL treated with mAb to β_2 alone.

further inhibition compared to treatment with the other two mAbs (2LPM19c + 3S3). As with the HSF, treatment of HDF with mAb (R6.5 $F(ab)_2$) to ICAM-1 did not inhibit PMNL migration, although ICAM-1 is constitutively expressed on HDF.[33,36] Expression of ICAM-1 on the HDF used here was confirmed by ELISA (see below) and immunofluorescence flow cytometry (not shown). Thus, the results are comparable with those observed with HSF, suggesting that Mac-1, rather than LFA-1 is involved in β_2 (CD18) integrin-mediated migration of PMNL through HDF monolayers as well.

Effect of monoclonal antibody to LFA-1 and Mac-1 on PMNL migration through endothelial monolayers

In order to determine whether Mac-1, rather than LFA-1 mediation of PMNL migration across fibroblast barriers was unique, we also investigated the role of LFA-1 and Mac-1 in PMNL migration through HUVE monolayers induced by C5a. As shown in Fig. 4, in response to C5a, PMNL migrated through HUVE monolayers. This migration was significantly and comparably inhibited by treatment of PMNL with either mAb to LFA-1 (TS1/22) or to Mac-1 (2LPM19c) alone. Treatment with a combination of mAbs to LFA-1 and to Mac-1 (2LPM19c) almost completely inhibited (95%) PMNL transendothelial migration and this was as effective as treatment with mAb to the β_2 chain (60.3) alone. The results suggest that LFA-1 on PMNL plays an important role in PMNL migration through HUVE monolayers, in marked contrast to PMNL migration across the fibroblast barriers.

Regulation by cytokines of an LFA-1/ICAM-1 pathway in PMNL migration through synovial and dermal fibroblast monolayers

ICAM-1, a ligand of LFA-1 is constitutively expressed at low levels on HSF and HDF, and is upregulated by proinflammatory cytokines.[34,36,37] A combination of TNF-α and IFN-γ is known to induce a synergistic increase in ICAM-1 expression on these cells[34,35] and this was also the case with the HSF and HDF employed here. Specifically, as measured by mAb R6.5 reactivity in ELISA, absorbance with unstimulated HDF was $0{\cdot}25 \pm 0{\cdot}02$; with 4 hr TNF-$\alpha$ (100 U/ml) + IFN-γ (200 U/ml) stimulation this increased to $1{\cdot}45 \pm 0{\cdot}15$ ($n = 3$), while non-binding isotype matched control mAb was $0{\cdot}11 \pm 0{\cdot}02$. Similarly, absorbances with HSF were $0{\cdot}28 \pm 0{\cdot}03$ on unstimulated and $1{\cdot}91 \pm 0{\cdot}23$ ($n = 3$) following TNF-α + IFN-γ stimulation and with control mAb absorbance was $0{\cdot}12 \pm 0{\cdot}01$. Analysis by immunofluorescence flow cytometry revealed a unimodal increase in expression of ICAM-1 on the entire HDF and HSF population after TNF-α + IFN-γ stimulation (not shown). We investigated whether such ICAM-1 upregulation may alter the mechanism of PMNL migration through HSF and HDF monolayers. As shown in Fig. 5, treatment of HSF or HDF monolayers with the cytokines TNF-α (100 U/ml) plus IFN-γ (200 U/ml) for 4 h, did not alter PMNL migration in response to C5a through HSF monolayers, but there was a small increase in PMNL migration through the HDF barrier ($P < 0{\cdot}05$). Cytokine treatment of the HDF resulted in slight inhibition by mAb to LFA-1 of PMNL migration. However, a role for LFA-1 was not clear until mAb to LFA-1 (TS1/22) was combined with mAb to Mac-1 (2LPM19c), which resulted in significant additive inhibitory effects on migration, comparable to inhibition by treatment with mAb (60.3) to β_2 integrin alone. Moreover, treatment of cytokine-activated fibroblasts with mAb (R6.5) to ICAM-1 also partially inhibited (by 25%) PMNL migration (Fig. 5).

Stimulation of HSF monolayers with TNF-α plus IFN-γ did not cause an increase in PMNL migration to C5a. The mechanisms of PMNL migration through cytokine-treated

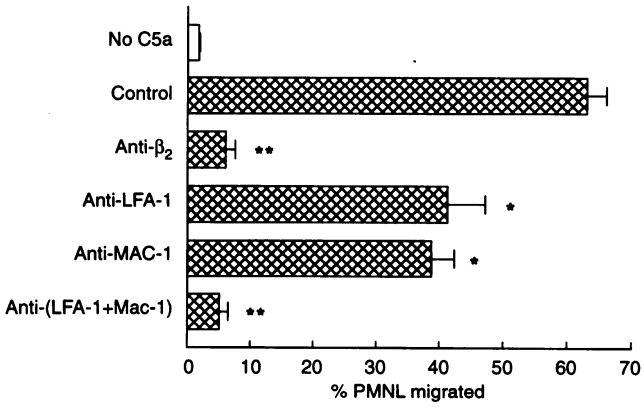

Figure 4. Effect of mAb to LFA-1 and Mac-1 on PMNL migration across human umbilical vein endothelium. The ^{51}Cr-labelled PMNL were treated (22°, 20 min) with mAb to β_2 integrin (60.3), LFA-1 (TS1/22), Mac-1 (2LPM19c) or control mAb to CR1 (543) alone, or in combination as indicated. The migration was induced by adding C5a (2×10^{-9} M) beneath the HUVE/filter unit. The migration time was 90 min. Values are mean ± SEM of five experiments performed with triplicates. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$, as compared to PMNL treated with control mAb.

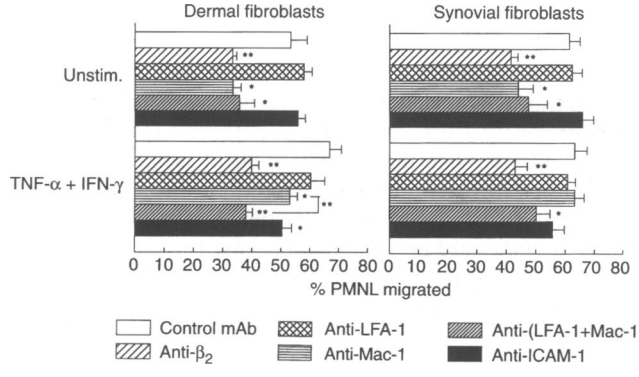

Figure 5. Regulation by cytokines of PMNL migration through HSF and HDF monolayers. HSF or HDF monolayers were treated (37°, 5% CO_2) with or without a combination of TNF-α (100 U/ml) and IFN-γ (200 U/ml) for 4 hr. The monolayers were washed to remove cytokines immediately before the migration assay. The ^{51}Cr-labelled PMNL were treated (22°, 20 min) with mAb to β_2 integrin (60.3), LFA-1 (TS1/22), Mac-1 (2LPM19c), CR1 (543) as control or with a combination of mAb to LFA-1 plus to Mac-1. In some cases, HSF or HDF were treated with mAb to ICAM-1 (R6.5 $F(ab)_2$; 20 μg/ml, 30 min) before addition of PMNL. All mAbs were present throughout the migration period. The migration was induced by C5a (3×10^{-9} M) added benath the HSF or HDF/filter unit. Values are mean ± SEM of three experiments performed with triplicates. *$P < 0{\cdot}05$; **0·01, by paired t-test compared to PMNL treated with control mAb, except where indicated otherwise.

HSF monolayers are less clear than with HDF. Across cytokine-activated HSF, PMNL migration was not inhibited by mAbs to LFA-1 or to Mac-1 alone. However, in contrast to PMNL migration through unstimulated HSF, migration was significantly inhibited by the combination of mAbs to LFA-1 plus to Mac-1 following cytokine stimulation of HSF. This migration was also slightly inhibited by mAb (R6.5) to ICAM-1, although this was not statistically significant. These results suggest that upon activation of HDF or HSF by the cytokines TNF-α and IFN-γ, LFA-1 and ICAM-1 become involved in mediating PMNL migration.

DISCUSSION

Recently, we observed that PMNL migration through a barrier consisting of either synovial or dermal fibroblasts is dependent on both β_2 (CD18) and β_1 (CD29) integrins.[11,12] Here we extended these observations to determine whether LFA-1 and Mac-1, the two β_2 integrins which are recognized to mediate adhesion and migration of PMNL on endothelium,[4,5,13,17,29,38] are also involved in PMNL migration across a barrier of connective tissue fibroblasts. Approximately one-third of the PMNL migration induced by C5a was dependent on the β_2 (CD18) integrins and a similar degree on the β_1 integrins (Fig. 1). Virtually all of the β_2 (CD18) integrin-mediated migration appeared to depend on Mac-1, as mAb 2LPM19c to Mac-1 inhibited PMNL migration across synovial and dermal fibroblasts (Figs 1 and 3), essentially as effectively as mAb to the common β_2 (CD18) chain, which blocks the function of LFA-1, Mac-1, and p150,95.[22,30] The same was the case when PMNL migration across HSF was induced by IL-8 (see text). It appears that LFA-1 does not play a role in this migration, because two different adhesion blocking mAbs to LFA-1 had no effect on PMNL migration through fibroblast barriers in response to C5a either alone or in combination with mAb to Mac-1 (Figs 1 and 2). This was despite the fact that the same mAb (TS1/22) preparation against LFA-1 inhibited PMNL migration across HUVE in this system and essentially all of the PMNL transendothelial migration could be accounted for by a combination of LFA-1 and Mac-1 pathways (Fig. 4). These results indicate that there are significant differences in the mechanisms involved in PMNL migration through fibroblast barriers and an endothelial cell barrier. It is unlikely that the observed effects are non-specific, because two other mAbs to Mac-1 reactive with the I-domain region of Mac-1 (CBRM 1/2 and 1/24; kind gifts from Dr T. A. Springer)[31] had comparable effect in inhibiting migration as did mAb 2LPM19c and two mAbs to Mac-1 (OKM-1 and LM2/1), which are weak inhibitors of the multiple ligand recognition by Mac-1,[31] did not inhibit PMNL transfibroblast migration (not shown).

As shown in Fig. 2, in order to observe β_1 integrin-mediated PMNL trans-synovial fibroblast migration, the β_2 integrin mediated pathway had to be blocked. In this context, blocking specifically the function of Mac-1 (with anti-α_M) had a similar effect to anti-β_2 chain treatment, i.e. by additively enhancing the inhibition of PMNL migration through HSF monolayers in combination with mAb to β_1 integrin (Fig. 2). In contrast, addition of mAb to LFA-1 to the mAb to β_1 integrin did not significantly inhibit PMNL migration when compared to mAb to β_1 integrin alone (Fig. 2). These results not only confirm that Mac-1 has a major role in β_2 integrin-mediated PMNL migration through HSF monolayers, but also suggest that Mac-1 is the predominant alternative mechanism to the β_1 integrins, of which both VLA-5 and VLA-6 contribute to PMNL transfibroblast migration.[11]

It is established that ICAM-1 functions as a ligand for both LFA-1 and Mac-1,[33,39] although LFA-1 binds to domain 1 and Mac-1 to domain 3.[32] Therefore, we evaluated the role of ICAM-1 in the Mac-1 mediated PMNL transfibroblast migration with mAb R6.5, which is known to block the interaction of not only LFA-1 with ICAM-1, but also of Mac-1 with ICAM-1.[31] This mAb, used as a $F(ab)_2$ fragment, did not inhibit the PMNL migration (see results and Fig. 3). The results suggest that ICAM-1 is not required for the Mac-1 mediated migration of PMNL through HSF monolayers in response to C5a. Mac-1 has numerous ligands, which may be relevant in this system, including fibrinogen, Arg–Gly–Asp sequence containing proteins, certain types of collagen, denatured proteins, glycosaminoglycan structures and an unidentified counter-receptor,[4,5,7,8,10,15,17,18,20,21,31,38,40,41] several of which may be available on the surface of fibroblasts, or the surrounding ECM. Which of these multiple ligands for Mac-1 may mediate the PMNL migration through connective tissue fibroblasts will require further study. To date in preliminary experiments with soluble forms of collagens, heparins, fibrinogen peptides and mAbs to alternative domains of ICAM-1, we have not observed any inhibition of Mac-1 mediated PMNL migration. Like Mac-1, CD11c/CD18 (p150,95), also can bind to denatured proteins,[7] and perhaps with lower affinity, to other ligands of Mac-1,[4,5,17] although the role of p150/95 in mediating PMNL migration appears to be minor.[4,5,29] In the system utilized here, mAb to Mac-1 blocked PMNL migration across HSF virtually as effectively as mAb 60.3 to the common β_2 integrin (CD18) chain (Figs 1 and 2), which blocks the function of LFA-1, Mac-1 and p150/95,[22,23] and mAb 60.3 blocked migration across HDF as well as anti-Mac-1 (Fig. 3). In fact, a mAb to p150/95 (CBRM-p150/2E1; kind gift from Dr T. A. Springer) did not inhibit PMNL migration through HSF or HDF and did not potentiate the inhibition by anti-Mac-1 treatment ($n=3$, not shown). Thus in this system, p150/95 appears to play a minor role, as is the case also for PMNL-transendothelial migration[4,5,29] (see also Fig. 4).

Expression of ICAM-1 on fibroblasts is upregulated by cytokines such as TNF-α and IFN-γ and these two cytokines appear to have synergistic effects in this respect.[34,35,37] These cytokines may be generated in inflamed connective tissue. The results in Fig. 5 show that stimulation of HDF or HSF with a combination of TNF-α and IFN-γ resulted in PMNL migration in response to C5a by not only a Mac-1 mechanism, but also an LFA-1 mediated mechanism. This was supported by the observation that under these conditions, mAb to LFA-1 significantly inhibited PMNL migration, when combined with mAb to Mac-1, across both HDF and HSF, an additive effect not seen when unstimulated HDF and HSF were used (Fig. 5). At least in part this difference in the role of LFA-1 appears to be mediated via an ICAM-1 mechanism, as mAb to ICAM-1 (R6.5, $F(ab)_2$) inhibited PMNL migration across the fibroblasts stimulated with TNF-α and IFN-γ (Fig. 5). Thus, inflammatory cytokines may significantly influence the mechanisms involved in chemotactic factor induced PMNL migration through connective tissues.

In summary, the findings here show that PMNL migration

through HSF and HDF monolayers in response to chemotactic factor is dependent on both β_2 and β_1 integrins and that Mac-1, but not LFA-1, is the predominant β_2 integrin involved in this process. This is distinct from PMNL transendothelial migration, in which both LFA-1 and Mac-1 are involved and where each can serve as an alternative mechanism. Some inflammatory cytokines, however, may activate fibroblasts so as to also support an LFA-1/ICAM-1 pathway of PMNL migration. Thus, PMNL migration through connective tissue likely involves a unique balance of integrin-mediated migration mechanisms, which are in part under cytokine control. Further studies are indicated to define the biologically relevant ligands for Mac-1 mediated PMNL migration through extravascular connective tissues.

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