The Journal of Immunology

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J Immunol 1998; 160:395-402; ;
http://www.jimmunol.org/content/160/1/395

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Human CD38 (ADP-Ribosyl Cyclase) Is a Counter-Receptor of CD31, an Ig Superfamily Member[1]

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Human CD38 is a cell surface molecule involved in the regulation of lymphocyte adhesion to endothelial cells. This suggests that HUVEC bear a ligand(s) for CD38 on the cell surface. By means of the mAb Moon-1, which specifically inhibits CD38-mediated cell adhesion, we have identified a *trans*-membrane 130-kDa molecule acting as a ligand for CD38. Here, we report that the molecule recognized by the Moon-1 mAb is CD31, a member of the Ig superfamily. This conclusion is based on 1) cross-inhibition assays between Moon-1 and reference anti-CD31 mAbs; 2) sequential immunoprecipitation experiments using Moon-1 and known anti-CD31 mAbs, and 3) reactivity of the Moon-1 mAb with CD31 transfectants. Further, CD31 and CD38 cognate interactions were found to modulate heterotypic adhesion as well as to implement cytoplasmic calcium fluxes identical to those obtained by means of agonistic anti-CD38 mAbs. Other effects tested included the synthesis of messages for a panel of cytokines, markedly increased upon receptor-ligand interactions. These results suggest that the interplay between CD38 and its ligand CD31 is an important step in the regulation of cell life and of the migration of leukocytes (and CD38$^+$ cancer cells) through the endothelial cell wall. ***The Journal of Immunology,*** **1998, 160: 395–402.**

Leukocyte migration and homing is an orchestrated process started by casual collisions with the endothelial layer surrounding vessels of every type. Loose and transient adhesion established between leukocytes and HUVEC leads to rolling (or tethering), a crucial event in the short term cell-to-cell contacts that enable the preparation of the following steps in the adhesion cascade (1, 2). This phenomenon is thought to be mediated by selectins, a family of molecules linked by structural and functional homologies.

Human CD38 is a single-chain 45-kDa type II glycoprotein with a unique pattern of surface expression, present on early hemopoietic cells, lost during maturation, and reexpressed during cell activation (3). As predicted based on its homology with *Aplysia californica* ADP-ribosyl cyclase, human CD38 was shown to act as a bifunctional ectoenzyme, which leads to the synthesis of cyclic ADP-ribose, a putative endogenous regulator of calcium-dependent calcium release (4, 5). Other clues to CD38 functioning have often seemed contradictory. On the one hand, CD38 seems to act as a channel that delivers activation signals in T, B, and NK cells (6), to induce cytokine production and secretion (7), and to prevent germinal center cells from undergoing apoptosis (8). On the other hand, CD38 is a messenger of death to human B cell precursors in the bone marrow, where ligation of the molecule inhibits proliferation and induces apoptosis through the activation of the *syk* tyrosine kinase, and phosphatidylinositol 3-kinase pathway (9).

Finally, the CD38 molecule has been shown to perform as a selectin, regulating the weak adhesion events that take place between circulating CD38$^+$ lymphocytes and HUVEC before integrin-mediated binding. This finding started the search for a putative ligand hidden on the HUVEC membrane (10). A putative CD38 ligand (CD38L)[3] was indeed identified by means of a mAb (Moon-1), selected by virtue of its ability to block CD38-mediated adhesion on the HUVEC side (11). The CD38L is a ~130-kDa single-chain protein constitutively expressed at high levels by HUVEC throughout the body, and variably present on T and B lymphocyte subsets, NK cells, granulocytes, and platelets (12). The availability of a recombinant soluble form of CD38 enabled us to prove the existence of a direct binding occurring with the CD38L immunoprecipitated from HUVEC lysates. The study also included the evaluation of some of the functional features of CD38L: ligation of the molecule by the specific mAb induced a spike in cytoplasmic Ca^{2+} levels ($[Ca^{2+}]_i$) in the Jurkat T cell line and increased [^{3}H]thymidine uptake in PBMC preparations.

We now report that the molecule recognized by Moon-1 mAb is CD31, a member of the Ig gene superfamily characterized by six Ig-like domains and by a unique adhesive ability mediated by homo- and heterophilic mechanisms (13–15). The initial hypothesis, based on coincidence of molecular weight, distribution pattern, and functional features, was confirmed first by modulation experiments using reference anti-CD31 mAbs and then at a molecular level using murine cells transfected with human CD31 cDNA as target for the Moon-1 mAb.

We observed that CD38/CD31 cognate interactions influence adhesion between HUVEC and CD38$^+$ cells and initiate a signaling pathway delivering messages to the cytosol and the nucleus,

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Received for publication May 23, 1997. Accepted for publication September 8, 1997.

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[1] This work was supported in part by grants from Telethon, Associazione Italiana Ricerca Cancro (AIRC), Special Projects ACRO (Consiglio Nazionale Ricerche (CNR)), AIDS, and TB (Istituto Superiore di Sanità, Roma, Italy), the Austrian Science Fund, and by the Cassa Risparmio Torino (CRT; Torino Italy) and Cariverona (Verona, Italy) Foundations.

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[3] Abbreviations used in this paper: CD38L, CD38 ligand; GAM, goat anti-mouse.

0022-1767/98/$02.00

characterized by rapid and complex effects, such as modulation of surface expression, variations in $[Ca^{2+}]_i$, and induction of messages for a panel of cytokines previously shown to be affected by CD38 signaling induced by agonistic mAbs.

Considered together, these findings shed some light on the dissection of leukocyte homing events and may lead to the development of new therapeutic drugs able to block adhesion in autoimmune diseases and chronic inflammatory states and to stop tumor diffusion in the body.

Materials and Methods

Monoclonal Abs

The Moon-1 hybridoma is derived from a fusion between the X63-Ag8.653 cell line and splenocytes obtained from female BALB/c mice immunized by injecting noncultured HUVEC directly into the spleen. The mAb was selected from a panel of 53 reagents reactive with HUVEC because of its ability to interfere with CD38-mediated lymphocyte adhesion to endothelium (11). mAbs specific for human CD31 that were used in this study are V21 (IgG2a, from T. Springer, Boston, MA), 1B5 (IgG1, from W. Knapp, Vienna, Austria), 5F4.9 (IgG1, from F. Bussolino, Torino, Italy), and MCA731 from Serotec (Oxford, U.K.). Anti-CD38 reagents included agonistic mAbs such as IB4 (IgG2a) (16) as well as IB6 mAb, an IgG2b able to bind CD38 without inducing any detectable transmembrane signaling. Other mAbs used were Moon-4 (IgG1 specific for CD29 and produced in the same fusion as Moon-1), Stroma-5 (IgG1 specific for a single chain of 70 kDa expressed at high levels by HUVEC), CB05 (anti-CD44), 10D12 (anti-CD11a), NL02 (anti-HLA class I), and 6F4 (anti-CD59); CB13 (anti-HLA class II), CB19 (anti-CD19), and CB20 (anti-CD20) were locally produced and purified, whereas 1E9 (anti-CD73) was provided by L. Thompson (Oklahoma City, OK). Other mAbs included were MCA883 (anti-CD62E), MCA796 (anti-CD62P), and MCA793 (anti-CD31) (all from Serotec). LECAM-1 (anti-CD62L) as well as anti-CD56 and anti-CD14 mAb were purchased from Becton Dickinson (San Jose, CA).

Cells and cell lines

HUVEC were isolated from segments of normal term umbilical cord veins and were cultured to confluence in medium containing PMA (5 ng/ml) for 2 days in 24-well plates (17). CD31 and CD38 transfectants were obtained by transfecting specific cDNAs into murine L fibroblasts and NIH-3T3 cells, respectively (18, 14). The cell lines used for this study were Jurkat and SUPT-1 (T lymphoblastoids) and the myelomonocytoid line U937. The majority of experiments performed on Jurkat were done on cells cloned by limiting dilution or selected by MACS (Miltenyi Biotech GmbH, Bergisch-Gladbach, Germany), to obtain populations displaying a homogeneous phenotype. Based on CD3, CD38, and CD31 surface expression, we have currently stabilized 3 main different Jurkat phenotypes: $CD3^+CD38^+CD31^+$, $CD3^+CD38^-CD31^-$ and $CD3^+CD38^+CD31^-$. PBMC were prepared as described in detail (11). Spleen samples from healthy adult individuals were obtained through the Department of Immunology and Transplantation, S. Giovanni Hospital, Torino, Italy. Thymuses and bone marrow samples were obtained through the Department of Pathology of Regina Margherita Hospital, Torino, Italy. Cell surface molecules were tested by direct or indirect immunofluorescence, using a FITC-labeled $F(ab')_2$ anti-mouse Ig antiserum (Silenus, Hawthorn, Australia) as staining Ab. The samples were analyzed using a FACSort cytometer (Becton Dickinson). Purified T cells were obtained from PBMC preparations from healthy donors by negative selection procedures, to avoid any subliminal activation triggered by mAb binding to T lymphocyte receptors. In brief, PBMC preparations underwent two cycles of a mixture (2 μg/10^6 cells) of anti-CD19 + anti-CD20, anti-CD16 + anti-CD56, and anti-HLA class II + anti-CD14: this protocol enabled us to obtain an ~98% purity, as assessed after checking CD3 and CD2 expression.

Radiolabeling, immunoprecipitation, and electrophoretic analysis

U937 cells (5×10^7) were labeled by lactoperoxidase-catalyzed surface iodination and lysed in 1 ml of buffer composed of 1% Nonidet P-40, and 140 mM NaCl plus 1 mM PMSF, aprotinin (15 μg/ml), and leupeptin (10 μg/ml), in 25 mM Tris-HCl, pH 8.0. Lysates were precleared by incubation with goat anti-mouse (GAM) IgG-agarose beads (Sigma Italia, Milan, Italy), and the precipitation conducted by incubation of precleared lysates with mAbs (Moon-1 or 5F4.9, used as reference anti-CD31 mAb)-coated GAM-agarose beads. Stroma-5, an isotype-matched mAb, specific for a 70-kDa single-chain structure, was included as the control of the experiments. In sequential immunoprecipitations, aliquots of the precleared lysate were first incubated with excess Moon-1 or 5F4.9 mAb-coated GAM-agarose beads. The residual lysates were then subjected to simultaneous immunoprecipitation for 4 h at 0°C with 5F4.9 or Moon-1 mAb, respectively. A final immunoprecipitation was conducted using the irrelevant Stroma-5 mAb. The material was eluted from the beads with sample buffer (2.3% SDS, 10% glycerol, 1 mM iodacetamide, and 65 mM Tris-HCl, pH 6.8) for analysis on SDS-PAGE, performed on 7.5% acryl/bis SDS slab gels. Following SDS-PAGE, gels were fixed in 10% acetic acid/30% methanol, dried in a Bio-Rad (Richmond, CA) gel drier, and placed on x-ray film (Kodak X-Omat AR, Rochester, NY) at −70°C for autoradiography, using Cronex Dupont (Sigma Italia) intensifying screens.

Binding assay

The dynamic binding assay adopted in this work was devised to minimize integrin-mediated adhesion. The assay relies upon binding evaluated at 4°C on 24-well plates lying on a rocking shelf to prevent static interactions between cells (10). HUVEC were plated in a 24-well plate, cultured to confluence in RPMI 1640 + 10% FCS containing PMA (5 ng/ml), and washed three times with the same medium. PMA treatment was found to greatly enhance HUVEC adherence to the culture wells and to reduce the risk of HUVEC detachment during the washings of the binding assay. Cells to be tested were labeled with 50 μl of ^{51}Cr (Amersham, Bucks, U.K.) for 1 h, washed twice with PBS, and dispensed (5×10^5 cells/well) on HUVEC-coated 24-well plates in RPMI 1640 + 10% FCS in the presence of the appropriate mAb (10 μg/ml). After a 15-min incubation, plates were spun at $120 \times g$ for 30 s, laid on a rocking shelf, and incubated for a further 20 min. The experiments were performed at 4°C working in a cold room, keeping cells on ice, and using cold medium (0.5 ml/well). The wells were then gently washed three times with 1 ml of cold RPMI 1640 + 10% FCS dispensed with a 10-ml pipette. Bound cells were lysed with 1 ml of 2% Triton X-100 and radioactivity measured in a gamma counter. The absolute binding was calculated as follows:

$$\frac{\text{sample counts} - \text{minimal control counts}}{\text{maximal control counts} - \text{minimal control counts}} \times 100.$$

Minimal control counts were obtained by dispensing the cells in wells without HUVEC, whereas maximal control counts were obtained by measuring the total radioactivity of the cell aliquots seeded in each well.

The relative percentage of binding used in this work was calculated as follows:

$$\frac{\text{absolute sample binding}}{\text{absolute control binding}} \times 100.$$

Sample and control bindings were measured in the presence (and in the absence) of the relevant mAb, respectively. The data reported in Figure 4 are representative of five to seven experiments: statistical analyzes were performed using the nonparametric Mann-Whitney test.

Down-modulation experiments

The effects of modulation of surface expression of CD38 was assessed in vitro by allowing the two molecules to interact and cross-talk in appropriate conditions, in which the interaction between receptor and ligand is usually followed by reduction of at least one of the two partners. Briefly, several $CD38^+$ cell populations were cocultured (RPMI 1640 + 10% FCS, 37°C, 5% CO_2,) with CD31 transfectants previously dispensed in six-well plates and left to adhere for 12 h. The optimal ratio of $CD38^+$ cells:CD31 transfectants was found to be 5:1, the most marked effects being seen after 5 h. CD38 surface expression was evaluated using an indirect immunofluorescence test, and the samples were analyzed using a cytofluorometer. Down-modulation of the molecule was observed in all of the cell populations examined, i.e., PBMC, thymocytes, splenocytes, and bone marrow cells. The same experiments were confirmed using different T and B cell lines expressing CD38 at high levels (e.g., SUPT-1 and Raji).

Determination of Ca^{2+} currents

Changes in intracellular Ca^{2+} concentrations in Jurkat cells were determined by flow cytometry. Jurkat cells (5×10^6 cells/ml) cloned to be $CD3^+/CD38^+/CD31^-$ were resuspended in HBSS and incubated for 30 min at 37°C with the detergent Pluronic F127 (1 μg/ml) and the Ca^{2+} binding fluorochrome Fluo-3/AM (2 μg/ml) (both from Sigma Italia). A variant clone of Jurkat cells not expressing CD38 (Jurkat $CD38^-$) was included as negative control. Cells were then washed twice in RPMI 1640 and kept in the dark at 4°C. CD31 transfected and untransfected cells were collected from the culture, washed twice in RPMI 1640, and similarly

maintained at 4°C. CD31 transfected and untransfected fibroblasts and target Jurkat cells were then mixed (10:1) in RPMI 1640 and centrifuged (1 min at 4°C, not exceeding 200 × *g*) to allow the formation of heteroconjugates. The cells were subsequently incubated for 1 or 2 min at 37°C. The pellets were finally resuspended and the conjugates gently disrupted for an immediate analysis on a FACSort cytometer (Becton Dickinson). Forward and right angle scatter were used to gate selectively on Jurkat cells after adopting an appropriate setting of the cytofluorograph. Quality controls of the gate specificity included immunofluorescence tests using species-specific markers (data not shown). Excitation was from an argon laser at 488 nm, and the emission at 525 nm was measured on a linear scale. Using the FACSort software, it was possible to acquire 2×10^3 events for each time point, while the analysis of the acquired data was performed using Lysis II software (Becton Dickinson). Fluorescence mean is representative of the $[Ca^{2+}]_i$ in Jurkat cells.

The same experiments were performed in the presence of anti-CD31 and anti-CD38 as blocking mAbs. A first set of tests was done by incubating CD31 transfected and untransfected cells with Moon-1 mAb or an irrelevant NL02 (anti-HLA class I) mAb (10 μg/10^6 cells) at 4°C for 30 min. After washing the unbound mAb, mouse fibroblasts were mixed with the specific Jurkat clones labeled with Fluo-3, and $[Ca^{2+}]_i$ levels were measured after a lag of 2 min.

A second set of experiments was devised using as blocking tool the IB6 mAb, a nonagonistic anti-CD38. The loaded Jurkat clones were resuspended in a medium containing IB6 at a concentration of 10 μg/ml, incubated briefly (10 min), and cocentrifuged with CD31 transfected or untransfected cells. The irrelevant NL02 mAb was added as control.

PCR-assisted mRNA amplification for selected cytokines

To determine whether CD31/CD38 cognate interactions were inducing mRNA for cytokines, purified T cells were incubated with CD31 transfected cells for 6 and 24 h, respectively, using untransfected cells as controls. After coculture at 37°C in a 5% CO_2 incubator, cells were collected and washed in PBS and the total cellular RNA extracted following the guanidium-isothiocyanate method (7). RNA (1 μg in a 20-μl reaction volume) was transcribed using Moloney murine leukemia virus reverse transcriptase, and PCR amplification was conducted starting from as low as 1 ng of original RNA. Cytokine-specific primer pairs were synthesized according to published sequences (DNA synthesizer, Applied Biosystems, Inc., Foster City, CA). PCR was performed in a 9600 Perkin-Elmer (Foster, CA) thermal cycler, as previously described (7). The reaction product was visualized by electrophoresis using 10 μl of the reaction mixture. The relative density of the ethidium bromide-stained PCR reaction products was determined by using a Pharmacia-LKB (Uppsala, Sweden) Ultrascan XL densitometer. The actual densitometric values for each cytokine mRNA were normalized using β-actin densitometry as the 100% reference with all other values expressed as the percentage of this figure.

Immunoenzymatic assay of cytokine production

The amounts of IL-6, IL-10, and IFN-γ produced by purified T cells (1×10^6) after 24 h of culture were measured by immunoenzymatic techniques, using commercially available kits (R&D Systems Inc., Abingdon, U.K.) following the manufacturer's instructions.

Results

Sequential immunoprecipitation

The initial findings of the modulation experiments were confirmed by sequential immunoprecipitation, which verified that Moon-1 and the reference anti-CD31 mAb 5F4.9 are reactive with the same molecule. As shown in Figure 1, cell lysates deprived of the fraction recognized by the Moon-1 mAb or anti-CD31 mAb 5F4.9 were subsequently used for a stepwise cycle of precipitations with 5F4.9 or Moon-1 mAb, respectively. Depletion of the Moon-1 Ag from a ^{125}I-surface labeled preparation of membranes from U937 cells is followed by the loss of any detectable 130-kDa band reactive with the 5F4.9 mAb. Reciprocal results were obtained by reversing the order of the precipitating mAb, in which the deprivation of the CD31 molecule by the reference 5F4.9 mAb causes the Moon-1 Ag to disappear. The isotype-matched Stroma-5 mAb was obtained as part of an ongoing effort to define new molecules expressed by HUVEC and stromal cells from the bone marrow of healthy individuals. This mAb was selected because of its isotype (IgG1) and because the epitope recognized displays a density on

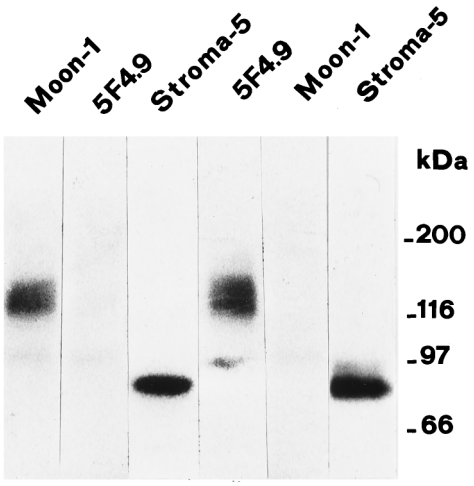

FIGURE 1. Sequential immunoprecipitation of Moon-1 and CD31 molecules and 7.5% SDS-PAGE analysis. When deprived of the 130-kDa single-chain band precipitated by Moon-1 mAb (*lane 1*), the lysate does not provide any apparent structure reactive with the reference anti-CD31 mAb (*lane 2*). However, the 70-kDa single-chain molecule recognized by the isotype-matched control, Stroma-5 mAb, is unaffected, and the relevant band is clearly apparent (*lane 3*). By reversing the order of the precipitating mAb, binding of the 130-kDa by 5F4.9 mAb (*lane 4*) fully depletes the pool of CD31 molecules: no chains are revealed in these conditions by Moon-1 mAb (*lane 5*). These conditions do not alter unrelated molecules precipitated by the isotype-matched control mAb (Stroma 5, *lane 3* and *6*).

HUVEC similar to that displayed by Moon-1 mAb. As clearly shown in Figure 1, neither condition alters the structure and precipitability of the control molecule.

Moon-1 mAb is specific for CD31

To formally prove that the epitope recognized by the Moon-1 mAb is located on human CD31, murine L fibroblasts and simian COS cells were transfected with CD31 cDNA produced in the laboratory of one of the authors (H.S.). Transfection of cDNA resulted in de novo cell surface expression of the human molecule by the recipient murine cells, clearly stained by Moon-1 and by the reference anti-CD31 mAbs. In contrast, mock-transfected cells or wild-type cells failed to show any detectable reactivity with the whole set of mAbs (Fig. 2).

Further, an epitope analysis using a panel of reference anti-CD31 reagents was performed. In short, experiments based on competition between the binding of ^{125}I-labeled Moon-1 and the unlabeled anti-CD31 mAbs to common target cells were designed. As shown in Figure 3, the use of excess amounts of all of the reference anti-CD31 mAbs hinders ^{125}I-labeled Moon-1 binding to its target; the most marked inhibition was obtained with the V21 mAb. The observation that all of the mAbs tested so far in competition with Moon-1 provide only a partial binding inhibition is confirmed by the finding that the Moon-1 epitope is located in the second Ig loop of CD31, while the majority of anti-CD31 mAbs react with loop 1. Importantly, the second loop is considered to be involved in heterophilic adhesion mechanisms (19, 20).

Adhesion assays between CD38$^+$ cell lines and CD31 transfectants

The ability of anti-CD31 mAbs to inhibit lymphocyte binding to HUVEC was assessed by means of a heterotypic cell binding test, a dynamic assay that minimizes integrin-mediated binding and highlights CD38-mediated adhesion (10). The Moon-1 mAb was

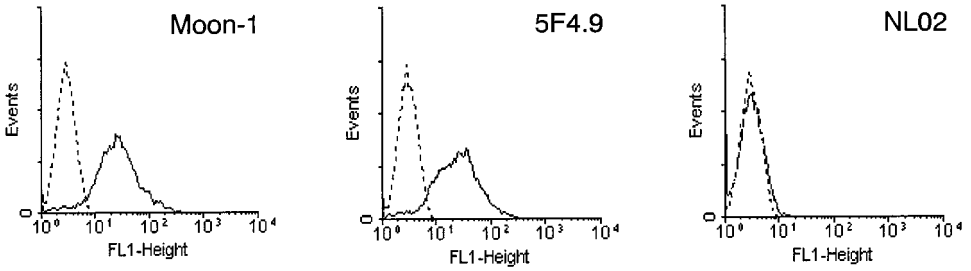

FIGURE 2. Flow cytometry analysis of mouse L cells transfected with the human CD31 cDNA and reacted with Moon-1 mAb, with control anti-CD31 5F4.9 mAb, and with isotype-matched NL02 mAb (anti-HLA class I). *x*-axis, fluorescence intensity/cells; *y*-axis, number of cells registered/channel.

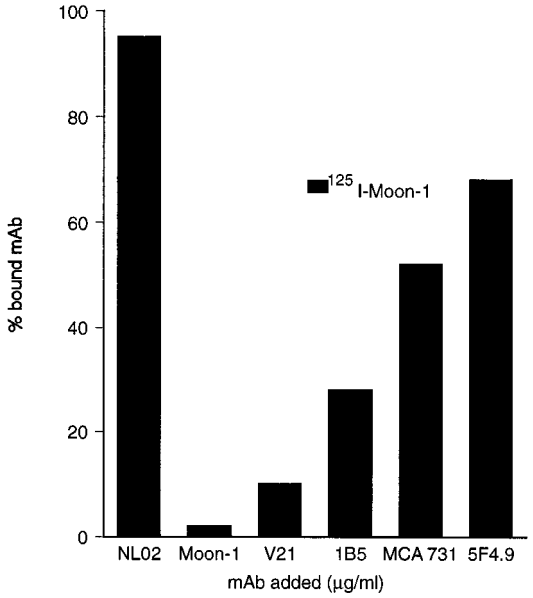

FIGURE 3. Competition assay between ^{125}I-labeled Moon-1 and different anti-CD31 mAbs. 2×10^5 CD31 transfectants were incubated at 4°C with 10 μg of different unlabeled mAbs specific for CD31, namely V21, 1B5, 5F4.9, and MCA731. NL02 (IgG1 anti-HLA class I) was the negative control (0%) and unlabeled Moon-1 the positive control (100%). After extensive washing of unbound mAbs, 2.5×10^5 cpm of ^{125}I-Moon-1 were added. Results are expressed as percentage of binding.

indeed selected because of its ability to block lymphocyte adhesion to HUVEC when this test is used. Briefly, SUPT-1 T cells were labeled with ^{51}Cr and plated on HUVEC-coated 24-well plates in the presence (and absence) of Moon-1 or mAbs specific for CD31, CD38, and other adhesion molecules (i.e., CD11a, CD44, CD62L, CD62P, CD62E). Of the panel of anti-CD31 mAbs, only MCA793 displayed adhesion inhibition levels quantitatively identical to that of Moon-1 mAb. Figure 4 (*Endothelium panel*) shows that SUPT-1 binding to HUVEC was inhibited by the addition of anti-CD31, CD38, and Moon-1 mAbs. Moreover, negative effects were also exerted by anti-CD62P and CD62E mAbs, not surprisingly so since these molecules are known to mediate lymphocyte/endothelium interactions that are effective in dynamic conditions and were induced by PMA under our experimental protocol. Lack of inhibition by the other mAbs was an expected finding, since SUPT-1 cells do not express CD62L and CD44, while CD11a-mediated adhesion is not detected by the binding assay adopted here. Confirmation that these interactions are mediated by CD31 on the HUVEC side and by CD38 on the T cells was derived from the ability of SUPT-1 to bind to plates coated with L cells transfected with human CD31 (CD31$^+$ L cells). Figure 4 (*L CD31$^+$ panel*) shows that SUPT-1 binding to CD31$^+$ L cells was inhibited by Moon-1 mAb and by mAbs to CD31 and to CD38, while mAbs to CD11a, CD59, CD73, CD62L, CD62P, and CD62E were not influential. No mAb inhibited SUPT-1 binding to control mock-transfected L cells (*L CD31$^-$ panel*).

These experiments also show that the blocking potential of anti-CD38 and CD31 mAbs is more marked on the binding of SUPT-1 to HUVEC than to CD31$^+$ L cells: this could probably be attributed to the ability of either CD38 or CD31 to interact with other ligands that take part in the adhesion to HUVEC and not to L cells (see *Discussion*).

Direct interactions between CD38 and CD31 in in vivo models measured by receptor down-modulation and $[Ca^{2+}]_i$ mobilization

The breakthrough in the definition of CD31 as ligand for CD38 was the reconstitution in vitro of a physiologic system in which the two molecules could interact with apparent and detectable effects. Cross-talk between the two molecules was shown by the effects induced by mixing CD38$^+$ cells with CD31 transfectants—a usual, even if indirect, way of highlighting a direct interaction between two molecules. Indeed, we observed a constant and significant down-modulation of the CD38 molecule, an effect not biased by other molecules, which was apparent and reproducible on cells from different lineages (PBMC, splenocytes, thymocytes, and bone marrow cells) (Fig. 5). The optimal effects were obtained 5 h after the interaction at a CD31 transfectant:CD38$^+$ cell ratio of 1:5.

CD38/CD31 interaction also gave rise to a relevant transmembrane signal: the experimental model was based on the contact between CD31 transfectants and Jurkat clones selected as CD38^{high} and CD31^{low} to nil expressors, to rule out any interference due to CD31-CD31 homotypic binding. The binding between CD38$^+$ and CD31$^+$ cells induced, in ~2 min, a significant and time-dependent increase of $[Ca^{2+}]_i$ in Jurkat cells (Fig. 6*A*). In contrast, interaction between Jurkat and untransfected cells did not modify the basal levels of $[Ca^{2+}]_i$ (Fig. 6*B*). Of relevance is the observation that the modulation of Ca^{2+} parameters in Jurkat (i.e., the ratio and time dependence) was quantitatively identical to those recorded in the same cells using the agonistic IB4 mAb (21). Also relevant in this context is the finding that Jurkat clones selected as CD38$^-$ did not provide any shift in $[Ca^{2+}]_i$ when matched with CD31 transfected or untransfected cells (not shown). This also seems to rule out the participation of other adhesion molecules in the implementation of the signal.

The effects were tested in their specificity by repeating the experiments in the presence of anti-CD38 and anti-CD31 reagents. The anti-CD38 selected to this aim was IB6 mAb, known to bind CD38 without inducing any transmembrane signaling, while the CD31 molecule was blocked using Moon-1 mAb. Results reported in Figure 6*C* indicate that the $[Ca^{2+}]_i$ mobilization induced upon CD38/CD31 interaction was almost completely blocked by using the anti-CD38 reagent, while it was significantly affected by the addition of anti-CD31 mAbs, even if at an amplitude lower than

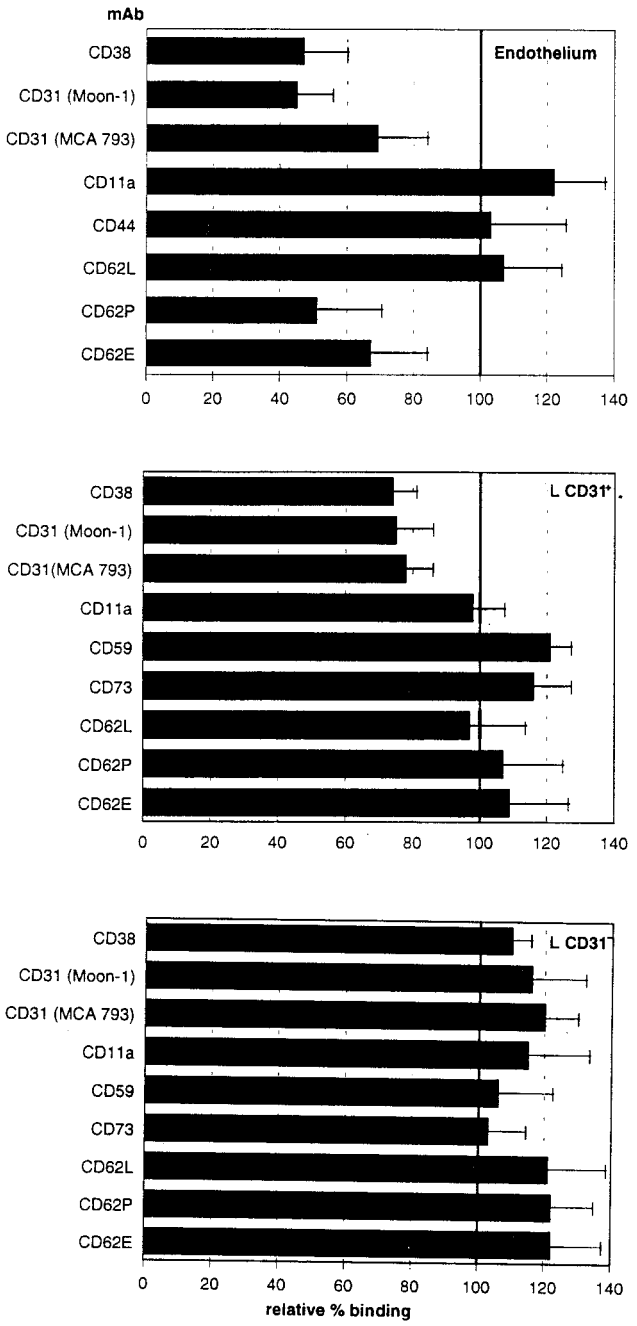

FIGURE 4. Dynamic binding of SUPT-1 cells to plates coated with HUVEC (*Endothelium panel*), CD31 transfectants (*L CD31$^+$ panel*), or control CD31$^-$ transfectants (*L CD31$^-$ panel*), performed in the presence of the relevant mAb (10 μg/ml). Binding to HUVEC is significantly inhibited by the Moon-1 mAb as well as by mAbs to CD38, CD31 (MCA 793), CD62P, and CD62E, while mAbs to CD11a, CD44 and CD62L were ineffective. As expected, binding to L CD31$^+$ cells is significantly blocked by Moon-1 and by mAbs to CD38 and CD31, but not by mAbs to CD11a, CD59, CD73, CD62L, CD62P, and CD62E. None of the mAbs tested inhibited the binding of SUPT-1 cells to L CD31$^-$ transfectants. Results are expressed as mean ± SD of the relative percentage of binding detected in five to seven experiments (see *Materials and Methods*). 100% relative binding is the cell binding in the absence of mAbs (control binding) and corresponds to a mean absolute cell binding (i.e., the % seeded cells that were bound to the cell-coated plates) of 20 ± 9% for HUVEC, of 42 ± 5% for L CD31$^+$ cells, and of 35 ± 8% for L CD31$^-$ cells. Statistical analysis was performed using the nonparametric Mann-Whitney test. The results of interactions obtained by using CD38$^+$ and CD38$^-$ heterotypes are reported elsewhere (10).

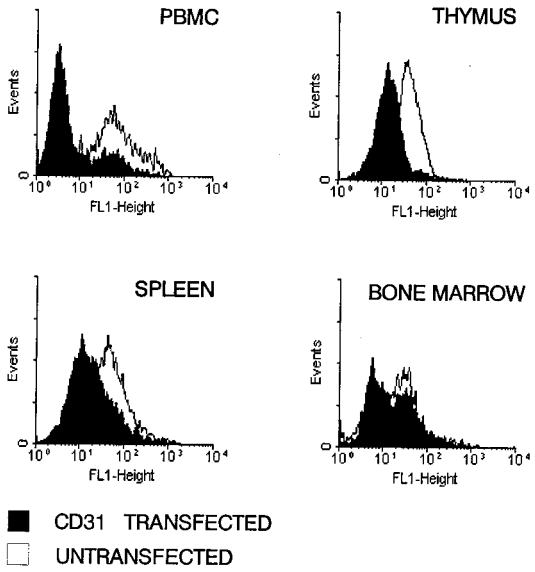

FIGURE 5. Down-modulation of surface CD38 observed upon interaction with CD31 transfectants in different cell populations (i.e., PBMC, thymocytes, splenocytes, and bone marrow cells). Target CD38$^+$ cells were coincubated with CD31 transfectants for 6 h (5:1 ratio) and analyzed using a FACSort (5000 events/sample). Linear profiles represent fluorescence levels of CD38$^+$ cells incubated with untransfected mouse L cells, while filled profiles show fluorescence levels upon interaction of the same cells with CD31 transfectants. *x*-axis, fluorescence intensity/cells; *y*-axis, number of cells registered/channel.

that of anti-CD38. The irrelevant isotype-matched NL02 mAb failed to alter in any way the profile obtained.

Direct interactions between CD38 and CD31 in the in vivo models measured by analysis of mRNA of selected cytokines and by quantitative evaluation in supernatants

These experiments were performed by exposing purified T cells to mouse transfectants expressing human CD31, as well as to control untransfected cells. The purified T cells were selected as a physiologic model in which CD38 signaling has been previously tested and analyzed in details (7). The cytokines selected for testing were those most influenced by the CD38-mediated signaling in T cells, namely IL-6, IFN-γ, IL-10, and granulocyte-macrophage CSF. As shown in Figure 7, every one of these cytokines showed increases ranging from 30 to 70% compared with basal levels. The message for IL-2, previously reported in different analytical tests as not affected by CD38 signaling, was unchanged in the present conditions. Also not significantly increased with respect to the basal levels were the messages obtained when reacting the purified T cells with untransfected fibroblasts (not shown).

The amounts of IL-6, IL-10, and IFN-γ released by purified T cells exposed to CD31 transfectants for 24 h were measured by specific immunoenzymatic kits. The results, expressed in pg/ml, are reported in Table I.

Discussion

Ever since its definition in 1981 as a lymphocyte activation marker, CD38 has proven an elusive molecule, as it is neither restricted to a definite lineage nor to mere activation only. It displays a broad distribution among cells of the immune system, its surface expression ranging from bone marrow precursors to terminally differentiated cells (i.e., plasma cells) or among cells outside the immunologic circuits (e.g., RBC) (22). Notwithstanding

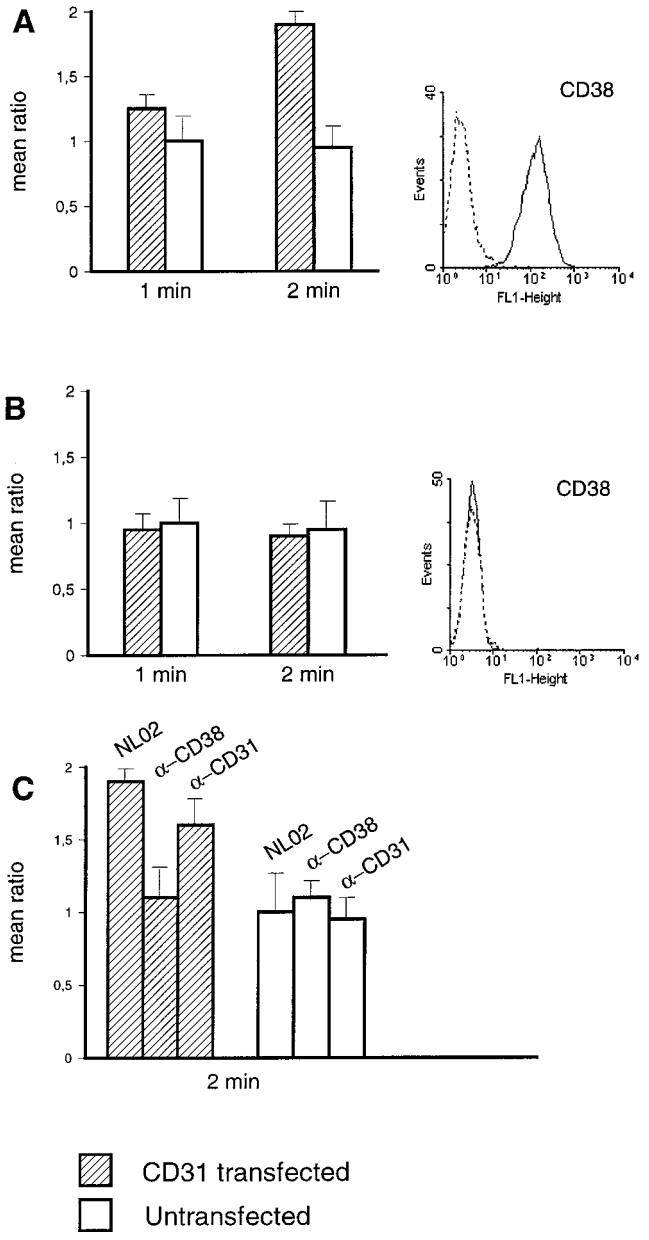

FIGURE 6. $[Ca^{2+}]_i$ modulation in Jurkat $CD3^+/CD38^+/CD31^-$ and Jurkat $CD3^+/CD38^-/CD31^-$ cells (cytofluorometric profile of CD38 expression is in the *inset*) upon binding with $CD31^+$ L fibroblasts (*A* and *B*). A lag of ~2 min is sufficient to deliver a signal inducing a significant increase of $[Ca^{2+}]_i$ (shaded bars). White bars show Ca^{2+} levels after incubation of Jurkat cells with untransfected L cells (representative of eight experiments). B section shows the same experiments performed using a Jurkat clone expressing undetectable amounts of surface CD38 (see phenotype, *inset*); no $[Ca^{2+}]_i$ modulation was observed upon interaction with $CD31^+$ or $CD31^-$ L cells. The effects observed in *A* were specifically blocked or reduced using nonagonistic IB6 mAb. Similar effects, even if reduced in amplitude, were observed when using Moon-1 mAb to bind CD31 (*C*). Jurkat cells (5×10^6 cells/ml) were loaded with the Ca^{2+}-binding fluorochrome Fluo-3/AM (2 μg/ml) mixed (10:1) with $CD31^+$ and $CD31^-$ transfectants. The cells were analyzed (2×10^3 events/sample in 5 s for each time point) on a FACSort, gating on Jurkat cells. Fluorescence mean is representative of the $[Ca^{2+}]_i$ in the Jurkat cells.

these limitations, the molecule has been used worldwide as a surface marker in many immunologic diseases and, just recently, in solid tumors (i.e., prostatic carcinoma) (23). Attention to the molecule was refueled when experiments proved its molecular and functional similarity to an enzyme purified from the mollusc *Aplysia Californica* (24). CD38 was then found to behave as a bifunctional ectoenzyme, synthesizing molecules involved in the regulation of cytoplasmic calcium levels. Further, experiments showed that CD38 can also operate as an adhesion molecule involved in cell-cell interactions, the most studied of which is the HUVEC/lymphocyte model. We recently identified a molecule displaying all of the characteristics of a ligand for CD38: ligation of this molecule by its specific mAb (Moon-1) blocks CD38-mediated adhesion of lymphocytes to HUVEC monolayers. CD38 ligand was initially described as a single-chain protein of ~120 kDa and successively characterized more accurately as a 130-kDa surface molecule. Proof of a direct interaction between the structure defined by the Moon-1 mAb and CD38 was obtained by immunochemical analyses exploiting the availability of a recombinant soluble CD38 molecule.

The identification of the molecule recognized by the Moon-1 mAb stemmed from an analysis of surface Ags sharing common features in terms of structure and tissue distribution. One of the candidates was CD31, a structure amply characterized in terms of surface expression, genetic regulation, and functional properties. Indeed, CD31 displays a structure of six iterative domains that constitute the hallmark of the Ig superfamily (15). Moreover, several studies from different groups have demonstrated that CD31 plays a key role in lymphocyte adhesion and extravasation, both through homo- and heterophilic mechanisms (19, 25, 26).

In this study, we present evidence that the CD38L recognized by Moon-1 mAb is CD31 and that CD38 and CD31 represent surface molecules linked by a functional relationship. A molecular coincidence between CD38L and CD31 was formally proved by the fact that murine fibroblasts expressing CD31 on the membrane are clearly stained by Moon-1 mAb. Supportive evidence came from modulation experiments as well as by sequential immunoprecipitations.

The adhesion assays subsequently performed demonstrated that CD38-mediated adhesion to HUVEC may be blocked either through engagement of CD38 or of CD31. The inhibitory role exerted by Moon-1 mAb, but not by all the CD31 mAbs in the adhesion assays, seems to confirm the hypothesis that domains critical for the binding do exist both on CD38 and on CD31. Information about CD31 is at the moment scant, and the only conclusion inferable at this point is that the binding region could be included in the last three domains (A. Horenstein, B.A. Imhof, and F. Malavasi, unpublished results). More precise information is available about the CD38 structure: recently defined are the domain bearing the catalytic site of the molecule as well as the domain involved in signaling and, likely, in adhesion (27). An updated map including all of the functional regions of CD38 can now be drawn. It is both interesting and intriguing that the limited region bearing the catalytic site also includes the epitopes (when different) of the agonistic mAbs, while those not giving phosphorylation or activation signals react outside this region.

To define the receptorial behavior of CD38 for CD31, we reconstituted an in vitro system in which the two molecules could interact in conditions mimicking the physiologic ones. By facing cells expressing CD31 or CD38 at high levels, we were able to induce CD38 to modulate after binding CD31 and to reconstitute a signal on living cells expressing only one of the two receptors. A significant mobilization of $[Ca^{2+}]_i$, one of the most sensitive and predictable indicator of trans-membrane signaling, was indeed observed in Jurkat clones expressing CD38 but not CD31. This confirmed previous observations based on the use of agonistic mAbs, whose binding with critical domains was believed to mimic the

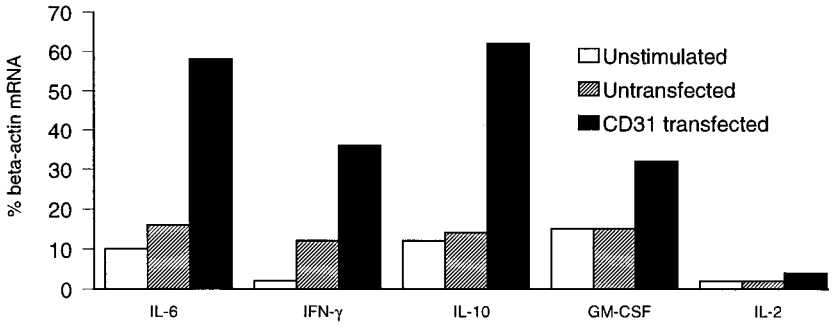

FIGURE 7. Comparative analysis of cytokine mRNA expression in purified T cells upon interaction with CD31 transfectants (black bars), untransfected cells (shaded bars), or at a baseline (white bars). After coculture at 37°C in a 5% CO_2 incubator, the cells were harvested and RNA extracted and reverse transcribed. The cDNA obtained was assayed for the presence of specific cytokine mRNA by PCR. Reaction products were run on a 1% agarose gel in the presence of appropriate controls. Data are expressed as percentage absorbance, using β-actin as reference, and represent mean values of five experiments.

Table I. *IL-6, IL-10, and IFN-γ secretion upon CD38-CD31 interactions*[a]

	None	Untransfected	CD31 Transfected
IL-6	610 ± 72	650 ± 68	820 ± 151
IL-10	44 ± 33	52 ± 24	88 ± 66
IFN-γ	800 ± 361	1100 ± 402	1900 ± 370

[a] Purified T cells (1×10^6) were incubated for 24 h with murine fibroblasts and with human CD31. Culture supernatants were then harvested and interleukin production evaluated by immunoenzymatic techniques using commercial kits. Data are representative of three experiments.

interactions with the true natural ligand. The interaction of CD38-CD31 is followed by a signal of biologic relevance not restricted to cells of tumor origin, such as Jurkat, but operative also in normal cells. The analysis of the messages for cytokines is preferred to the quantitative assay, since it is a sensitive indicator of the transduction of a biologic signal and is not affected by the external microenvironment. Indeed, after exposing resting T cells to CD31 transfectants, we observed a significant increase in the mRNA level for IL-6, IL-10, IFN-γ, and granulocyte-macrophage CSF. In line with the results on the release of cytokines are preliminary observations indicating that the NF-κB complex is also affected by CD38 signaling induced upon CD31 binding (T. Musso, Torino, Italy, and B. Aggarwal, Houston, TX, unpublished observations). Other effects currently under analysis concern the ability to upmodulate other ectoenzymes (e.g., PC-1 and CD73) for which a structural and functional relationship with CD38 has already been described (28–30), to induce synthesis of other cytokines, or to give rescue from apoptosis in selected models (e.g., in germinal center cells and also from lymphocytes derived from lamina propria). Another point to be taken into account is the role played by CD38 in association with B and TCRs (31).

Our findings indicate that CD31 acts as a counter-receptor for CD38, answering the question raised when CD38 was reported as ruling lymphocyte adhesion to HUVEC. However, unresolved issues about CD38 remain, the first one concerning the topologic paradox of an ectoenzyme working in an environment where the substrate is not present and the product of which is used inside the cell. A report on the crystal structure of *Aplysia* ribosyl cyclase has offered clues that can likely be transferred to its homologue, CD38 (32). Indeed, the enzyme contains two spatially separated pockets composed of sequence-conserved residues, suggesting that the cyclization reaction may entail the use of distinct sites. This would imply that CD38 works as a dimer, with the extracellular portions enclosing a cavity to entrap ADP-ribose, while hinge motions may be the basis for signaling mechanism(s). Alternatively, one may envision that CD38 interaction with CD31 is the first physiologic step of internalization of the CD38 molecule, which could find its substrate inside the cell and yield the final product of the reaction (R. Di Primio, manuscript in preparation). As a further alternative, the molecule could be shed with the production of an enzymatically active soluble form of CD38 in biologic fluids (33). Other mechanisms that could be involved are a flip-flop sequence or a caveolae-like system, even though there is still a debate on the existence of caveolae in lymphocytes. Open issues also concern CD31, a molecule exerting homotypic as well as heterotypic adhesion, the last one being mediated by $\alpha_v\beta_3$ integrin in man and in mouse and by a yet unidentified ligand of 120 kDa (34–36). However, the finding of a receptor with multiple ligands has become the rule rather than the exception in recent leukocyte biology and is also a lesson presented by several pathogens, such as EBV and HIV.

Finally, the process of tumor cell diffusion in the body may provide important clues as to where to test the validity of the receptor/ligand roles proposed for these molecules: if this hypothesis holds true, one may suggest the use of soluble forms of CD31 and CD38 as a tool for blocking or hampering the metastatic process or for locally blocking the adhesion between $CD38^+$ cells and pulmonary HUVEC, as is the case for retinoic acid syndrome in acute promyelocytic leukemia (37).

Acknowledgments

The authors are grateful to J. Banchereau and H. C. Lee, and to S. Albelda, who performed the epitope mapping of the Moon-1 mAb. Thanks are also given to B. A. Imhof for providing reagents and constructive criticism. This paper is dedicated to the memory of Prof. Alberto P. M. Cappa, M.D., mentor and friend.

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