Integrins mediate adherence and migration of T lymphocytes on human peritoneal mesothelial cells

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We previously showed that a local immune response largely composed of type 1 T cells correlated with a favorable outcome of the peritonitis associated with peritoneal dialysis. To clarify how these subsets are recruited to the peritoneal cavity during inflammation, we measured integrin-mediated interactions between the T cells and human peritoneal mesothelial cells. Direct microscopy showed that lipopolysaccharide or peritoneal dialysis effluent stimulated the adherence of T cells to mesothelial cells, a process mediated by the integrins α6β1 and α4β1. Further, the migration of Th1 cell across human mesothelial cell monolayers grown on transwell surfaces was reduced by anti-α6β1 integrin antibody while that of Th2 cell was inhibited by an anti-α4 integrin antibody. Pretreatment with either lipopolysaccharide or rapid response peritoneal dialysis effluent stimulated T cell migration and this was significantly decreased by the α6β1 compared to the α4 antibody. These results suggest that integrins may play an important role in mediating selective T cell subset adhesion and migration across human peritoneal mesothelial cell monolayers and differential integrin expression and selective T cell subset recruitment during peritonitis may affect outcome.

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Peritoneal dialysis (PD) is an established treatment for end-stage renal disease.[1] Peritonitis, a local inflammatory disorder, is the most serious complication of PD.[2–7] All the resident peritoneal cavity cells, as well as the leukocytes that are recruited and infiltrate, coordinate the immune response associated with peritoneal infection.[8–10]

Human peritoneal mesothelial cells (HPMCs) line the surface of the peritoneal membrane. Following stimulation by bacteria-secreted products or pro-inflammatory cytokines, HPMCs secrete inflammatory cytokines and chemokines, which activate and control peritoneal inflammatory processes and attract leukocytes into the peritoneal cavity.[11–15] HPMCs constitutively express intracellular adhesion molecule-1 and vascular cell adhesion molecule-1; expressions of both are increased with *in vivo* inflammation or after cytokine stimulation *in vitro*.[11,16–19] Thus, HPMCs play an important role in mediating leukocyte trafficking into the peritoneal cavity.

In PD patients, leukocyte infiltration into the peritoneal cavity is important for eradicating the invading organisms.[20,21] The mechanisms that control leukocyte extravasation from the circulation have been extensively studied. However, how leukocytes recognize and traverse the peritoneal mesothelial lining and enter the peritoneal cavity has yet to be determined. Previous investigations have addressed the role of intracellular adhesion molecule-1 expression in leukocyte migration across HPMCs,[11,16,22] and the role of interferon-γ and interleukin (IL)-17 in controlling neutrophil migration across the mesothelium.[23,24] The mechanisms that control T-lymphocyte migration across HPMCs have not been addressed.

The T-cell/subsets response plays a role in peritoneal immunity during peritonitis. Previous studies showed that interferon-γ levels in PD effluent (PDE) were often elevated in patients with bacterial peritonitis.[25] We previously found that a progressive increase in the CD4/CD8 ratio in PDE indicated a favorable outcome in patients with PD-related peritonitis.[26] We recently found that high IL-12 and IL-18 levels in PDE during the early phase of peritonitis were correlated with a predominant type I T-cell immune response

and a favorable outcome.[27] Some studies show the link between T cells and HPMCs in peritoneal immunity. Hausmann *et al.*[28] found that HPMCs participated in the peritoneal immune response by antigen presentation and contributed to T-cell activation by secreting IL-15.[28] Basok *et al.*[29] reported that CD40, whose ligand is mainly expressed on the membrane of activated CD4 lymphocytes, is expressed on HPMCs. However, the interaction between T cells and HPMCs needs further study.

Integrins mediate cell–cell and cell–extracellular matrix adhesion.[30–33] Integrins are not simply adhesion sites on T cells; they play prominent roles in the migration of T cells to peripheral lymph node and inflammatory sites, in antigen presentation, cytotoxic killing, and transduction of co-stimulatory signals in T cells.[34–38] T helper (Th) subtype 1 (Th1) and subtype 2 (Th2) cells have different integrin expression patterns; these differences contribute to the differential recruitment of Th1 and Th2 cells[39] and promote a predominant type I or type II T-cell immune response. Previous studies showed preferential expression of integrin α6β1 on Th1 cells; IL-12 upregulates their expression and promotes Th1 cell migration.[39–41] Th2 cells express elevated levels of α4β1 integrins, which mediate Th2 cell adhesion and migration.[35,39,41,42] The role of T-cell integrin as an adhesion, migration, and co-stimulatory molecule suggests that integrins may be involved in the interaction between T cells and HPMCs. However, the role and regulation of T-cell integrins during the interaction with HPMCs are poorly defined.

To clarify the mechanisms of T-lymphocyte adherence and migration across HPMCs, we developed an *in vitro* adhesion and migration assay to determine whether integrins are involved in T lymphocyte–mesothelial recognition under various conditions. The various integrins involved in the different T-cell subsets and in their interaction with HPMCs were also investigated to elucidate the mechanism regulating the polarized T-cell immune response. Our data indicate that integrins may play an important role in mediating T-lymphocyte adhesion and migration across HPMCs; the presence of differential integrins promotes the selective recruitment of T-cell subsets and correlates with peritonitis treatment outcome.

RESULTS

Adherence of T lymphocytes to HPMCs

To determine whether T lymphocytes are capable of adhering to HPMCs under different environmental stimuli, the adhesion relationships were observed directly under a microscope. HPMCs have a small, round, often eccentric, nucleus and a large amount of cytoplasm (Figure 1a). When HPMCs were not pre-stimulated, 3.0 ± 1.6% of HPMC–T-lymphocytes formed rosettes (Figure 1b). When HPMCs were pre-exposed to lipopolysaccharide (LPS), 20.2 ± 3.1% formed rosettes (Figure 1c). When HPMCs were pre-exposed to rapid and delayed-response PDE, 19.1 ± 2.9% (Figure 1d) and 18.2 ± 3.3% (Figure 1e) formed rosettes, respectively. After stimulation with LPS or PDE, HPMCs showed a polarized morphology with multiple elongated cytoplasmic processes (Figure 1c–e). Compared with HPMCs without pre-stimulation, significantly more LPS- and PDE-pretreated HPMCs formed rosettes with T lymphocytes ($P<0.05$). These data show that T lymphocytes exhibit low-level specific binding to HPMCs under control conditions, and that stimulation enhances adhesion.

Integrin expression during HPMC–T-lymphocyte adhesion

To study integrin expression during HPMC–T-lymphocyte adhesion, immunofluorescence staining was carried out. The expression of α6β1 and α4β1 integrins was examined due to

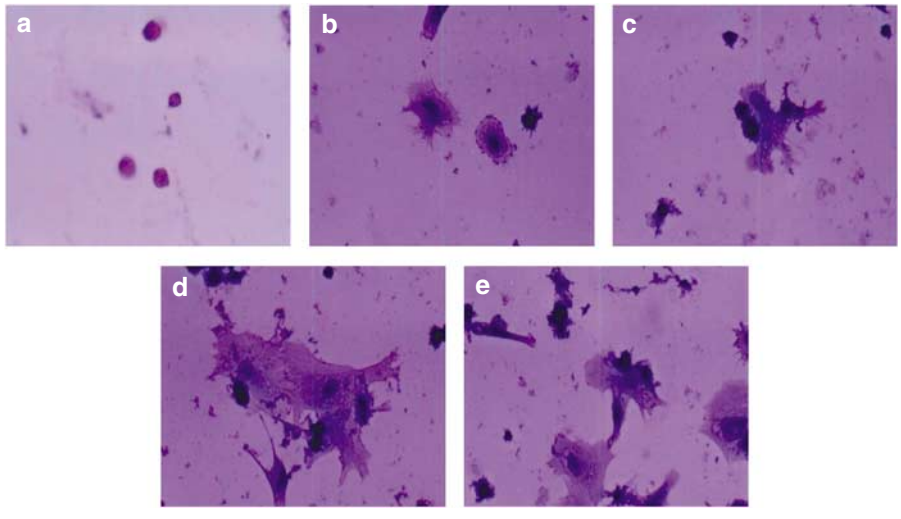

Figure 1 | Adhesion between T lymphocytes and HPMCs. (**a**) The morphology of unstimulated HPMCs. HPMC–T-lymphocyte rosette formation was seen in (**b**) 3.0 ± 1.6% when the HPMCs were not pre-stimulated; (**c**) 20.2 ± 3.1% when HPMCs were pre-stimulated with LPS 10 μg/ml; (**d**) 19.1 ± 2.9% when HPMCs were pre-stimulated with rapid-response PDE 1000 μg/ml; and (**e**) 18.2 ± 3.3% when HPMCs were pre-stimulated with delayed-response PDE 1000 μg/ml.

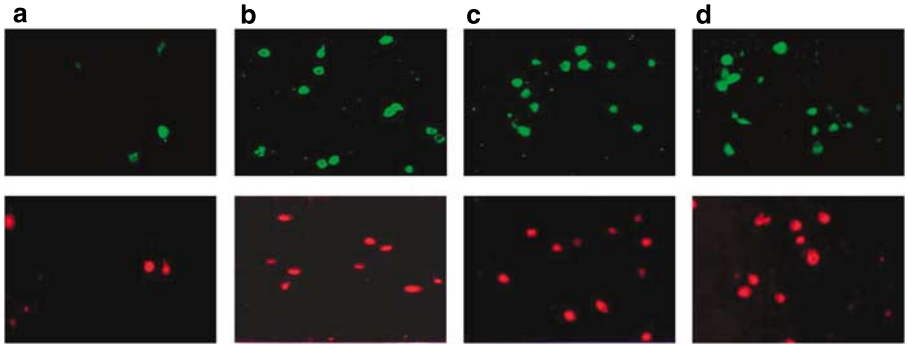

Figure 2 | Immunofluorescence staining of integrin expression during HPMC–T-lymphocyte adhesion. (**a**) T lymphocytes adhered to unstimulated HPMCs. Upper panel: 4.7 ± 1.1% of T lymphocytes were stained with anti-α6β1 antibody and showed green fluorescence. Lower panel: 3.2 ± 0.7% of T lymphocytes were stained with anti-α4 antibody and showed red fluorescence. (**b**) T lymphocytes adhered to HPMC pre-stimulated with LPS 10 μg/ml. Upper panel: 36.4 ± 5.3% of T lymphocytes were stained with anti-α6β1 antibody and showed green fluorescence. Lower panel: 32.2 ± 4.3% of T lymphocytes were stained with anti-α4 antibody and showed red fluorescence. (**c**) T lymphocytes adhered to HPMCs pre-stimulated with rapid-response PDE 1000 μg/ml. Upper panel: 38.6 ± 4.4% of T lymphocytes were stained with anti-α6β1 antibody and showed green fluorescence. Lower panel: 35.9 ± 5.3% of T lymphocytes were stained with anti-α4 antibody and showed red fluorescence. (**d**) T lymphocytes adhered to HPMCs pre-stimulated with delayed-response PDE 1000 μg/ml. Upper panel: 35.2 ± 4.2% of T lymphocytes were stained with anti-α6β1 antibody and showed green fluorescence. Lower panel: 36.4 ± 3.7% of T lymphocytes were stained with anti-α4 antibody and showed red fluorescence.

their potential role in Th subset adhesion and migration. When T lymphocytes interacted with unstimulated HPMCs, the expressions of integrins α6β1 and α4β1 were 4.7 ± 1.1% and 3.2 ± 0.7%, respectively (Figure 2a). When HPMCs were pre-stimulated with LPS, the T-lymphocyte expressions of integrins α6β1 and α4β1 increased to 36.4 ± 5.3% and 32.2 ± 4.3%, respectively (Figure 2b). Compared with the control group of HPMCs without pre-stimulation, T-lymphocyte expressions of integrins α6β1 and α4β1 increased significantly ($P<0.05$). When HPMCs were pre-stimulated with rapid-response PDE, T-lymphocyte expressions of α6β1 and α4β1 integrins increased significantly ($P<0.05$) compared with the control groups (38.6 ± 4.4%, 35.9 ± 5.3%, respectively; Figure 2c). T-lymphocyte expressions of α6β1 (35.2 ± 4.2%) and α4β1 (36.4 ± 3.7%) integrins increased significantly ($P<0.05$) when they interacted with HPMCs pretreated with delayed-response PDE (Figure 2d), compared with when they interacted with unstimulated HPMC controls. Blank controls with HPMC only were negative for the expression of both α6β1 and α4β1 integrins. Thus, integrins α6β1 and α4β1 are involved in HPMC–T-lymphocyte adhesion, and their expression increases after HPMC pre-stimulation.

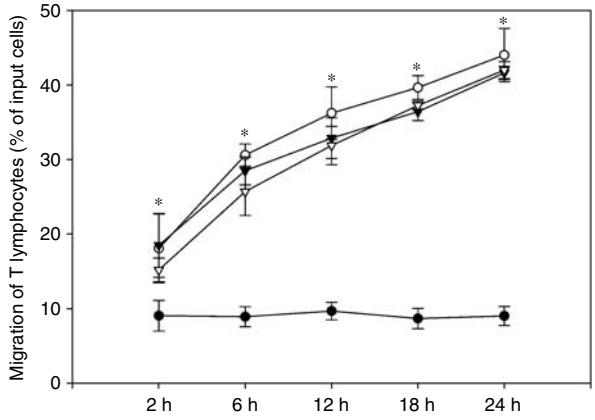

Figure 3 | Effect of HPMC pre-stimulation on T-lymphocyte migration. Apical pre-stimulation of the HPMC monolayer with LPS or PDE resulted in a time-dependent increase in the migration of T lymphocytes across HPMC. ●, control; ○ LPS 10 μg/ml; ▼ rapid-response PDE 1000 μg/ml; ▽ delayed-response PDE 1000 μg/ml. Data are expressed as mean ± s.d. from eight separate experiments. *$P<0.05$ versus control.

T-lymphocyte migration across HPMCs and the effect of LPS and PDE

To determine whether T lymphocytes migrate across HPMCs under various environmental stimuli, an *in vitro* migration system was used to assess the time course of changes (Figure 3). Apical pre-stimulation of the HPMC monolayer with LPS or PDE resulted in a time-dependent increase in T-lymphocyte migration across HPMCs. T-lymphocyte migration across pre-stimulated HPMCs was significantly greater than control after 2 h of stimulation, and continued to increase ($P<0.05$; Figure 3). No significant difference in T-lymphocyte migration was observed for HPMCs pre-stimulated with LPS or PDE (rapid or delayed clinical response).

Integrins mediate T-lymphocyte migration across HPMCs

To determine whether integrins had an effect on T-lymphocyte migration across HPMCs, antibody-blocking of T-lymphocyte migration was studied. In the control group, T-lymphocyte migration was not significantly reduced by pretreatment with anti-α6β1 or anti-α4 antibody (Figure 4a). T lymphocytes pretreated with anti-α6β1 or anti-α4 antibody had significantly reduced migration across HPMCs in the LPS and PDE groups (Figure 4b–d). When HPMCs were pre-stimulated with LPS, T lymphocytes pretreated with anti-α6β1 antibody showed significantly reduced migration

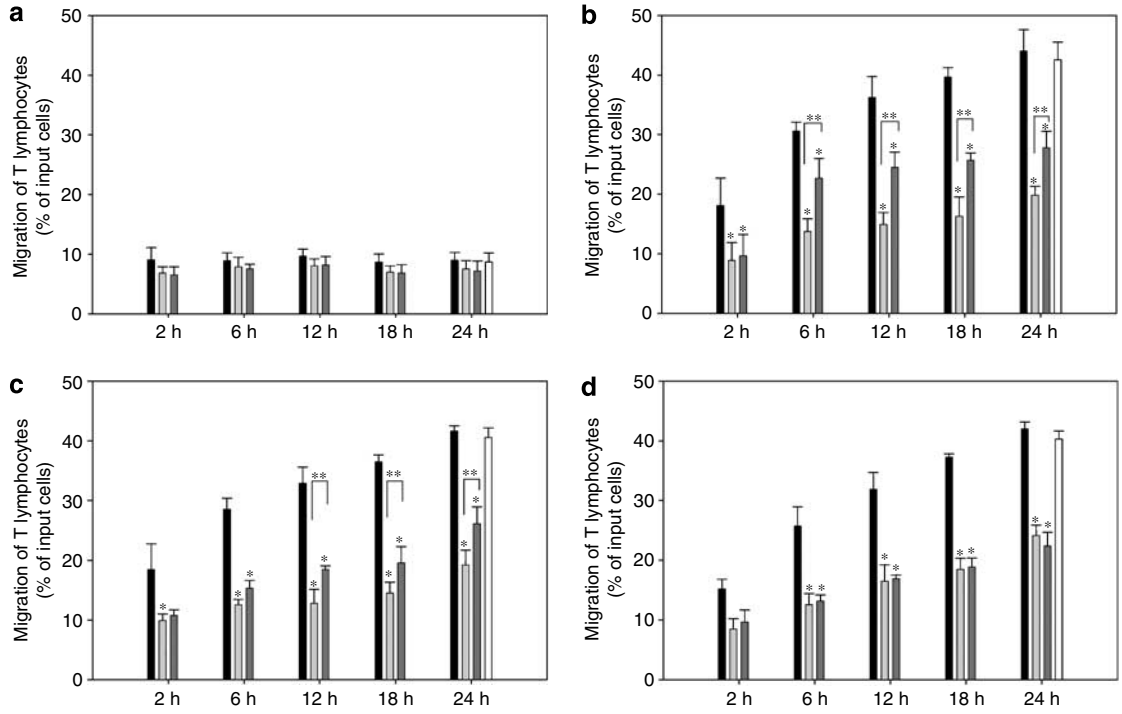

Figure 4 | Inhibition of T-lymphocyte migration across HPMCs by anti-integrin antibodies. (**a**) HPMCs without pre-stimulation at different time periods; HPMCs pre-stimulated with (**b**) LPS 10 μg/ml; (**c**) rapid-response PDE 1000 μg/ml; (**d**) delayed-response PDE 1000 μg/ml in different time periods. ■, no antibody; □, T lymphocytes pretreated with anti-α6β1 antibody; ▩, T lymphocytes pretreated with anti-α4 antibody; □, T lymphocytes pretreated with isotype-matched control antibody. Data are expressed as mean ± s.d. from eight separate experiments. *$P<0.05$ versus control, **$P<0.05$ anti-α6β1 antibody versus anti-α4 antibody.

after 6 h stimulation and continued throughout the entire duration of the study, compared to T lymphocytes pretreated with anti-α4 antibody ($P<0.05$; Figure 4b). Similar results were noted in HPMCs pre-stimulated with rapid-response PDE (Figure 4c). However, when HPMCs were pre-stimulated with delayed-response PDE, the reduction in T-lymphocyte migration was similar to that seen with anti-α6β1 and anti-α4 antibody pretreatment ($P>0.05$; Figure 4d). These data suggest that integrins play a role in T-lymphocyte migration across HPMCs. However, the importance of α6β1 and α4β1 integrins depends on the conditions used to stimulate HPMCs.

Migration of Th1 cells across HPMCs is primarily mediated by α6β1 integrin

Polarized human Th lymphocytes were generated (Figure 5) to study how different integrins mediate the migration of the various T-cell subsets across HPMCs. In the control group, there was no significant reduction of Th1-cell migration following anti-α6β1 or anti-α4 antibody treatment (Figure 6a). In the LPS and PDE groups, Th1 cells that were pretreated with anti-α6β1 antibody showed decreased migration across HPMCs compared with untreated Th1 cells (Figure 6b–d). When Th1 cells were pretreated with anti-α4 antibody, Th1-cell migration was mildly decreased compared with untreated Th1 cells, but the difference was not statistically significant (Figure 6b–d). In the LPS- and PDE-stimulated groups, there was a significant decrease in Th1-cell migration with anti-α6β1 antibody treatment compared with anti-α4 antibody treatment throughout the experiment ($P<0.05$; Figure 6b–d). These data suggest that α6β1 integrin is more important than α4β1 integrin in Th1-cell migration across HPMCs.

Migration of Th2 cells across HPMCs is primarily mediated by α4β1 integrin

In the control group, there was no significant reduction in Th2-cell migration after pretreatment with either anti-α6β1 or anti-α4 antibody (Figure 7a). In the LPS- and PDE-stimulated groups, pretreatment with anti-α4 antibody inhibited Th2-cell migration across HPMCs compared with untreated Th2 cells ($P<0.05$; Figure 7b–d). The reduction in Th2-cell migration occurred after 2 h of stimulation and continued throughout the entire experiment. Th2-cell migration was somewhat reduced with anti-α6β1 antibody pretreatment, but, compared with untreated Th2 cells, the difference was not statistically significant (Figure 7b–d). In the LPS- and PDE-stimulated groups, there was a significant decrease in Th2-cell migration with anti-α4 antibody treatment compared with anti-α6β1 antibody treatment ($P<0.05$; Figure 7b–d). Thus, α4β1 integrin appears to be more important than α6β1 integrin in Th2-cell migration across HPMCs.

DISCUSSION

The present study used *in vitro* systems to examine the adhesion relationship and migration behavior of HPMCs and

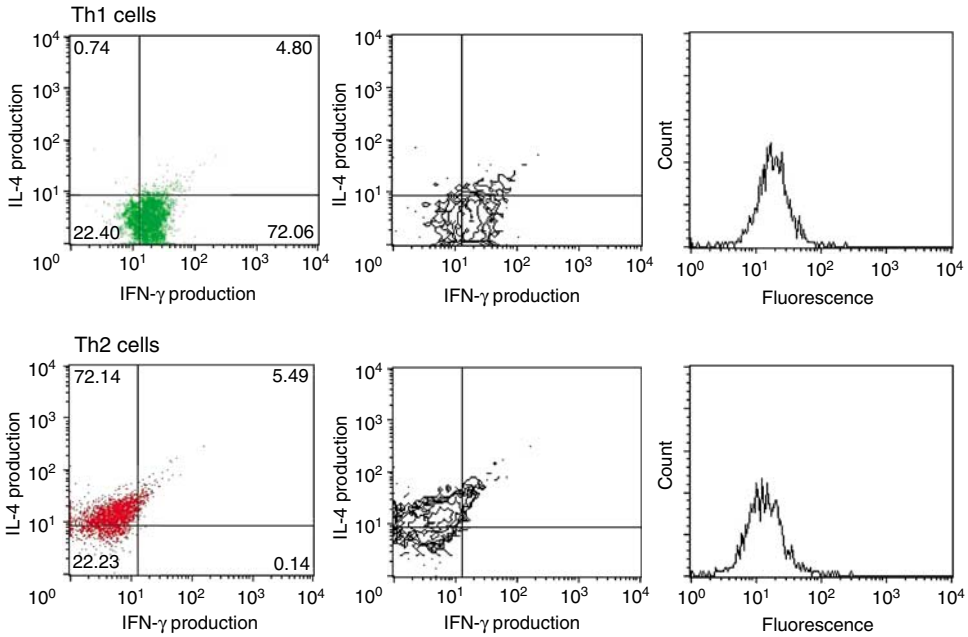

Figure 5 | Flow cytometry analyses of Th1 and Th2 cells phenotypes generated *in vitro*. The phenotype of the *in vitro* differentiated Th1 and Th2 cells was determined by two-color intracytoplasmic staining for interferon (IFN)-γ and IL-4-producing cells. The fraction of cells located in each quadrant is indicated in the inset. One representative experiment of the eight that were done is shown.

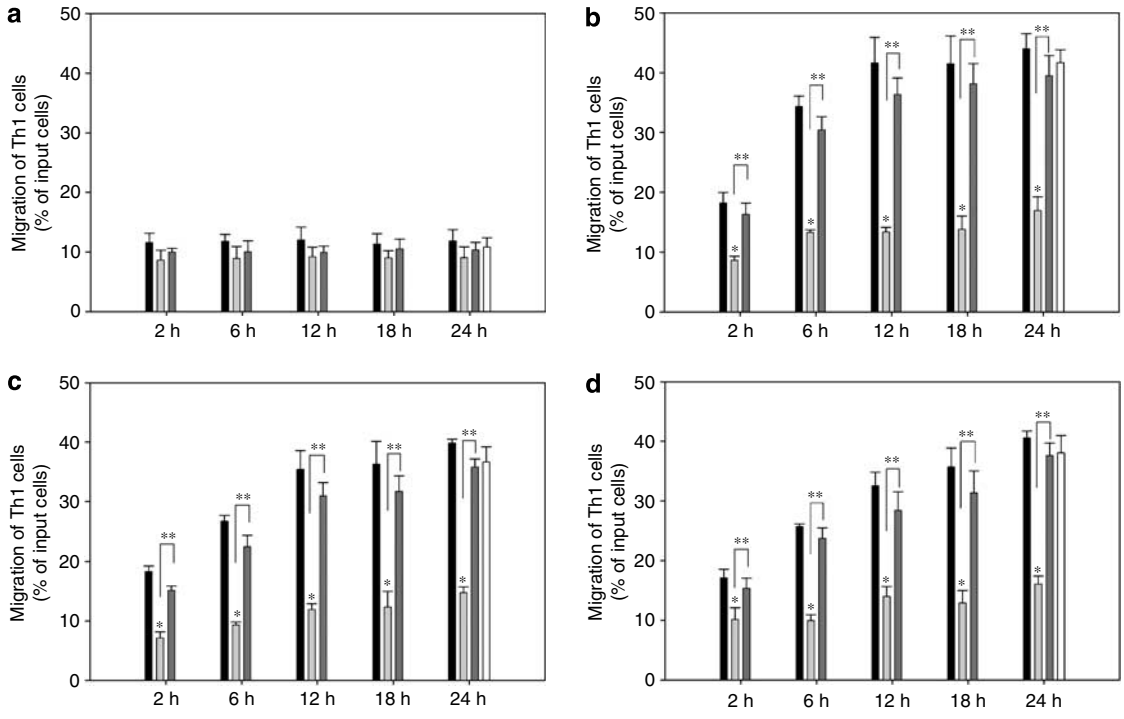

Figure 6 | Inhibition of Th1-cell migration across HPMCs by anti-α6β1 antibodies. (**a**) HPMCs without pre-stimulation at different time periods; HPMCs pre-stimulated with (**b**) LPS 10 μg/ml; (**c**) rapid-response PDE 1000 μg/ml; (**d**) delayed-response PDE 1000 μg/ml in different time periods. ■, no antibody; □, Th1 cells pretreated with anti-α6β1 antibody; ▩, Th1 cells pretreated with anti-α4 antibody; □, Th1 cells pretreated with isotype-matched control antibody. Data are expressed as mean ± s.d. from eight separate experiments. *$P<0.05$ versus control, **$P<0.05$ anti-α6β1 antibody versus anti-α4 antibody.

T lymphocytes. The morphology of the T-lymphocyte–mesothelial cell interaction and the role that integrins play in the interaction have not been previously reported with respect to peritoneal immunity.

Microscopic observation showed direct adhesion of HPMCs and T lymphocytes through rosette formation. This provides evidence that the mesothelium plays an active role in peritoneal inflammatory processes. Based on

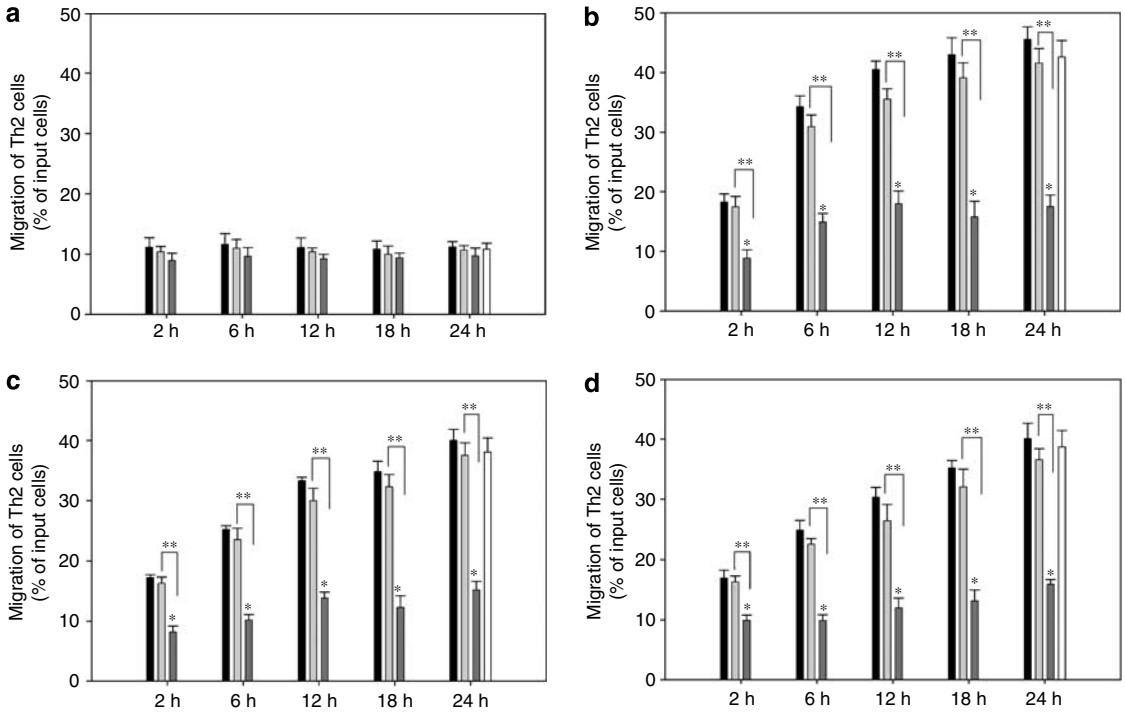

Figure 7 | Inhibition of Th2-cell migration across HPMCs by anti-α4 antibodies. (**a**) HPMCs without pre-stimulation at different time periods; HPMC pre-stimulated with (**b**) LPS 10 μg/ml; (**c**) rapid-response PDE 1000 μg/ml; (**d**) delayed-response PDE 1000 μg/ml in different time periods. ■, no antibody; □, Th2 cells pretreated with anti-α6β1 antibody; ▩, Th2 cells pretreated with anti-α4 antibody; □, Th2 cells pretreated with isotype-matched control antibody. Data are expressed as mean ± s.d. from eight separate experiments. *$P < 0.05$ versus control, **$P < 0.05$ anti-α6β1 antibody versus anti-α4 antibody.

immunofluorescence staining, it was found that integrins α6β1 and α4β1 were involved in the interaction and that their expression increased after stimulation. The data suggest that both integrin and its ligand are important in peritoneal inflammatory and immune reaction. The present study also found morphological evidence that HPMCs may act as antigen-presenting cells,[28] and that integrins may act as co-stimulatory molecules that activate T lymphocytes and promote the adaptive peritoneal immune response.

The migration assay study indicated the importance of integrins α6β1 and α4β1 in the migration process of T lymphocytes across HPMCs. Furthermore, α6β1 integrin predominantly mediates Th1-cell migration across HPMCs and α4β1 integrin predominantly mediates Th2-cell migration. To the best of our knowledge, this is the first time that Th-subset integrin expression and function have been demonstrated in peritonitis. The differential expression of integrins on Th subsets is compatible with previous studies under other conditions.[35,39–42] These data suggest that integrins are expressed on T lymphocytes and play an important regulatory role in controlling the phenotype of infiltrating T lymphocytes during peritoneal inflammation.

In our previous study,[27] a type I T-cell response was found to be critical for favorable outcome following peritonitis treatment. In the present study, stimulation with rapid-response PDE caused a profound decrease in T-cell migration when the T cells were pretreated with anti-α6β1 antibody. These data indicate that, with rapid-response PDE stimulation, adaptive immunity predominantly shifted toward Th1 immune response, which is clinically correlated with a favorable treatment outcome. The maintenance of a functionally polarized immune response requires different T-cell subsets to localize in sites of inflammation. The present data may explain how different T-cell subsets are preferentially recruited to sites of peritoneal inflammation; selective modulation of these integrins illustrates an additional level of control for specific T-cell subset homing. Manipulating T-lymphocyte integrin expression could modulate the Th1/Th2 balance and affect the outcome of peritonitis treatment.

The present study shows that under LPS stimulation, a Th1 immune response was predominant; T-lymphocyte migration was significantly decreased with anti-α6β1 antibody pretreatment compared with anti-α4 antibody pretreatment. In previous studies, LPS from *Escherichia coli* was found to induce a Th1 response,[43,44] whereas LPS from *Porphyromonas gingivalis* and schistosome egg antigen induced Th2 responses.[43,45,46] The present data are consistent with previous studies,[43,44,47–52] showing that LPS induces Th1 polarization. Toll-like receptor 4 principally recognizes LPS and mediates LPS transduction. Kato *et al.*[53] found that Toll-like receptor 4 induces chemokine expression in murine peritoneal mesothelial cells; this may play a role in inducing peritoneal inflammation and fibrosis. Further studies to delineate the interaction of Toll-like receptor 4 in HPMCs

and LPS, which results in various levels of α6β1 expression by peritoneal T cells, will provide new insights into how peritoneal inflammation and, possibly, even fibrosis are modulated.

Environmental stimuli are important for HPMC–T-lymphocyte interaction. Our results show that exposure to bacterial products or PDE modulates the adhesion and migration properties of T-lymphocytes. This phenomenon is compatible with the characteristics of integrins. Under normal conditions, integrins are inactive. Integrins provide strong adhesion and migration only after activation by other stimuli, such as exposure to cytokines or chemokines; the engagement of other cell-surface receptors results in rapid integrin activation and ligand binding.[30,34] The strength with which integrins bind to their ligands can be rapidly and precisely regulated; this is called 'affinity modulation'.[54] Our data support the findings that T-cell integrins play important roles in the interaction between HPMC and T cell only in the presence of environmental stimuli.

As the integrin antibody-blocking experiments did not show complete inhibition in this *in vitro* migration assay, it is likely that some other molecules may also be involved in T-lymphocyte migration across HPMCs. Although the concentration of these other molecules appears to be less than that of integrins, these other adhesion molecules should be identified. Combined with our current results, additional studies delineating other adhesion molecules involved in T-lymphocyte migration across HPMCs will enhance our understanding of the T-lymphocyte-trafficking mechanism during peritoneal inflammation.

In conclusion, the present study found that integrins play an important role in T-lymphocyte adherence and migration across HPMCs, which are important in regulating the traffic of T lymphocytes into the peritoneal cavity. Different T-cell subsets express different integrins; this may mediate the polarized Th1/Th2 immune reaction in peritonitis. Th-subset recruitment and the outcome of peritonitis could be affected by manipulating the expression of different integrins or by blocking the function of different integrins. These findings enhance our understanding of the peritoneal immune response and facilitate the development of new strategies for peritonitis immunotherapy and prevention.

METHODS

Peritonitis and response to treatment

The definitions related to peritonitis and response to treatment were as previously described.[27] A rapid clinical response was defined as the resolution of the symptoms of peritonitis, including disappearance of abdominal pain and clearing of the peritoneal dialysate within 72 h of the initiation of antibiotic therapy, followed by complete recovery within 7–10 days of treatment initiation. A delayed clinical response refractory to treatment was defined as the persistence of symptoms, positive dialysate cultures, and an elevated white blood cell count in the dialysate beyond 72 h after the initiation of appropriate antibiotic treatment, followed by a fluctuating and protracted course lasting $\geqslant$10–14 days.

PDE collection

On day 0, after the onset of symptoms and prior to antibiotic treatment, bags of PDE were collected and sent to the research lab. The PDE was centrifuged at 400 *g*, 4°C for 10 min. After centrifugation, the supernatants were collected, refrigerated, and then dried to powder using Freeze Dryer (Eyela, Tokyo, Japan). The powder was stored at −70°C until analysis.

Isolation and culture of HPMCs

Human peritoneal mesothelial cells obtained from the omental tissue of consenting patients undergoing abdominal surgery were isolated and characterized as described previously.[55] The identity of HPMCs was confirmed by their uniform cobblestone appearance at confluence; presence of surface microvilli; absence of factor VIII-related antigen staining; and presence of cytokeratin and vimentin. All experiments were performed with confluent HPMCs from the second passage. All cells were washed and growth arrested for 48 h in serum-free, hydrocortisone-free culture medium prior to use.

T-lymphocyte preparation

Mononuclear cells were obtained from citrated venous blood obtained from healthy human donors using dextran sedimentation and Ficoll-Hypaque (Sigma-Aldrich Inc., St Louis, MO, USA) density gradient centrifugation. To remove phagocytes, the mononuclear cells were incubated on a Petri dish for 60 min at 37°C. Lymphocytes were obtained from the non-adherent fraction. The lymphocytes were separated into T and B cells based on the formation of E-rosettes and differential centrifugation of rosetting and non-rosetting cells, as described previously.[56]

HPMC–T-cell adhesion

Sterile coverslides placed in six-well plates were prepared. A total of $1–2 \times 10^5$ HPMCs suspended in culture medium were added to each well and incubated at 5% CO_2, 37°C for 60 min, for attachment to occur. Then, HPMCs were stimulated with (1) *Escherichia coli* 0111:B4 LPS (10 μg/ml; Chemicon, Temecula, CA, USA), (2) rapid-response PDE (1000 μg/ml), (3) delayed-response PDE (1000 μg/ml), or (4) no stimulation for 30 min at 5% CO_2, 37°C incubation. For T-lymphocyte adhesion, 2×10^5 T lymphocytes were added to each well and incubated for 60 min at 5% CO_2 at 37°C. The slides were washed gently with phosphate-buffered saline three times to remove non-adherent cells, followed by Liu staining. The slides were mounted and examined under an Olympus microscope. The percentage of HPMC–T-cell rosette formation was determined. Rosette formation was defined as $\geqslant$2.5 lymphocytes attached to each mesothelial cell. Approximately 200 HPMCs were counted blindly in each experiment; the same observer assessed all slides.

Evaluation of integrin expression by immunofluorescence

HPMC–T-cell adhesion was stimulated and achieved under four conditions, as described above. For immunofluorescence staining, anti-α6β1 antibody (5A; Chemicon) or anti-α4 antibody (AB1924; Chemicon) in a 1:50 dilution was added and the slides were incubated for 45 min at room temperature. The slides were washed three times in phosphate-buffered saline, and then fluorescein isothiocyanate-labeled donkey anti-mouse IgG (AP192F; Chemicon) or rhodamine-labeled donkey anti-rabbit IgG (AP182R; Chemicon) at 1:200 was added. After 60 min, the slides were washed again and mounted with buffered glycerol. The proportion of green-fluorescing $\alpha6\beta1^+$ T cells and the proportion of red-fluorescing $\alpha4^+$ T cells could be determined. These proportions were quantified

by counting multiple high-powered fields using an Olympus fluorescence microscope with epi-illumination and appropriate barrier filters for fluorescein isothiocyanate and rhodamine. Approximately 200 T cells were counted blindly in each experiment; the same observer assessed all slides.

Generation of polarized human Th lymphocytes

Polarized Th cell lines were generated by stimulation with 2 μg/ml phytohemagglutinin in the presence of various combinations of cytokines and neutralizing anti-cytokine antibodies.[40,41,57] Briefly, 5 ng/ml IL-12 (R&D Systems, Minneapolis, MN, USA) and 200 ng/ml neutralizing anti-IL-4 antibody (R&D Systems) were added to the Th1 cell cultures. A 10 ng/ml quantity of IL-4 (R&D Systems) and 2 μg/ml neutralizing anti-IL-12 antibodies (R&D Systems) were added to the Th2 cell cultures. The cells were washed and expanded in complete medium with 100 U/ml IL-2 (R&D Systems) after 72 h. The T cells were harvested after 10 days of culture, centrifuged to remove dead cells, and used immediately.

The phenotype of the differentiated T cells was confirmed by intracellular cytokine staining.[41,57] The permeabilized cells were double-stained with fluorescein isothiocyanate-labeled anti-interferon-γ (R&D Systems) and phycoerythrin-labeled anti-IL-4 antibodies (R&D Systems). Samples were analyzed using flow cytometry with a FACScan (Becton Dickinson, Mountain View, CA, USA).

Establishment of a HPMC monolayer on tissue culture inserts

To assess T-lymphocyte migration, confluent HPMCs were subcultured and seeded onto the bottom side of polycarbonate tissue culture inserts (5 μm pore size, diameter 6.5 mm; Corning Costar, Cambridge, MA, USA). The HPMC monolayers were established as previously described.[11,23] Briefly, the tissue culture inserts coated with human type IV collagen (Sigma-Aldrich Inc.) on the upper surface of the inverted insert were kept for 1 h at room temperature. Then, neutralized rat tail collagen I plugs were set in the inner chamber of the upright insert. HPMCs (5×10^5 cells/ml, 200 μl) in serum-free culture medium were added to the upper (collagen IV-coated) surface of the inverted insert and allowed to attach for 4 h at 37°C. Then, the inserts were placed upright in 24-well plates containing 1 ml of medium supplemented with 10% (vol/vol) fetal calf serum; 0.4 ml of serum-free medium was added to the inner chamber of the insert. The medium was replaced daily, and a confluent monolayer was obtained after 5–10 days. Once the monolayer reached confluence, a 48 h growth arrest period in serum-free medium was maintained prior to the experiments.

T-lymphocyte/subset migration experiments

Collagen I plugs were removed from the growth-arrested HPMC insert cultures before the experiments. The HPMC insert cultures were washed with serum-free medium apically and basolaterally to remove constitutively secreted cytokines. Then, HPMCs were apically pre-incubated with (1) *Escherichia coli* 0111:B4 LPS (10 μg/ml; Chemicon), (2) rapid-response PDE (1000 μg/ml), (3) delayed-response PDE (1000 μg/ml), or (4) no stimulation for 2, 6, 12, 18, 24 h prior to the addition of T lymphocytes, Th1, or Th2 cells. T lymphocytes, Th1, or Th2 cells were washed and suspended in RPMI 1640 (Gibco, Carlsbad, CA, USA) with 0.5% bovine serum albumin; 0.1 ml of cell suspension ($1–2 \times 10^6$ cells) was added to the upper chamber. T-lymphocyte migration experiments were performed over a 6 h period at 37°C with 5% CO_2.

Antibody-blocking experiments

To determine the inhibitory effects that integrin-blocking antibodies had on the migration of T lymphocytes and T-lymphocyte subsets across HPMCs, blocking experiments were performed. T lymphocytes, Th1, and Th2 cell suspensions were incubated for 15 min with anti-α6β1 blocking antibody (1:50 dilution; 5A; Chemicon) or anti-α4 blocking antibody (1:50 dilution; P1H4; Chemicon) before the cells were added to the upper chamber. At the 24 h time point, the experiments included isotype-matched control monoclonal antibodies.

Quantitation of migrated T lymphocytes and subsets

The migration of T lymphocytes and subsets across HPMCs was assessed in the suspension isolated from the lower chamber. The number of transmigrated cells relative to the input was measured using flow cytometry with a FACScan (Becton Dickinson) based on a 60 s acquisition at a flow rate of 100 μl/min.

Statistics

All data are presented as mean ± s.d. Differences between groups were analyzed by the Mann–Whitney *U*-test. Values of $P < 0.05$ were considered to be statistically significant different.

DISCLOSURE

The authors declare no conflict of interest.

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