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The Role of Integrin $\alpha_D\beta_2$ (CD11d/CD18) in Monocyte/Macrophage Migration

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Abstract

Integrin $\alpha_D\beta_2$ (CD11d/CD18) is a multiligand macrophage receptor with recognition specificity identical to that of the major myeloid cell-specific integrin $\alpha_M\beta_2$ (CD11b/CD18, Mac-1). Despite its prominent upregulation on inflammatory macrophages the role of $\alpha_D\beta_2$ in monocyte and macrophage migration is unknown. In this study, we have generated model and natural cell lines expressing different densities of $\alpha_D\beta_2$ and examined their migration to various extracellular matrix proteins. When expressed at a low density, $\alpha_D\beta_2$ on the surface of recombinant HEK293 cells and murine IC-21 macrophages cooperates with β_1/β_3 integrins to support cell migration. However, its increased expression on the $\alpha_D\beta_2$ -expressing HEK293 cells and its upregulation by PMA on the IC-21 macrophages results in increased cell adhesiveness and inhibition of cell migration. Furthermore, ligation of $\alpha_D\beta_2$ with anti- α_D blocking antibodies restores β_1/β_3 -driven cell migration by removing the excess $\alpha_D\beta_2$ -mediated adhesive bonds. Consistent with *in vitro* data, increased numbers of inflammatory macrophages were recovered from the inflamed peritoneum of mice after the administration of anti- α_D antibody. These results demonstrate that the density of $\alpha_D\beta_2$ is critically involved in modulating macrophage adhesiveness and their migration, and suggest that low levels of $\alpha_D\beta_2$ contribute to monocyte migration while $\alpha_D\beta_2$ upregulation on differentiated macrophages may facilitate their retention at sites of inflammation.

Keywords

integrin $\alpha_D\beta_2$; CD11d/CD18; macrophage; cell migration; inflammation

Introduction

The β_2 integrins are heterodimeric adhesion receptors expressed on leukocytes which play an important role in the immune-inflammatory response. The group consists of four members, $\alpha_L\beta_2$ (CD11a/CD18, LFA-1), $\alpha_M\beta_2$ (CD11b/CD18, Mac-1), $\alpha_X\beta_2$ (CD11c/CD18, p150, 95) and $\alpha_D\beta_2$ (CD11d/CD18), which have a common β_2 subunit and different, albeit homologous, α subunits. The two related members of the group, $\alpha_M\beta_2$ and $\alpha_D\beta_2$, are expressed predominantly

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on myeloid leukocytes and are strongly upregulated after agonist stimulation on neutrophils and monocytes, respectively. A large number of studies have been performed both *in vitro* and *in vivo* to understand the role of the major myeloid cell-specific integrin $\alpha_M\beta_2$ in neutrophil migration [1]. In the current model of leukocyte trafficking during the inflammatory reaction, $\alpha_M\beta_2$ does not appear to be important for adhesion to the endothelium but cooperates with $\alpha_L\beta_2$ in leukocyte emigration from the vessel [2,3]. The same integrins, together with $\alpha_4\beta_1$, have also been implicated in migration of monocytes although $\alpha_M\beta_2$ and $\alpha_L\beta_2$ appear to be less important than $\alpha_4\beta_1$ [4,5]. While $\alpha_M\beta_2$ is required for transendothelial extravasation, it does not support neutrophil migration through the interstitial extracellular matrix (ECM). Instead, neutrophils appear to utilize β_1 integrins for migration through tissues [6,2,7,8]. Nevertheless, $\alpha_M\beta_2$ may have a unique role in neutrophils migration. As studies in different models of inflammation have shown, neutrophils from the $\alpha_M\beta_2$ -deficient mice had enhanced migratory properties since the leukocyte influx was increased ~2–3-fold in the $\alpha_M\beta_2$ -deficient mice compared with wild-type counterparts [9,10,3,11]. Furthermore, using model cells expressing different levels of $\alpha_M\beta_2$, we have demonstrated that the progressive increase of $\alpha_M\beta_2$ density inhibited cell migration mediated by β_1 integrins [12]. These observations suggest that the major function of $\alpha_M\beta_2$ is not to support migration but rather to serve as a brake during neutrophil migration through the interstitial space. The basis for this function of $\alpha_M\beta_2$ is thought to be its unusual stickiness and its ability to bind the same ECM proteins as the β_1 integrins. For example, both $\alpha_M\beta_2$ and $\alpha_5\beta_1$ can adhere to fibronectin [12]. Another important characteristic of $\alpha_M\beta_2$ is that this receptor can be upregulated ~7–10-fold on the surface of neutrophils in a stepwise manner [1] while the levels of β_1 integrins increase modestly [7]. Accordingly, when the density of $\alpha_M\beta_2$ exceeds that required for optimal β_1 -driven cell locomotion, migration halts. These observations would be consistent with the idea that the major function of $\alpha_M\beta_2$ is not migration but neutrophil adhesion and the control of adhesion-dependent leukocyte responses such as degranulation, oxidative burst and phagocytosis at sites of inflammation [10].

We have recently demonstrated that another member of the subfamily, the most recently discovered integrin $\alpha_D\beta_2$, exhibits multiligand binding properties and has recognition specificity overlapping that of $\alpha_M\beta_2$ [13]. Specifically, $\alpha_D\beta_2$ is capable of supporting cell adhesion to various ECM proteins, including fibronectin, vitronectin, fibrinogen, CCN1 (Cyr61) and others. We have also shown that in $\alpha_D\beta_2$, the α_D I-domain is responsible for the binding function and that the mechanism whereby α_D I-domain recognizes its ligands is similar to that utilized by $\alpha_M\beta_2$ [13]. The finding that $\alpha_D\beta_2$ and $\alpha_M\beta_2$ have similar recognition specificity and bind a broad assortment of proteins in the ECM suggests that $\alpha_D\beta_2$ might perform an analogous function in leukocyte migration. Like $\alpha_M\beta_2$, integrin $\alpha_D\beta_2$ is poorly expressed on peripheral blood leukocytes [14] and can be rapidly upregulated in response to chemotactic stimulation as a result of its transport to the cell surface from internal secretory granules [14]. However, its expression in neutrophils is much lower than that of $\alpha_M\beta_2$ [14]. In contrast, $\alpha_D\beta_2$ levels are gradually increased by *de novo* synthesis upon monocyte differentiation into macrophages, and oxidized LDL and AcLDL further upregulate its expression [15]. Furthermore, $\alpha_D\beta_2$ (but not $\alpha_M\beta_2$) is strongly upregulated on macrophages within atherosclerotic plaques [14]. Such a difference in upregulation of $\alpha_M\beta_2$ and $\alpha_D\beta_2$ on neutrophils and monocytes/macrophages, respectively, might indicate their distinct roles in acute and chronic inflammatory responses. The unique pattern of $\alpha_D\beta_2$ up-regulation by atherogenic lipoproteins suggests a potential role for this integrin in the regulation of monocyte/macrophage migration to extravascular sites and, thus, in the development of atherosclerosis. However, exactly how $\alpha_D\beta_2$ contributes to monocyte/macrophage migration is still unknown.

In the present study we have examined the migratory properties of $\alpha_D\beta_2$. We have generated model and natural monocytic cell lines expressing different levels of $\alpha_D\beta_2$ and tested their

migration to various ECM proteins. In addition, a mouse model of inflammation has been used to further clarify the role of $\alpha_D\beta_2$ in monocyte/macrophage migration *in vivo*.

Materials and Methods

Proteins and antibodies

Human fibrinogen was obtained from Enzyme Research Laboratories (South Bend, IN). Fibronectin was isolated from fresh human plasma by gelatin-agarose affinity chromatography [16]. Vitronectin was isolated from outdated plasma treated with 8M urea using heparin-agarose affinity chromatography [17]. CCN1 (connective tissue growth factor, Cyr61) was purified as described [18]. The mAb IB4 directed against the β_2 integrin subunit and mAb M1/70 which recognizes the mouse α_M integrin subunit were purified from the conditioned media of the hybridoma cell line obtained from American Type Culture Collection (Manassas, VA) using protein A agarose (GE Healthcare, Piscataway, NJ). F(ab')₂ preparations of Mab M1/70 were isolated after digestion with pepsin (1:200 w/w). F(ab')₂ retained its full immunoreactivity as assessed by FACS analyses and did not contain intact mAb molecules or its Fc fragments as assessed with goat anti-rat IgG Fc alkaline phosphatase-conjugated antibody (Pierce). Polyclonal antibodies 1950 and 1932 directed against the β_1 and β_3 integrin subunits, respectively, were purchased from Chemicon Int. (Temecula, CA). MAbs against murine CD14 (PE conjugated), F4/80 (PE conjugated) and α_X integrin (FITC conjugated) were from eBioscience (San Diego, CA). Monoclonal antibody against the mouse α_V integrin (clone RMV-7) was purchased from Fitzgerald Industries International Inc. (Concord, MA). Polyclonal antibody against the α_D I-domain was described previously [13]. The antibody recognizes both human and mouse α_D I-domains and has no cross-reactivity with recombinant human and mouse α_M , α_X and α_L I-domains. The antibody was isolated from rabbit serum by affinity chromatography using α_D I-domain-Sepharose. Fab preparations were isolated after enzymatic digestion with papain (Sigma-Aldrich, St. Louis, MO) followed by purification by protein A agarose.

Cells

The IC-21 macrophage cell line was obtained from the ATCC. Cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 0.1 mg/ml streptomycin, 0.1 unit/ml penicillin, and 2 mM L-glutamine. The $\alpha_D\beta_2$ -expressing HEK 293 cells were generated as described previously [13] and maintained in DMEM/F-12 (Invitrogen) supplemented with 10% FBS, 2 mM glutamine, 15 mM HEPES, 0.1 mg/ml streptomycin, and 0.1 unit/ml penicillin. Populations of cells expressing low and high density of $\alpha_D\beta_2$ integrin were selected by fluorescence activated cell sorting using a BD Biosciences FACS Vantage Instrument.

Flow cytometry

FACS analyses were performed to assess the expression of integrins on the surface of the $\alpha_D\beta_2$ transfected HEK 293 cells and the mouse IC-21 macrophage cell line. The cells were incubated with integrin-specific antibodies and analyzed using a FACScan™ (Beckton Dickinson) as described previously [19]. To determine integrin expression on murine peripheral blood monocytes, a population of cells isolated from whole blood by Ficoll fractionation which contained both monocytes and lymphocytes was incubated with a mixture of phycoerythrin-conjugated anti-CD14 (5 μ g/ml) and selected FITC-conjugated anti-integrin antibodies (10 μ g/ml). Likewise, cells isolated from the mouse peritoneal cavity were incubated with a mixture of the phycoerythrin-conjugated macrophage-specific marker F4/80 (5 μ g/ml) and FITC-conjugated anti-integrin antibodies. The levels of integrins were determined using back-gating with anti-CD14 and F4/80 mAbs for peripheral blood monocytes and peritoneal macrophages, respectively.

Adhesion and migration assays

For adhesion assays, 96-well tissue culture plates (Costar, Cambridge, MA) were coated with different concentrations of vitronectin for 3 h at 37°C. The wells were post-coated with 0.5% PVA for 1 h at 22°C. The $\alpha_D\beta_2$ -expressing cells were labeled with 10 μ M Calcein AM (Molecular Probes, Eugene, OR) and assays were performed as described previously. [12]

Cell migration assays with calcein-labeled cells were performed under sterile conditions using 24-well transwell plates (Costar, Corning, NY) with 8- μ M pore size uncoated polycarbonate filters as previously described [20,12]. Lower wells contained 600 μ l of vitronectin or other ECM proteins in DMEM/F-12 which during the assay coat both sides of the filter. Upper wells, inserted into the lower wells, contained a final volume of 250 μ l after the addition of cells. To begin the assay, 150 μ l of cells (2.5×10^6 /ml) in medium, with or without mAbs, was added to upper wells and cells were allowed to migrate for 18 h at 37°C in a 5% CO₂ humidified atmosphere. In this format, migration is largely directional and cells migrate from the upper chamber to the underside of the filter coated with a ligand [20]. Two hours prior to the completion of the migration assay, cells were labeled with calcein AM which was added to the lower chamber at 10 μ M final concentration. Assays were stopped by removing cells from the upper surface of the polycarbonate membrane by wiping the surface twice with a cotton-tipped applicator. Cells migrating to the underside of the filter were detected using a CytoFluorII fluorescence plate reader (Applied Biosystems, Farmington, MA).

Peritoneal Inflammation

Peritonitis was induced by intraperitoneal injection of 0.5 ml of 3% Brewer thioglycollate medium (Sigma-Aldrich, St. Louis, MO) in C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). After 72 hours the mice were euthanized by isoflurane inhalation and the peritoneal cavities were lavaged with 5 ml PBS. In experiments with antibodies, 100 μ g of purified anti- α_D polyclonal antibody, Mab M1/70 or normal mouse IgG diluted in PBS were injected intraperitoneally 4 hours before thioglycollate injection. In selected experiments, mice were injected with F(ab')₂ preparations of Mab M1/70 (100 μ g). Macrophages were counted by nonspecific esterase assay as described [21]. In selected experiments, the number of cells in total lavage fluid was counted in a hemacytometer and macrophage number was determined by performing differential counts after cytopspin and Wright stain. In each experiment, 7 mice were used for a control (non-treated) and 7 mice were used for the treated group.

Statistical analysis

Results are presented as the means $\pm$ SD. Statistical analyses were performed using Student's *t* test. The value of $P < 0.05$ was considered significant.

Results

Generation of the $\alpha_D\beta_2$ expressing cells with different densities of receptors and characterization of their adhesive and migratory properties

To examine the role of $\alpha_D\beta_2$ in migration, we initially examined HEK 293 cells stably transfected with α_D and β_2 integrin subunits [13]. To generate cells with different levels of $\alpha_D\beta_2$, the initial heterogeneous population of the $\alpha_D\beta_2$ -expressing cells was sorted into two fractions. Surface expression of $\alpha_D\beta_2$ was determined by flow cytometry using mAbs directed against the α_D and β_2 subunits (Fig.1). The mean fluorescence intensities of the two cell lines differed by ~10-fold, indicating that $\alpha_D\beta_2$ was expressed at different densities. Moderate levels of endogenous integrins $\alpha_5\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_6\beta_1$ and $\alpha_V\beta_1$ were detected on the surface of transfected cells in agreement with previous data [12] (not shown). Because adhesion is necessary for cells to obtain traction upon migration, we examined the adhesive capacity of

the generated cell lines. In these experiments, vitronectin was selected as a representative ECM ligand and the maximal levels of adhesion were determined. Consistent with the $\alpha_D\beta_2$ density differential, adhesion of cells expressing low levels of integrin was ~ 2-fold lower than that of high expressors (Fig.2). In previous studies we demonstrated that in the $\alpha_D\beta_2$ -expressing cells both $\alpha_D\beta_2$ and β_1 integrins (apparently $\alpha_V\beta_1$, a vitronectin receptor in HEK 293 cells) contribute to adhesion to vitronectin [13]. In agreement with these observations, anti- α_D function blocking antibody partially inhibited cell adhesion of both cell lines and this effect correlated with the level of $\alpha_D\beta_2$ expression. Accordingly, anti- α_D inhibited adhesion of cells expressing high and low densities of $\alpha_D\beta_2$ by ~80% and 50%, respectively (Fig.2). Furthermore, mock-transfected HEK 293 cells adhered to vitronectin; however, the adhesion was low because these cells rely only on endogenous $\alpha_V\beta_1$ for binding to this ligand.

To investigate the relationship between the $\alpha_D\beta_2$ density and cell migration, we compared the ability of cells expressing different levels of integrin to migrate toward vitronectin in a Transwell system. In these experiments, vitronectin (50 $\mu\text{g/ml}$) was placed in the lower chamber and migration was initiated by adding cells to the upper chamber. Migration was allowed to proceed for 18 h at 37°C, at which time the number of cells adherent to the underside of the filter coated with vitronectin was determined. Migration of the two cell lines to vitronectin was very different (Fig.3A). Both mock-transfected cells and the cell line expressing a low density of $\alpha_D\beta_2$ migrated efficiently, with migration of the latter cells being 1.7-fold higher. However, migration of cells expressing a high density of $\alpha_D\beta_2$ was suppressed (by ~3-fold) compared to mock-transfected cells. To examine the contribution of $\alpha_D\beta_2$ and $\alpha_V\beta_1$ to migration, cells were preincubated with 20 $\mu\text{g/ml}$ of either anti- α_D or anti- β_1 function-blocking antibodies before their addition to the upper chamber of the transwell system. Migration of cells with a low density of $\alpha_D\beta_2$ was inhibited by ~50% and 85%, respectively (Fig.3B). Thus, both $\alpha_D\beta_2$ and $\alpha_V\beta_1$ support migration of these cells. However, preincubation of cells expressing a high-density of $\alpha_D\beta_2$ with anti- α_D produced the opposite effect (Fig.3C), i.e., the antibody did not inhibit migration but instead enhanced migration by ~4-fold. At the same time, anti- β_1 still blocked migration. Hence, ligation of $\alpha_D\beta_2$ with anti- α_D in these cells could restore migration to the level observed with mock-transfected cells. Since the level of β_1 integrins in the two cell lines expressing different densities of $\alpha_D\beta_2$ is the same, the difference in their migratory ability appears to arise from the dissimilarity in the density of $\alpha_D\beta_2$. Thus, these results suggest that in both cell lines migration is mediated by $\alpha_V\beta_1$, and that $\alpha_D\beta_2$ expressed at a low density contributes to migration. However, when the density of $\alpha_D\beta_2$ increases it suppresses migration.

To confirm that the rate of migration in this system depends on the density of $\alpha_D\beta_2$ but not on the nature of the adhesive ligand, we examined cell migration to different ECM proteins. Previous studies have demonstrated that fibronectin, fibrinogen and CCN1 are ligands for $\alpha_D\beta_2$ [13]. Furthermore, fibronectin and fibrinogen can interact with the β_1 integrin $\alpha_5\beta_1$ [22, 23], and CCN1 can engage $\alpha_6\beta_1$ [24]. As shown in Fig.4, pretreatment of cells expressing a high density of $\alpha_D\beta_2$ with anti- α_D antibody significantly enhanced migration to all proteins while the anti- β_1 specific antibody inhibited migration. These results indicate that as with vitronectin, the increased density of $\alpha_D\beta_2$ results in the decline of β_1 -driven migration to other ligands and that the retarded migration could be restored by the removal of the restraint imposed by the excess of the $\alpha_D\beta_2$ - ligand bonds. However, the extent to which ligation of $\alpha_D\beta_2$ by function-blocking anti- α_D increased migration was different, which may reflect the relative contribution of $\alpha_D\beta_2$ and β_1 integrins to adhesion. For instance, cell adhesion to CCN1 is mediated predominantly by $\alpha_D\beta_2$ with a small contribution of β_1 integrins [13], and, accordingly, anti- α_D exerted a strong stimulating effect on cell migration. In contrast, anti- α_D caused a moderate effect on cell migration to fibronectin because both $\alpha_5\beta_1$ and $\alpha_D\beta_2$ mediate cell adhesion to this ligand [13]. Nevertheless, regardless of these differences the

stimulating effect of anti- α_D was detected with all ligands suggesting that the increased density of $\alpha_D\beta_2$ inhibits cell migration to various ECM proteins.

Migratory properties of $\alpha_D\beta_2$ expressed on the macrophage cell line IC-21

Because macrophage-derived foam cells that are present in atherosclerotic lesions express high levels of $\alpha_D\beta_2$ [14], we examined the role of $\alpha_D\beta_2$ in monocyte/macrophage migration. In previous studies, we have demonstrated that the mouse macrophage cell line IC-21 expresses similar levels of $\alpha_M\beta_2$ and $\alpha_D\beta_2$ [13]. After stimulation of these cells with 100 ng/ml PMA for 48 h, the level of $\alpha_D\beta_2$ was upregulated by more than 5-fold while expression of $\alpha_M\beta_2$ did not change (Fig.5). After 72 h, expression of $\alpha_D\beta_2$ remained high and gradually returned to the basal levels after 7 days in culture.

Since resting and PMA-stimulated IC-21 cells can model the two cell lines with low and high densities of $\alpha_D\beta_2$, respectively, these cells were tested for their ability to support migration to vitronectin. At least three other integrins on the surface of IC-21 cells, including $\alpha_V\beta_1$, $\alpha_V\beta_3$, and $\alpha_M\beta_2$, can potentially mediate migration to vitronectin. Expression of α_M , α_V , β_1 and β_3 integrins did not change significantly after 72 hours of stimulation with PMA (not shown) suggesting that only up-regulation of $\alpha_D\beta_2$ can modulate cell adhesiveness. Both cell lines were able to migrate to vitronectin but migration of resting IC-21 was reproducibly higher than that of PMA-stimulated cells (Fig. 6A,B) and when compared in the same experiment (not shown). Preincubation of cells with the Fab preparation of the anti- α_D polyclonal antibody inhibited migration of resting macrophages indicating that $\alpha_D\beta_2$ support migration (Fig.6A). No inhibition of migration by control Fab of rabbit IgG was detected. In these studies, we have utilized Fab preparations of anti- α_D because preliminary experiments demonstrated that intact rabbit IgG at the concentration 20 μ g/ml inhibited migration of IC-21 cells, an effect often encountered in such assays that is attributed to the Fc portion of the antibody molecule. Pretreatment of resting macrophages with either intact anti- α_M mAb M1/70 or its F(ab')₂ inhibited migration by ~45%, indicative of the contribution of $\alpha_M\beta_2$ to cell migration. As a control, rat IgG did not inhibit cell migration. Function blocking antibodies against murine α_V , the common subunit for integrins $\alpha_V\beta_1$ and $\alpha_V\beta_3$, also inhibited cell migration indicating that either $\alpha_V\beta_1$ or $\alpha_V\beta_3$ or both can support migration (Fig. 6A).

In contrast to resting cells, preincubation of PMA-stimulated macrophages with Fab preparations of anti- α_D resulted in an increase of their migration which was significant (Fig. 6B). These results are consistent with the effect of anti- α_D on migration of HEK 293 cells expressing a high density of $\alpha_D\beta_2$ and suggest that this integrin can be responsible for the increased adhesiveness of stimulated macrophages as well. By contrast, anti- α_M mAb M1/70 produced the opposite effect, i.e., it inhibited macrophage migration. Hence, like in resting macrophages, $\alpha_M\beta_2$ contributes to migration. Collectively, these data demonstrate that upregulation of $\alpha_D\beta_2$ in a cell line which naturally expresses this receptor leads to decreased cell migration. In addition, these results distinguish the opposite roles of two integrins in PMA-stimulated macrophages: while $\alpha_M\beta_2$ promotes migration, $\alpha_D\beta_2$ inhibits it.

Effect of anti- α_D antibody on migration of macrophages to mice peritoneum

To assess the role of $\alpha_D\beta_2$ in monocyte migration *in vivo*, we examined the effect of anti- α_D antibody on emigration of monocytes into the peritoneal cavity of mice with thioglycollate-induced inflammation. Previous studies demonstrated that significant numbers of monocytes arrive into the peritoneum by 72 h after thioglycollate injection [25], where they apparently differentiate into macrophages. To determine whether $\alpha_D\beta_2$ upregulation occurs in an environment such as that in inflamed peritoneum, we characterized expression of the integrin on monocytes at 72 h after thioglycollate injection and on monocytes in the circulation. Expression of several other macrophage integrins, including α_M , α_D , α_X , β_1 and β_3 have also

been determined. Fig.7 shows that the levels of $\alpha_D\beta_2$ and $\alpha_M\beta_2$ were increased by ~4- and 1.5-fold, respectively, compared to basal levels on peripheral blood monocytes. No increase in the expression of other integrin subunits was detected. Resident macrophages, which constitute approximately 5% the total number of recruited macrophages, contained a low level of $\alpha_D\beta_2$ and elevated levels of $\alpha_M\beta_2$ (data not shown). Thus, $\alpha_D\beta_2$ is the major integrin on the surface of stimulated macrophages elicited in response to inflammatory stimulation.

To analyze the role of $\alpha_D\beta_2$ in accumulation of macrophages in the peritoneum, mice were injected with 100 μ g of anti- α_D antibody 4 hours before injection of thioglycollate. After 72 hours, macrophages in the peritoneal exudate were counted and compared with macrophage levels in a control group injected with rabbit IgG or PBS. The amount of macrophages in the mice treated with anti- α_D antibody was increased by $44 \pm 8\%$ compared to the control group. Fig.8 shows a representative of four independent experiments. In each experiment, 7 mice were used for the control (non-treated) and 7 mice were used for the treated group. The difference between the groups in each experiment was statistically significant. The results indicate that the blockade of $\alpha_D\beta_2$ with anti- $\alpha_D\beta_2$ antibody resulted in the increased numbers of monocyte/macrophage recovered from the peritoneum (Fig.8). In contrast, both mAb M1/70 or its F(ab')₂ preparation inhibited monocyte migration slightly and this effect was not significant (data not shown). These data suggest that up-regulation of $\alpha_D\beta_2$ on activated macrophages might either suppress their migration into the peritoneum or enhance their adhesion to the peritoneal wall. Furthermore, $\alpha_D\beta_2$ and $\alpha_M\beta_2$ may have different roles in monocyte migration and macrophage accumulation in the peritoneum.

Discussion

The β_2 integrins play a central role in the inflammatory response by mediating adhesive reactions of leukocytes during their migration from the peripheral blood to extravascular sites of injury. In a previous study we have demonstrated that two members of the group, $\alpha_M\beta_2$ and the most recently discovered integrin $\alpha_D\beta_2$, have similar recognition specificity in that they are capable of supporting adhesion to numerous ECM proteins [13]. In addition to their salient multiligand binding properties, another distinctive feature of both integrins is that they are upregulated to high densities on the surface of leukocytes. When coming into play, these qualities may influence adhesive and migratory properties of leukocytes in unusual ways. Indeed, previous studies proposed that a gradual increase of $\alpha_M\beta_2$ density on the surface of stimulated neutrophils can serve as a “brake” in the overall process of neutrophil migration [3,12]. The commonality in properties between $\alpha_M\beta_2$ and its sister integrin $\alpha_D\beta_2$ suggests that upregulation of $\alpha_D\beta_2$ may perform a similar function in monocyte/macrophage migration. To define the specific contribution of $\alpha_D\beta_2$ to the process of macrophage migration, we have utilized $\alpha_D\beta_2$ -expressing HEK 293 cells as well as a murine IC-21 macrophage cell line expressing different levels of $\alpha_D\beta_2$ and we also compared $\alpha_D\beta_2$ function with that of $\alpha_M\beta_2$. In addition, using anti- α_D blocking antibodies we have examined the role of $\alpha_D\beta_2$ in a mouse model of peritonitis. The main findings of our study are that: 1) when expressed at a low density, integrin $\alpha_D\beta_2$ cooperates with β_1/β_3 integrins in supporting cell migration; 2) when expressed at a high density, $\alpha_D\beta_2$ inhibits β_1/β_3 -governed cell migration; and 3) activation of macrophages *in vitro* and *in vivo* is accompanied by strong upregulation of $\alpha_D\beta_2$.

Inhibition of cell migration by integrins expressed at high density is well established. As shown experimentally in studies with transfected cells and based on generated mathematical models, cell migration exhibits a bell-shaped dependence on the cell-substratum adhesiveness with the maximal speed occurring at its intermediate value [26,27,28]. This is an adhesiveness at which the cell can form new attachments at the cell front but break bonds at the rear. The total cell-substratum adhesiveness is a resultant of three variables, including ligand concentration, integrin activation and integrin density [28]. Under conditions when two other parameters are

constant, a moderate increase in integrin density enhances the strength of cell adhesion and promotes migration. However, as the receptor density continues to raise, this leads to an increased number of adhesive bonds between integrins and the substratum, which inhibits migration. The behavior of $\alpha_D\beta_2$ observed in our experiments fits this general model well, i.e. $\alpha_D\beta_2$ expressed at a low level promotes migration while high density $\alpha_D\beta_2$ inhibits migration. The conclusion that the increase in the $\alpha_D\beta_2$ -mediated adhesiveness is responsible for inhibition of migration is based on the effect of anti- α_D function-blocking antibodies which enhance cell migration to multiple $\alpha_D\beta_2$ ligands. Thus, disruption the excessive integrin-substrate bonds diminish cell adhesion and restores cell migration.

Although the relationships between integrin density and the rate of migration are evident, they have been established for recombinant cells transfected with only one integrin [28]. A unique feature of myeloid leukocytes (which is also modeled in our experiments with the $\alpha_D\beta_2$ -expressing HEK 293 cells) is that they express different types of integrins, β_1 and β_2 , both of which can engage the same proteins in the ECM. Furthermore, these receptors are expressed differently on various leukocyte populations. While circulating neutrophils express low levels of β_1 integrins and their levels are only modestly induced on stimulated cells [29,7], $\alpha_M\beta_2$ is strongly upregulated on neutrophils migrated into inflamed tissues [10]. Furthermore, as we demonstrate here, β_1 and β_3 integrins are not induced on migrating monocytes whereas the levels of $\alpha_D\beta_2$ are increased several fold. Therefore, although both β_1/β_3 and β_2 integrins can support adhesion to the same ligands their function in migration appears to be different: β_1/β_3 integrins drive migration whereas β_2 integrins, through alterations in their density, modulate the β_1/β_3 -driven migration. The role of β_1 integrins as primary migratory receptors is also supported by our data with anti- β_1 function-blocking mAbs which invariably inhibited migration of the $\alpha_D\beta_2$ -transfected HEK 293 cells to various ECM proteins. In addition, blocking the common α_V subunit of $\alpha_V\beta_1$ and $\alpha_V\beta_3$ blocked migration of IC-21 macrophages. However, $\alpha_D\beta_2$, when expressed at a low density, can contribute to migration as evidenced by the following data: 1) anti- α_D antibodies partially inhibited cell migration; and 2) migration of the $\alpha_D\beta_2$ -transfected cells expressing a low density of integrin was enhanced compared to mock-transfected cells (Fig.3A). In this regard, previous *in vivo* studies demonstrated that anti- $\alpha_D\beta_2$ antibody inhibited the accumulation of monocytes and neutrophils at sites of spinal cord injury [30]. Since infiltration of leukocytes was determined 48 h after injury, the period when $\alpha_D\beta_2$ seems not to be significantly upregulated, the anti- $\alpha_D\beta_2$ antibody might exert the inhibitory effect on leukocyte extravasation. Thus, depending on its density, $\alpha_D\beta_2$ may have a dual role in monocyte/macrophage migration, both pro-migratory and anti-migratory.

Previous studies demonstrated that expression of $\alpha_D\beta_2$ requires *de novo* synthesis and occurs during *in vitro* differentiation of human peripheral blood monocytes to macrophages in the presence of selected cytokines [15]. Moreover, oxLDL and AcLDL have been identified as stimuli that augment $\alpha_D\beta_2$ gene expression [15]. Also, activation with PMA can induce α_D mRNA in HL60 monocytic cells [15] and upregulate $\alpha_D\beta_2$ surface expression in isolated eosinophils [31]. Consistent with these findings, culturing murine macrophages with PMA led to a 5-fold increase in $\alpha_D\beta_2$ levels after 48–72 h whereas the levels of $\alpha_M\beta_2$ remained unaltered (Fig.5). Our data that both anti- α_D and anti- α_M antibodies inhibited cell migration of non-stimulated cells *in vitro* suggest that both $\alpha_M\beta_2$ and $\alpha_D\beta_2$ support migration. However, on PMA-stimulated cells, over-expressed $\alpha_D\beta_2$ suppressed migration while $\alpha_M\beta_2$ still supported migration. Thus, in these cells $\alpha_M\beta_2$ appears to provide no added contribution to the total cell adhesiveness which arises mainly from the excess of $\alpha_D\beta_2$.

An important finding of the present study is that macrophages elicited by thioglycollate injection upregulated $\alpha_D\beta_2$ by ~4-fold after 72 h indicating that physiologic activators in the inflamed peritoneum also induce $\alpha_D\beta_2$ synthesis *in vivo*. The anti- α_D antibodies increased the number of macrophages recruited in the peritoneal cavity after 72 h of inflammatory

stimulation. Thus, the data with blocking anti- α_D antibodies *in vitro* and *in vivo* are entirely consistent and, therefore, the blockade of excessive adhesive bonds formed between $\alpha_D\beta_2$ and its ligands may explain the increased recovery of macrophages from the peritoneal cavity. However, different mechanisms may account for this effect. First, the anti- α_D antibodies potentially may inhibit increased $\alpha_D\beta_2$ -mediated monocyte adhesion to the ECM proteins in the mesenteric wall as monocytes move from circulation into the peritoneal cavity, thus resulting in their augmented migration. Nevertheless, a pattern of $\alpha_D\beta_2$ upregulation seems to argue against the involvement of $\alpha_D\beta_2$ during post-endothelial monocyte migration. Since $\alpha_D\beta_2$ synthesis occurs slowly over a 72 h period it is reasonable to assume that monocytes upregulate $\alpha_D\beta_2$ only after they have traversed the peritoneal wall and develop into inflammatory macrophages. Therefore, the most probable interpretation is that $\alpha_D\beta_2$ is responsible for enhanced macrophage adhesion to the peritoneal wall and their retention in the peritoneal cavity. Hence, the increased number of macrophages recovered from the peritoneal fluid likely reflects the inhibitory effect of anti- α_D antibodies on adhesion of inflammatory macrophages to the peritoneal wall. Further studies using different models of inflammation may help to define how $\alpha_D\beta_2$ contributes to monocyte/macrophage recruitment and their retention at sites of inflammation.

The increased expression of $\alpha_D\beta_2$ on macrophage foam cells [14] suggests that $\alpha_D\beta_2$ may play roles in the development of atherosclerosis. Based upon our present results, it is tempting to speculate that upregulation of $\alpha_D\beta_2$ on differentiated macrophages can increase cell adhesiveness, resulting in the retention of macrophages in the atherosclerotic lesions and thereby evoking the development of atherosclerosis. It appears that $\alpha_D\beta_2$ upregulation on inflammatory macrophages contrasts that of $\alpha_M\beta_2$. Analogous to our data that $\alpha_M\beta_2$ is not upregulated on IC-21 cells, surface expression of this integrin on elicited peritoneal macrophages was augmented to a lesser extent than that of $\alpha_D\beta_2$ (1.5- versus 4-fold). Furthermore, expression of $\alpha_M\beta_2$ has been downregulated in atherosclerotic lesion-derived macrophages [32] and $\alpha_M\beta_2$ -deficiency has not influenced the development of atherosclerosis [33]. However, since β_2 -deficiency does protect against atherosclerosis [34], other β_2 integrins appear to play roles in lesion progression. Given the remarkable pattern of $\alpha_D\beta_2$ upregulation by atherogenic lipids, as well as overexpression of $\alpha_D\beta_2$ ligands such as CCN1 in the atherosclerotic lesions [35] the potential role of this integrin in disease progression merits further investigation.

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References

1. Kishimoto, TK.; Baldwin, ET.; Anderson, DC. Inflammation: Basic Principles and Clinical Correlates. 3rd. 1999. p. 537-569.
2. Henderson RB, Lim LHK, Tessier PA, Gavins FNE, Mathies M, Perretti M, Hogg N. The use of lymphocyte function-associated antigen (LFA)-1-deficient mice to determine the role of LFA-1, Mac-1, and α_4 integrin in the inflammatory response of neutrophils. *J.Exp.Med* 2001;194:219–226. [PubMed: 11457896]
3. Ding ZM, Babensee JE, Simon SI, Lu HF, Perrard JL, Bullard DC, Dai XY, Bromley SK, Dustin ML, Entman ML, Smith CW, Ballantyne CM. Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and migration. *J.Immunol* 1999 Nov 1;163:5029–5038. [PubMed: 10528208]
4. Issekutz TB. In vivo blood monocyte migration to acute inflammatory reactions, IL-1 α , TNF- α , INF- γ , and C5a utilize LFA-1, Mac-1, and VLA-4. *J.Immunol* 1995;154:6533–6540. [PubMed: 7759886]
5. Henderson RB, Hobbs JAR, Mathies M, Hogg N. Rapid recruitment of inflammatory monocytes is independent of neutrophil migration. *Blood* 2003;102:328–335. [PubMed: 12623845]

6. Shang XZ, Issekutz AC. β_2 (CD18) and β_1 (CD29) integrin mechanisms in migration of human polymorphonuclear leucocytes and monocytes through lung fibroblast barriers: shared and distinct mechanisms. *Immunology* 1997;92:527–535. [PubMed: 9497495]
7. Werr J, Johansson J, Eriksson EE, Hedqvist P, Ruoslahti E, Lindbom L. Integrin $\alpha_2\beta_1$ (VLA-2) is a principal receptor used by neutrophils for locomotion in extravascular tissue. *Blood* 2000;95:1804–1809. [PubMed: 10688841]
8. Ibbotson GC, Doig C, Kaur J, Gill V, Ostrovsky L, Fairhead T, Kubes P. Functional α_4 -integrin: a newly identified pathway of neutrophil recruitment in critically ill septic patients. *Nature Med* 2002;7:465–470. [PubMed: 11283674]
9. Coxon A, Rieu P, Barkalow FJ, Askari S, Sharpe AH, Von Andrian UH, Arnaout MA, Mayadas TN. A novel role for the beta 2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. *Immunity* 1996;5:653–666. [PubMed: 8986723]
10. Lu H, Smith CW, Perrard J, Bullard D, Tang L, Entman ML, Beaudet AL, Ballantyne CM. LFA-1 is sufficient in mediating neutrophil emigration in Mac-1 deficient mice. *J.Clin.Invest* 1997;99:1340–1350. [PubMed: 9077544]
11. Prince JE, Brayton CF, Fosset MC, Durand JA, Kaplan SL, Smith CW, Ballantyne CM. The differential roles of LFA-1 and Mac-1 in host defense against systemic infection with *Streptococcus pneumoniae*. *J.Immunol* 2001;166:7362–7369. [PubMed: 11390487]
12. Lishko VK, Yakubenko VP, Ugarova TP. The interplay between Integrins $\alpha_M\beta_2$ and $\alpha_5\beta_1$ during cell migration to fibronectin. *Exp.Cell Res* 2003;283:116–126. [PubMed: 12565824]
13. Yakubenko VP, Yadav SP, Ugarova TP. Integrin $\alpha_D\beta_2$, an adhesion receptor up-regulated on macrophage foam cells, exhibits multiligand binding properties. *Blood* 2006;107:1643–1650. [PubMed: 16239428]
14. Van der Vieren M, Le Trong H, St.John T, Staunton DE, Gallatin WM. A novel leukointegrin, $\alpha_d\beta_2$, binds preferentially to ICAM-3. *Immunity* 1995;3:683–690. [PubMed: 8777714]
15. Noti JD. Expression of the myeloid-specific leukocyte integrin gene CD11d during macrophage foam cell differentiation and exposure to lipoproteins. *International Journal of Molecular Medicine* 2002;10:721–727. [PubMed: 12429998]
16. Vuento M, Vahery A. Purification of fibronectin from human plasma by affinity chromatography under non-denaturing conditions. *Biochem.J* 1979;183:331–337. [PubMed: 534500]
17. Yatohgo T, Izumi M, Kashiwagi H, Hayashi M. Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct.Funct* 1988;13:281–292. [PubMed: 2460263]
18. Kireeva ML, Mo FE, Yang GP, Lau LF. Cyr61, a product of a growth factor-inducible immediate-early gene, promotes cell proliferation, migration, and adhesion. *Mol.Cell.Biol* 1996;16:1326–1334. [PubMed: 8657105]
19. Yakubenko VP, Lishko VK, Lam SCT, Ugarova TP. A molecular basis for integrin $\alpha_M\beta_2$ ligand binding promiscuity. *J.Biol.Chem* 2002;277:48635–48642. [PubMed: 12377763]
20. Forsyth CB, Solovjov DA, Ugarova TP, Plow EF. Integrin $\alpha_M\beta_2$ -mediated cell migration to fibrinogen and its recognition peptides. *J.Exp.Med* 2001;193:1123–1133. [PubMed: 11369784]
21. Tang L, Ugarova TP, Plow EF, Eaton JW. Molecular determinants of acute inflammatory responses to biomaterials. *J.Clin.Invest* 1996;97:1329–1334. [PubMed: 8636446]
22. Pytela R, Pierschbacher MD, Ruoslahti E. Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell* 1985;40:191–198. [PubMed: 3155652]
23. Suehiro K, Gailit J, Plow EF. Fibrinogen is a ligand for integrin $\alpha_5\beta_1$ on endothelial cells. *J.Biol.Chem* 1997;272:5360–5366. [PubMed: 9030612]
24. Leu SJ, Lam SC, Lau LF. Pro-angiogenic activities of Cyr61 (CCN1) mediated through integrins $\alpha_v\beta_3$ and $\alpha_6\beta_1$ in human umbilical vein endothelial cells. *J.Biol.Chem* 2002;277:46248–46255. [PubMed: 12364323]
25. Ploplis VA, French EL, Carmeliet P, Collen D, Plow EF. Plasminogen deficiency differentially affects recruitment of inflammatory cell populations in mice. *Blood* 1998;91:2005–2009. [PubMed: 9490683]
26. DiMilla PA, Barbee K, Lauffenburger DA. Mathematical model for the effects of adhesion and mechanics on cell migration speed. *Biophys.J* 1991;60:15–37. [PubMed: 1883934]

27. DiMilla PA, Stone JA, Quinn JA, Albelda SM, Lauffenburger DA. Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. *J.Cell Biol* 1993;122:729–737. [PubMed: 8335696]
28. Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, Horwitz AF. Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature* 1997;385:537–540. [PubMed: 9020360]
29. Werr J, Xie X, Hedqvist P, Ruoslahti E, Lindbom L. β_1 integrins are critically involved in neutrophil locomotion in extravascular tissue in vivo. *J.Exp.Med* 1998;187:2091–2096. [PubMed: 9625769]
30. Mabon PJ, Weaver LC, Dekaban GA. Inhibition of monocyte/macrophage migration to a spinal cord injury site by an antibody to the integrin α_D : a potential new anti-inflammatory treatment. *Exp.Neurol* 2000;166:52–64. [PubMed: 11031083]
31. Grayson MH, Van der Vieren M, Sterbinsky SA, Gallatin WM, Hoffman PA, Staunton DE, Bochner BS. $\alpha_D\beta_2$ Integrin is expressed on human eosinophils and functions as an alternative ligand for vascular cell adhesion molecule 1 (VCAM-1). *J.Exp.Med* 1998;188:2187–2191. [PubMed: 9841932]
32. Gray JL, Shankar R. Down regulation of CD11b and CD18 expression in atherosclerotic lesion-derived macrophages. *Am.Surg* 1995;61:674–679. [PubMed: 7618805]
33. Kubo N, Boisvert WA, Ballantyne CM, Curtiss LK. Leukocyte CD11b expression is not essential for the development of atherosclerosis in mice. *J.Lipid Res* 2000;41:1060–1066. [PubMed: 10884286]
34. Nageh MF, Sandberg ET, Marotti KR, Lin AH, Melchior EP, Bullard DC, Beaudet AL. Deficiency of inflammatory cell adhesion molecules protects against atherosclerosis in mice. *Arterioscler.Thromb.Vasc.Biol* 1997;17:1517–1520. [PubMed: 9301629]
35. Schober JM, Chen N, Grzeszkiewicz T, Emeson EE, Ugarova TP, Ye RD, Lau LF, Lam SCT. Identification of integrin $\alpha_M\beta_2$ as an adhesion receptor on peripheral blood monocytes for Cyr61 and connective tissue growth factor, immediate-early gene products expressed in atherosclerotic lesions. *Blood* 2002;99:4457–4465. [PubMed: 12036876]

Fig.1. Characterization of HEK 293 cells expressing different levels of $\alpha_D\beta_2$

Binding of anti- α_D polyclonal antibody (10 $\mu\text{g/ml}$) and anti- β_2 mAb IB4 (10 $\mu\text{g/ml}$) to mock-transfected and the $\alpha_D\beta_2$ -expressing cells was analyzed by flow cytometry. Results are presented as histograms with the logarithm of fluorescence intensity on the *abscissa* and the cell number on the *ordinate*. The level of integrin expression is shown as filled histograms. Control cells incubated with the Alexa Fluor-488 conjugated secondary antibody are shown as open histograms.

Fig.2. Adhesion of HEK 293 cells expressing different densities of $\alpha_D\beta_2$ and mock-transfected HEK 293 cells to vitronectin

Aliquots of calcein-labeled cells (5×10^4) were allowed to adhere to wells of 96-well plates coated with 5 $\mu\text{g/ml}$ vitronectin in the absence or presence of blocking anti- $\alpha_D\beta_2$ antibody (10 $\mu\text{g/ml}$). After incubation for 30 min at 37°C in 5% CO_2 , the nonadherent cells were removed by two washes with PBS and fluorescence was measured. The number of adherent cells was calculated by using the fluorescence of aliquots with a known number of labeled cells. Data are expressed as a percentage of added cells and are the mean $\pm$ SD of three individual experiments (* denotes $P < 0.05$; ** denotes $P < 0.01$).

Fig.3. Migration of the $\alpha_D\beta_2$ -expressing HEK 293 cells to vitronectin

A, HEK 293 cells expressing different densities of $\alpha_D\beta_2$ were analyzed for their ability to migrate through the Transwell inserts having a pore size of 8 μ M and 6.5 mm in diameter. Cells were allowed to migrate toward 50 μ g/ml of vitronectin placed in the lower chamber of the Transwell system for 18 hours at 37°C in 5% CO₂. Two hours prior to the completion of the migration assay, cells were labeled with calcein AM and fluorescence of cells that migrated to the undersurface of the filter was determined. The cells expressing low (**B**) and high (**C**) densities of $\alpha_D\beta_2$ were analyzed for their ability to migrate to 50 μ g/ml of vitronectin in the presence of function blocking polyclonal anti- α_D antibody or blocking anti- β_1 mAb. Cells were preincubated with 20 μ g/ml of each antibody for 20 min before their addition to the upper

chamber of Transwell plates. Migration data are expressed as a percentage of the migration of mock-transfected cells (A) or control $\alpha_D\beta_2$ transfected cells in the absence of antibodies (B, C). Results are the mean $\pm$ SD from three to five separate experiments, each with duplicate measurements. (*, $P<0.05$; **, $P<0.01$ compared to mock transfected cells in (A) or control (B,C)).

Fig.4. Migration of HEK 293 cells expressing a high density of $\alpha_D\beta_2$ to various ECM proteins
 HEK 293 cells expressing a high density of $\alpha_D\beta_2$ were analyzed for their ability to migrate to 50 μ g/ml fibrinogen, fibronectin and CCN1 in the absence or in the presence of blocking polyclonal anti- α_D antibody or blocking anti- β_1 mAb. Cells were preincubated with 20 μ g/ml antibodies for 20 min before their addition to the upper chamber of Transwell plates. Cells were allowed to migrate toward ligands for 18 hours at 37°C in 5% CO₂ and the extent of cell migration was assessed as described in Materials and Methods. Data are expressed as a percentage of migration; cell migration in the absence of blocking antibodies was assigned a value of 100%. Values represent the means $\pm$ SD with duplicate wells for each experiment from three or more individual experiments (*, P<0.05; **, P<0.01 compared to control).

Fig.5. Analyses of integrin $\alpha_D\beta_2$ and $\alpha_M\beta_2$ expression on mouse IC-21 macrophages after PMA stimulation

IC-21 cells were stimulated with 100ng/ml PMA for different periods of time and the levels of $\alpha_M\beta_2$ (open bars) and $\alpha_D\beta_2$ (black bars) integrins were assessed by flow cytometry using anti- α_M mAb M1/70 and polyclonal anti- α_D antibody. Results are the mean $\pm$ SD values of mean fluorescence from three independent experiments (*, $P<0.05$; **, $P<0.01$ compared to α_D expression before PMA treatment.)

Fig.6. Migration of IC-21 cells expressing different levels of $\alpha_D\beta_2$ to vitronectin

Unstimulated (**A**) and PMA-stimulated for 72 h (**B**) IC-21 cells were analyzed for their ability to migrate through the Transwell inserts to 50 $\mu\text{g/ml}$ of vitronectin either in the absence or presence of either Fab preparations of anti- α_D or mAb M1/70. Cells were preincubated with 20 $\mu\text{g/ml}$ of each antibody for 20 min before their addition to the upper chambers of Transwell plates. Cells were allowed to migrate toward vitronectin coated on the undersurface of the filter for 18 hours at 37°C in 5% CO_2 . Data are expressed as a percentage of migrated cells in the absence of blocking antibodies. Results are the mean $\pm$ SD values from three to five individual experiments with duplicate wells in each experiment for each reagent tested (*, $P < 0.04$; **, $P < 0.01$ compared to control).

Fig.7. Analyses of integrin expression on mouse monocytes isolated from peripheral blood and on peritoneal macrophages

Peripheral blood monocytes were isolated from mouse blood collected by cardiac puncture. Monocytes were obtained as a monocyte-lymphocyte phase after separation using a Ficoll-Paque gradient. Peritoneal macrophages were collected at 72 hours after injection of 0.5 ml 3% thioglycollate by washing peritoneum cavity with 5 ml of PBS. The integrin densities on monocytes (open bars) and macrophages (black bars) were determined by flow cytometry using selected FITC-conjugated mAbs against specific integrin subunits. Monocytes isolated from the peripheral blood and macrophages in the peritoneal lavage were detected using back-gating with phycoerythrin-conjugated mAbs against CD14 and F4/80, respectively. Results are the mean $\pm$ SD values of mean fluorescence in three or more independent experiments (**, $P < 0.01$ compared to α_D and α_M expression on monocytes).

Fig.8. Effect of anti- α_D antibodies on macrophage accumulation in the peritoneum

Mice were treated with affinity purified anti- α_D polyclonal antibody (100 μ g) or rabbit IgG 4 hours before the development of peritonitis. Peritonitis was induced by intraperitoneal injection of 3% Brewer thioglycollate medium (0.5 ml). 72 hours later, the mice were euthanized by isoflurane inhalation, and their peritoneal cavities were lavaged with 5 ml PBS. The numbers of migrated macrophages in the peritoneum were determined by nonspecific esterase assay. The data shown are representative of four separate experiments. The data represent the means $\pm$ SD for the control and the treated groups with seven mice in each group. *Asterisk* denotes the statistically significant difference between the control and treated groups ($P < 0.02$). This experiment was replicated 4 times with the same results.