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

Apart 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with a poor antigen-presenting cell (APC) function (7,8), but an enhanced inflammatory phenotype (T. Nikolic and G.B., unpublished observations).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 E_2 (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 blood stream 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

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Received for publication 12 November 2003 and accepted in revised form 29 April 2004.

APC, antigen-presenting cell; DC, dendritic cell; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; FN, fibronectin; IL, interleukin; LFA-1, leukocyte function antigen-1; MRP, myeloid-related protein; NF-κB, nuclear factor-κB; VLA, very late antigen.

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of circulating monocytes might be explained by a difference in the apportioning between different subsets of circulating monocytes. In humans, the circulating monocyte pool can be divided into $CD14^+CD16^-$ and $CD14^+CD16^+$ monocytes (22), which display distinct trafficking and adhesion properties (23,24). Monocyte chemoattractant protein-1 upregulates CD11b expression on $CD14^+CD16^-$ monocytes, but not on $CD14^+CD16^+$ 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 $CD14^+CD16^+$ monocyte subset has been observed in autoimmune rheumatoid arthritis and inflammatory diseases, such as HIV infection and sepsis (25–27). We therefore investigated whether the increased adhesion of the circulating monocyte pool of type 1 diabetic patients to FN could be ascribed to a difference in adhesion of the monocyte populations. We could not, however, detect any differences in the adhesion to FN of the two populations, nor could we find a difference in the size of these populations.

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.

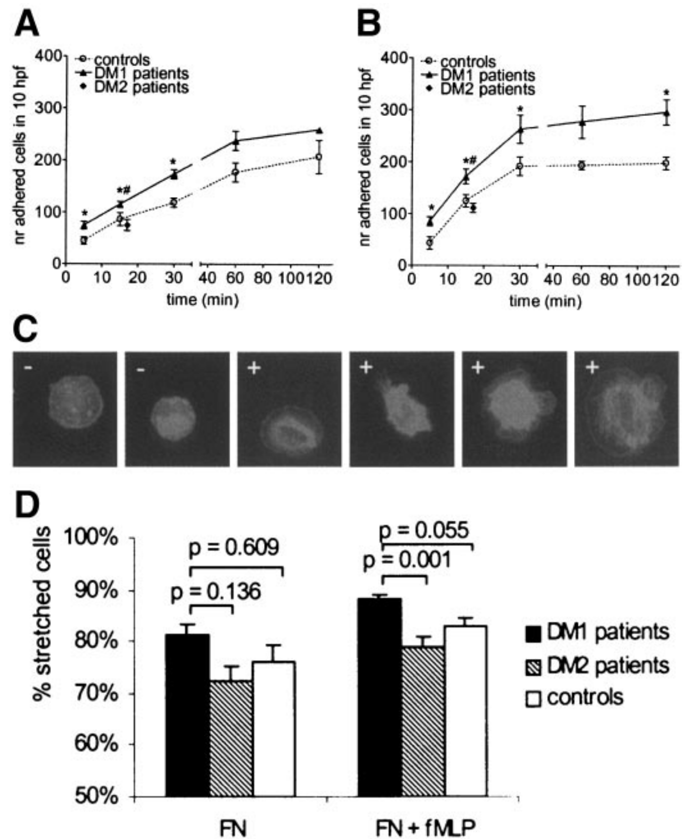

FIG. 1. Time-dependent adhesion of monocytes to FN in the absence (*A*) and presence (*B*) of fMLP. At 15 min, data represent means ± SE of 10 type 1 patients (DM1), 6 type 2 diabetic patients (DM2), and 8 healthy control subjects; at the other time points, $n = 3$ (Student's *t* test). For 15-min data, one-way ANOVA with Bonferroni correction was used. *$P < 0.05$ vs. controls, #$P < 0.05$ vs. type 2 patients. *C*: Examples of spreading and stretching (−, nonstretched; +, stretched or spread). The percentage of spread and stretched cells in the different test groups at 15 min is given in *D* (means ± SE, same *n* as in *A* and *B*, one-way ANOVA with Bonferroni correction).

RESEARCH DESIGN AND METHODS

Heparinized blood was drawn from type 1 diabetic patients (30 men: average age 35.0 ± 3.1 years [± SE], HbA_{1c} 8.5 ± 0.5%, duration of diabetes after initial diagnosis 15.0 ± 2.9 years; and 22 women: age 41.7 ± 2.5 years, HbA_{1c} 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 38.8 ± 3.4 years; 9 women: age 33.8 ± 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, HbA_{1c} 7.8 ± 0.3%; and 14 women: age 56.9 ± 3.6 years, HbA_{1c} 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 Fluks (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; Pharmacia, Uppsala, Sweden), and centrifuged (1000*g*, 15 min, room temperature). The cells at the interface were collected, washed, layered on a Percoll gradient (1.063 g/ml; Pharmacia), and centrifuged (400*g*, 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 stretching of patient and control monocytes. Monocytes were suspended in RPMI 1640 containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Biowhittaker), plated on Chambertek glass slides (0.1×10^5 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. 1*C*). Two individuals counted at least 200 cells independently.

Integrin expression and cell sorting. The expression of CD11b, CD18, CD29, CD49a, CD49d, and CD49e on $CD14^+$ monocytes in heparinized blood was determined using flow cytometry on a FACSscan (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.

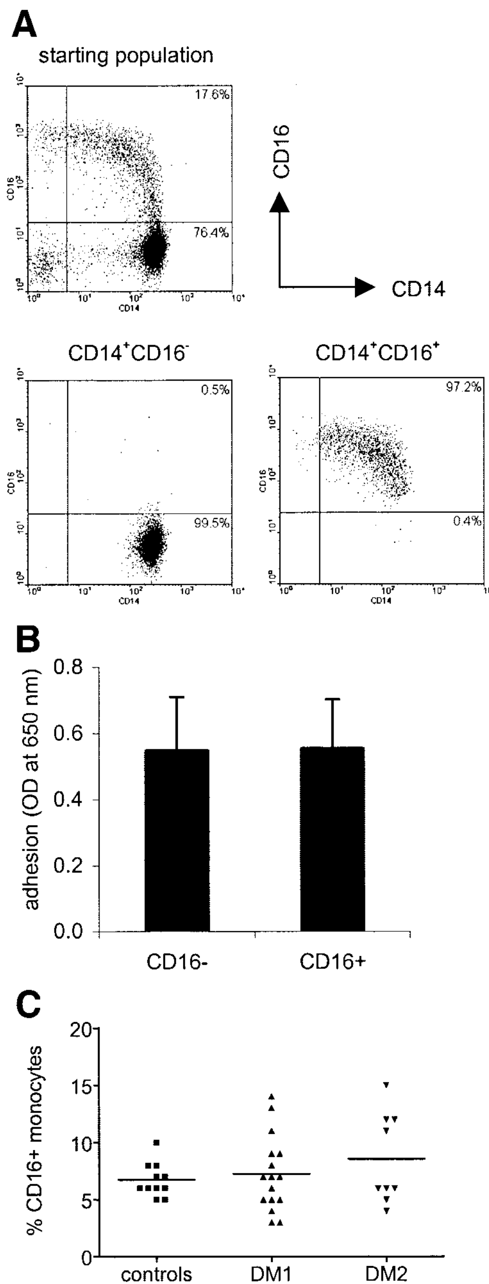

FIG. 2. $CD14^+CD16^+$ and $CD14^+CD16^-$ monocyte subpopulations were sorted and purities >95% were reached. *A*: Representative flow cytometric analyses of starting and sorted populations. *B*: Adhesion to FN of both subsets was determined; differences were not observed (means ± SD, $n = 3$). *C*: Differences were also not observed among type 1 diabetic patients, type 2 diabetic patients, and healthy control subjects regarding the percentage of the $CD14^+CD16^+$ monocyte subpopulation in the peripheral blood.

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 (note that this assay differs from the above-described adhesion assay in which adhesion was determined by individual cell counting). Cells were fixed using 20% formaldehyde (Sigma) and stained with 1% methylene blue (BDH Chemicals, Poole, England). Staining intensity was determined by measuring absorption at a wavelength of 650 nm using an ELISA reader (Thermo Lab Systems, Amersfoort, Netherlands) and a rate of cell adhesion. The assay was validated by comparing the staining intensities to direct cell counts (data not shown). In some cases, serum was depleted of MRP-8/14 by immunoprecipitation with an antibody against MRP-8/14 (Bachem); an unrelated monoclonal antibody with the same isotype was used as the control (Santa Cruz, Heerhugowaard, Netherlands). Sera were incubated with the antibodies for 60 min at 4°C and then protein A sepharose (Pharmacia) for 60 min at 4°C, and then centrifuged briefly at 12,000*g*. The supernatant was used to expose the cells, as described above.

Statistical analyses. Data were analyzed using one-way ANOVA with Bonferroni correction, unpaired Student's t test, or Mann-Whitney U test. Correlation was examined using Spearman's rho test. All data were tested for two-tailed significance. $P < 0.05$ was considered to be statistically significant.

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. 1*A* 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. 1*A* and *B*).

After monocytes adhere to FN, a rearrangement of the cortical actomyosin cytoskeleton takes place, leading to a stretching of the cells. Figure 1*C* 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. 1*D*).

$CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes and adhesion to FN. To study whether a putative shift in the apportioning 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. 2*A*) and their adhesion to an FN-coated surface studied. Differences could not be detected in the adhesion to FN between the two populations (Fig. 2*B*). 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. 2*C*).

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

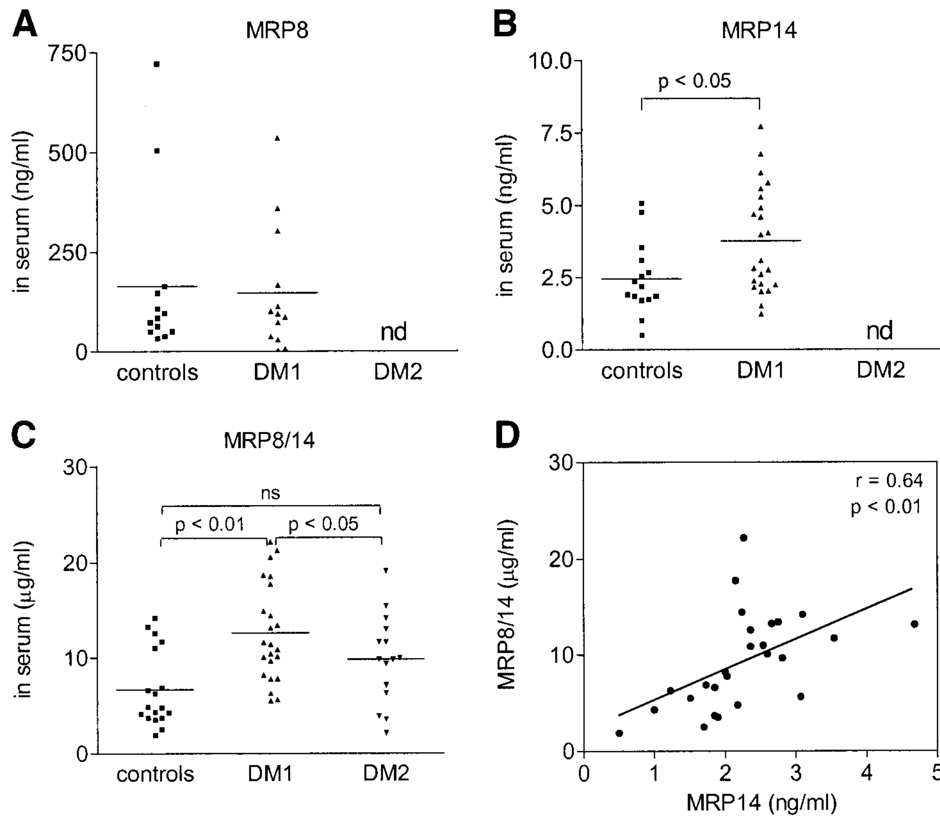

FIG. 3. Serum levels of MRP-8 of 13 control subjects and 13 type 1 patients (*A*; Student's *t* test), MRP-14 of 15 control subjects and 23 type 1 patients (*B*; Student's *t* test), and MRP-8/14 in 18 control subjects, 23 type 1 patients, and 15 type 2 patients (*C*; one-way ANOVA). nd, not determined. A positive correlation (Spearman's rho) was observed between MRP-14 and MRP-8/14 in the serum (*D*).

serum levels of MRP-8, -14, and -8/14. As shown in Fig. 3*A*–*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. 3*D*). 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. 3*C*).

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. 4*A*).

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. 4*B*).

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 5*A* 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. 5*B*).

In addition, ex vivo monocytes of type 1 diabetic patients were studied to investigate whether an upregulation of CD11b could indeed be detected. Figure 6*A* 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. 5*C*) 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. 5*D*). 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. 6*B* and Table 1). Moreover, monocytes of type 2 diabetic patients showed a higher expression level of the CD11b and CD18 integrins

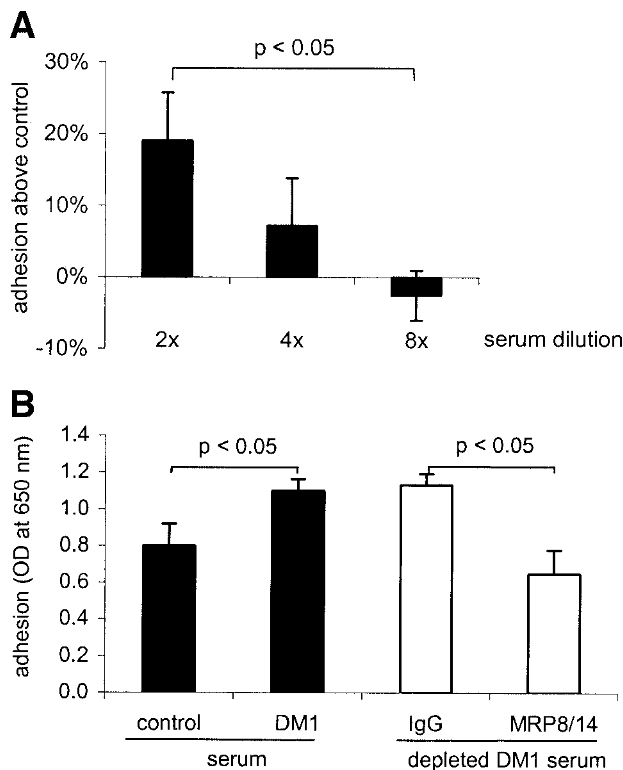

FIG. 4. *A*: Adhesion of healthy donor monocytes to FN after exposure to dilutions of serum samples of type 1 diabetic patients (tests were performed with 11 sera). The adhesion is expressed as a value relative to values of healthy control sera (n = 10, percentage above these controls). *B*: Increased adhesion of monocytes to FN by the serum samples of type 1 diabetic patients (■, means ± SD, n = 3) and the effect of serum depletion of MRP-8/14 by monoclonal antibodies (□, 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. 6*C–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 HbA_{1c} 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 chemoattracted (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 aberrances 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

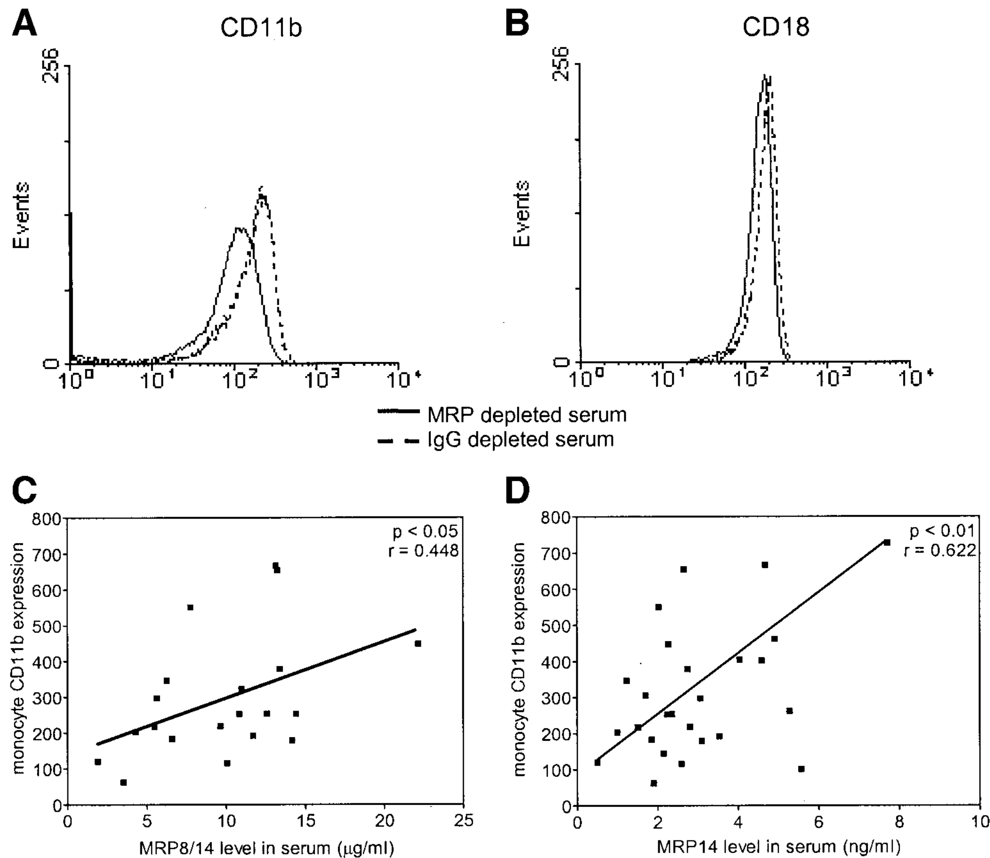

FIG. 5. ***A* and *B*: Flow cytometric analysis of the effect of MRP-8/14 in the serum of a type 1 diabetic patient on the surface expression of CD11b and CD118. The experiment was performed four times, always showing the same results by MRP-8/14. *C* and *D*: CD11b surface expression on monocytes of type 1 diabetic patients and healthy control subjects in correlation (Spearman's rho) to the serum level of MRP-8/14 and -14.**

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 diabetes 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 HbA_{1c} 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

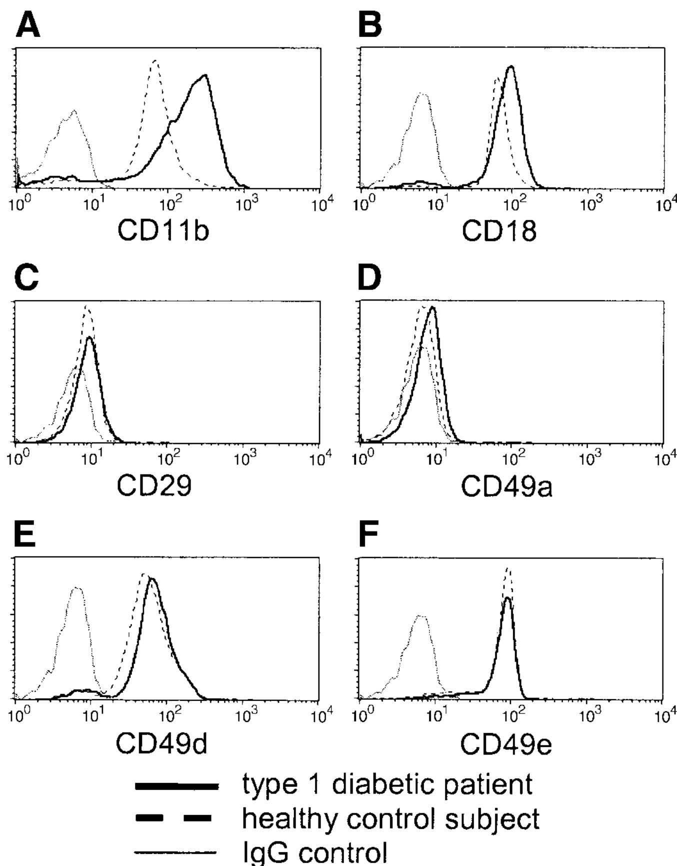

FIG. 6. A representative flow cytometric analysis of the surface expression of CD11b (*A*), CD18 (*B*), CD29 (*C*), CD49a (*D*), CD49d (*E*), and CD49e (*F*) on monocytes of a type 1 diabetic patient and an age- and sex-matched healthy control subject.

animal models of type 1 diabetes before islet infiltration (6–9). The determination of MRP production by monocytes of islet antibody−positive nondiabetic individuals and patients with established prediabetes needs 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.

TABLE 1
Integrin expression on circulating monocytes

Integrins	Fold expression*	MFI type 1 diabetic patients	MFI control subjects	*n*	*P*†
CD11b α_M	3.24 ± 0.52	429.44 ± 93.09	218.11 ± 59.93	20	0.002
CD18 β_2	1.65 ± 0.13	122.93 ± 19.03	86.19 ± 11.45	20	0.046
CD29 β_1	1.10 ± 0.03	16.64 ± 2.11	16.03 ± 1.77	20	0.930
CD49a α_1	1.16 ± 0.06	7.32 ± 0.51	7.19 ± 0.54	17	0.855
CD49d α_4	1.25 ± 0.08	64.27 ± 5.25	57.93 ± 4.56	21	0.273
CD49e α_5	1.05 ± 0.05	77.42 ± 4.29	79.2 ± 4.47	21	0.599

Data are means ± SE. MFI, mean fluorescence intensity. *Mean fold expression relative to healthy control subjects. †Statistical significance tested using the Mann-Whitney test.

ACKNOWLEDGMENTS

This study was supported by research grants from the European Union QLRT-1999-00276 (MONODIAB) and the Dutch Diabetes Research Foundation (96.606).

We are grateful to Prof. Rob Benner for critically reading the manuscript and to Edwin de Haas for assistance with the cell sorting.

REFERENCES

1. Jansen A, Voorbij HA, Jeucken PH, Bruining GJ, Hooijkaas H, Drexhage HA: An immunohistochemical study on organized lymphoid cell infiltrates in fetal and neonatal pancreases: a comparison with similar infiltrates found in the pancreas of a diabetic infant. *Autoimmunity* 15:31–38, 1993
2. Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA: Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and β-cell destruction in NOD mice. *Diabetes* 43:667–675, 1994
3. Ziegler AG, Erhard J, Lampeter EF, Nagelkerken LM, Standl E: Involvement of dendritic cells in early insulitis of BB rats. *J Autoimmun* 5:571–579, 1992
4. Voorbij HA, Jeucken PH, Kabel PJ, De Haan M, Drexhage HA: Dendritic cells and scavenger macrophages in pancreatic islets of prediabetic BB rats. *Diabetes* 38:1623–1629, 1989
5. Rosmalen JG, Martin T, Dobbs C, Voerman JS, Drexhage HA, Haskins K, Leenen PJ: Subsets of macrophages and dendritic cells in nonobese diabetic mouse pancreatic inflammatory infiltrates: correlation with the development of diabetes. *Lab Invest* 80:23–30, 2000
6. Steptoe RJ, Ritchie JM, Harrison LC: Increased generation of dendritic cells from myeloid progenitors in autoimmune-prone nonobese diabetic mice. *J Immunol* 168:5032–5041, 2002
7. Strid J, Lopes L, Marcinkiewicz J, Petrovska L, Nowak B, Chain BM, Lund T: A defect in bone marrow derived dendritic cell maturation in the nonobese diabetic mouse. *Clin Exp Immunol* 123:375–381, 2001
8. Lee M, Kim AY, Kang Y: Defects in the differentiation and function of bone marrow-derived dendritic cells in non-obese diabetic mice. *J Korean Med Sci* 15:217–223, 2000
9. Poligone B, Weaver DJ Jr, Sen P, Baldwin AS Jr, Tisch R: Elevated NF-kappaB activation in nonobese diabetic mouse dendritic cells results in enhanced APC function. *J Immunol* 168:188–196, 2002
10. Weaver DJ Jr, Poligone B, Bui T, Abdel-Motal UM, Baldwin AS Jr, Tisch R: Dendritic cells from nonobese diabetic mice exhibit a defect in NF-kappa B regulation due to a hyperactive I kappa B kinase. *J Immunol* 167:1461–1468, 2001
11. Lety MA, Coulaud J, Bens M, Dardenne M, Homo-Delarche F: Enhanced metabolism of arachidonic acid by macrophages from nonobese diabetic (NOD) mice. *Clin Immunol Immunopathol* 64:188–196, 1992
12. Luo C, Kallajoki M, Gross R, Mulari M, Teros T, Ylinen L, Makinen M, Laine J, Simell O: Cellular distribution and contribution of cyclooxygenase COX-2 to diabetogenesis in NOD mouse. *Cell Tissue Res* 310:169–175, 2002
13. Liu J, Beller D: Aberrant production of IL-12 by macrophages from several autoimmune-prone mouse strains is characterized by intrinsic and unique patterns of NF-kappa B expression and binding to the IL-12 p40 promoter. *J Immunol* 169:581–586, 2002
14. Delemarre FG, Simons PJ, de Heer HJ, Drexhage HA: Signs of immaturity of splenic dendritic cells from the autoimmune prone biobreeding rat: consequences for the in vitro expansion of regulator and effector T cells. *J Immunol* 162:1795–1801, 1999
15. Lau A, Ramanathan S, Poussier P: Excessive production of nitric oxide by macrophages from DP-BB rats is secondary to the T-lymphopenic state of these animals. *Diabetes* 47:197–205, 1998
16. Takahashi K, Honeyman MC, Harrison LC: Impaired yield, phenotype, and function of monocyte-derived dendritic cells in humans at risk for insulin-dependent diabetes. *J Immunol* 161:2629–2635, 1998
17. Jansen A, van Hagen M, Drexhage HA: Defective maturation and function of antigen-presenting cells in type 1 diabetes. *Lancet* 345:491–492, 1995
18. Springer TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76:301–314, 1994
19. Springer TA: Adhesion receptors of the immune system. *Nature* 346:425–434, 1990
20. Ventura MA, Ribier A, Delbourg I, Chambaut-Guerin AM, Thomopoulos P:

The adhesiveness of monocytic U937 cells is stimulated by pro-inflammatory agents and inhibited by adenosine 3′:5′-cyclic monophosphate. *Biochem Pharmacol* 39:677–683, 1990

21. Amato PA, Unanue ER, Taylor DL: Distribution of actin in spreading macrophages: a comparative study on living and fixed cells. *J Cell Biol* 96:750–761, 1983
22. Ziegler-Heitbrock HW: Heterogeneity of human blood monocytes: the CD14+ CD16+ subpopulation. *Immunol Today* 17:424–428, 1996
23. Weber C, Belge KU, von Hundelshausen P, Draude G, Steppich B, Mack M, Frankenberger M, Weber KS, Ziegler-Heitbrock HW: Differential chemokine receptor expression and function in human monocyte subpopulations. *J Leukoc Biol* 67:699–704, 2000
24. Ancuta P, Rao R, Moses A, Mehle A, Shaw SK, Luscinskas FW, Gabuzda D: Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. *J Exp Med* 197:1701–1707, 2003
25. Kawanaka N, Yamamura M, Aita T, Morita Y, Okamoto A, Kawashima M, Iwahashi M, Ueno A, Ohmoto Y, Makino H: CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. *Arthritis Rheum* 46:2578–2586, 2002
26. Thieblemont N, Weiss L, Sadeghi HM, Estcourt C, Haeffner-Cavaillon N: CD14low CD16high: a cytokine-producing monocyte subset which expands during human immunodeficiency virus infection. *Eur J Immunol* 25:3418–3424, 1995
27. Fingerle G, Pforte A, Passlick B, Blumenstein M, Strobel M, Ziegler-Heitbrock HW: The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients. *Blood* 82:3170–3176, 1993
28. Hogg N, Allen C, Edgeworth J: Monoclonal antibody 5.5 reacts with p8,14, a myeloid molecule associated with some vascular endothelium. *Eur J Immunol* 19:1053–1061, 1989
29. Rammes A, Roth J, Goebeler M, Klempt M, Hartmann M, Sorg C: Myeloid-related protein (MRP)-8 and MRP-14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. *J Biol Chem* 272:9496–9502, 1997
30. Eue I, Pietz B, Storck J, Klempt M, Sorg C: Transendothelial migration of 27E10+ human monocytes. *Int Immunol* 12:1593–1604, 2000
31. Kerkhoff C, Eue I, Sorg C: The regulatory role of MRP-8 (S100A8) and MRP-14 (S100A9) in the transendothelial migration of human leukocytes. *Pathobiology* 67:230–232, 1999
32. Fluks AJ: Three-step isolation of human blood monocytes using discontinuous density gradients of Percoll. *J Immunol Methods* 41:225–233, 1981
33. Mahnke K, Bhardwaj R, Sorg C: Heterodimers of the calcium-binding proteins MRP-8 and MRP-14 are expressed on the surface of human monocytes upon adherence to fibronectin and collagen: relation to TNF-alpha, IL-6, and superoxide production. *J Leukoc Biol* 57:63–71, 1995
34. Robinson MJ, Tessier P, Poulsom R, Hogg N: The S100 family heterodimer, MRP-8/14, binds with high affinity to heparin and heparan sulfate glycosaminoglycans on endothelial cells. *J Biol Chem* 277:3658–3665, 2002
35. Frosch M, Strey A, Vogl T, Wulffraat NM, Kuis W, Sunderkotter C, Harms E, Sorg C, Roth J: Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. *Arthritis Rheum* 43:628–637, 2000
36. Takahashi M, Ikeda U, Masuyama J, Kitagawa S, Kasahara T, Saito M, Kano S, Shimada K: Involvement of adhesion molecules in human monocyte adhesion to and transmigration through endothelial cells in vitro. *Atherosclerosis* 108:73–81, 1994
37. Meerschaert J, Furie MB: The adhesion molecules used by monocytes for migration across endothelium include CD11a/CD18, CD11b/CD18, and VLA-4 on monocytes and ICAM-1, VCAM-1, and other ligands on endothelium. *J Immunol* 154:4099–4112, 1995
38. Hasegawa Y, Yokono K, Taki T, Amano K, Tominaga Y, Yoneda R, Yagi N, Maeda S, Yagita H, Okumura K, et al: Prevention of autoimmune insulin-dependent diabetes in non-obese diabetic mice by anti-LFA-1 and anti-ICAM-1 mAb. *Int Immunol* 6:831–838, 1994
39. Hutchings P, Rosen H, O'Reilly L, Simpson E, Gordon S, Cooke A: Transfer of diabetes in mice prevented by blockade of adhesion-promoting receptor on macrophages. *Nature* 348:639–642, 1990
40. Baron JL, Reich EP, Visintin I, Janeway CA Jr: The pathogenesis of adoptive murine autoimmune diabetes requires an interaction between alpha 4-integrins and vascular cell adhesion molecule-1. *J Clin Invest* 93:1700–1708, 1994
41. Fabien N, Bergerot I, Orgiazzi J, Thivolet C: Lymphocyte function associated antigen-1, integrin α4, and L-selectin mediate T-cell homing to the pancreas in the model of adoptive transfer of diabetes in NOD mice. *Diabetes* 45:1181–1186, 1996
42. Sanchez-Madrid F, del Pozo MA: Leukocyte polarization in cell migration and immune interactions. *EMBO J* 18:501–511, 1999
43. Canning MO, Grotenhuis K, De Haan-Meulman M, De Wit HJ, Berghout A, Drexhage HA: An abnormal adherence of monocytes to fibronectin in thyroid autoimmunity has consequences for cell polarization and the development of veiled cells. *Clin Exp Immunol* 125:10–18, 2001
44. Newton RA, Hogg N: The human S100 protein MRP-14 is a novel activator of the beta 2 integrin Mac-1 on neutrophils. *J Immunol* 160:1427–1435, 1998
45. Yednock TA, Cannon C, Vandevert C, Goldbach EG, Shaw G, Ellis DK, Liaw C, Fritz LC, Tanner LI: Alpha 4 beta 1 integrin-dependent cell adhesion is regulated by a low affinity receptor pool that is conformationally responsive to ligand. *J Biol Chem* 270:28740–28750, 1995
46. Setiadi H, Wautier JL, Courillon-Mallet A, Passa P, Caen J: Increased adhesion to fibronectin and Mo-1 expression by diabetic monocytes. *J Immunol* 138:3230–3234, 1987
47. Martin S, Rothe H, Tschope D, Schwippert B, Kolb H: Decreased expression of adhesion molecules on monocytes in recent onset IDDM. *Immunology* 73:123–125, 1991
48. Lundahl J, Hallden G, Skold CM: Human blood monocytes, but not alveolar macrophages, reveal increased CD11b/CD18 expression and adhesion properties upon receptor-dependent activation. *Eur Respir J* 9:1188–1194, 1996
49. Litherland SA, Xie XT, Hutson AD, Wasserfall C, Whittaker DS, She JX, Hofig A, Dennis MA, Fuller K, Cook R, Schatz D, Moldawer LL, Clare-Salzler MJ: Aberrant prostaglandin synthase 2 expression defines an antigen-presenting cell defect for insulin-dependent diabetes mellitus. *J Clin Invest* 104:515–523, 1999