The CD157-Integrin Partnership Controls Transendothelial Migration and Adhesion of Human Monocytes*

CD157, a member of the CD38 gene family, is an NAD-metabolizing ectoenzyme and a signaling molecule whose role in polarization, migration, and diapedesis of human granulocytes has been documented; however, the molecular events underpinning this role remain to be elucidated. This study focused on the role exerted by CD157 in monocyte migration across the endothelial lining and adhesion to extracellular matrix proteins. The results demonstrated that anti-CD157 antibodies block monocyte transmigration and adhesion to fibronectin and fibrinogen but that CD157 cross-linking is sufficient to overcome the block, suggesting an active signaling role for the molecule. Consistent with this is the observation that CD157 is prevalently located within the detergent-resistant membrane microdomains to which it is implicated in tumor dissemination (9). Further, it is endowed with receptor-like features observed in different cell types (10) and transduces signals by interacting with transmembrane partner molecules (11, 12), a strategy shared by other GPI-anchored molecules (13).

Along with CD38, human CD157 constitutes the NAD glycohydrolase/ADP-ribosyl cyclase gene family (6). These two molecules share 36% of their protein sequence, including 10 cysteine residues forming intrachain disulfide bonds. However, they differ in structure, with CD157 being a glycosylphosphatidylinositol (GPI)-anchored glycoprotein and CD38 being a transmembrane type II glycoprotein (7). CD157 is mainly expressed by cells of the myeloid lineage, bone marrow stroma, and vascular endothelium (8). CD157 is also expressed by ovarian cancer epithelium and by peritoneal mesothelial cells, where it is implicated in tumor dissemination (9). Ectoenzymes include a heterogeneous group of surface molecules with multiple functions. In addition to interacting with specific substrates, many of them participate in leukocyte activation and migration. In some cases, ectoenzymes exert enzymatic control of leukocyte trafficking (1). Other functions are independent of their enzymatic ability, relying on the activation of signaling cascades that lead to calcium fluxes, cytoskeletal remodeling, and release of inflammatory mediators (2, 3). In selected cases, ectoenzymes associate with adhesion molecules to regulate their signaling (4, 5).

Monocytes play key functions in innate and adaptive immunity, immune surveillance, host defense, and inflammation (15). Furthermore, monocyte trafficking is a critical factor in inflammatory and autoimmune diseases, such as atherosclerosis, multiple sclerosis, and rheumatoid arthritis (16).

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CD157 is involved in neutrophil polarization, adhesion, and motility (11) and controls transendothelial migration (14). This study extends the analysis of the functional role of CD157 to human monocytes, adopted as a physiological model in which to explore the molecular mechanism underlying the role of the molecule in the control of cell motility.

Monocytes originate from hematopoietic precursors in the bone marrow and circulate in the blood for 24–48 h before extravasating into the connective tissues. In inflammation, monocytes are recruited to the affected tissues, where they differentiate into macrophages and dendritic cells (17). The continuous traffic from blood to tissue and from tissue to lymph nodes relies on orchestrated sequential interactions between leukocytes and vascular or lymphatic endothelium (18). Each

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step is a prerequisite for the next one. Cell adhesion molecules (such as selectins and integrins) have well-established roles in supporting tethering, rolling, and firm adhesion to the vascular endothelium (19). Chemokines and selected molecules at the endothelial junctions (including CD31, JAM-1, vascular endothelial cadherin, and CD99) are critical in the control of the transmigration process (20). These central components are flanked by an increasing number of molecules from different families, which contribute to distinct steps of the extravasation cascade (21).

The results obtained in this study indicate that CD157 is involved in the control of monocyte extravasation and adhesion to ECM proteins. CD157 establishes a structural interaction with $\beta_1$ and $\beta_2$ integrin, and, following antibody-induced cross-linking, it promotes their relocation into detergent-resistant domains, thus driving the dynamic reorganization of signaling-competent membrane microdomains. Moreover, CD157 effectively contributes to the integrin-driven signaling network that is critical during monocyte transmigration.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The anti-CD157 (SY/11B5, IgG1), anti-$\beta_1$ integrin/CD29 (Moon-4, IgG1), anti-CD71 (CB26, IgG2a) mAb, and irrelevant murine monoclonal IgG (mlgG1) were produced in-house. Anti-CD11b (I07, IgG1) and anti-$\beta_2$ integrin/CD18 (TS1/18, IgG1) mAbs were provided by A. Arnout (Harvard Medical School, Boston, MA). All mAbs were assigned to the manufacturer’s instructions. This yielded affinity-purified, F(ab’2) fragments of protein G, and the F(ab’2) fragments were obtained using immobilized papain (Sigma–Aldrich). RF3 (anti-CD157, IgG2a) mAb was from MBL International, D01P (anti-CD29, IgG2a) mAb was from Santa Cruz Biotechnologies, and anti-CD157, IgG2a) mAb was from Abnova, 3H1192 (anti-CD29, IgG2a) mAb was from Sigma-Aldrich. Selected mAbs were coupled to Alexa Fluor 488 (Molecular Probes) or Chromis-550 (CyAnMagenta) or FITC. Fluorescence was determined using a FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences). Background mAb binding was estimated by means of isotype-matched negative control mAb. Ten thousand events were considered for each analysis.

Transendothelial Migration Assay—HPMEC were grown to confluence on 5-μm pore Transwell chambers (Corning–Costar) coated with fibronectin (10 μg/ml) and either left unstimulated or stimulated for 4 h at 37 °C with TNFα (20 ng/ml) or IL-1β (10 units/ml). Peripheral blood mononuclear cells (5 × 10^6) were treated with the indicated mAbs (10 μg/ml) for 20 min before the assay and then seeded on the HPMEC monolayers. To induce CD157 cross-linking, F(ab’2) RoMlgG (50 μg/ml) was added to the desired samples. After a 2-h incubation at 37 °C, the transmigrated (suspended and adhered) monocytes were collected and counted by FACS at medium speed for 3 min. The transmigration index (TMI) was determined using the formula: TMI = (number of cells migrated in the presence of blocking mAb)/(number of cells migrated in the presence of mlgG).

Cell Adhesion to ECM Proteins—Purified monocytes or THP-1 cells (5 × 10^5) pretreated with selected mAbs (10 μg/ml) for 20 min) were plated onto 96-well plates coated with FN (10 μg/ml) or fibronogen (50 μg/ml) and blocked with 2% BSA. To induce CD157 cross-linking, F(ab’2) RoMlgG (50 μg/ml) was added to the indicated samples. After a 1-h incubation at 37 °C, non-adherent cells were removed by washing, and adherent cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Adherent cells were assessed by light microscopy, and five randomly selected fields (×10) for each well were photographed and counted. Each condition was carried out in quadruplicate, and experiments were repeated at least three times.

Isolation of Detergent-insoluble Microdomains (Lipid Rafts) by Sucrose Gradient Centrifugation and Western Blot Analysis—THP-1 cells (5 × 10^5) were lysed in 0.7 ml of ice-cold MES lysis buffer (25 mM MES, pH 6.5, 5 mM NaF, 1 mM Na3VO4, 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 50 μg/ml aprotinin, and leupeptin). Where indicated, cells were pretreated (10 min at 37 °C) with FN (10 μg/ml), with anti-CD157 mAb (5 μg/ml), or with anti-CD157 mAb followed by F(ab’2) RoMlgG (20 μg/ml for 1 min at 37 °C). Cell lysates were mixed (v/v) with 80% sucrose (w/v in MES buffer), and a step gradient was prepared by overlaying with 30 and 5% sucrose. After centrifugation at 200,000 × g for 16 h at 4 °C, 12 fractions (0.4 ml each) were collected starting from the top of the gradient. Equal amounts of each fraction were resolved on 10% SDS-PAGE under non-reducing conditions, transferred to polyvinylidene difluoride (PVDF) membranes, and subjected to Western blotting with the indicated mAbs followed by RoMlgG-HRP and then developed using an ECL–based system (PerkinElmer Life Sciences). The lipid raft marker GM1 was detected by dot blot analysis using HRP-labeled Ctx.

Immunofluorescence and Confocal Microscopy—Serum-starved cells were fixed in Hanks’ balanced salt solution (pH 6.5) containing 2% paraformaldehyde and 1 mM ZnCl2 for 20 min, washed twice in Hanks’ balanced salt solution, and treated for 30 min with 50 mM glycine in Hanks’ balanced salt solution.
supplemented with 1% FCS to quench the aldehyde groups (23). Fixed cells were double-stained with anti-CD157-Chromis-550 and anti-CD18- or CD29-Alexa Fluor 488 mAbs or Ctx-FITC. In selected experiments, cells were incubated with anti-CD157-biotin (5 μg/ml for 10 min on ice), washed, and reacted with streptavidin-Dylight-549 (20 μg/ml for 10 min on ice) and then placed at 37 °C for 2 min to induce capping, blocked by ice-cold PBS with 0.5% BSA and 0.1% NaN₃, and fixed as above. Counterstaining was performed with anti-CD18-, CD29-, or CD71-Alexa Fluor 488 mAbs or Ctx-FITC. Engagement of integrins was induced by incubating THP-1 cells (5 × 10⁶/ml) in Hanks’ balanced salt solution with FN (10 μg/ml) at 37 °C for 10 min. Cells were fixed, stained with the indicated mAbs, and analyzed by confocal microscopy, using an Olympus FV300 laser scanning confocal microscope equipped with two helium neon (543 and 582 nm) lasers, a blue argon (488 nm) laser, and Fluoview 300 software (Olympus Biosystems). Cells were imaged using a ×60 oil immersion objective (1.4 NA). Images of optical sections (512 × 512 pixels) were digitally recorded and processed using Adobe Photoshop CS4 (Mountain View, CA) software. For co-localization analysis, all image data were preprocessed prior to quantification by means of an iterative constrained Tikhonov-Miller algorithm (DeconvolutionLab ImageJ plugin (24)) to reduce the blurring from nearby bright objects and the out-of-focus noise. Co-localization was evaluated using the Colocalization Colormap script, an ImageJ plugin for automated quantification and visualization of co-localized fluorescent signals (25). The method computes correlation of intensities between pairs of individual pixels in two different channels. Results are presented as mean correlation index (Icorr) ± S.E. Icorr indicates the fraction of positively correlated pixels in the image.

Co-immunoprecipitation Assays—THP-1 cells were treated with 0.5 mm membrane-impermeable cross-linker dithiobis (sulfosuccinimidyldiacetate) (Pierce) for 30 min at 20 °C. The reaction was stopped with 20 mm Tris for 15 min, and then cells were washed and lysed in ice-cold MES buffer. Cell lysates were centrifuged at 14,000 × g for 30 min, precleared overnight with protein G-Sepharose beads, and then incubated overnight at 4 °C with protein G-Sepharose beads conjugated to 2 μg of anti-CD157, anti-CD18, anti-CD29, or anti-CD71 mAbs. The beads were washed with PBS, and proteins were eluted by adding non-reducing Laemmli sample buffer and boiling for 5 min. Eluted proteins were resolved on 10% SDS-PAGE under non-reducing conditions, transferred to PVDF membranes, and probed with mAbs to CD157, CD18, CD29, or CD71 followed by HRP-conjugated secondary antibodies and then detected using ECL.

Phosphorylation Assay—THP-1 cells (2 × 10⁶/ml) were incubated with anti-CD157, anti-CD18, and anti-CD29 mAb (5 μg/ml) for 10 min at 4 °C, washed in cold PBS, cross-linked with F(ab’)₂, RaMlgG (20 μg/ml), and incubated at 37 °C for 1 min. Cells were placed on ice, washed in cold PBS supplemented with 1 mm Na₂VO₃ to stop the reaction, and lysed in ice-cold radioimmune precipitation buffer (50 mm Tris HCl, 150 mm NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mm EDTA, 0.1% SDS supplemented with 1 mm Na₂VO₃, 5 mm NaF, 50 μg/ml aprotinin and leupeptin). After centrifugation, total lysates (30 μg/lane) were run on 10% SDS-PAGE under reducing conditions and transferred to PVDF membranes. Membranes were blocked and probed with HRP-labeled anti-phosphotyrosine (PY20) polyclonal antibody, anti-phospho-ERK1/2 (Tyr-204/Tyr-187) (BD Biosciences), anti-phospho-c-Src (Tyr-530), anti-phospho-FAK (Tyr-397), and anti-phospho-ACK (Ser-493) (Santa Cruz Biotechnologies). The membranes were stripped and reprobed with anti-ERK2, anti-c-Src, anti-FAK, and anti-AKT1 mAb (Santa Cruz Biotechnologies) followed by incubation with the appropriate HRP-conjugated antibodies. Specific bands were visualized by ECL.

Statistical Analysis—Unless otherwise indicated, values are expressed as means ± S.E. Comparisons between two groups were carried out using the Student’s t test for normal distributed variables. One-way analysis of variance with Bonferroni post test correction was used for multiple comparisons. Statistical analyses were performed using Prism 5, GraphPad software (San Diego, CA). Differences were considered statistically significant for p values ≤0.05.

RESULTS

CD157 expression on circulating monocytes is up-regulated by MCP-1—Flow cytometric analysis confirmed that CD157 is expressed by virtually all the circulating monocytes (Fig. 1A) and demonstrated that its expression rapidly increases upon activation with MCP-1, reaching a plateau after 30 min of stimulation (Fig. 1, A and B). As expected, CD11b expression was enhanced by MCP-1 (Fig. 1B) (26). Immunoblot further proved that CD157 is expressed by monocytes (data not shown) as well as the human monocytic THP-1 cells at the expected molecular weight (see below, Fig. 5A). Up-regulation of monocytic CD157 expression by MCP-1, a chemoattractant factor that promotes monocyte migration and diapedesis, suggested the potential involvement of CD157 in monocyte trafficking.

CD157 is involved in monocyte transendothelial migration—A central aspect of leukocyte trafficking is the continuous transition from the blood circulation into the tissues and back. The vascular endothelium constitutes the interface between the two tissue compartments, so transendothelial migration represents a critical step of leukocyte trafficking.

The potential role of CD157 in regulating transmigration was explored in a conventional in vitro assay using primary monocytes treated with a blocking anti-CD157 mAb or with an anti-CD31 mAb used as a positive control for blocking transmigration (27). To avoid interfering with the Fc receptors expressed by monocytes, F(ab’)₂ mAbs were used. Because the majority of leukocyte traffic occurs in vivo primarily through the microvessels, we used HPMEC as monolayers to reproduce a more physiological environment. These experiments were initially done with resting HPMEC monolayers across which monocytes migrate in response to basal concentrations of chemoattractants, such as MCP-1 (28). The results demonstrated that CD157 ligation by a specific mAb resulted in significant inhibition of transmigration of monocytes, comparable with that obtained with a blocking mAb to CD31. Anti-CD157-mediated inhibition was dose-dependent with significant effects down to 0.4 μg/ml (Fig. 1C).
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In concert with neutrophils, monocytes and tissue macrophages participate in inflammatory responses. Therefore, the role of CD157 in transendothelial migration was investigated using cytokine-activated HPMEC monolayers, thereby simulating an inflammatory microenvironment. Stimulation with TNFα or IL-1β had no effect on the surface expression or distribution of CD157 on endothelial cells (14). Ligation of CD157 with a specific mAb significantly reduced transmigration across TNFα-activated HPMEC (Fig. 1D) or across IL-1β-activated HPMEC (data not shown). To mimic the extent of CD157 clustering likely occurring on monocytes during transmigration, monocytes were treated with anti-CD157 mAb, and then cross-linking of CD157 was induced by the addition of F(ab′)2 RuMlgG prior to their addition to the HPMEC monolayer (t = 0). Alternatively, monocytes treated with anti-CD157 mAb were seeded on HPMEC monolayers for 30 min (a sufficient time for monocytes to get to endothelial junctions), and then CD157 cross-linking was performed. Antibody-induced clustering of CD157 overcome the block and significantly restored monocyte transmigration. The effect was independent of the timing of cross-linking (Fig. 1D). Antibody-induced cross-linking of CD31 at the start of the assay (t = 0) failed to reverse the blockade, whereas it was effective when performed at t = 30 min (Fig. 1D), as described (29). These data gave raise to the hypothesis that clustering of CD157 might function as a switch that controls transmigration by eliciting intracellular signaling.

CD157 Controls Monocyte Adhesion to Extracellular Matrix Proteins—Adhesion of leukocytes to the extracellular matrix is essential for migration across blood vessels and subsequently into the stroma of inflamed tissues (30). It is regulated by a wide variety of cell surface receptor systems, the most relevant of which belong to the β1 and β2 integrin subfamilies.

The contribution of CD157 in regulating the adhesion of monocytes to fibronectin and fibrinogen (i.e. the elective extracellular ligands of β1 and β2 integrin, respectively) was assessed by performing in vitro experiments using monocytes or THP-1 cells treated with anti-CD157 mAbs. Ligation of CD157 significantly reduced the ability of monocytes (Fig. 2, A and B) and THP-1 cells (Fig. 2C) to adhere to both ECM proteins. As expected, cell binding to fibrinogen and fibronectin was inhibited by neutralizing antibodies to CD29 (β1 integrin) (Fig. 2, A and C) and CD18 (β2 integrin) (Fig. 2B), respectively. Combined

![Flow Cytometric Analysis of Surface Expression](image-url)

**FIGURE 1.** CD157 is expressed in human monocytes and is involved in transendothelial migration. A, representative flow cytometric analysis of surface expression of CD157 on monocytes (black peak) or on monocytes stimulated with MCP-1 for 30 min (white peak). The gray peak indicates the isotype-matched control mAb. Ten thousand events were considered for each analysis. The results presented are representative of three experiments. x axis = fluorescence intensity (FL-1-height), and y axis = number of cells (events). B, monocytes were treated with 10 μM MCP-1 for the indicated time at 37 °C, fixed, and incubated with anti-CD157 or anti-CD11b mAb followed by F(ab′)2 RuMlgG-FITC. Results showing the increasing rate of mean fluorescence intensity (MFI) are expressed as MCP-1-induced expression of CD157/basal or of CD11b/basal surface expression of each molecule at 37 °C. The results are expressed as means of three separate experiments ± S.E. C, transmigration assays across resting HPMEC grown on fibronectin-coated Transwell filters were performed toward a 10 μM MCP-1 Transwell gradient in the presence of increasing concentrations of F(ab′)2, anti-CD157 (black bars), anti-CD31 mAb (gray bars), or mlgG (white bars) (10 μg/ml). After a 2-h incubation at 37 °C, the number of transmigrated monocytes was assessed by FACS at medium speed for 3 min. Results are the mean ± S.E. of three separate experiments and are expressed as TMI. Anti-CD157 blocked transmigration in a dose-dependent manner. **, p < 0.01 and ***, p < 0.001. D, cross-linking monocyte CD157 reverses the anti-CD157-induced block in transmigration. Monocytes pretreated with F(ab′)2, anti-CD157 (black bars), anti-CD31 mAb (gray bars), or mlgG (white bars) (10 μg/ml) were added to HPMEC monolayers activated with TNF-α (20 ng/ml) for 2 h at 37 °C. Where indicated, CD157 and CD31 were cross-linked (at time 0 or 30 min after the beginning of the transmigration) by the addition of a F(ab′)2, RuMlgG (50 μg/ml). After a 2-h incubation at 37 °C, transmigrated monocytes were counted as above. Results are expressed as TMI and are the mean ± S.D. of three replicates. One representative experiment of five is shown. **, p < 0.01 and ***, p < 0.001.
ligation of CD157 and CD29 or CD157 and CD18 resulted in an increased inhibition on both monocyte and THP-1 cell adhesion. However, none of the experimental conditions analyzed entirely abrogated cell adhesion, indicating that other molecules must also participate. Strikingly, CD157 clustering induced by cross-linking with a secondary antibody reversed the blockade and virtually completely restored monocyte (Fig. 3, A and B) and THP-1 cell (Fig. 3C) adhesion. Taken together, these data suggested the existence of a functional interplay between CD157 and both β1 and β2 integrin orchestrating critical steps of leukocyte trafficking and corroborate the hypothesis that CD157 is involved in intracellular signal transduction.

CD157 Engagement Recruits β1 and β2 Integrins to Detergent-resistant (Lipid Raft) Domains—Next, the possibility that CD157 was engaged in the transduction of activation signals regulating monocyte functions was investigated. The GPI-mediated anchorage to the membrane, indicating the lack of a transmembrane domain of CD157, fostered the hypothesis that it could fulfill receptor functions in the context of membrane microdomains, as part of multimolecular complexes. To address this issue, THP-1 cells (used as a monocyte model) were subjected to lysis and discontinuous sucrose gradient centrifugation to separate detergent-resistant (lipid rafts) from detergent-sensitive (non-rafts) membrane fractions. To validate the fractionation procedure, Ctx was used to identify ganglioside GM1, a major constituent of lipid rafts, whereas CD71 (a raft-excluded protein) was chosen as a marker of the detergent-sensitive fractions. In standard culture conditions and like many other GPI-anchored molecules (31), CD157 almost exclusively associated to detergent-insoluble fractions. A substantial portion of both β1 (CD29) and β2 (CD18) integrins was found outside the detergent-insoluble fractions (Fig. 3A). When THP-1 cells were treated with anti-CD157 mAb, which can cross-link two CD157 molecules, the membrane compartmentalization of CD157, CD18, and CD29 was similar to that observed in untreated cells (Fig. 3B). To exclude that the observed membrane compartmentalization of CD157 and integrins represented an artifact of detergent extraction, confocal microscopy analysis was performed in parallel on live THP-1 cells. This approach showed that CD157 had a characteristic patched pattern in basal conditions and largely co-localized with GM1 gangliosides. In contrast, CD18 and CD29 were uniformly localized on the cell membrane (Fig. 4, A and D, left, and E).

To establish whether cross-linking of many CD157 molecules accompanied the reorganization of the membrane topology, the distribution of CD157, CD18, and CD29 molecules on the cell membrane was monitored after CD157 clustering, using biochemical approaches and confocal microscopy.

FIGURE 2. Effect of CD157 on the adhesion of monocytes to fibronectin and fibronectin. A–C, monocytes (A and B) or THP-1 cells (C) were treated with the indicated mAbs (10 μg/ml for 20 min). CD157 cross-linking was induced by the addition of F(ab')2 RU-MiG (50 μg/ml for 20 min) where indicated. Cells were plated onto 96-well plates coated with FN or fibronectin and incubated for 1 h at 37 °C, and then washed, fixed, and stained with crystal violet. The number of monocytes that had adhered to ECM proteins was counted in five randomly selected fields in each well. Results represent the mean ± S.E. of three experiments performed in quadruplicate. **, p < 0.01 and ***, p < 0.001.
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CD157 cross-linking by means of a mAb followed by the addition of a secondary antibody triggered the recruitment into the raft fractions of significant amounts of CD18 and CD29 (Fig. 3C). Confocal microscopy analysis on live cells confirmed these results. CD157 molecules were induced to cap by mAb cross-linking at 37 °C, and the membrane fluidity and energy intake (required to induce clustering of molecules) were blocked by incubation of the cells on ice in the presence of NaN₃ and successive fixation. Upon primary and secondary antibody binding, CD157 molecules and the area of the cell membrane with the highest concentration of GM1 gangliosides localized at the same pole of the cell membrane (Fig. 4, bottom). Co-capping experiments highlighted that polar aggregation of CD157 induced CD18 and CD29 to localize in identical areas of the membrane in the majority of cells (Fig. 4, B and D, middle, and E). These results indicated that CD157 clustering in human monocytes led β₁ and β₂ integrins into the rafts.

Increasing evidence suggests that ligand-induced oligomerization causes the activation of integrins and provides the stimulus for their association with rafts (32, 33). It was reasonable to expect that ligand-induced integrin oligomerization would cause their lateral association with CD157. To test this hypothesis, clustering of integrins was induced by incubating THP-1 cells with fibronectin. As anticipated, integrin engagement by fibronectin shifted a significant fraction of both CD18 and CD29 from the non-raft to the raft compartment (Fig. 3D). Next, the effects of interaction with fibronectin on integrin compartmentalization into lipid rafts were examined by confocal microscopy. Capping of CD29 and CD18 induced by THP-1 incubation with fibronectin was accompanied by co-localization with CD157 (Fig. 4, C and D, right) in 84 and 74% of the cells, respectively (Fig. 4E). No co-localization of CD157 with CD71 was observed in any of the experimental conditions adopted (Fig. 4, D and E). Together, these results confirmed that CD157 and integrins are interdependent partners in a functional biological network.

CD157 Is Physically Associated with β₁ and β₂ Integrins on Monocyte Membrane—The observation that cross-linking of CD157 induced co-localization of β₁ and β₂ integrins in the same areas on the membrane of THP-1 cells suggested that they might be physically associated. To test this hypothesis, we performed co-immunoprecipitation experiments. The results showed that the anti-CD157 mAb recognized its 45-kDa target protein in the anti-CD18 as well as in the anti-CD29 eluates. Reciprocally, anti-CD29 and anti-CD18 mAb were able to react with the expected 130- and 95-kDa bands, respectively, in the anti-CD157 mAb eluates. However, the anti-CD71 mAb failed to recognize its target protein in any of the eluates; reciprocally, anti-CD157, anti-CD18, and anti-CD29 mAbs failed to react with their target proteins in the anti-CD71 eluates, demonstrating specificity (Fig. 5A). These results indicated that, as we demonstrated in granulocytes (12), CD157 is likewise physically bound to both β₁ and β₂ integrin at the cell surface of human monocytes. These observations strongly support the hypothesis of functional cross-talk between CD157 and integrins instrumental to the control of monocyte transmigration.

CD157 Exerts Receptor Activities in Human Myeloid Cells—Topographic relocation of activated receptors, including integrins, into lipid rafts provides foci of intracellular signaling for the cell and may be considered a general mechanism that guarantees leukocyte functions. Therefore, molecules (such as CD157) that promote raft association are likely to effectively...
contribute to the transduction of intracellular signals that trigger the effector functions.

Further insights into signaling events driven by CD157 in monocytic cells were gathered by exploring the phosphorylation pathway induced on THP-1 cells through antibody-mediated cross-linking. Experiments were performed in parallel using anti-CD18 and anti-CD29 mAbs. Western blot analysis of the proteins tyrosine phosphorylated after short term stimulation at 37 °C demonstrated that CD157 clustering induced a rapid and transient increase of phosphorylation of a broad range of molecules, largely shared with the target substrates of CD18- and/or CD29-mediated activation pathways (Fig. 5B). The increase in phosphorylation reached a peak at 1 min and persisted for ~5 min, declining thereafter (data not shown). The most relevant bands featuring increased intensity as compared with the basal condition (i.e. cells treated with F(ab')2 RaMlgG for 1 min at 37 °C) were at 130, 110, 65, 44, and 42 kDa (as indicated by arrows) (Fig. 5B). Western blot analysis performed using substrate-specific antibodies indicated that target substrates included FAK (p130), c-Src (p65), the serine/threonine kinase Akt (a key downstream effector of p110 phosphoinositide 3-kinase), and MAPK ERK1/2 (p42–44) (Fig. 5C).
CD157 is a GPI-anchored molecule with no intrinsic signaling capacity; it has been hypothesized that the molecule establishes physical association with transmembrane receptors to transduce signals. The structural association between CD157 and both $\alpha_\text{H9252}1$ and $\alpha_\text{H9252}2$ integrin and the coexistence of CD157 with selected integrins in specific membrane domains provided the rationale for identifying integrins as likely candidates for being CD157 partner proteins. Evidence was sought by inducing combined clustering of CD157 and $\alpha_\text{H9252}1$ integrin or CD157 and $\alpha_\text{H9252}2$ integrin on the plasma membrane of THP-1 cells by means of antibody cross-linking. The results showed that concurrent ligation of CD157 with either CD18 or CD29 is accompanied by a sharp increase in phosphorylation of c-Src, Akt, and ERK1/2, with no variations in the absolute amount of c-Src, Akt, and ERK1/2 in the cell lysates (Fig. 5, B–D). These results indicated that CD157 can use both $\alpha_\text{H9252}1$ and $\alpha_\text{H9252}2$ integrins as tools in signal transduction. This leads to the activation of distinct downstream signaling pathways converging on the MEK/ERK pathway, which is responsible for controlling crucial aspects of monocyte extravasation.
DISCUSSION

Monocyte recruitment to extravascular sites is an important component of the inflammatory response to a variety of stimuli such as bacterial infections, tumor growth, and atherosclerotic plaque formation. The surface expression and release (as soluble proteins or membrane vesicles) of a number of molecules serves to coordinate and fine-tune the local inflammatory response according to the particular circumstances. Here we present evidence that CD157 is one of these molecules. We used antibody-mediated cross-linking of CD157 to mimic its natural clustering (a strategy commonly adopted for initiating signal transduction, although the actual mechanism of how this works remains unclear) and demonstrated that CD157 cross-linking promotes \( \beta_1 \) and \( \beta_2 \) integrin relocation and orchestrates the assembly of signaling-competent membrane domains. The outcome is regulation of monocyte behavior. This unique role of CD157 accounts for its involvement in critical functions of monocytes, including (i) transendothelial migration and (ii) adhesion to ECM proteins. CD157 operates both in non-inflammatory as well as in inflammatory conditions. Indeed, the extent of the inhibitory effects of blocking mAb on transmigration across quiescent and activated endothelium is comparable. The results obtained in adhesion assays strengthened this assumption. Indeed, clustering of CD157 by the addition of a secondary cross-linking antibody at the beginning of or during the process is sufficient to significantly restore transmigration, suggesting that CD157 cross-linking triggers signal transduction. The affinity for lipid rafts is an inherent feature of integrins as it is shared by integrins activated in several ways (43). Conceivably, in addition to the interaction with their ligand, integrins could be held within the lipid rafts as a result of their lateral association with membrane proteins residing within them (44, 45). Our results demonstrate that this is the case for CD157 on monocytes. Indeed, following CD157 aggregation, a considerable amount of both CD18 and CD29 moves to the lipid raft compartment of the cell membrane, resulting in subsequent activation of downstream signaling. The ability to form large raft patches with signaling activity following cross-linking is a feature shared by GPI-anchored molecules (46); instead, the promotion of integrin compartmentalization into lipid rafts is not a prerogative of all GPI-anchored molecules. For example, CD87 cross-linking on myeloid cells is unable to promote \( \beta_2 \) integrin redistribution into lipid rafts (47). The existence of physical interactions between CD157 and integrins provides a compelling basis for motivating their functional interplay; however, the molecular bases supporting the structural interaction of CD157 with different integrins remain unclear and deserve further investigation. These findings indicate that CD157 (i) behaves as a molecular organizer of membrane microdomains and (ii) is part of the integrin-driven molecular machinery that regulates crucial functions of monocytes, including transendothelial migration and adhesion to ECM proteins.

To explore the signal transduction cascade raised by CD157 engagement, we assessed the phosphorylation status of selected proteins upon CD157 antibody cross-linking and demonstrated that CD157-mediated intracellular signaling relies on integrin/Src/FAK, leading to increased activity of downstream MAPK/ERK1/2 and PI3K/Akt pathways. These pathways are core components of the integrin-mediated signaling network coordinating complex biological responses of monocytes, such as cell growth, adhesion, and migration, in normal and pathological conditions (48).
Role of CD157 in the Control of Monocyte Functions

In this dynamic interplay between CD157 and integrins, not only does CD157 share effector targets with $\beta_1$ and $\beta_2$ integrins, which represent crucial checkpoints of leukocyte trafficking, but it also acts as a stimulatory molecule and cooperates with both integrins in the control of cell migration and adhesion. Indeed, concurrent engagement of CD157 and integrins resulted in increased tyrosine kinase receptor phosphorylation and PI3K and MAPK signaling cascade activation. This is consistent with previous reports demonstrating the need to aggregate integrins on monocyte plasma membrane to achieve maximal tyrosine phosphorylation leading to optimal effector functions (49, 50).

It is easy to imagine that in a physiological context, CD157 engagement by its non-substrate ligand (so far unknown) recruits pre-existing $\beta_1$ and $\beta_2$ integrin nanoclusters (50) into lipid rafts, thus influencing their three-dimensional organization and promoting the transduction of intracellular signals, which drive efficient cytoskeletal rearrangements, cell adhesion, and transmigration. These conclusions are further supported by the observation that neutrophils from patients with paroxysmal nocturnal hemoglobinuria (which express neither CD157 nor other GPI-anchored molecules as a result of an acquired genetic mutation) (51) are characterized by defective transendothelial migration and adhesion to ECM proteins (11, 14).

In conclusion, these results begin to outline a model where CD157 controls leukocyte extravasation through relocation of integrins into signaling-competent microdomains (i.e. signalosomes), which are required to efficiently recruit protein tyrosine kinases and scaffolding proteins and to switch on the complex network of interconnected signaling pathways (21, 52).

Integrins are attractive targets for the treatment of inflammatory and autoimmune diseases, and several specific antibodies have been tested in clinical trials. However, the integrin-blocking approaches have significant side effects related to impaired host response (53). The existence of CD157 as an integrin-associated molecule that mediates specific functions offers the potential for fine-tuning integrin functions and permitting the development of improved therapeutic strategies in inflammatory diseases such as arthritis and atherosclerosis.

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