Ligand binding of leukocyte integrin very late antigen-4 involves exposure of sulfhydryl groups and is subject to redox modulation

Si-Yen Liu1, Min-Yi Tsai2, Kuo-Pin Chuang*1, Ya-Fang Huang1 and Chi-Chang Shieh1,2,3,4

1 National Cheng Kung University Medical College, Graduate Institute of Basic Medicine, Tainan, Taiwan
2 National Cheng Kung University Medical College, Graduate Institute of Microbiology and Immunology, Tainan, Taiwan
3 National Cheng Kung University Medical College, Department of Pediatrics, Tainan, Taiwan
4 National Health Research Institute, Tainan, Taiwan

Activation of leukocyte integrins is important for selective recruitment of cells from the circulation to tissues. Our previous studies showed that the binding between the integrin very late antigen-4 (VLA-4) and vascular cell adhesion molecule-1 (VCAM-1) is modulated by reactive oxygen species. In this study, we investigated the molecular nature of redox modulation on the activation states of VLA-4 on human leukocytes. We found that ligand binding of VLA-4 induced exposure of sulfhydryl groups on the ε4 peptide. Low concentrations (5–10 μM) of exogenous hydrogen peroxide in the presence or absence of added glutathione enhanced the ligand binding ability of VLA-4 to VCAM-1 and cell rolling on VCAM-1, while higher concentrations (>100 μM) of hydrogen peroxide inhibited the binding. Exogenous hydrogen peroxide and glutathione induced molecular modification of S-glutathionylation on the ε4 peptide. The redox regulation of the VLA-4 binding activity required outside-in signaling and cytoskeleton rearrangement. Our results indicate that ligand binding of VLA-4 involves redox modulations which may play a pivotal role in regulating the activation states of VLA-4 in inflammatory tissues and hence direct leukocyte trafficking.

Introduction

Leukocytes use cell surface adhesion molecules including integrins, immunoglobulin (Ig) superfamily proteins and selectins to mediate the complex cell binding activity required for leukocyte recruitment to inflammatory sites [1, 2]. Integrins are heterodimeric, transmembrane receptor molecules formed by the
association of one α and one β subunit. Among the leukocyte integrins, very late antigen-4 (VLA-4; α4β1, CD49d/CD29) is of particular importance in leukocyte trafficking of monocytes, lymphocytes, and eosinophils in a variety of inflammations. The interaction of integrin VLA-4 and vascular cell adhesion molecule-1 (VCAM-1), an Ig superfamily molecule expressed on activated endothelium and fibronectin, an extracellular matrix protein, has been suggested to be among the most important factors in the selective recruitment of eosinophils in allergic diseases and also for the trafficking of monocytes and memory/activated lymphocytes to inflammatory tissues [3, 4].

The location and timing of integrin activation are crucial for effective leukocyte recruitment, which should be coordinated with the process of leukocyte activation. Meanwhile, the activation of leukocytes usually leads to activation of NADPH oxidase and production of reactive oxygen species (ROS), which are crucial for effective immune defense [5–7]. Our previous studies showed that ROS produced by activated granulocytes affect eosinophil binding to VCAM-1 [8]. Moreover, ligation of lymphocyte function-associated antigen-1 (LFA-1) integrin on monocytes can reduce VLA-4-mediated adhesion through a ROS-dependent pathway [9]. These findings suggest that ROS produced in inflammatory tissues may be important for regulating the activation of VLA-4 and hence leukocyte trafficking in a tissue-specific manner.

Previous studies showed that integrin conformation may be altered by mutations or redox changes on the cysteine residues of their subunit peptides [10–17]. Here, we investigated how VLA-4 integrin ligand binding activity can be regulated by redox modulation.

Results

Sulfhydryl blockers inhibit the cell adhesion of HL-60 clone 15 cells to VCAM-1 and suppress the active state of VLA-4

We first tested the effect of blockers for redox-sensitive free sulfhydryl groups for VLA-4-mediated leukocyte adhesion using HL-60 clone 15 cells on recombinant VCAM-1. We performed cell adhesion assays to investigate whether blocking of sulfhydryl groups affects cell binding to VCAM-1. We found that pretreatment with sulfhydryl blockers, 5,5′-dithiobis(2-nitrobenzoic acid (DTNB; 100 μM), which is membrane impermeable, and N-ethylmaleimide (NEM; 10 μM), which is membrane permeable, significantly reduced HL-60 clone 15 cell adhesion to VCAM-1 (Fig. 1A). We then examined the effects of sulfhydryl blockers on expression of the activation conformation of β1 integrin using a ligand-induced binding site-specific antibody (HUTS-4) for β1 integrins. We found that treatment of HL-60 clone 15 cells with sulfhydryl blockers decreased the level of activated β1 integrin when compared with untreated controls (Fig. 1B). The total VLA-4 cell surface level, as detected by a conformation-independent mAb, was not
changed after the pretreatment with sulphydryl blockers (Fig. 1C). As VLA-4 is the counter-receptor of VCAM-1 on HL-60 clone 15 cells, these findings suggested that the presence of free sulphydryl groups on HL-60 clone 15 cells may be required for cellular adhesion mediated by VLA-4 integrin.

**Ligand binding of VLA-4 involves changes in exposed sulphydryl groups on the VLA-4 α4 chain**

We then directly tested whether the ligand binding of VLA-4 involves the alteration of free-sulphydryl exposure on VLA-4. HL-60 clone 15 cells treated with a recombinant soluble VLA-4 ligand, VCAM-1-Fc or fibronectin, were labeled with the free sulphydryl-binding chemical amido-4-[4-(maleimidomethyl) cyclohexanecarboximido] butane (BMCC), resolved by SDS-PAGE and then analyzed for peptide-bound BMCC to measure the level of exposed sulphydryl groups on VLA-4 integrin (Fig. 2A). In resting cells and cells treated with Fc control protein, there was almost no detectable BMCC label on the α4 chain (Fig. 2A, lanes 1 and 2). However, in the presence of VCAM-1-Fc or fibronectin, the α4 chain was significantly labeled with BMCC (Fig. 2A, lanes 5 and 9). In the presence of VCAM-1-Fc or fibronectin and Mn2+, the α4 chain was even more strongly labeled with BMCC (Fig. 2, lanes 4 and 8) while Mn2+ alone did not increase the labeling (Fig. 2A, lane 3). The HL-60 clone 15 cells pretreated with NEM or DTNB did not show the increase in exposed sulphydryl groups in the α4 chain, even in the presence of the ligand (Fig. 2A, lanes 6, 7, 10 and 11). The relative intensities of the BMCC signals on α4 are shown semi-quantitatively as column heights. To test whether the effect of sulphydryl blocking is due to indirect effects of the blockers on the ligands, rather than on the integrin peptides, we pretreated the VCAM-1-Fc and fibronectin with NEM and then tested their effects on ligand binding-induced sulphydryl exposure on α4. No apparent changes on biotin-BMCC labeling in these experiments were found (Fig. 2B). Under the same ligand binding experimental conditions to VCAM-1-Fc, we did not find detectable BMCC-labeled β1 subunits (Fig. 2C). However, biotin-BMCC labeling was found on both α4 and β1 peptides when the cells were pre-reduced with 10 mM dithiothreitol (DTT) (Fig. 2B, C). These data thus showed that ligand binding conditions alter the state of sulphydryl group exposure on the α4 but not the β1 chain and that the exposure is different from non-specific reduction by DTT.

**Redox modification of VLA-4 by exogenous ROS modulates its ligand binding activity**

We next tested whether changing the redox state of VLA-4 by exogenous ROS modulates its binding ability, using a ligand binding assay to soluble recombinant ligands. We first tested the dose-response of FITC-labeled VCAM-1-Fc recombinant protein to cells in order to determine the appropriate concentration for ligand binding assays. We found that the binding of FITC-labeled VCAM-1-Fc to HL-60 clone 15 cells increased with higher concentrations of the recombinant protein in the range of 0.2–1 μg/mL, but plateaued at higher concentrations. We hence used the recombinant proteins at the concentration of 1 μg/mL in subsequent ligand binding assays. The ligand binding of FITC-labeled VCAM-1-Fc (1 μg/mL) can be suppressed with excessive unlabeled VCAM-1-Fc (10 μg/mL) but not by Fc (10 μg/mL) (Fig. 3A). These experiments confirmed the validity of the specific ligand binding assay using the FITC-labeled VCAM-1-Fc protein. We then evaluated the effects of hydrogen peroxide (H2O2) on the ligand binding ability of VLA-4 by measuring the binding of FITC-labeled VCAM-1-Fc and β1 integrin activation on both HL-60 clone 15 cells (Fig. 3B). Our results showed that at a lower concentration (10 μM), hydrogen peroxide enhanced the binding ability of VLA-4 with FITC-labeled VCAM-1-Fc while at a higher concentration hydrogen peroxide (100 μM) markedly decreased the ligand binding activity of VLA-4. The activating effect of low-concentration hydrogen peroxide, however, was inhibited by the presence of the membrane-impermeable sulphydryl group blocker DTNB (100 μM) (Fig. 3B, left upper panel). We also examined the effect of hydrogen peroxide on the activation conformation of VLA-4, using Mn2+ as a positive control. Similar to ligand binding, we found that pretreatment of HL-60 clone 15 cells with hydrogen peroxide (10 μM) up-regulated the expression of the activation conformation of VLA-4 (Fig. 3B, left middle panel). DTNB pretreatment inhibited the activating effect of hydrogen peroxide on expression of the activated β1 conformation, even though the levels of cellular staining with conformation-independent anti-VLA-4 antibody were not affected (not shown). We then used FITC-labeled recombinant ICAM-1 protein to test the effects of different concentrations of hydrogen peroxide on the ligand binding activity on the same cells. Different from the experiments using recombinant VCAM-1, no apparent changes in ligand binding to ICAM-1 was found in these experiments (Fig. 3B, left lower panel). We then tested the activating effects of hydrogen peroxide and DTNB on freshly isolated human eosinophils. Like the responses in HL-60 clone 15 cells, we found that low concentration of hydrogen peroxide activates cellular
ligand binding with VCAM-1, but not ICAM-1 recombinant protein, in the primary human eosinophils (Fig. 3B, right panels). Moreover, the hydrogen peroxide-mediated activation also was reversed by pretreatment with DTNB. The effects of hydrogen peroxide on eosinophil integrin-mediated ligand binding hence appear to be integrin specific.

In order to investigate whether the enhancement of ligand binding of VLA-4 by hydrogen peroxide is through affecting the cell surface clustering of the integrin, we examined the distribution of cell surface VLA-4 under different conditions with confocal microscopy. We found that the clustering of VLA-4 on the polarized cells surface decreased with increasing

![Confocal microscopy images showing VLA-4 clustering](image)

**Figure 2.** Ligand binding triggered the exposure of sulfhydryl groups on the VLA-4 α4 chain. HL-60 clone 15 cells under different ligand binding conditions were treated with biotin-BMCC and analyzed for the exposed sulfhydryl groups on the α4 and β1 peptides. (A) Cells were treated with medium only (lane 1), recombinant Fc (50 μg/mL, lane 2), 1 mM Mn²⁺ (lane 3), 50 μg/mL recombinant VCAM-1-Fc and 1 mM Mn²⁺ (lane 4), 50 μg/mL VCAM-1-Fc (lane 5), 50 μg/mL VCAM-1-Fc and sulfhydryl group blockers (lanes 6 and 7), 50 μg/mL fibronectin and 1 mM Mn²⁺ (lane 8), 50 μg/mL fibronectin (lane 9), 50 μg/mL fibronectin and sulfhydryl group blockers (lanes 10 and 11). (B) Cells were treated with medium only (lane 1), recombinant VCAM-1-Fc (50 μg/mL, lane 2), NEM-treated recombinant VCAM-1-Fc (50 μg/mL, lane 3), 50 μg/mL fibronectin (lane 4), 5 μg/mL NEM-treated fibronectin (lane 5) and 10 mM DTT (lane 6) in the presence of biotin-BMCC (50 μM) for 60 min. The cell lysates were then subjected to immunoprecipitation with anti-α4 antibody and analyzed with immunoblotting with anti-biotin (upper panel) or anti-α4 antibody (middle panel). (C) Cells (lanes 1–6) were treated under the same conditions as in (A) lanes 1–5 and (B) lane 6 as indicated. Cells lysates were then subjected to immunoprecipitation with anti-β1 mAb and analyzed by immunoblotting with anti-biotin (upper panel) or anti-β1 (lower panel) antibodies. The experiment was repeated twice with similar results.
Figure 3. Redox modification of VLA-4 by exogenous ROS modulated its ligand binding activity. (A) Ligand binding assays for HL-60 clone 15 cells were performed at the indicated concentrations of FITC-VCAM-1-Fc. The competitive assays were performed in the presence of 10 μg/mL unlabeled Fc (black) or VCAM-1-Fc (purple) and 1 μg/mL FITC-VCAM-1-Fc and subjected to flow cytometric analysis. One representative of three experiments is shown. (B) HL-60 clone 15 cells (left panel) or human eosinophils (right panel) were pretreated with different concentrations of H2O2 (10 μM, green; 100 μM, black) or 10 μM H2O2 with DTNB (100 μM, blue) for 10 min at room temperature and analyzed for ligand binding function of VLA-4 integrin by FITC-labeled VCAM-1-Fc binding assay (top panels). The DTNB-H2O2-treated cells were stained with a ligand-induced binding site-specific β1 antibody and analyzed by flow cytometry to detect the change in integrin activation states (middle panels). Cells pretreated with the indicated concentrations of H2O2 were analyzed for ligand binding function of LFA-1 integrin by direct FITC-labeled ICAM-1-Fc binding assay (lower panels). (C) HL-60 clone 15 cells treated with VCAM-1 and H2O2 were stained for VLA-4 expression with mouse anti-α4 antibody followed by FITC-labeled goat anti-mouse secondary antibody. The approximate percentages of polarized cells under each condition are shown at the bottom. Fc protein was used as a negative control in experiments. These experiments were repeated five times with similar results.
concentrations of exogenous hydrogen peroxide (Fig. 3C). The enhancement of ligand binding of VLA-4 by low concentration of hydrogen peroxide hence does not appear to be caused by increased clustering of the cell surface integrin but is likely due to the conformation-dependent integrin activation.

Glutathione and hydrogen peroxide enhance S-glutathionylation on α4 integrin and affect VLA-4 ligand binding

Glutathione (GSH) serves a major role in maintaining the reduced state of cellular protein sulfhydryl groups by forming mixed disulfides with protein sulfhydryl groups under oxidation conditions, causing reversible S-glutathionylation [18]. As the exposure and redox changes on sulfhydryl groups on the VLA-4 α4 peptide had been shown to be critical for modulation of the activation state of this integrin, we went on to examine the state of S-glutathionylation of VLA-4 on HL-60 clone 15 cells after treatment with hydrogen peroxide in the presence or absence of added glutathione. Treatment of cells with low concentrations (5 and 10 μM) of hydrogen peroxide induced significant S-glutathionylation (Fig. 4A, compare lane 1 with lane 3 and lane 4). Higher concentration (100 μM) of hydrogen peroxide, on the contrary, reduced the S-glutathionylation level (Fig. 4A, lane 5). In the presence of added glutathione (10 μM), cells treated with lower concentration of hydrogen peroxide had more S-glutathionylation when compared with cells without added glutathione (Fig. 4A, lanes 6 and 7, compare with lanes 3 and 4). However, in cells treated with 100 μM hydrogen peroxide, S-glutathionylation did not increase with added glutathione (Fig. 4A, compare lane 8 with lane 5). Under the same experimental conditions, there was no detectable S-glutathionylation on the β1 chain (not shown). We then tested the ligand binding activity of the cells treated with different concentrations of hydrogen peroxide with or without glutathione using the VCAM-1-Fc recombinant protein. We found that glutathione alone and 5 μM hydrogen peroxide alone did not significantly increase VCAM-1 ligand binding. However, 10 μM hydrogen peroxide with or without added glutathione and 5 μM hydrogen peroxide with added glutathione significantly increased the ligand binding on HL-60 clone 15 cells (Fig. 4B, upper panel). We also tested the effect of another small-molecule sulfhydryl provider, cysteine, on ligand binding. No apparent changes in binding to soluble FITC-VCAM-1-Fc were found in ligand binding assays in cells with or without cysteine (10 μM) in the medium (Fig. 4B, middle panel). High concentration of hydrogen peroxide (100 μM) with or without added glutathione, however, suppressed the ligand binding to recombinant VCAM-1 (Fig. 4B, lower panel). The levels of cellular staining with conformation-independent anti-VLA-4 antibody on the cell surface were not affected by GSH and hydrogen peroxide treatment (not shown).

Glutathione and hydrogen peroxide regulate the cell rolling of HL-60 clone 15 on VCAM-1

We next examined the influence of exogenous hydrogen peroxide and glutathione on VLA-4-mediated cell adhesion in a dynamic manner using HL-60 clone 15 cells in VCAM-1-coated flow chamber assays (Fig. 5). Under a wall shear stress of 1.0 dyne/cm², cells passing through the chamber in the time period of 60 s on the protein-coated surface were recorded. While there was no rolling cell detected on chambers coated with Fc recombinant protein, cells rolling at speeds <1100 μm/s were detected in experiments using chambers with VCAM-1-Fc recombinant protein. We found that cells without pretreatment and cells treated with glutathione (10 μM) or cysteine (10 μM) only rolled at a higher velocity with a median speed of 923, 1029 and 1069 μm/s, respectively (Fig. 5A, upper panels). Cells treated with 5 μM hydrogen peroxide had a slower rolling with a median speed of 846 μm/s. Cells treated with 5 μM hydrogen peroxide in combination with 10 μM glutathione rolled even more slowly with a median speed of 533 μm/s (Fig. 5A, middle panels). In cells treated with 10 μM hydrogen peroxide, the cells rolled at a speed of 711 μm/sec. Added glutathione did not further decrease the speed and the cells rolled at a speed of 886 μm/s (Fig. 5A, lower panels). In experiments using higher concentrations of hydrogen peroxide (100 μM), no cell can be observed to roll in the flow chamber (data not shown). Different from glutathione, added cysteine did not appear to decrease the rolling speed in either 5 or 10 μM hydrogen peroxide (Fig. 5A, right panels). The changes in median rolling speeds in comparison with medium-alone condition are summarized in Fig. 5B. These data indicate that different concentrations of hydrogen peroxide in association with glutathione may modulate VLA-4-mediated adhesion and lead to changes in leukocyte rolling under flow conditions.

Cytoskeleton reorganization and focal adhesion kinase signaling are involved in hydrogen peroxide-mediated enhancement of VLA-4 integrin ligand binding

We then tested whether intracellular events induced by integrin ligand binding, including cytoskeleton reorganization and protein kinase activation, are involved in ROS-mediated integrin activation. The HL-60 clone 15 cells were pretreated with the cytoskeleton organization inhibitor, cytochalasin B, or focal adhesion kinase (FAK)
small interfering RNA (siRNA) and then analyzed for the hydrogen peroxide-induced enhancement of cellular binding to FITC-labeled VCAM-1-Fc. Neither of these cellular treatments affected the cell surface expression of VLA-4 (Fig. 6A). We found that pretreatment with cytochalasin B (5 µg/mL) abolished the activating effect in VLA-4 ligand binding by hydrogen peroxide (10 µM) (Fig. 6B). These findings suggested that hydrogen peroxide-enhanced ligand binding of VLA-4 involves cytoskeleton rearrangement. We then examined

Figure 4. Glutathione and hydrogen peroxide enhanced S-glutathionylation on α4 integrin and affected VLA-4 ligand binding. HL-60 clone 15 cells treated with buffer only (lane 1), GSH (10 µM, lane 2), H2O2 (5 µM, lane 3), H2O2 (10 µM, lane 4), H2O2 (100 µM, lane 5), 5 µM H2O2 and 10 µM GSH (lane 6), 10 µM H2O2 and 10 µM GSH (lane 7), 100 µM H2O2 and 10 µM GSH (lane 8) were lysed and subjected to immunoprecipitation with anti-α4 mAb and analyzed by immunoblotting with anti-glutathione (upper panel) or anti-α4 antibody (middle panel). The ratios of the signals are shown as relative density in the lower panel. (B) HL-60 clone 15 cells treated with the indicated concentrations of H2O2 with or without 10 µM GSH (upper panel), 10 µM cysteine (middle panel) or 10 µM GSH (lower panel) were analyzed for ligand binding function of VLA-4 integrin by direct FITC-labeled VCAM-1-Fc binding assay. The experiments were repeated three times with similar results.
whether FAK, which is essential for integrin signaling, is involved in redox modulation of VLA-4. We downregulated the cellular level of FAK using siRNA and tested the activating effect of hydrogen peroxide on VLA-4 ligand binding. The results showed that HL-60 clone 15 cells partially silenced for FAK expression could not be activated by hydrogen peroxide (10 μM) for VLA-4 ligand binding (Fig. 6B, C). These findings suggest that both the cytoskeleton rearrangement and FAK-mediated signaling are important in redox modulation of the VLA-4 activation state.

Discussion

The trafficking of leukocytes from the blood into peripheral tissues through a multiple-step intercellular adhesion process is essential for selective recruitment of leukocytes to sites of inflammation [1, 2, 19]. Integrins on leukocytes are noted for their capability to adopt active or inactive conformations to determine their adhesiveness in response to different environmental signals [20–23]. VLA-4, an integrin selectively expressed on subpopulations of lymphocytes, monocytes and...
eosinophils, binds to VCAM-1 and mucosal addressin cell adhesion molecule-1 (MadCAM-1) on the cell surface and the CS1 region of fibronectin in the extracellular matrix [24, 25]. In addition to mediating tight adhesion for cell arrest, VLA-4 is active in mediating tethering and facilitates leukocyte rolling on the endothelial surface [26, 27]. Given the variety of adhesion functions, the modulation of activation states on VLA-4 in different microenvironments may play a pivotal role in leukocyte trafficking and hence critically affect immune responses.

Previous investigations revealed that conformational changes, in addition to cell surface expression, determine the integrin-mediated cellular adhesion [28, 29]. The high- and low-affinity states of VLA-4 defined by traditional adhesion assays, however, appear inadequate to explain the complex regulation of integrin binding activity in different environments, including the rapid transition from cell rolling in shear flow of circulation to static binding in the tissue [30–32]. It has been proposed that distinct VLA-4 ligand binding conformations exist to mediate functional transition from cellular rolling to tight adhesion [33, 34]. We previously reported that the binding activity of leukocyte integrins is affected by ROS, which are usually abundant in inflammatory microenvironments such as allergic airways, characterized by infiltration of activated leukocytes including eosinophils [8]. We hence used an eosinophil-like cell line, HL-60 clone 15, which highly expresses VLA-4 on the cell surface, and a new recombinant adhesion protein ligand binding assay and flow chamber cell rolling assays to investigate the role of redox modulation of cell adhesion in this study. We first used conformation-dependent and conformation-independent mAbs in flow cytometric analysis to detect the activation-deactivation states of VLA-4 in this study. The results of the experiments, however, are limited by the facts that (1) functionally important intermediate conformations other than activation or deactivation are likely to exist and cannot be differentiated by these mAbs; and (2) the expression of β1 integrins other than VLA-4 may complicate the interpretation of VLA-4 activation states on the cell surface. We hence investigated the regulation of VLA-4 in the context of biochemical changes, ligand binding assays and cell rolling assays, which unraveled the redox modification of VLA-4 in a more functional manner. Our experiments revealed the novel results that exogenous hydrogen peroxide and glutathione may affect VLA-4-mediated leukocyte ligand binding and cell rolling in a dynamic manner. In addition to the effects of H$_2$O$_2$ on ligand binding to VCAM-1 protein, a marked decrease in the speed of cell rolling in the flow chamber assay with shear stress close to physiological conditions implicates that redox modulation may provide a tissue-specific molecular change on VLA-4 and facilitates the cellular transition from suspension to tight adhesion in inflammatory conditions.

Based on structural and functional studies on different integrins, a model for bidirectional signal transmission across the plasma membrane to mediate the modulation of integrin function was proposed [35]. Three different conformations (closed headpiece and bent, closed headpiece and extended, and open headpiece and extended) which correlate with ligand binding activity were envisioned in the model. The molecule changes to activated conformations involve separation

---

**Figure 6.** Cytoskeleton reorganization and FAK signaling are involved in hydrogen peroxide-mediated modulation of VLA-4 integrin ligand binding. (A) The cytochalasin B-treated and FAK siRNA-transfected HL-60 clone 15 cells were stained with conformation-independent antibody and analyzed by flow cytometry. (B) HL-60 clone 15 cells treated with inhibitors were analyzed for H$_2$O$_2$ (10 μM)-induced enhancement of ligand binding activity. The cells treated with H$_2$O$_2$ alone (purple) were compared with cells pretreated with cytochalasin B (5 μg/mL, black) for 20 min at room temperature or cells transfected with FAK siRNA (green). The ligand binding activity of VLA-4 integrin was measured with FITC-labeled VCAM-1-Fc binding and flow cytometric analysis. (C) The expression level of FAK in FAK siRNA-transfected cells was detected by Western blotting (left panel) and RT-PCR (right panel). The experiments were repeated three times with similar results.
of the α and β chains at transmembrane and membrane-proximal segments, extension of the bent extracellular heterodimer and activation of ligand-binding I domains. As VLA-4 integrin has an α chain (α4) that does not contain an I domain, the conformational changes on the α4 peptide depend on intra- and inter-molecular interactions which lead to the exposure and stabilization of VLA-4 ligand-binding domain(s). Using a site-directed mutagenesis approach on αIIbβ3 integrin, Luo et al. found that mutations containing disulfide bridges that prevent the separation of transmembrane domains of α and β chains abolish the activating effect of cytoplasmic mutations [36]. Intriguingly, this mutant with an inter-subunit disulfide bond between the α and β chains in the integrin retains Mn2+-stimulated ligand binding but shows a profound defect in outside-in signaling including FAK activation. These results are in line with our results showing the lack of sulphydryl exposure on VLA-4 treated with Mn2+ (Fig. 2A). Another recent structural study by Shi et al. showed that in LFA-1 (αLβ2 integrin), a disulfide bond introduced between the PSI and 1-EGF2 domains in the β2 subunit locks the integrin in a bent conformation [37]. These experiments clearly showed the potential role of inter- and intra-peptide disulfide bonds in regulating integrin binding and signaling functions in αIIbβ3 and LFA-1 integrins. As different integrins apparently need molecule-specific regulation mechanisms to regulate their activity on the same cell, disulfide bonds formed between cysteines may participate in this redox modulation of extracellular conformation in an integrin-specific manner, which involves both inside-out and outside-in signaling, and determine the distinct activation states of different integrins. Very little structural information, however, is available regarding the structural basis for redox modulation of VLA-4 integrin.

As the primary structure of the α4 integrin peptide contains no cysteine in the cytoplasmic and transmembrane domains whereas there are 24 cysteines in its extracellular domain [38], the sensitivity of α4 peptide conformation to redox modification may be mediated by modifications on these extracellular cysteines. In this study, we first showed that the exposure of free sulphydryl groups on the α4 chain of VLA-4 can be induced by ligand binding. Moreover, the membrane-impermeable sulphydryl blocker DTNB inhibited both HL-60 clone 15 cell binding to VCAM-1 and the expression of the ligand-induced binding site on VLA-4. These data indicate that changes in the redox status of exposed cysteine residues on the extracellular domain of the α4 peptide are critical for the regulation of the ligand binding ability of VLA-4 integrin. Previous studies established that at least two of the α4 extracellular-domain cysteines (C278 and C717) are important for VLA-4 ligand binding [39]. Alignment of the protein sequence of the α4 chain and two other integrin α chains, αIIb and αV, revealed that the α4 peptide contains unique cysteine residues at positions 80, 84, 278, 717, 767 and 828 (Supporting Information). The cysteines 80, 84, and 278 are located in the propeller domain while the cysteines 717, 767 and 828 are in membrane-proximal extracellular domains. Differential modification of these integrin-specific redox-sensitive site(s) on α4 peptides thus may determine the conversion between resting and active states on VLA-4 and confers different molecular conformations which may be positively or negatively regulated by ROS in each stage of leukocyte trafficking. The conformational changes affecting the membrane-proximal domains, as well as the ligand-binding N-terminal domains, of integrins thus may affect the ligand binding activity of the integrin [40].

Glutathione, the tripeptide γ-glutamyl-cysteiny1-glycine, has been documented to be essential for redox regulation of certain intracellular protein functions [41]. Although previously considered as mainly an intracellular component of the low-molecular-weight sulphydryl pool, glutathione is present extracellularly at levels comparable to intracellular concentrations in some cell types in tissues including the respiratory lining fluid [42, 43], which is the milieu of interest of this study. The formation of mixed disulfides between proteins and glutathione causes protein S-glutathionylation. Using a systematic approach to identify cell surface proteins susceptible to redox modifications, Ghezzi et al. identified α4 as one of the most susceptible membrane-bound proteins to be reduced by N-acetyl-cysteine to express free sulphydryl. Moreover, the intracellular glutathione level appears to be important for the maintenance of the expression of cell surface sulphydryl groups, as pretreating the cells with a glutathione synthesis inhibitor induced a parallel decrease in surface sulphydryl groups [44]. Proposed mechanisms of protein S-glutathionylation involve the oxidation of cysteine to form sulfenic acid (Cys-SOH), which may then react with glutathione to the mixed disulfide. As S-glutathionylation confers protection against further oxidation on peptides, it maintains their oxidized state, but at the same time keeps the modification reversible. Given the redox regulation revealed in this study and the reversible nature of integrin activation, the peptide modification by S-glutathionylation thus may be instrumental for the functional regulation of VLA-4 integrin. At a lower concentration, hydrogen peroxide, in association with glutathione, may reversibly oxidize the α4 peptide and confer a conformation for higher affinity. At a higher concentration, hydrogen peroxide may irreversibly oxidize critical cysteine residues, which render the
integran unfit for S-glutathionylation and hence unable to acquire an active conformation. This may explain the suppressive effects of hydrogen peroxide on integrin binding activity. Higher concentrations of hydrogen peroxide, however, also might deliver more oxidation stress on other extracellular and intracellular structures and affect complex cellular functions including signal transduction, molecular clustering and cytoskeleton reorganization. These indirect effects may explain the slightly different effects of redox modification on ligand binding assays and cell rolling assays (Fig. 4, 5).

Ligand binding of VLA-4 induces the association of actin filaments and signaling proteins with the cytoplasmic domain of integrin peptides [45]. Previous studies showed that binding of paxillin to the α4 integrin cytoplasmic domain is essential for cellular responses to integrin-mediated cell adhesion [46]. Meanwhile, the structural basis of integrin activation through binding of talin to the tyrosine-phosphorylated β chain was recently revealed [47]. Ligated integrins then induce assembly of actin filaments and stress fibers and form focal adhesion complexes to maintain the integrin in an active state. FAK, a paxillin-binding tyrosine kinase, is a pivotal molecule in focal adhesion complexes activated by the integrin conformational changes and transmits intracellular signals. Although complete suppression of FAK expression in HL-60 clone 15 cells affected cell viability in our experiments (data not shown), we found that the effect on redox enhancement of VLA-4 activity disappeared in partial silencing of FAK (Fig. 6). These results, showing that suppression of cytoskeleton rearrangement and down-regulation of FAK abolished the enhancing effect of hydrogen peroxide on the ligand binding ability of VLA-4, further suggest that outside-in integrin signals are essential for the ROS-induced VLA-4 activation. It is thus reasonable to propose a working hypothesis that after ligand binding, free sulphhydryl groups on the α4 peptide are exposed and hence become subject to oxidative modification by low concentrations of exogenous hydrogen peroxide and glutathione. This modification may subsequently lead to separation of the transmembrane domains of the α and β subunits and the subsequent outside-in signaling involving FAK-mediated tyrosine phosphorylation and cytoskeleton reorganization, which are compatible with the prediction of the open headpiece and extended conformation model [35]. These intracellular changes hence may stabilize an activated VLA-4 conformation that facilitates ligand binding and cell rolling.

In conclusion, the role of redox regulation on leukocyte integrin VLA-4 binding activity was revealed in this study. The exposure and redox changes on sulphhydryl groups on the VLA-4 α4 peptide appear to confer a specific molecular modulation mechanism for the binding activity of this integrin. Our results hence suggest that ROS in the tissue microenvironments may participate in the regulation of immune responses by changing peptide conformation and affecting the dynamic process of selective leukocyte recruitment in inflammatory tissues.

Materials and methods

Cells, reagents and mAb

HL-60 clone 15 cells (ATCC-CRL-1964) were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum and 40 U/mL penicillin/streptomycin. The HL-60 clone 15 cells were treated with 0.5 mM butyric acid for 4 days to differentiate to an eosinophil-like phenotype [48]. The differentiated HL-60 clone 15 cells expressed VLA-4 and LFA-1 integrins on the cell surface at comparable levels (MFI: 19.47 and 19.06 for VLA-4 and LFA-1, respectively). Human eosinophils were purified from peripheral blood of patients with allergy with dextran sedimentation and Ficoll-Hypaque centrifugation, followed by negative selection with magnetic cell sorting (MACS) with anti-CD16 antibody-coated beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Biotin-BMCC and FITC protein labeling kits were from Pierce (Rockford, IL). NEM, DTNB, hydrogen peroxide (H₂O₂) and GSH were purchased from Sigma (St. Louis, MO). Mouse anti-VLA-4 antibody (HP2/1) was purchased from Immunotech (Marseille, France). Mouse ligand-induced binding site-specific VLA-4 antibody (HUTS-4), anti-glutathione antibody (clone D8), anti-β1 antibody (clone 12G10), anti-FAK antibody (clone-4A), and anti-α4 chain rabbit serum were purchased from Chemicon (Temecula, CA). A control irrelevant mouse mAb (IgG, clone 1-64.1) was a gift from Dr. Y. S. Lin. Fibronectin protein was purchased from Millipore (Bedford, MA). DTT was purchased from Sigma.

Cell adhesion assay

Adhesion assays were performed as previously described [8, 9] with modifications. Briefly, soluble VCAM-1-Fc at 15 μg/mL in 0.1 M sodium phosphate buffer (pH 7.5) was immobilized overnight at 4°C in 96-well plates. Nonspecific binding was blocked with 0.5% BSA. HL-60 clone 15 cells were preincubated on ice for 10 min with heat-inactivated human serum to block Fc receptors, and 1 × 10⁶ cells/well were used. The cells were then labeled with 1.5 μg/mL 2,7′-bis-(2-carboxyethyl)-5(and-6) carboxyfluorescein (BCECF/AM; Molecular Probes, Eugene, OR) and incubated at 37°C for 1 h. The BCECF/AM-labeled HL-60 clone 15 cells were washed with phosphate-buffered saline (pH 7.2) for three times. Next, BCECF/AM-labeled HL-60 clone 15 cells in HHMC (HEPES-buffered Hank’s buffer, 1 mM Mg²⁺/1 mM Ca²⁺, 0.5% BSA) were allowed to adhere to VCAM-1-Fc-coated plates for 60 min at 37°C. Cells (1 × 10⁶/well) were analyzed for binding in the absence or presence of sulphhydryl blockers. Nonadherent cells were removed by a flick wash. The fluorescence of input and adherent cells was determined with a fluorescence plate reader.
VCAM-1-Fc, ICAM-1-Fc, and Fc recombinant proteins were prepared as described [8]. Recombinant proteins were labeled using FITC according to the manufacturer's protocol (Pierce).

**Cell surface labeling, immunoprecipitation and Western blotting**

The unpaired cysteines within peptides were tagged using the sulfhydryl group modification reagent, biotin-BMCC, as described [49]. Briefly, HL-60 clone 15 cells were incubated with heat-inactivated human serum on ice for 10 min, pelleted by centrifugation, and washed three times with phosphate-buffered saline. Then, 1 × 10⁶ cells were incubated with 50 μM biotin-BMCC, with or without pretreatment of 50 μg/mL recombinant VCAM-1-Fc or fibronectin and hydrogen peroxide (10 or 100 μM) in serum-free RPMI 1640 medium at room temperature for 1 h with rotation. After removal of unbound biotin-BMCC by centrifugation, cells were washed with phosphate-buffered saline pH 7.2 for three times. The cells were then lysed with 0.2 mL lysis buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris-base, 1 mM EDTA, pH 7.4, and protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany)] and kept on ice for 30 min. The cell lysate was centrifuged to remove insoluble material (12 000 × g, 4°C) for 10 min. For immunoprecipitation, the lysates, after preclearing with 10 μL protein G agarose beads at 4°C for 4 h, were incubated with 0.2 μg/mL primary anti-VLA-4 mAb overnight at 4°C with gentle rocking. Next, the lysates were incubated with 10 μL protein G agarose beads with gentle agitation at 4°C for 4 h and washed three times before analysis. The proteins were then resolved on 12% reducing SDS-PAGE gels, electrophoresed onto PVDF membranes and blocked overnight at 4°C with 5% non-fat milk. After treatment of the membrane with 10 μL protein G agarose beads and washing, mouse anti-VLA-4 antibody (HP2/1) was used to stain the Western blots. HRP-conjugated antibody complexes were detected with Renaissance Chemiluminescence Reagent (Amersham Biosciences, Piscataway, NJ) and images were exposed to autoradiography films.

**FITC-labeled recombinant protein binding assay**

VCAM-1-Fc, ICAM-1-Fc, and Fc recombinant proteins were analyzed using the two-tailed Student's t-test.

In some experiments, the recombinant VCAM-Fc or fibronectin was treated with 10 mM NEM for 1 h to block the free sulfhydryl groups. The excess NEM was then removed by ultrafiltration spin columns (Millipore, Bedford, MA). For direct ligand binding studies, 1 × 10⁶ cells/mL were pre-incubated with heat-inactivated human serum on ice for 10 min and washed with Hank's buffer for three times. Next, the cells were incubated with 1 μg FITC-VCAM-1-Fc or FITC-ICAM-1-Fc in 0.1 mL Hank's buffer at room temperature for 10 min. To assess the effect of sulfhydryl blocker (0.1 mM DTNB), hydrogen peroxide (5, 10 or 100 μM) or glutathione (10 μM) or cysteine (10 μM) on VLA-4-VCAM-1-Fc interaction, HL-60 clone 15 cells (1 × 10⁶ cells/mL) were pre-incubated with Hank's buffer containing the tested reagents at room temperature for 10 min. The cells were then washed with phosphate-buffered saline for three times. Next, the cells were incubated with 1 μg FITC-VCAM-1-Fc or FITC-ICAM-1-Fc in 0.1 mL Hank's buffer at room temperature for 10 min and analyzed by flow cytometry. FITC-Fc was used as the negative control.

**Fluorescent microscopy**

For visualization of cell surface VLA-4 clustering, HL-60 clone 15 cells were incubated with 1 μg VCAM-1-Fc in 0.1 mL Hank's buffer for 10 min and fixed with 1% formaldehyde in PBS for 10 min at room temperature. After the cells were washed, mouse anti-VLA-4 antibody (HP2/1) was used to stain the cells. FITC-conjugated goat anti-mouse antibody was then used as the secondary antibody. Images were photographed with a Leica fluorescent microscope (TCS SPS; Wetzlar, Germany) and analyzed with MetaMorph software (Universal Imaging Corporation, Downingtown, PA).

**Cell rolling assay**

Recombinant human VCAM-1-Fc (5 μg/mL) or Fc (5 μg/mL) was immobilized overnight at 4°C onto 30-mm polystyrene petri dishes. The dishes were washed with phosphate-buffered saline and blocked with 1% BSA (w/v) for 1 h at room temperature. The dishes were assembled into parallel plate flow chambers (GlycoTech, Rockville, MD). HL-60 clone 15 cells (1 × 10⁶ cells/mL) in Hank's buffer were injected into the flow chamber, incubated with hydrogen peroxide (5 or 10 μM) with or without 10 μM glutathione or cysteine for 10 min, then flown through at a constant flow rate with a shear stress of 1.0 dynes/cm² for 180 s at 37°C. The numbers of rolling cells were recorded and quantified. Cells moving at a speed less than 1100 μm/s were counted as rolling cells. The cell rolling velocity in a time period of 60 s (30–180 s) was calculated and compared under different experimental conditions.

**Transfection of FAK siRNA**

To inhibit FAK protein expression, HL-60 clone 15 cells were transfected with siRNA. The siRNA duplex, 5’-GCAUGUGGCC-GCAUGUGGCCUGCUAAUGGAdTdTdTdTdTdTGCACCACG-GUAUCCU-3’, directed toward the mRNA target, 5’-AAGCAUGUGGCCUGCUAUGGA-3’, was synthesized by (HTS7000 Bioassay Reader; Perkin-Elmer, Norwalk, CT). As previously reported [8], the adhesion between VCAM-1 and HL-60 clone 15 cells can be blocked with anti-VLA-4 antibody, which confirmed that this adhesion assay detects VLA-4-VCAM-1-dependent cell binding. The results of the adhesion assays were analyzed with the two-tailed Student's t-test.
Dharmacon (Lafayette, CO). A scrambled RNA duplex (5’-GGGCGCGGGCUGGAGGCGAGTGTGTAATGTTGCGCGGAAAACGCUGCUAAC-3’) was used as a control. Transfections of duplex siRNA (100 nM) were performed using Oligofectamine (Gibco, Gaithersburg, MD) according to the manufacturer’s protocol. At 24 h after transfection, cells were analyzed by Western blotting and RT-PCR analysis and for ligand binding assays. For RT-PCR, FAK primers (forward 5’-GGCCTGCTGTTAAGGAACG–T3; reverse 5’-TGGCTGTG-TGTGCTGGTGTTAGG-3’) and GAPDH primers (forward 5’-ATGGAAAACTCCATCCACTCT-3’; reverse 5’-GTTGAG-GGTGAGCAC-AGGGTACTTTATT-3’) were used. Thirty-five amplification cycles consisting of 15 s of denaturation at 94°C, 30 s of annealing at 58°C, and 30 s of extension at 72°C were performed. The PCR products were then visualized with agarose gel electrophoresis and ethidium bromide staining.

Acknowledgements: This project was supported by grants from the National Science Council and National Health Research Institute, Taiwan. The authors want to thank Drs. Ming-Shi Chang, Woei-Jer Chuang, Sam Hwang, and Trai-Ming Yeh for helpful discussions.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1. Butcher, E. C. and Picker, L. J., Lymphocyte homing and homostasis. Science 1995, 272: 60-66.
2. Springer, T. A., Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm. Cell 1994, 76: 301-314.
3. Yang, G. X. and Hagmann, W. K., VLA-4 antagonists: Potent inhibitors of lymphocyte migration. Med. Res. Rev. 2003, 23: 369-392.
4. Boussque, J., Chanez, P., Lacoste, J. Y., Barneo, G., Ghavalian, N., Enander, I., Venge, P. et al., Eosinophilic inflammation in asthma. N. Engl. J. Med. 1990, 323: 1033-1039.
5. Babior, B. M., NADPH oxidase. Curr. Opin. Immunol. 2004, 16: 42-47.
6. Roos, D., van Bruggen, R. and Meischl, C., Oxidative killing of microbes by neutrophils. Microbes. Infect. 2003, 5: 1307-1315.
7. Lin, S. J., Huang, Y. F., Chen, J. Y., Heyworth, P. G., Noack, D., Wang, J. Y., Lin, C. Y. et al., Molecular quality control machinery contributes to the leukocyte NADPH oxidase deficiency in chronic granulomatous disease. Biochem. Biophys. Acts 2002, 1586: 275-286.
8. Chuang, K. P., Tsai, W. S., Wang, Y. J. and Shiue, C. C., Superoxide activates very late antigen-4 on an eosinophil cell line and increases cellular binding to vascular cell adhesion molecule-1. Eur. J. Immunol. 2003, 33: 645-655.
9. Chuang, K. P., Huang, Y. F., Hsu, Y. L., Liu, H. S., Chen, H. C. and Shiue, C. C., Ligation of lymphocyte function-associated antigen-1 on monocytes decreases very late antigen-4-mediated adhesion through a reactive oxygen species dependent pathway. Blood 2004, 104: 4046-4053.
10. Lu, C., Shimaoka, M., Zhang, Q., Takagi, J. and Springer, T. A., Locking in alternate conformations of the integrin alpha beta 2 domain with disulfide bonds reveals functional relationships among integrin domains. Proc. Natl. Acad. Sci. USA 2001, 98: 2393-2398.
11. Chen, P., Melchior, C., Brons, N. H., Schlegel, N., Caen, J. and Kieffer, N., Probing conformational changes in the I-like domain and the cysteine-rich repeat of human beta 3 integrins following disulfide bond disruption by cysteine mutations: Identification of cysteine 598 involved in alphabeta3 activation. J. Biol. Chem. 2001, 276: 38628-38635.
Yednock, T. A., Cannon, C., Vandevert, C., Goldbach, E. G., Shaw, G., Ellis, D. K., Liaw, C., et al., Alpha4beta1 integrin-dependent cell adhesion is regulated by a low affinity receptor pool that is conformationally responsive to ligand. *J. Biol. Chem.* 1995. 270: 28740–28750.

Chen, C., Mobley, J. L., Dwir, O., Shimron, F., Grabovsky, V., Lobb, R. R., Shimizu, Y. and Alon, R., High affinity very late antigen-4 subsets expressed on T cells are mandatory for spontaneous adhesion strengthening but not for rolling on VCAM-1 in shear flow. *J. Immunol.* 1999. 162: 1084–1095.

Luo, B. H., Carman, C. V. and Springer, T. A., Structural basis of integrin regulation and signaling. *Annu. Rev. Immunol.* 2007. 25: 619–647.

Luo, B. H., Springer, T. A. and Takagi, J., A specific interface between integrin transmembrane helices and affinity for ligand. *PLoS Biol.* 2004. 2: e153.

Shi, M., Foo, S. Y., Tan, S. M., Mitchell, E. P., Law, S. K. and Lescar, J., A structural hypothesis for the transition between bent and extended conformations of the leukocyte beta 2 integrins. *J. Biol. Chem.* 2007. 282: 30198–30206.

Takada, Y., Elices, M. J., Crouse, C. and Hemler, M. E., The primary structure of the alpha 4 subunit of VLA-4: Homology to other integrins and a possible cell-cell adhesion function. *EMBO J.* 1989. 8: 1361–1368.

Pujades, C., Teixido, J., Bazzoni, G. and Hemler, M. E., Integrin alpha 4 cysteines 278 and 717 modulate VLA-4 ligand binding and also contribute to alpha 4/180 formation. *Biochem. J.* 1996. 313: 899–908.

Luque, A., Gomez, M., Puzon, W., Takada, Y., Sanchez-Madrid, F. and Callahan, C., Activated conformations of very late activation integrins detected by a group of antibodies (HUTS) specific for a novel regulatory region (355–425) of the common beta 1 chain. *J. Biol. Chem.* 1996. 271: 11067–11075.