Increased Serum Levels of MRP-8/14 in Type 1 Diabetes Induce an Increased Expression of CD11b and an Enhanced Adhesion of Circulating Monocytes to Fibronectin

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The recruitment of monocytes from the bloodstream is crucial in the accumulation of macrophages and dendritic cells in type 1 diabetic pancreases. Adhesion via integrins to endothelium and extracellular matrix proteins, such as fibronectin (FN), and the production of myeloid-related protein (MRP)-8, -14, and -8/14 by recently transmigrated monocytes are thought to be instrumental in such recruitment. We determined the FN-adhesive capacity and integrin expression of monocytes of type 1 and type 2 diabetic patients and related them to the subjects' serum levels of MRP-8, -14 and -8/14. Monocytes of type 1 diabetic patients displayed an increased adhesion to fibronectin in comparison with type 2 patients and healthy control subjects but had a normal expression of the FN binding integrins CD29, CD49a, CD49d, and CD49e (although CD11b and CD18 expression was increased). MRP-8/14, which was increased in the sera of type 1 diabetic patients, induced healthy donor monocytes to adhere to FN and upregulate CD11b expression in a dosage-dependent manner. The observed MRP-induced increased adhesion of monocytes to FN and upregulation of CD11b most likely contributed to a facilitated accumulation of monocytes and monocyte-derived cells at the site of inflammation, in this case the pancreatic islets. Diabetes 53: 1979–1986, 2004

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Part from lymphocytes, dendritic cells (DCs) and various types of macrophages accumulate in the type 1 diabetes pancreas (1). In the NOD mouse and the BB-DP rat, animal models of the disease, DCs and macrophages are among the first cells to accumulate in and around the islets (2–5), suggesting that these cells play a key role in the development of islet autoimmunity.

Various abnormalities have been described in the development and function of DCs, macrophages, and monocytes (important precursors for DCs and macrophages) in type 1 diabetes. In the NOD mouse, there is evidence that before insulitis onset, the development of DCs and macrophages from bone marrow precursors is disturbed (6,7), resulting in cells with a poor antigen-presenting cell (APC) function (7,8), but an enhanced inflammatory phenotype (T. Nikolic and G.B., unpublished observations). DCs have raised nuclear factor-κB (NF-κB) activity (9,10), and NOD macrophages produce higher quantities of proinflammatory compounds such as prostaglandin E2 (11,12) and interleukin (IL)-12 (13). Also in the BB-DP rat, spleen DCs are poor APCs (14), with macrophages exhibiting an elevated production of NO (15). Human studies have focused mainly on circulating monocytes. As in the animal models, cells show poor APC function and an enhanced proinflammatory cytokine production (16,17). There are, however, few studies on adhesion molecule expression and chemotactic ability of diabetic monocytes.

The recruitment of monocytes from the bloodstream must play a crucial role in the accumulation of DCs; macrophages in the pancreas and aberrances in this process may play an additional role in local abnormalities of the cells. Recruitment involves the expression of adhesion molecules on the vascular endothelium and activated monocytes, as well as the adhesion and transmigration of the monocytes (18,19). In the islets, monocytes will interact with extracellular matrix (ECM) proteins, such as fibronectin (FN), and will differentiate into macrophages and dendritic cells.

We first explored the adhesion of circulating monocytes of type 1 and type 2 diabetic patients to FN in the absence or presence of N-formyl-methionyl-leucyl-phenylalanine (fMLP), a cleavage product of bacterial and mitochondrial proteins that is generally used as a potent stimulatory agent for neutrophils and monocytes (20). After monocytes adhere to FN, a rearrangement of the cortical actomyosin cytoskeleton takes place, resulting in stretching and spreading of the monocytes on the FN-coated surface (21). We found an increased adhesion to and stretching on FN-coated surfaces of type 1 diabetes circulating monocytes in both the absence and presence of fMLP.

The increased adhesion and stretching of the total pool...
of circulating monocytes might be explained by a difference in the partitioning between different subsets of circulating monocytes. In humans, the circulating monocyte pool can be divided into CD14<sup>+</sup>CD16<sup>−</sup> and CD14<sup>+</sup>CD16<sup>+</sup> monocytes (22), which display distinct trafficking and adhesion properties (23,24). Monocyte chemokine-stimulating protein-1 upregulates CD11b expression on CD14<sup>+</sup>CD16<sup>−</sup> monocytes, but not on CD14<sup>+</sup>CD16<sup>+</sup> monocytes, as the latter are low in C-C chemokine receptor 2. This illustrates the different potential of the subpopulations to express adhesion molecules. It is notable that an expansion of the CD14<sup>+</sup>CD16<sup>−</sup> monocytes was excluded from this study.

Myeloid-related protein (MRP)-8 and -14 are calcium-binding proteins of the S100 family. These proteins form a heterodimeric complex in a calcium-dependent manner and are specifically expressed by recently transmigrated monocytes and granulocytes (28). MRP-8- and MRP-14-expressing cells dominate inflammatory reactions. Stimulation of monocytes with granulocyte-macrophage colony-stimulating factor, IL-1β, or lipopolysaccharide induces the expression and secretion of the MRP-8/14 heterodimer (29). When monocytes are stimulated with MRP-14 or -8/14, a rapid increase in their CD11b surface expression is observed (30). Although the precise function of the MRPs is still unclear, they are thought to be involved in leukocyte-endothelium interactions and to play an important role in leukocyte trafficking (30,31).

We therefore also investigated the level of MRPs in the sera of type 1 and type 2 diabetic patients and explored whether an association exists between the serum levels of MRP proteins and the adhesion of circulating monocytes of type 1 and type 2 diabetic patients to FN. In addition, we studied the relation of the MRP levels in the serum with the surface expression of various adhesion molecules (CD11b, CD18, CD29, CD49a, CD49d, and CD49e) on monocytes of type 1 and type 2 diabetic patients. We showed that the enhanced adhesion of the type 1 diabetic circulating monocyte pool to FN is due to an increased level of MRP-8/14 and that the increased serum levels of MRP-8/14 in type 1 diabetic patients correlate with an increased CD11b expression on circulating monocytes.

**RESEARCH DESIGN AND METHODS**

Heparinized blood was drawn from type 1 diabetic patients (30 men: average age 35.0 ± 3.1 years [± SE], HbA1c 8.5 ± 0.5%, duration of diabetes after initial diagnosis 15.0 ± 2.9 years; and 22 women: age 41.7 ± 2.5 years, HbA1c 8.6 ± 0.4%, duration of diabetes after initial diagnosis 22.7 ± 3.2 years) and healthy control subjects matched for age and sex (12 men: age 39.8 ± 3.4 years, 9 women: age 38.8 ± 7.7 years). In addition, blood was drawn from type 2 diabetic patients as disease control subjects (8 men: age 52.9 ± 4.0 years, HbA1c 7.8 ± 0.3%; and 14 women: age 56.9 ± 3.6 years, HbA1c 8.0 ± 0.4%). Patients with obvious vascular complications and/or recent surgical interventions were excluded from this study.

Sera were collected and used for the detection of MRP-8, -14, and -8/14. Monocytes were isolated using an adaptation of the protocol described by Fluits (32). Briefly, blood was diluted 1.5 times with PBS containing 0.1% BSA (Biowhittaker, Verviers, Belgium), layered on a Ficoll gradient (1.077 g/ml; Biowhittaker, Verviers, Belgium), and centrifuged (1000 g; Pharmacia, Uppsala, Sweden), and centrifuged (1000g, 15 min, room temperature). The cells at the interface were collected, washed, and layered on a Percoll gradient (1.063 g/ml; Pharmacia), and centrifuged (400g, 40 min, room temperature). The monocytes were collected from the interface and washed to a final purity of 85–95%.

The Medical Ethics Committee of the Erasmus Medical Center (Rotterdam) approved this study on collected blood monocytes. All subjects gave their written informed consent.

**Adhesion and spreading of patient and control monocytes.** Monocytes were suspended in RPMI 1640 containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% heat-inactivated fetal bovine serum (Biowhittaker), plated on Chambertek glass slides (0.1 × 10<sup>6</sup> cells/chamber; Nalge Nunc, Naperville, IL), and coated with 10 μg/ml fibronectin (Sigma, Steinheim, Germany). After a 15-min incubation at 37°C in the absence or presence of the stimulatory agent fMLP, the cells were washed with PBS and fixed with 4% paraformaldehyde (Sigma) supplemented with 3% glucose. The cells were permeabilized using 0.5% Triton X-100 (Sigma) and stained with 0.1 μg/ml phalloidin-fluorescein isothiocyanate (Sigma) for 30–45 min. After cells were washed and mounted on slides, adhesion was determined as the number of cells in 10 high-power fields using a fluorescence microscope at 200× magnification. Stretching was recorded as the percentage of cells with a stretched morphology (Fig. 1C). Two individuals counted at least 200 cells independently.

**Integrin expression and cell sorting.** The expression of CD11b, CD18, CD29, CD49a, CD49d, and CD49e on CD14<sup>+</sup> monocytes in heparinized blood was determined using flow cytometry on a FACScan (Becton Dickinson, San Jose, CA) and CellQuest software (BD Pharmingen). Whole blood samples were stained with the appropriate antibodies (BD Pharmingen, Alphen aan den Rijn, Netherlands), and then washed with PBS containing 0.1% BSA; the erythrocytes were lysed using BD lysing buffer (BD Pharmingen) for 10 min at room temperature. Samples were subsequently washed and analyzed.
For cell sorting, monocytes were labeled with CD14 and CD16 antibodies (BD Pharmingen). After sorting (FACS Vantage; Becton Dickinson, Amsterdam, Netherlands), the purity of the cell suspensions was >95%. Myeloid-related protein detection. Levels of MRP-8, -14, and -8/14 were measured in serum by a commercially available enzyme-linked immunosorbent assay (ELISA; Bachem, Heidelberg, Germany) according to the manufacturer’s protocol.

Adhesion assay in the presence of subject or control serum. Monocytes were obtained from a buffy coat of a healthy control donor (Sanquin Blood Bank, Rotterdam, Netherlands) using Ficoll and Percoll gradients. Donor monocytes were exposed for 4 h at 37°C to dilutions of sera of either type 1 diabetic patients or healthy control subjects. After being exposed, cells were washed and allowed to adhere to FN-coated 96-well plates for 60 min at 37°C. Proliferation of the monocytes with fMLP resulted in an increased adhesion at all time points studied, we concluded that the monocytes of type 1 diabetic patients display a higher, rather than a faster, adhesive capacity. Monocytes of type 2 diabetic patients were studied as disease controls at time point 15 min only; they had adhesion properties equal to those of healthy control monocytes (Fig. 1A and B).

After monocytes adhere to FN, a rearrangement of the cortical actomyosin cytoskeleton takes place, leading to a stretching of the cells. Figure 1C shows representative stretched monocytes to illustrate the changes in shape encountered. After 15 min of incubation, more type 1 diabetic monocytes displayed a stretched morphology than type 2 diabetic and healthy control monocytes, in both the absence and the presence of fMLP (Fig. 1D).

CD14⁺CD16⁺ and CD14⁺CD16⁻ monocytes and adhesion to FN. To study whether a putative shift in the proportioning between the CD14⁺CD16⁺ and CD14⁺CD16⁻ monocyte populations would be responsible for the above-described differences in FN adhesion of the total monocyte pool, the two monocyte populations were sorted (Fig. 2A) and their adhesion to an FN-coated surface studied. Differences could not be detected in the adhesion to FN between the two populations (Fig. 2B). We also determined the percentages of both populations in the peripheral blood of type 1 and type 2 diabetic patients and healthy control subjects. Differences in the percentages of the CD14⁺CD16⁺ monocyte populations in relation to the total monocyte population also could not be detected (Fig. 2C).

Serum levels of MRP-8, -8/14, and -14. To study the role of MRP-8/14 and -14 in the increased adhesion of monocytes of type 1 diabetic patients to FN, we determined the

RESULTS

Adhesion, stretching, and spreading of monocytes. Monocytes of type 1 and type 2 diabetic patients and healthy control subjects were isolated from the blood and allowed to adhere to an FN-coated surface in the absence or presence of fMLP. Adhesion was determined at several time points (5–120 min); monocytes reached maximal adhesion at 60–120 min. As shown in Fig. 1A and B, monocytes of type 1 diabetic patients displayed an increased adhesion to FN in comparison with monocytes of healthy control subjects at all time points studied and in the absence and presence of fMLP (although stimulation of the monocytes with fMLP resulted in an increased adhesion to FN). Because the monocytes showed an increased adhesion at all time points studied, we concluded that the monocytes of type 1 diabetic patients display a higher, rather than a faster, adhesive capacity. Monocytes of type 2 diabetic patients were studied as disease controls at time point 15 min only; they had adhesion properties equal to those of healthy control monocytes (Fig. 1A and B).
serum levels of MRP-8, -14, and -8/14. As shown in Fig. 3A–C, the serum levels of MRP-14 and -8/14 were significantly higher in type 1 diabetic patients compared with the levels found in healthy control subjects (MRP-8/14 was the most significant). MRP-8 levels were normal in type 1 diabetic patients. A close and positive correlation was found between the levels of MRP-14 and -8/14 ($r = 0.64$, $P < 0.01$) (Fig. 3D). Neither a correlation between the serum levels of both MRPs and HbA$_1c$, nor a correlation between serum levels of MRPs and patients’ age or duration of type 1 diabetes could be found (data not shown). In type 2 diabetic patients, only the level of MRP-8/14 was determined and was found to be normal (Fig. 3C).

**Serum MRP-8/14 levels in relation to monocyte adhesion to FN.** To investigate whether the increase in MRP-8/14 in the serum of type 1 diabetic patients could play a direct role in the adhesion of monocytes to FN, monocytes of a healthy control donor were exposed to sera of type 1 diabetic patients or that of healthy control subjects. Figure 4 shows that a higher induction of adhesion to FN was observed using the sera of type 1 diabetic patients in comparison with sera of healthy control subjects. This higher induction of adhesion declined with the dilution of the serum (Fig. 4A).

To demonstrate a specific role for MRP-8/14 in the induction of adhesion, sera of type 1 diabetic patients and control subjects were depleted of MRP-8/14 using a specific monoclonal antibody. Depletion of the sera was confirmed using immunoblotting (data not shown). After exposing the monocytes of a healthy control donor to the depleted sera, it was found that the adhesion-inducing capacity of the sera declined. With an irrelevant isotype control antibody, such an effect was not observed (Fig. 4B).

**Serum MRP-8/14 levels and integrin expression on monocytes.** To investigate if the high serum MRP-8/14 levels of type 1 diabetic patients could influence the expression of integrins, monocytes of healthy donors were exposed to sera that were depleted of MRP-8/14 or depleted using an irrelevant isotype control antibody. Figure 5A shows that the presence of MRP-8/14 in sera specifically upregulated the CD11b expression on healthy donor monocytes. The expression of CD18 was not influenced (Fig. 5B).

In addition, ex vivo monocytes of type 1 diabetic patients were studied to investigate whether an upregulation of CD11b could indeed be detected. Figure 6A and Table 1 show that monocytes of type 1 diabetic patients displayed a higher expression level of CD11b. Furthermore, a positive and significant correlation was found between the serum levels of MRP-8/14 ($r = 0.448$, $P < 0.05$) (Fig. 5C) and the CD11b expression on the monocytes. Such a positive correlation was also present between the MRP-14 level and the CD11b expression ($r = 0.622$, $P < 0.01$) (Fig. 5D). However, it must be noted that the expression of CD18 was also (albeit just significantly) raised on ex vivo monocytes of type 1 diabetic patients as compared with those of healthy control subjects (Fig. 6B and Table 1). Moreover, monocytes of type 2 diabetic patients showed a higher expression level of the CD11b and CD18 integrins.
means /H11550 /H18554 effect of serum depletion of MRP-8/14 by monoclonal antibodies (to values of healthy control sera (performed with 11 sera). The adhesion is expressed as a value relative to controls). The adhesion is expressed as a value relative to values of healthy control sera (n = 10, percentage above these controls). B: Increased adhesion of monocytes to FN by the serum samples of type 1 diabetic patients (W, means ± SD, n = 3) and the effect of serum depletion of MRP-8/14 by monoclonal antibodies (C, means ± SD, n = 3). The experiment was carried out three times, always showing the same phenomenon by MRP-8/14 depletion. Statistics were performed using Mann-Whitney test.

(CD11b: 569 ± 278 vs. 152 ± 78; CD18: 154 ± 59 vs. 76 ± 31).

With regard to the expression levels of the CD49d, CD49e, and CD29 integrins (combined forming the FN-binding adhesion molecules very late antigen [VLA]-4 and VLA-5), a statistically significant difference between monocytes of type 1 diabetic patients (Fig. 6C–F and Table 1), type 2 diabetic patients (data not shown), and healthy control monocytes could not be found.

DISCUSSION

In inflammatory conditions, circulating monocytes change from a quiescent, nonadhesive state into an adhesive state characterized by an increased expression of adhesion molecules and an increase in functional adhesion (18,19). This activation is associated with a higher secretion of MRP-8, -14, and -8/14, which contributes to a further adhesion of the monocytes to the endothelium and a transmigration into the tissue (30). Contact of transmigrated monocytes with the ECM induces a further expression of MRP-8/14 (33). MRP-8/14 is bound by the vascular endothelium (28,34), and secretion of MRP-8/14 by monocytes is inhibited by resting endothelial cells (35).

In this study we demonstrated that increased levels of MRP-8/14 and -14 are present in the sera of type 1 diabetic patients and are specific for this subtype of diabetes (type 2 diabetic patients had normal levels). An effect of the

hyperglycemic state can be ruled out, as HbA1c levels did not influence serum MRP-8/14. High serum levels of MRP-8/14 and -14 are also present in rheumatoid arthritis, in which the serum level of MRP-8/14 correlates with the presence of MRP-8/14 in the synovial fluid and with disease activity (35). Our data thus indicate that MRP-8/14 levels in the peripheral blood of type 1 diabetic patients may be used as a marker for the activity of the islet inflammatory response.

Proinflammatory-activated monocytes in the circulation attach to the endothelium via an interaction between adhesion molecules like Mac-1 (the heterodimer of CD11b and CD18) and leukocyte function antigen-1 (LFA-1) (CD11a and CD18) and the endothelial adhesion molecules intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (36). This interaction is the first in a sequence of events resulting in the transmigration of monocytes into the tissue (37). Blocking LFA-1 and Mac-1 on leukocytes and ICAM-1 on endothelial cells in NOD mice prevents the onset of diabetes (38–41), indicating the important role of these adhesion molecules in the development of diabetes. When monocytes have transmigrated through the endothelium, they encounter the ECM, of which FN is a major component. The monocytes attach to the ECM via integrins and a complex conjunctional action between their integrins and components of the ECM enables them to adhere, change shape, migrate, and differentiate into macrophages and DCs (42).

We have shown that circulating monocytes of type 1 diabetic patients display increased CD11b and CD18 surface expression, adhesion to FN-coated surfaces, and stretching on such surfaces. The latter observation suggests increased activity of the actomyosin cytoskeleton under circumstances of FN adhesion. Previously we reported an aberrant cytoskeletal function of FN-adherent monocytes of autoimmune thyroiditis patients when the cells are chemotacted (43). Hence there is increasing evidence that integrin expression and function are abnormally regulated in “autoimmune” monocytes. In this study, the FN adhesion and spreading of the type 1 diabetic monocytes did not appear to be due to a difference in the size of the two important subsets in the circulating monocyte pool (i.e., the CD14+CD16- and CD14+CD16+ monocyte subsets). Our data showed that these aberrations were most likely due to the raised serum MRP-8/14 levels, because serum of type 1 diabetic patients was able to induce an increased adhesion of healthy donor monocytes to FN-coated surfaces and because this activity could be blocked by specific antibodies to MRP-8/14. Although in our experiments only MRP-8/14 was depleted from the serum, MRP-14 might also contribute to the increased adhesion, as exposure of monocytes to MRP-14 has been shown to increase the expression of adhesion molecules (i.e., CD11b) (30) and stimulation of neutrophils with MRP-14 has been shown to increase both their adhesion to fibrinogen and their CD11b expression (44).

The increased adhesion of monocytes of type 1 diabetic patients to FN could be explained by a change in the affinity of the FN-specific VLA-4 and -5 integrins, given that we did not observe an increase in the molecular expression levels of these integrins. Such an increase in affinity has been shown for VLA-4, of which a pool of low-affinity...
integrins is present that can change into a high-affinity conformation upon activation, thus mediating adhesion without changing the overall surface expression (45). Determining the surface expression of the activated forms of VLA-4 and VLA-5 using specific antibodies for such activated forms could provide support this idea and should be pursued.

An increased adhesion of type 1 diabetic monocytes to FN has been previously reported, but was restricted to monocytes of patients with poorly regulated type 1 diabetics and severe vascular complications (46). None of our patients had poorly regulated diabetes or had obvious signs of vascular complications. Moreover, monocytes of type 2 diabetic patients (with similar HbA1c levels and a similar tendency to vascular complications) did not display an increased adhesion to FN.

It is interesting that the serum MRP-8/14 of type 1 diabetic patients was able to not only increase the adhesion of healthy donor monocytes to FN, but also increase the expression level of CD11b on these monocytes. In accordance with such induction, we were able to detect an increase in CD11b expression on monocytes of type 1 diabetic patients. However, we also found the CD18 expression on such monocytes raised, whereas MRP-8/14 in sera of type 1 diabetic patients was not able to enhance the expression of this integrin. Moreover, on monocytes of type 2 diabetic patients, the CD11b and CD18 surface expression was increased (and in this study in the absence of increased serum MRP-8/14 levels). This showed that the increased CD11b and CD18 surface expression on monocytes of type 1 and type 2 diabetic patients cannot be due solely to MRPs and must at least be partially explained by consequences of the disease itself, most likely poor glycemic control.

An increased expression of Mac-1 (CD11b and CD18) on monocytes of type 1 diabetic patients has been previously reported (46). There has also been, however, a reported decreased expression of CD18 on isolated monocytes in type 1 diabetic patients (47). In this latter study, monocytes were tested in isolation by density gradient centrifugation. We tested monocytes in “whole blood” samples after lysis of the erythrocytes, thus reducing stimulation of cells by the isolation procedure to a minimum. Monocytes release Mac-1 from preexisting vesicles after stimulation, which results in a rapid increase in surface expression (48). Therefore, isolation procedures may influence expression levels of Mac-1 on monocytes.

If the level of MRP-8/14 is indicative of the activity of the islet inflammatory response, it is of note that the type 1 diabetic patients used in this study had long-standing diabetes (>10 years). In most of these patients, the pancreatic inflammation must have subsided by the time of our study. Could it be that intrinsic (inborn) abnormalities in the monocytes of type 1 diabetic patients or in their endothelial cells force the cells to an intrinsic high production of MRPs and other proinflammatory substances? Such “intrinsic” abnormal “proinflammatory” monocytes have indeed been detected in prediabetes (49) and in
animal models of type 1 diabetes before islet infiltration (6–9). The determination of MRP production by blood monocytes of islet antibody–positive nondiabetic individuals and patients with established prediabetes would need to be done to answer this question (as would the simple determination of MRP-8/14 in the sera of such individuals).

In conclusion, our results showed raised levels of MRP-8/14 and -14 in the sera of type 1 diabetic patients. These factors are responsible for an increased adhesion of circulating monocytes of type 1 diabetic patients to FN and are at least partially responsible for an increased expression of CD11b on such monocytes. Such MRP-induced increased adhesion most likely contributes to a facilitated accumulation of monocytes and monocyte-derived cells at the site of inflammation, in this case, the pancreatic islets.

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**TABLE 1**

<table>
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<th>Integrins</th>
<th>Fold expression*</th>
<th>MFI type 1 diabetic patients</th>
<th>MFI control subjects</th>
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<td>CD11b</td>
<td>3.24 ± 0.52</td>
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<td>CD18</td>
<td>1.65 ± 0.13</td>
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<td>CD29</td>
<td>1.10 ± 0.03</td>
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<td>CD49a α1</td>
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<td>CD49d α4</td>
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<td>79.2 ± 4.47</td>
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</table>

Data are means ± SE. MFI, mean fluorescence intensity. *Mean fold expression relative to healthy control subjects. †Statistical significance tested using the Mann-Whitney test.
The adhesiveness of monocytic U937 cells is stimulated by pro-inflammatory agents and inhibited by adenosine 3’,5’-cyclic monophosphate. Biochim Pharmacol 39:677–683, 1990


