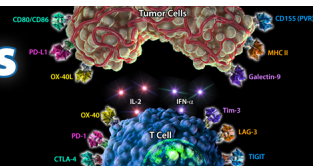




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Human Thy-1 (CD90) on Activated Endothelial Cells Is a Counterreceptor for the Leukocyte Integrin Mac-1 (CD11b/CD18)

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Human Thy-1 (CD90) on Activated Endothelial Cells Is a Counterreceptor for the Leukocyte Integrin Mac-1 (CD11b/CD18)¹

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Leukocyte recruitment in response to inflammatory signals is in part governed by interactions between endothelial cell receptors belonging to the Ig superfamily and leukocyte integrins. In our previous work, the human Ig superfamily glycoprotein Thy-1 (CD90) was identified as an activation-associated cell adhesion molecule on human dermal microvascular endothelial cells. Furthermore, the interaction of Thy-1 with a corresponding ligand on monocytes and polymorphonuclear cells was shown to be involved in the adhesion of these leukocytes to activated Thy-1-expressing endothelial cells. In this study, we have identified the specific interaction between human Thy-1 and the leukocyte integrin Mac-1 (CD11b/CD18; $\alpha_M\beta_2$) both in cellular systems and in purified form. Monocytes and polymorphonuclear cells were shown to adhere to transfectants expressing human Thy-1 as well as to primary Thy-1-expressing human dermal microvascular endothelial cells. Furthermore, leukocyte adhesion to activated endothelium as well as the subsequent transendothelial migration was mediated by the interaction between Thy-1 and Mac-1. This additional pathway in leukocyte-endothelium interaction may play an important role in the regulation of leukocyte recruitment to sites of inflammation. *The Journal of Immunology*, 2004, 172: 3850–3859.

Leukocyte extravasation into perivascular tissue plays a key role in inflammatory diseases. This recruitment requires leukocyte interaction with vascular endothelium and consists of multiple consecutive processes, including the capture of circulating leukocytes, subsequent leukocyte rolling, arrest, firm adhesion, and ensuing diapedesis (1, 2). This multistep paradigm is realized by sequential activation-dependent interactions between endothelial cell adhesion molecules and their specific ligands on leukocytes. The first step of transient adhesion and rolling is known to be mediated by an interaction of leukocyte or endothelial cell selectins and their oligosaccharide-bearing ligands (3, 4). Arrest and firm adhesion of leukocytes to endothelium is dependent on the activation of β_2 integrins like Mac-1 (CD11b/CD18; $\alpha_M\beta_2$) or LFA-1 (CD11a/CD18; $\alpha_L\beta_2$) on the leukocyte cell surface, followed by interaction with endothelial cell proteins belonging to the Ig superfamily (IgSF)³ such as ICAM-1 (5–9). The specific activation factors regulating these consecutive steps are not completely understood, but involve inside-out signaling initiated by inflammatory mediators as well as outside-in signaling by ligand binding of leukocyte adhesion molecules (10–12). Furthermore, the extravasation process cannot be viewed as a sequence of

separate steps mediated by several individual families of adhesion molecules, but rather as a series of overlapping synergistic interactions among adhesion molecules resulting in an adhesion cascade (1).

Understanding the process of leukocyte extravasation is mandatory for biomedical research aimed at alleviating inflammatory diseases, including atherosclerosis, arthritis, asthma, or psoriasis (12). However, despite numerous earlier results, recent studies have shown evidence for additional leukocyte-endothelium adhesion pathways left to define (13–15).

The involvement of additional adhesion molecules would provide an explanation for the persistence of inflammatory responses and leukocyte recruitment upon blocking ICAM-1 with specific Abs as well as seen in ICAM-1-deficient mice (15–17). Recently, we showed that the human Thy-1 is an activation-associated cell adhesion molecule on human dermal microvascular endothelial cells (HDMEC). Thy-1 is a highly glycosylated GPI-anchored cell surface protein with a molecular mass of ~35 kDa, belonging to the IgSF (18–21). The expression of Thy-1 in humans is restricted to neuronal cells, a subset of CD34⁺ blood stem cells, fibroblasts, and activated endothelial cells (18, 22). In this respect, the human Thy-1 (CD90) could be one of these molecules that have so far not been appreciated to play a role in leukocyte adhesion and transendothelial migration.

In this study, we demonstrate that human Thy-1 specifically interacts with Mac-1 (CD11b/CD18; $\alpha_M\beta_2$) on the human leukocyte cell surface. Furthermore, this interaction is involved in leukocyte adhesion to activated Thy-1-expressing endothelial cells as well as in the subsequent transendothelial migration. These results suggest an additional pathway of β_2 integrin-mediated leukocyte adhesion processes, possibly playing an important role in leukocyte invasion into inflamed tissues.

Materials and Methods

Monoclonal Abs

The following murine mAbs directed against human Ags were used as purified IgG and purchased from Beckman Coulter (Krefeld, Germany): 25.3 (anti-CD11a), Bear1 (anti-CD11b), Mo1 (anti-CD11b), FITC-labeled

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³ Abbreviations used in this paper: IgSF, Ig superfamily; HDMEC, human dermal microvascular endothelial cell; DTAF, dichlorotriazinyl-amino-fluorescein; PMNC, polymorphonuclear cell; CHO, Chinese hamster ovary.

Bear1 (anti-CD11b), BU15 (anti-CD11c), 7E4 (anti-CD18), IgG1 isotype control, and dichlorotriazinyl-amino-fluorescein (DTAF)-labeled goat anti-mouse.

AS02 (anti-Thy1) was used as hybridoma cell culture supernatants; characterization has been described previously (23, 24). LPM19c (anti-Mac1) was kindly provided by Dr. A. May (Deutsches Herzzentrum, Munich, Germany); L15 (anti-LFA1) was kindly provided by Dr. Y. van Kooyk (Amsterdam, The Netherlands). PE- and CY3-labeled streptavidin was purchased from Beckman Coulter.

Cell culture

Polymorphonuclear cells (PMNC) were prepared as follows. Whole blood was incubated with Dextran M70 (2% final concentration; Sigma-Aldrich, Taufkirchen, Germany) for 60 min at room temperature. The plasma was removed, and PMNC were separated by Ficoll density-gradient centrifugation (Lymphocyte Separation Medium; $\rho = 1.077$ g/ml; ICN, Aurora, OH) at $800 \times g$ for 20 min. The pellet containing PMNC and erythrocytes was incubated with distilled water for 1 min to lyse the remaining erythrocytes. Finally, PMNC were washed twice with PBS.

Mononuclear cells were prepared by Ficoll density-gradient centrifugation of whole blood diluted with PBS 1/1 at $800 \times g$ for 20 min at room temperature.

Subsequently, the interphase containing monocytes and lymphocytes was pelleted and washed three times with PBS. Monocytic and lymphocytic cell populations could be diversified during flow cytometry analysis.

Monocytes, purified from buffy coat by countercurrent elutriation (85–90% purity) were kindly provided by Prof. S. Hauschildt (Department of Immunology, University of Leipzig).

THP1 cells were cultured in RPMI 1640 and Glutamax II (Biochrom, Berlin, Germany), supplemented with 10% FCS (Biochrom) and 1% penicillin/streptomycin (Biochrom) at 37°C and 5% CO₂.

Dermal fibroblasts were obtained after outgrowth from human skin biopsies, cultured in DMEM (Biochrom) with 10% FCS, 1% ascorbic acid, and 1% penicillin/streptomycin at 37°C and 5% CO₂, and passaged after reaching confluence using 0.05% trypsin and 0.02% EDTA (Biochrom).

Chinese hamster ovary (CHO) cells were cultured in DMEM/RPMI 1640 (1:1) with 10% FCS, 1% ascorbic acid, and 1% penicillin/streptomycin at 37°C and 5% CO₂, and passaged after reaching confluence using 0.05% trypsin and 0.02% EDTA.

Transiently Thy-1 transfected CHO cells were established by electroporation with Thy-1 cDNA cloned into the expression vector pCMV5 (Invitrogen, Carlsbad, CA) and cultured in DMEM/RPMI 1640 (1:1) with 10% FCS, 1% ascorbic acid, and 1% penicillin/streptomycin. Expression of Thy-1 was controlled by flow-cytometric assay using mAb AS02.

HDMEC were prepared from human foreskin as previously described (24), and cultured using the EGM-MV BulletKit (Promocell, Heidelberg, Germany) according to the manufacturer's protocol.

Stably Mac-1 transfected CHO cells, kindly provided by Dr. R. Ingalls (Evans Biomedical Research Center, Boston University School of Medicine, Boston, MA) were cultured in Ham's F12 with 10% FCS, 1% ascorbic acid, 1% penicillin/streptomycin, and 0.5 mg/ml G418 at 37°C and 5% CO₂, and passaged after reaching confluence using 0.05% trypsin and 0.02% EDTA.

Protein purification of human Thy-1

Thy-1 was purified from cell extracts of human dermal fibroblasts by immunoaffinity chromatography using mAb AS02 as previously described (24).

Briefly, the Thy-1-specific mAb AS02 was immobilized on cyanogen bromide-activated Sepharose (Pharmacia, Freiburg, Germany) according to the manufacturer's protocol. Human dermal fibroblasts were solubilized in 20 mM phosphate buffer with 0.5% Triton X-100, 1 mM PMSF, 25 mM EDTA, and 10 μ g/ml leupeptin and aprotinin for 30 min and were cleared by centrifugation at $10,000 \times g$ for 10 min at 4°C. The resulting supernatant was incubated with the cyanogen bromide-activated Sepharose coupled with mAb AS02 in batch overnight at 4°C. After extensive washing with PBS, the bound proteins were eluted with 0.2 M triethylamine (pH 11.5) and neutralized with 10 mM phosphate buffer (pH 6.0). The eluted fractions containing purified Thy-1 were pooled and concentrated by ultrafiltration (Amicon, Witten, Germany). Subsequently, purified Thy-1 was labeled with D-biotinoyl ϵ -amidocaproic acid hydroxysuccinimide ester (Roche Biochemicals, Mannheim, Germany) according to manufacturer's protocol.

Immunohistochemistry

Thy-1 Ag was detected by the incubation of cryostat sections with the mAb AS02 and avidin-biotin complex technique according to manufacturer's protocol (supersensitive multilink alkaline phosphatase ready-to-use detec-

tion system; Biogenix, San Ramon, CA). Washing was performed with 0.9% NaCl/0.05 M Tris-HCl (pH 7.4). Bound proteins were detected by color reaction applying New Fuchsin substrate system (DAKO, Hamburg, Germany). To analyze the expression of Thy-1 ligand, cryostat sections from skin of patients with bullous pemphigoid were incubated for 1 h with the biotinylated purified Thy-1 or a labeled albumin as negative control followed by incubation with streptavidin-alkaline phosphatase (Sigma-Aldrich).

Flow cytometry analysis

Cells were harvested and washed twice in PBS, and $\sim 2 \times 10^5$ cells were pelleted in 96-well plates. Subsequently, cells were incubated with 1 μ g of the different mAbs (mAb against CD11a, CD11b, CD11c, CD18, or IgG1 isotype control) or alternatively with biotinylated purified Thy-1 or biotinylated BSA as negative control for 60 min at 4°C. After washing twice with PBS/Gelafusal (Serumwerke, Bernburg, Germany)/sodium azide, cells were incubated with DTAF-labeled goat anti-mouse secondary Ab (diluted 1/200 in PBS) or PE-labeled streptavidin (1/100 in PBS), respectively. Finally, cells were washed twice with PBS, and Ab or Thy-1 binding to cells was analyzed by flow cytometry (Epics XL; Beckman Coulter). For blocking experiments, THP1 cells, monocytes, or PMNC were preincubated with purified unlabeled Thy-1 for 30 min at 4°C, followed by incubation with biotinylated mAbs against CD11a, CD11c, or CD18, and subsequently PE-labeled streptavidin as described, or directly DTAF-labeled mAb against CD11b. Binding ability after different preincubation of cells was analyzed by flow cytometry.

In vitro ligand-receptor interactions

Binding of biotinylated fibrinogen to immobilized Mac-1 (a kind gift from Dr. S. Bodary (Genetech, Richmond, CA)) or BSA (each 5 μ g/ml) was performed as previously described (25, 26). Alternatively, binding of purified Thy-1 (0–1000 nM) to immobilized Mac-1 or the immobilized I domain of Mac-1 (kindly provided by Dr. D. Tuckwell (Manchester, U.K.)) or BSA (each 5 μ g/ml) was performed in TBS containing 0.1% BSA, 0.05% Tween 20, and 1 mM Mn²⁺. For inhibition studies, the binding of Thy-1 (400 nM) to immobilized Mac-1 was performed in the absence or presence of mAb to Mac-1, of mAb to LFA-1, of fibrinogen, or of the ligand-binding I domain of Mac-1, as indicated in the figures. Following incubation for 2 h at 22°C, the biotinylated mAb AS02 against Thy-1 (23, 24) was added, followed by addition of peroxidase-conjugated streptavidin (DAKO) and the substrate ABTS, and binding was quantitated at 405 nm. Nonspecific binding to BSA-coated wells was used as blank and was subtracted to calculate specific binding.

Cell adhesion assays

Cell adhesion of monocytes and PMNC to immobilized purified human Thy-1 was tested. Maxisorp plates (Nunc, Wiesbaden, Germany) were coated with mAb AS02 (20 μ g/well) directed against human Thy-1 overnight at 4°C and then washed three times with PBS. Subsequently, purified Thy-1 (100 ng/well) and accordingly BSA (10 μ g/well) as control were added to the wells coated with the Thy-1-specific mAb AS02 and incubated overnight at 4°C. Monocytes and PMNC were biotinylated with D-biotinoyl ϵ -aminocaproic acid hydroxysuccinimide ester (Roche Biochemicals) according to manufacturer's protocol, washed three times with PBS, and incubated with mouse IgG1 or mAb against CD11b (5 μ g/500 μ l) for 30 min at 4°C. Subsequently, 1×10^5 of the respective cells were added to each well and incubated for 60 min at 37°C.

Unbound cells were removed by several washings, and the number of the respective adherent cells was counted in 10 microscopic fields.

To perform cell adhesion assays with Thy-1-expressing cells, CHO cells were transiently transfected with Thy-1 cDNA cloned into pCMV5 expression vector (CHO.Thy-1) or vector alone (CHO.vec). Expression of Thy-1 on cell surface was tested by flow cytometry using mAb AS02 (24); Thy-1 transfectants were used, when surface expression of Thy-1 was detectable on >50% of the transfected cells. Vector-transfected CHO cells and Thy-1 transfected CHO cells were seeded on chamber slides (Nunc) and cultured 24 h to obtain a confluent cell monolayer. Monocytes or PMNC were biotinylated, and 5×10^5 of the respective cells were added to each well of chamber slides. After incubation for 60 min at 37°C, unbound cells were removed by several washes with PBS. Adherent cells were fixed with ice-cold methanol for 10 min at –20°C, air-dried, and blocked by incubation with PBS/1% BSA. Subsequently, adherent biotinylated cells were visualized by incubation with CY3-conjugated streptavidin (Beckman Coulter; 1:500 in PBS/1% BSA) for 60 min at room temperature, and finally washed twice with PBS. The number of cells that adhered to the confluent cell monolayer was counted in 10 microscopic fields.

In blocking experiments, biotinylated monocytes or PMNC were preincubated with mAb against CD11a (27), CD11b, CD11c, or CD18 (28) (10 $\mu\text{g}/\text{ml}$), with IgG1 as control or without any mAb for 30 min at 4°C, and subsequently added to the confluent monolayer of CHO.Thy-1. The adhesion assay was performed as described above, and the number of cells adhering to the confluent transfected CHO cells was counted in 10 microscopic fields.

To perform cell adhesion assays using primary Thy-1-expressing endothelial cells, HDMEC were cultured and stimulated with PMA (Life Technologies, Grand Island, NY) as previously described (29). Treatment of HDMEC with PMA or inflammatory mediators such as TNF- α results in the induction of Thy-1 in a subpopulation of HDMEC.

In cell adhesion assays, stimulation by PMA was used because of the induction of higher amounts of Thy-1 cell surface expression. Thy-1-negative HDMEC were separated via an immunomagnetic separation using the mAb AS02 as described (29). The negative cells were analyzed for the contamination with Thy-1-positive cells by flow cytometry. Only cell preparations with <10% Thy-1-positive cells were used, whereas PMA-stimulated HDMEC contained 40–60% Thy-1-positive cells detected by binding of mAb AS02. PMA-stimulated HDMEC containing both Thy-1-positive and Thy-1-negative HDMEC as well as stimulated HDMEC depleted of Thy-1-positive cells were seeded on chamber slides and cultured 24 h at 37°C to obtain a confluent cell monolayer. Subsequently, cell adhesion of monocytes and PMNC was performed as described above.

Thus, the adhesion of leukocytes to PMA-stimulated HDMEC containing Thy-1-expressing cells was compared with the adhesion to Thy-1-negative HDMEC. Positively selected endothelial cells were not used, because the fragmentary secession of the beads used in positive separation affected the cell growing and adhesion. Thus, a confluent monolayer of cells is not reached until a couple of days because of the small number of available cells. Admittedly, the expression of Thy-1 on the cell surface of activated endothelial cells is down-regulated toward 5 days after stimulation. For blocking experiments, monocytes and PMNC were preincubated in the absence or presence of an IgG1 as negative control or mAb to CD11b, followed by the cell adhesion assay as described above.

Transendothelial migration assay

HDMEC were stimulated with PMA, and subsequently, the Thy-1-negative activated HDMEC were separated from the whole cell population as described above.

Both activated HDMEC containing ~40–60% Thy-1-positive cells and activated Thy-1-negative cells were seeded into Transwell culture inserts (pore size, 8 μm ; Falcon; BD Biosciences, Heidelberg, Germany) ($5\text{--}8 \times 10^4$ cells/insert) in 24-well culture plates and cultured overnight to reach a

confluent cell monolayer. PMNC and monocytes were prepared from the whole blood by density-gradient centrifugation as described and incubated with mAb against CD11b or an IgG1 isotype control for 30 min at 4°C. After washing the HDMEC monolayer, a cell suspension of 8×10^4 monocytes or PMNC in 200 μl of RPMI 1640/1% FCS was given to the upper compartment of the Transwell culture inserts, whereas 600 μl of RPMI 1640/1% FCS containing 100 nM fMLP (Sigma-Aldrich) was given to the lower compartment. The transmigration was performed for 3 h at 37°C and 5% CO₂. Subsequently, the Transwell culture inserts were removed; cells migrated into the lower compartment were resuspended; and cell number was analyzed using flow cytometry.

Statistical analysis

Statistical analysis was performed using the Wilcoxon *t* test; values of *p* < 0.01 were regarded as significant.

Results

A specific ligand of human Thy-1 is expressed on PMNC and monocytes

The human Thy-1 was detected only on dermal fibroblasts in healthy skin using an anti-Thy-1 Ab (mAb AS02) in immunohistochemical staining of cryostat sections (Fig. 1A). In contrast, in tissue sections of inflammatory diseases such as bullous pemphigoid, the Thy-1 Ag was also detected on endothelial cells (Fig. 1B). The expression of the corresponding ligand of human Thy-1 was analyzed using the purified, biotinylated Thy-1 protein in immunohistochemical staining. In tissue sections of bullous pemphigoid, a ligand of human Thy-1 was detected on leukocytes present within microvessels (Fig. 1D) as well as in the blister fluid (E), whereas no binding to fibroblasts, keratinocytes, or endothelial cells was detectable. To underline these results, binding of purified Thy-1 was analyzed in flow cytometry. THP1 cells, monocytes, and PMNC were able to bind purified, biotinylated Thy-1, whereas only minor binding to lymphocytes was detectable (data not shown) (30). These results indicate the expression of a ligand of Thy-1 on human monocytes and PMNC.

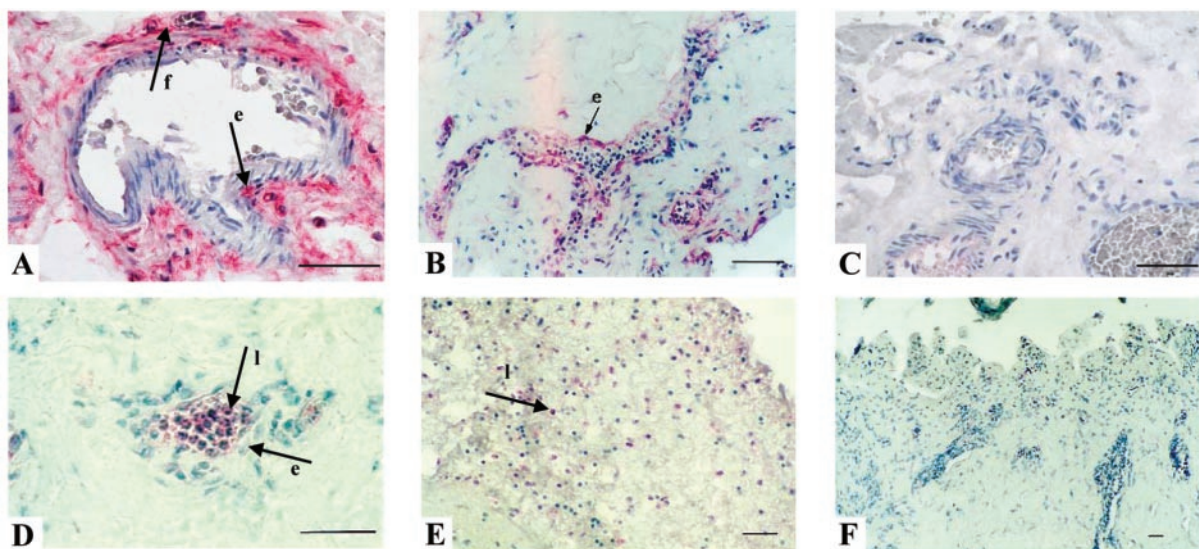


FIGURE 1. Expression of human Thy-1 and Thy-1 ligand in situ. Cryostat sections of healthy skin as well as of bullous pemphigoid were used for immunohistological detection of human Thy-1 and of the corresponding Thy-1 ligand (red staining). A, In healthy skin, Thy-1 detected by mAb AS02 was expressed only on fibroblasts (f), whereas endothelial cells (e) were not stained. B, In bullous pemphigoid, endothelial cells (e) as well as fibroblasts (f) showed an expression of Thy-1. D and E, The corresponding Thy-1 ligand was detected on leukocytes (l) present within microvessels (D) or the blister fluid (E) by binding of purified and labeled Thy-1. C and F, Both IgG isotype Ab (C) used as control for the mAb AS02 and a biotinylated albumin (F) used as negative control for Thy-1 binding showed no staining.

Human Thy-1 is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18)

To further identify the Thy-1 ligand, the binding pattern of purified Thy-1 was compared with the binding abilities of Abs against different integrin chains. We could demonstrate similarities in the expression of Thy-1 ligand and the integrin chains CD11b and CD11c, because the corresponding mAbs interacted with both monocytes and PMNC, but only with a subpopulation of lymphocytes (data not shown). Subsequently, blocking experiments were performed to specify possible interactions of Thy-1 with these integrins. Thus, we could demonstrate that binding of a commercially obtained mAb against CD11b to the cell surface of THP1 cells was clearly inhibited by previous binding of purified Thy-1 (Fig. 2).

In contrast, the binding of mAb against CD11a, CD11c, and CD18 on THP1 cells was not influenced significantly by Thy-1 (Fig. 2). Blocking experiments conducted in the same way with PMNC and monocytes also revealed an inhibition of the binding of a mAb against CD11b by preincubation with purified Thy-1 (data not shown). In conclusion, these results suggest interactions between Thy-1 and Mac-1 (CD11b/CD18; $\alpha_M\beta_2$) on THP1, monocytes, and PMNC.

To corroborate these findings, binding of purified, human Thy-1 to Mac-1-transfected CHO cells was observed, whereas no binding to vector-transfected CHO cells used as control was detectable (Fig. 3). The expression of CD11a, CD11b, CD11c, and CD18 on the cell surface of the transfected CHO cells was controlled by binding of the respective mAbs before.

Because the previous observations indicated an interaction between Thy-1 and Mac-1, the direct interaction between the purified proteins Thy-1 and Mac-1 was examined in a cell-free system. Thus, a saturable dose-dependent binding of purified human Thy-1 to immobilized Mac-1 or to the immobilized isolated I domain of Mac-1 as well could be demonstrated (Fig. 4A). The binding of Thy-1 to immobilized Mac-1 was inhibited in the presence of the function-blocking mAb LPM19c against Mac-1, whereas there was no inhibition caused by the mAb to LFA-1 (Fig. 4B). Further-

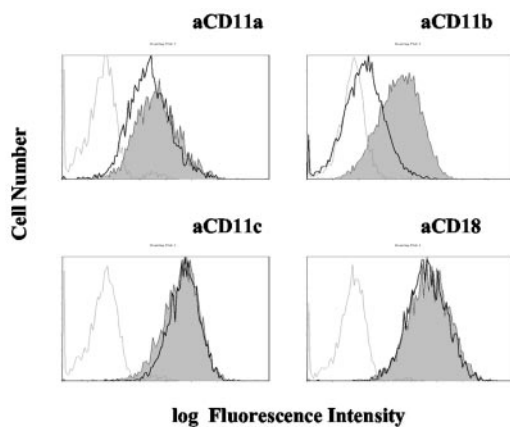


FIGURE 2. Binding of a mAb against CD11b is inhibited by purified human Thy-1. The binding of mAbs against CD11a, CD11b, CD11c, and CD18 to the cell surface of THP1 cells was analyzed without or following preincubation with purified Thy-1 by flow cytometry. The gray filled curves represent the binding of the respective mAb without any preincubation, whereas the binding of the respective mAb after previous binding of purified human Thy-1 is expressed as a black line. Each thin line represents the binding of an IgG1 control. The figure represents a typical experiment, and similar results were obtained in at least four separate experiments.

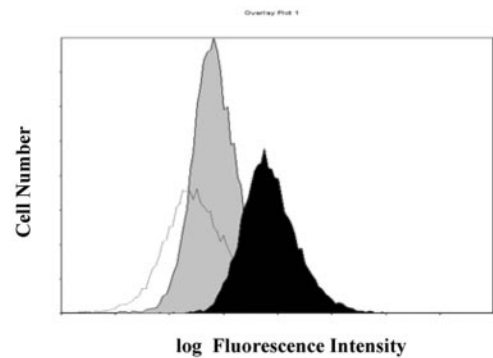


FIGURE 3. Thy-1 binds to the cell surface of Mac-1 (CD11b/CD18) transfectants. Binding of purified, biotinylated human Thy-1 to the cell surface of Mac-1 (CD11b/CD18)-transfected CHO cells (black filled curve) in comparison with the binding to vector-transfected CHO cells (gray filled curve) used as control was analyzed by flow cytometry. A thin line represents the binding of biotinylated albumin to Mac-1-transfected CHO cells used as negative control. The figure represents a typical experiment, and similar results were obtained in at least three separate experiments.

more, the binding of Thy-1 to immobilized Mac-1 is inhibited by the isolated I domain of Mac-1 as well as by fibrinogen, an established ligand of Mac-1 (Fig. 4B). In addition, fibrinogen and Thy-1 competed with each other for the binding to immobilized Mac-1, indicating that they interact with overlapping sites on Mac-1 probably located within the I domain, the major ligand-binding domain of Mac-1 (Fig. 4, C and D).

Adhesion of monocytes and PMNC to human Thy-1 is mediated by Mac-1

To examine the functional relevance of the Thy-1/Mac-1 interaction, we studied the ability of monocytes and PMNC to adhere to immobilized purified Thy-1. We could detect an adhesion of monocytes and PMNC to immobilized Thy-1, whereas there was an explicitly lower binding to plates coated with mAb AS02 and subsequent albumin as control (Fig. 5).

Furthermore, the adhesion of both monocytes and PMNC to immobilized Thy-1 was inhibited by preincubation of the respective cells with a mAb against CD11b, whereas the basal binding of leukocytes to the control was not influenced by preincubation with the mAb (Fig. 5). These results indicate that specific adhesion of leukocytes to Thy-1 can occur, mediated by interaction with Mac-1.

Subsequently, the contribution of the demonstrated Thy-1/Mac-1 interaction in leukocyte adhesion to Thy-1-expressing cells was investigated. CHO cells were transfected with Thy-1 cDNA cloned into pCMV5 expression vector (CHO.Thy1) or with vector alone (CHO.vec). The cell surface expression of Thy-1 was detected by flow cytometry using the mAb AS02, whereas other known human cell adhesion molecules such as ICAM-1, VCAM-1, and α_4 integrin were not expressed on these cells (data not shown). As shown in Fig. 6, monocytes and PMNC, known to express Mac-1 on their cell surface, strongly adhered to Thy-1 transfectants, but not to CHO.vec control cells (Fig. 6A). Binding of monocytes and PMNC to Thy-1 expressed on the cell surface of transfectants was distinctly inhibited by ~50% following preincubation with a mAb against CD11b as compared with adhesion of untreated cells (Fig. 6B). mAbs against CD11a, CD11c, and CD18 used here did not show any influence on the adhesion of CHO.Thy-1 similar to the IgG1 isotype control (Fig. 6B). In contrast,

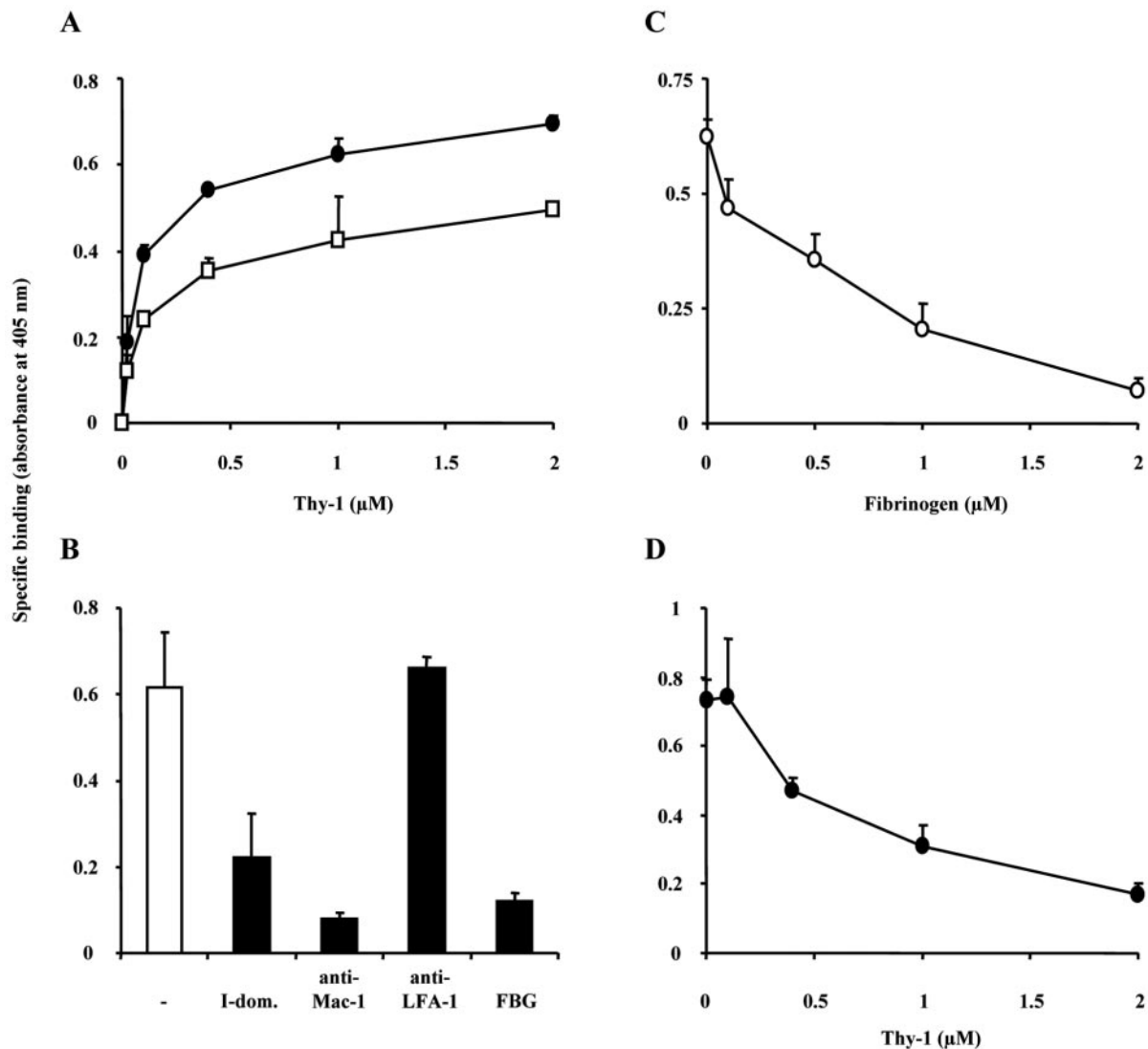


FIGURE 4. Thy-1 interacts with purified Mac-1 in a cell-free system. *A*, The specific binding of purified Thy-1 to immobilized Mac-1 (●) or to the immobilized I domain (□) is shown. *B*, The specific binding of purified Thy-1 (1 μM) to immobilized Mac-1 is shown in the absence (-) or presence of mAb against Mac-1, mAb against LFA-1 (each at 10 $\mu\text{g}/\text{ml}$), of fibrinogen (FBG), or of the isolated I domain (each 1 μM). *C*, The binding of Thy-1 (1 μM) to immobilized Mac-1 in the absence or presence of increasing concentrations of fibrinogen was analyzed. *D*, The binding of fibrinogen to immobilized Mac-1 in the absence or presence of increasing concentrations of Thy-1 was analyzed. Specific binding is expressed as absorbance at 405 nm. Data are mean \pm SD of a typical experiment; similar results were observed in three independent experiments.

the low binding of monocytes and PMNC to CHO.vec control cells is not inhibited by any of the Abs used (data not shown).

These results indicate that the specific interaction demonstrated between Thy-1 and Mac-1 results in an adhesion of leukocytes to Thy-1-expressing transfected cells. Additionally, the adhesion of monocytes and PMNC has been analyzed after stimulation of the respective cells with PMA as well as with fMLP. Both unstimulated or PMA- and fMLP-stimulated monocytes and PMNC showed an explicit adhesion to Thy-1-transfected CHO cells, whereas binding to vector-transfected CHO cells was negligible (data not shown).

Thy-1/Mac-1 interaction mediates leukocyte adhesion to activated endothelial cells and subsequent transendothelial migration

The binding of leukocytes to Thy-1-expressing HDMEC was analyzed to examine a possible involvement of human Thy-1 in the physiologic process of leukocyte adhesion to activated endothe-

lium as well. Treatment of HDMEC with PMA resulted in the induction of Thy-1 expression in a subpopulation of ~40–60% of the stimulated HDMEC. Thus, the adhesion of leukocytes to PMA-stimulated HDMEC containing both Thy-1-positive and Thy-1-negative cells was compared with the binding to PMA-activated Thy-1-negative HDMEC subpopulation. Monocytes and PMNC strongly adhered to PMA-activated HDMEC containing Thy-1-expressing cells, whereas the number of cells adherent to the Thy-1-negative HDMEC subpopulation was reduced by ~50% (Fig. 7A). Additionally, the adhesion of monocytes or PMNC to Thy-1-expressing endothelial cells was inhibited by previous binding of the mAb against CD11b to the cell surface of the leukocytes (Fig. 7B).

Finally, the influence of Thy-1 on the transmigration of monocytes and PMNC across a confluent monolayer of PMA-activated HDMEC was examined. Thus, the transmigration of both monocytes and PMNC across a monolayer of activated Thy-1-negative

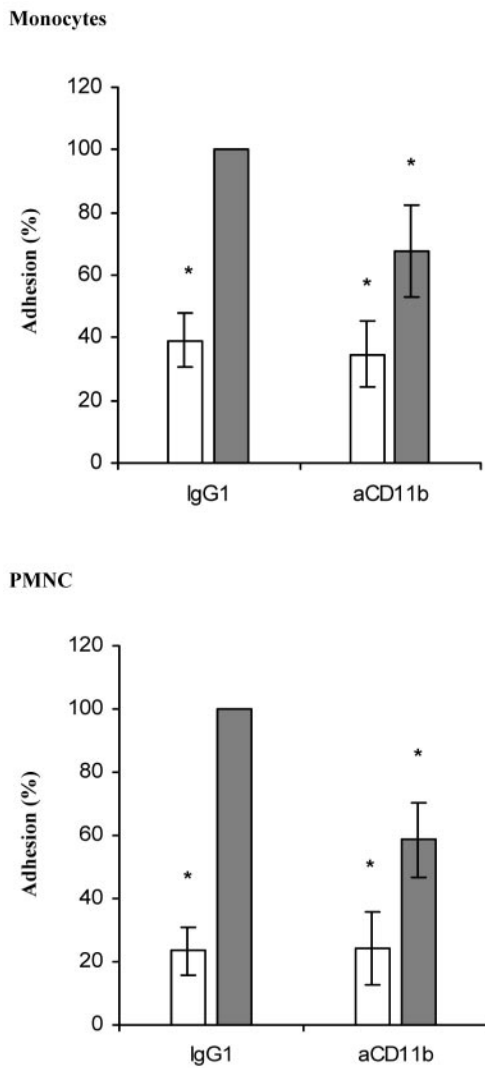


FIGURE 5. Monocytes and PMNC adhere to purified immobilized Thy-1. The adhesion of monocytes and PMNC to immobilized Thy-1 (■) or BSA (□) as control is shown after preincubation of the cells with a mAb against CD11b or an IgG1 control. The number of adherent cells is expressed as percentage of the number of IgG1-treated cells adhering to immobilized Thy-1. Data are mean \pm SD of three independent experiments. *, $p < 0.001$ compared with adhesion of IgG1-treated cells to Thy-1.

HDMEC was reduced by ~ 40 – 50% compared with activated partially Thy-1-expressing HDMEC (Fig. 8).

Moreover, the transendothelial migration of monocytes and PMNC across activated partially Thy-1-expressing HDMEC was inhibited by preincubation with a mAb to CD11b (data not shown). Thus, the specific interaction between Thy-1 and leukocyte Mac-1 contributes to both the adhesion and transendothelial migration of circulating PMNC and monocytes.

Discussion

Leukocyte recruitment into perivascular tissue to sites of inflammation, tissue injury, or infection plays a key role in initiating the function of leukocytes in host defense or inflammatory processes. The emigrating leukocytes need to interact with the activated endothelium and with subendothelial and extracellular matrix components, a multistep cascade that is realized by the sequential interaction between different leukocyte adhesion molecules and their specific ligands. A number of studies have contributed much

knowledge about the specific interactions determining leukocyte extravasation, including the selectin-mediated rolling of leukocytes as well as the firm adhesion and following transendothelial migration realized in part by β_2 integrins and proteins of the IgSF such as ICAM-1 (1, 2). Indeed, besides well-characterized adhesion molecules, few studies indicated the involvement of further yet-unidentified proteins in additional pathways of adhesion processes (13–17).

In this study, we have identified the human Thy-1 (CD90) expressed on activated endothelial cells as a new counterreceptor for the leukocyte β_2 integrin Mac-1 ($\alpha_M\beta_2$; CD11b/CD18). Furthermore, we demonstrated for the first time the involvement of the Thy-1/Mac-1 interaction in firm adhesion of leukocytes to activated endothelium and in the subsequent transendothelial migration.

Recently, the human Thy-1 could be identified as an activation-associated cell adhesion molecule on HDMEC (29, 30). The human Thy-1 (CD90) is not detectable on resting endothelial cells, but its expression is induced by phorbol ester and inflammatory mediators in vivo and in vitro (22, 31, 32).

In situ, the Thy-1 expression could be detected exclusively on endothelial cells in tissues in which cell activation, inflammation, or angiogenesis occurs, like synovial membrane of joints from patients suffering from active rheumatoid arthritis or the stromal environment of melanoma, psoriasis, or bullous pemphigoid, whereas resting endothelial cells in healthy tissues showed no Thy-1 expression (30). In vitro, Thy-1 expression is induced on a subpopulation of HDMEC, possibly because of the occurrence of a mixed population of both vascular and lymphatic endothelial cells (29, 30).

The binding pattern of purified human Thy-1 revealed the expression of a corresponding ligand of human Thy-1 on monocytes and PMNC in situ and in vitro. In contrast, only a poor binding of human Thy-1 to a subpopulation of lymphocytes could be detected, indicating a corresponding ligand restricted to myeloid leukocytes. Consequently, there is a tight colocalization of Thy-1-expressing endothelial cells and Thy-1 ligand-expressing leukocytes within microvessels, maybe resulting in leukocyte-endothelial cell interaction. In the following, the involvement of the Thy-1/Thy-1 ligand interaction in adhesion of monocytes and PMNC to Thy-1-expressing endothelial cells and fibroblasts could be demonstrated in cell adhesion assays (30).

In this study, we could unequivocally identify the leukocyte β_2 integrin Mac-1 as the corresponding ligand of human Thy-1 by several criteria: 1) the binding of a mAb against CD11b to the cell surface of monocytes and neutrophils was inhibited by the previous binding of purified Thy-1; 2) the binding of purified Thy-1 to Mac-1 transfectants as well as the saturable dose-dependent binding of Thy-1 to immobilized purified Mac-1 and to the isolated I domain of Mac-1 could be demonstrated; 3) the binding of Thy-1 to immobilized Mac-1 was inhibited in the presence of the isolated I domain of Mac-1 and of mAb against Mac-1; and 4) in addition, fibrinogen, an established ligand of Mac-1 interacting within the I domain, competed with Thy-1 for the binding to immobilized Mac-1. In conclusion, these results demonstrate an interaction between Thy-1 and Mac-1. Moreover, the binding of Thy-1 to the isolated I domain of Mac-1 as well as the competition of Thy-1 and fibrinogen for an overlapping binding site suggest that the binding of Thy-1 to Mac-1 is probably located within the major ligand-binding I domain of Mac-1.

The leukocyte β_2 integrin Mac-1 has been shown to be responsible for myeloid cell adhesion to and transmigration across endothelium (14, 33), or epithelium (34), as well as for adhesion-dependent functions such as neutrophil homotypic aggregation and

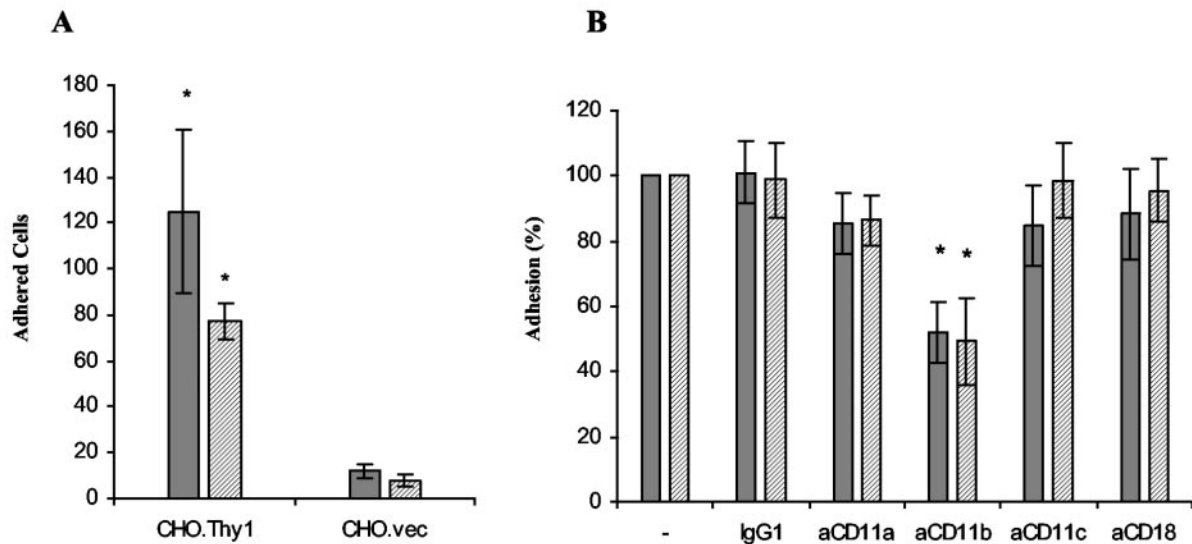


FIGURE 6. Adhesion of PMNC and monocytes to Thy-1 transfectants is mediated by Mac-1. *A*, The number of monocytes (▨) and PMNC (▩) adhering to Thy-1-transfected CHO cells (CHO.Thy-1) was analyzed in comparison with adhesion to vector-transfected CHO cells (CHO.vec). Data represent the mean \pm SD of at least three independent experiments. *, $p < 0.001$ compared with adhesion to CHO.vec as control. *B*, The adhesion of monocytes (▨) and PMNC (▩) to CHO.Thy-1 was analyzed in the absence (-) or presence of mAb against CD11a, CD11b, CD11c, CD18, or an IgG1 control. Cell adhesion is expressed as percentage of the number of untreated cells adhering to CHO.Thy-1, which was set as 100%. All data are mean \pm SD of at least three independent experiments. *, $p < 0.001$ compared with adhesion of untreated cells.

chemotaxis (35–37), and phagocytosis of opsonized particles (38, 39). These Mac-1-dependent interactions are mediated or affected by binding to several cell surface ligands including ICAM-1 (40, 41), ICAM-2 (42), platelet glycoprotein Ib α (43), junctional adhesion molecule-3 (44), and the L/W blood group protein ICAM-4 (45, 46), as well as to soluble ligands such as C3bi (38, 39), complement factor H (47), coagulation factor X (48), fibrinogen (49), fibronectin (50), collagen (51), heparin (52), haptoglobin (53), or kininogen (27). Furthermore, Mac-1 was demonstrated to associate with a number of cell surface proteins such as urokinase receptor (54–56), Fc γ RIII (57), and Fc α RI (58), or LPS binding protein

(59), and to cross talk with other integrins such as very late Ag-4 (60), very late Ag-5 (50, 61), or $\alpha_v\beta_3$ (62). These various interactions reveal Mac-1 as a multiligand receptor playing a key role in initiating and regulating inflammatory processes and innate immunity. The interaction between Mac-1 and ICAM-1 is supposed to be a predominant adhesion pathway mediating the firm adhesion of leukocytes to endothelial cells at sites of inflammation, because the expression of ICAM-1 is induced or increased upon cell activation by proinflammatory mediators.

However, the presence of additional leukocyte-endothelium adhesion pathways mediated by interaction of Mac-1 (CD11b/CD18)

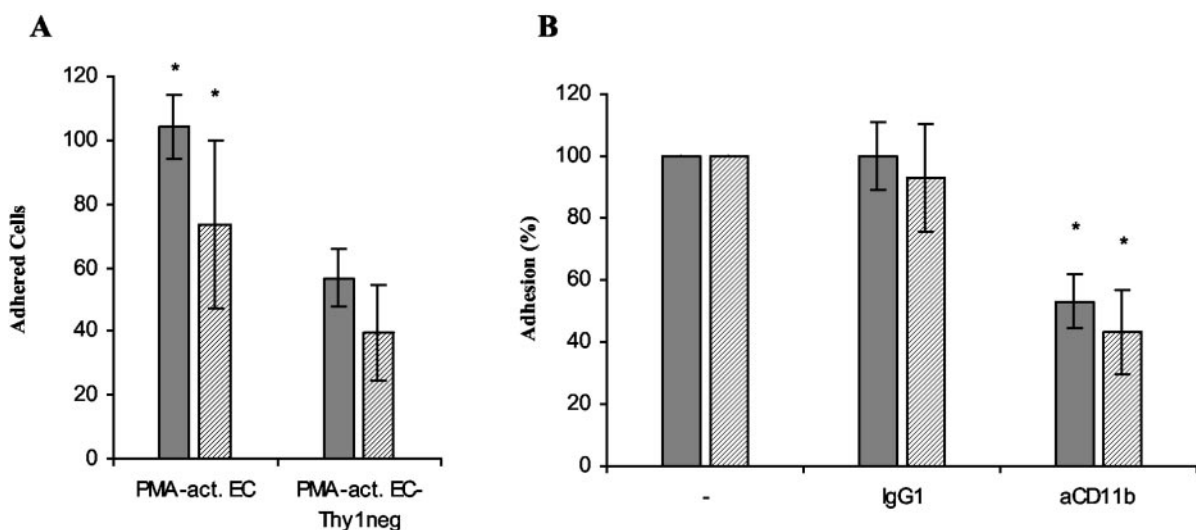


FIGURE 7. Adhesion of leukocytes to activated Thy-1-expressing endothelial cells is mediated by Mac-1. *A*, The adhesion of monocytes (▨) or PMNC (▩) to PMA-activated HDMEC containing 40–60% Thy-1-positive cells was analyzed in comparison with adhesion to separated PMA-activated Thy-1-negative HDMEC. Data are mean \pm SD of three independent experiments. *, $p < 0.001$ compared with adhesion of the respective cells to activated Thy-1-negative HDMEC. *B*, The adhesion of monocytes (▨) or PMNC (▩) to PMA-activated HDMEC containing 40–60% Thy-1-positive cells was studied in the absence (-) or presence of mAb against CD11b or an IgG1 control. Cell adhesion is expressed as percentage of the number of untreated adherent cells, which was set as 100%. Data are mean \pm SD of three independent experiments. *, $p < 0.001$ compared with adhesion of the respective untreated cells.

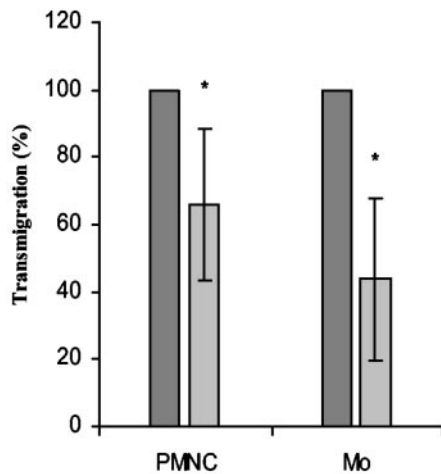


FIGURE 8. Thy-1 is involved in transendothelial migration of PMNC and monocytes. The transmigration of PMNC and monocytes across a monolayer of PMA-activated Thy-1-negative HDMEC (■) was compared with the transmigration across PMA-activated HDMEC containing 40–60% Thy-1-positive endothelial cells (▨), which was set as 100%. The number of transmigrated cells was analyzed by flow cytometry. Data are mean \pm SD of five independent experiments. *, $p < 0.001$ compared with transmigration of the respective cells through a monolayer of PMA-activated HDMEC containing Thy-1-positive cells.

with a ligand distinct from ICAM-1 was suggested in different studies using ICAM-1-deficient mice or Ab-blocking experiments (13–17, 63). In contrast, ICAM-2 is expressed on resting endothelial cells and is down-regulated by inflammatory mediators (64, 65). Thus, a role for ICAM-2 in the early recruitment of leukocytes during initiation of the immune response, in lymphocyte recirculation, and in dendritic cell trafficking is suggested (64, 66). However, recently, Lehmann et al. (67) showed that ICAM-2 solely contributes to lymphocyte recirculation through lymph nodes and Peyer's patches, but not in other organs. Furthermore, the interaction between Mac-1 and junctional adhesion molecule-3, expressed on resting endothelial cells and platelets, is suggested to mediate not only the binding of leukocytes to surface-adherent platelets, but also to play a role in leukocyte adhesion to and transmigration through the endothelium (44, 68, 69).

Our present findings indicate that human Thy-1 is a further endothelial cell receptor for the leukocyte integrin Mac-1 that is up-regulated by inflammatory mediators and contributes to leukocyte recruitment to sites of inflammation, tissue injury, or infection. Cell adhesion assays revealed an adhesion of PMNC and monocytes to purified Thy-1 as well as to Thy-1-transfected cells, mediated by the specific interaction between Thy-1 and Mac-1, because adhesion was inhibited by mAb against CD11b. The leukocyte adhesion to Thy-1 seems to be mediated specifically by interaction between Thy-1 and the α_M chain/CD11b of Mac-1, because binding of a mAb against CD18 described as function blocking could not inhibit the examined adhesion. Interestingly, previous binding of a mAb against CD11b blocked the adhesion of PMNC and monocytes to human Thy-1 only partially, indicating a possible involvement of additional unidentified leukocyte adhesion molecules. Moreover, a ligand of human Thy-1 could be demonstrated on cell surface of melanoma cells, mediating the binding of melanoma cells to activated Thy-1-expressing HDMEC and fibroblasts (29).

The identification of this ligand or of the possible occurrence of an identical ligand on leukocytes and melanoma cells, respectively, as well as an involvement of this interaction in melanoma

metastasis, is under investigation. Leyton et al. (70) could identify an interaction between murine Thy-1 and integrin $\alpha_V\beta_3$ on astrocytes. Thus, evidence for an involvement of the integrin $\alpha_V\beta_3$ in Thy-1-mediated adhesion of leukocytes or melanoma cells to activated endothelial cells remains to be further examined.

Furthermore, as shown by the first preliminary results, adhesion of PMNC and monocytes to Thy-1 transfectants occurred to approximately the same extent as binding of those cells to stably ICAM-1-transfected cells, suggesting a possibly important role for human Thy-1 in mediating leukocyte adhesion besides ICAM-1-mediated adhesion processes (data not shown). Because the extent of adhesion of stimulated monocytes and PMNC is comparable with binding of unstimulated leukocytes, the Thy-1/Mac-1-mediated adhesion process seems to be not distinctly influenced by affinity and avidity changes of the leukocyte integrin Mac-1. The physiological relevance of the Thy-1/Mac-1 interaction was substantiated by demonstrating its role in mediating leukocyte adhesion to activated endothelium and subsequent transendothelial migration. We could show that leukocyte adhesion to, as well as transmigration through, activated endothelial cells containing Thy-1-expressing cells is increased compared with activated Thy-1-negative endothelial cells. Considering that both activated endothelial cell populations do not differ in expression of other adhesion molecules besides human Thy-1, leukocyte adhesion and transmigration are distinctly facilitated by Thy-1-mediated interactions. Furthermore, both leukocyte adhesion to activated endothelial cells and subsequent transmigration are mediated by the specific interaction between Thy-1 and Mac-1, because these processes are inhibited by the blocking of CD11b (30).

Thy-1 expression on endothelial cells induced by inflammatory mediators was detected on the whole cell surface, but not reinforced at intercellular contacts between endothelial cells. Thus, the enhanced transendothelial migration of leukocytes through Thy-1-expressing endothelial cells may be a result of the previous increased adhesion of leukocytes rather than a specifically Thy-1-mediated process. On this account, further adhesion and transmigration assays under flow conditions are required to examine the particular function of Thy-1 more closely in comparison with other integrin ligands.

Taken together, our results demonstrate human Thy-1, a cell surface glycoprotein of the IgSF, expressed on activated endothelial cells, as a counterreceptor for the leukocyte β_2 integrin Mac-1 (CD11b/CD18; $\alpha_M\beta_2$). Furthermore, this interaction is involved in the adhesion of leukocytes to activated endothelial cells as well as in subsequent transendothelial migration of leukocytes. Thus, Thy-1/Mac-1 interaction possibly plays an important role in mediating leukocyte recruitment into perivascular tissue to sites of inflammation, tissue injury, or infection. Consequently, investigations aimed at the molecular biology as well as the regulation of leukocyte emigration into perivascular tissue should include the Thy-1/Mac-1 interaction as well, besides well-characterized adhesion processes.

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