

Full-length article

## Engagement of PSGL-1 enhances $\beta_2$ -integrin-involved adhesion of neutrophils to recombinant ICAM-1<sup>1</sup>

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### Key words

P-selectin ligand protein; intercellular signaling peptides and proteins; intercellular adhesion molecule-1; CD18 antigens

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### Abstract

**Aim:** The interactions of selectins and their ligands initiate the process of leukocyte migrating into inflamed tissue. P-selectin glycoprotein ligand 1 (PSGL-1) is the best characterized ligand of selectins, and has been demonstrated to mediate the adhesion of leukocytes to all three selectins *in vivo*. PSGL-1 not only functions as an anchor molecule to capture the leukocytes to the activated endothelial cells by its interaction with selectins, but also transduces the signals to activate leukocytes. Our present work aimed to investigate the mechanism by which PSGL-1-mediated signal activates neutrophils and enhances the adhesion to the endothelial cells. **Methods:** We detected the effects of the engagement of PSGL-1 with monoclonal antibodies (mAb) or P-selectin on the adhesion of neutrophils to the recombinant intercellular adhesion molecule-1 (ICAM-1), and on the expression of  $\beta_2$ -integrin. Additionally, the role of cytoskeleton in these process was studied by using inhibitor cytochalasin B. **Results:** The engagement of PSGL-1 increased the expression of  $\beta_2$ -integrin on the surface of neutrophils and enhanced the adhesion of neutrophils to the recombinant ICAM-1. mAb against CD18 impaired the adhesion of PSGL-1-engaged neutrophils to ICAM-1. Moreover, the inhibitor cytochalasin B largely blocked the increase of CD18 expression as well as the adhesion of PSGL-1-engaged neutrophils to ICAM-1. **Conclusion:** The PSGL-1-transduced signals can enhance  $\beta_2$ -integrin-involved adhesion of neutrophils to the recombinant ICAM-1, and this process depends on the dynamics of cytoskeleton.

### Introduction

Inflammation is a multiple-step process initiated by the adhesion of leukocytes to the activated endothelial cells. The recruitment of leukocytes from the flowing bloodstream is a rapid event that requires special mechanisms to establish the contact of leukocyte with endothelium. Selectins represent a class of cell adhesion molecules that are specialized for this purpose<sup>[1]</sup>. P-selectin glycoprotein ligand 1 (PSGL-1), the best characterized ligand of the selectins, has been demonstrated to mediate the adhesion of leukocytes to all three selectins *in vivo*<sup>[2-5]</sup>.

PSGL-1 is a highly extended mucin expressed on microvilli

of almost all types of leukocytes. In addition to its direct role in the capture of leukocytes from the bloodstream, PSGL-1 has also been demonstrated to function as a signal-transduced receptor and initiate a series of intracellular signal events during the activation of leukocytes, including activation of  $\beta_2$ -integrin<sup>[6-8]</sup>, tyrosine-protein phosphorylation<sup>[9,10]</sup>, secretion of cytokines<sup>[9]</sup> and transcriptional activation<sup>[11]</sup>. Our previous work suggests that PSGL-1-mediated signals induce the alteration of F-actin-based cytoskeleton in neutrophils, and non-receptor tyrosine kinase c-Abl may be involved in the process<sup>[12]</sup>. A recent study showed that PSGL-1 stimulated lymphocyte function-associated antigen (LFA)-1-mediated Th1 cell binding to intercellular adhesion

molecule-1 (ICAM-1)<sup>[13]</sup>.

In the present work, we demonstrated that the engagement of PSGL-1 with mAb or P-selectin induced the increased expression of  $\beta_2$ -integrin and enhanced the adhesion of neutrophils to the recombinant ICAM-1. TS1/18, the monoclonal antibody against CD18, impaired the adhesion of the PSGL-1-engaged neutrophils to the ICAM-1, suggesting the contribution of  $\beta_2$ -integrins to the adhesion of neutrophils to ICAM-1 is caused by the engagement of PSGL-1. Moreover, cytochalasin B (an inhibitor for the assembly of F-actin-based cytoskeleton) largely blocked the increased expression of  $\beta_2$ -integrin and the adhesion of PSGL-1-engaged neutrophils to ICAM-1, which implies an important role of the F-actin-based cytoskeleton. Our combined results suggest that the PSGL-1-transduced signal can enhance  $\beta_2$ -integrin-involved adhesion of neutrophils to the recombinant ICAM-1 through the increase of  $\beta_2$ -integrin expression, and this process depends on the dynamics of the F-actin-based cytoskeleton.

## Materials and methods

**Recombinant proteins, antibodies and reagents** The recombinant human P-selectin (ADP3) and ICAM-1 (ADP4) were purchased from R&D Systems (Minneapolis, MN, USA).

PL2 (the non-blocking mAb against PSGL-1, mouse IgG<sub>1</sub>, sc-18856) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). W6/32 (the anti-HLA-A,B,C mAb, mouse IgG<sub>1</sub>, 14-9983) was purchased from Ebioscience (San Diego, CA, USA). 9E1 (the mAb recognizing the extra-cellular domain of recombinant human P-selectin, mouse IgG<sub>1</sub>, BBA30) was purchased from R&D Systems. TS1/18 (the mAb against CD18, which can block the ligation of  $\beta_2$ -integrin with ICAM-1, mouse IgG<sub>1</sub>, MA1810) was purchased from Pierce Biotechnology (Rockford, IL, USA). Fluorescein isothiocyanate (FITC)-conjugated  $\beta_2$ -integrins antibody IB4 was purchased from Ancell Corporation (Bayport, MN, USA) (167-040). Dextran T-500 used for neutrophil isolation was purchased from Pharmacia (Uppsala, Sweden). Cytochalasin B (an inhibitor for assembly of F-actin) was purchased from Sigma (Saint Louis, MO, USA).

**Neutrophil isolation** Neutrophils were isolated by standard procedures after sedimentation of erythrocytes by dextran T-500 and centrifugation of leukocytes over Ficoll-Hypaque gradients as described in our previous work<sup>[12]</sup>. After lysis of contaminating erythrocytes with hypotonic saline (0.17 mol/L Tris, 0.16 mol/L NH<sub>4</sub>Cl, pH 7.2), the neutrophils were washed and re-suspended in cold PBS containing 0.1% bovine serum albumin (BSA). The isolated neutrophils

were adjusted at a concentration of  $0.5 \times 10^6$  cells/mL and stored at 4 °C until use. More than 95% of the isolated cells were polymorphonuclear leukocytes, and viability was determined to be 98% by Trypan blue exclusion.

**Engagement of PSGL-1** Preceding the interaction with ICAM-1 monolayer, the isolated neutrophils were ligated with PL2, an mAb against PSGL-1 (or control antibody W6/32) at a concentration of 10  $\mu$ g/mL at 4 °C for 20 min. In other experiments, the isolated neutrophils were subjected to flow on the P-selectin/ICAM-1 monolayer to encounter and engage with P-selectin.

**Monolayer preparation** To prepare the ICAM-1 monolayer, the petri dishes were coated with 3  $\mu$ g/mL recombinant ICAM-1 overnight and then blocked with 1% BSA at 37 °C for 2 h, and in control groups, recombinant ICAM-1 was substituted with BSA. In other experiments, the petri dishes were simultaneously coated with 3  $\mu$ g/mL of each recombinant P-selectin and ICAM-1 overnight and then blocked with 1% BSA at 37 °C for 2 h.

**Cell adhesion assay** The adhesion of PSGL-1-engaged neutrophils to the recombinant ICAM-1 monolayer was detected by using a parallel-plate flow chamber (GlycoTech, Rockville, MD, USA). Briefly, the neutrophils were washed twice and re-suspended in RPMI 1640 medium. At a shear stress of 1.2 dyn/cm<sup>2</sup>, cells were infused over the ICAM-1 (or P-selectin/ICAM-1) monolayer via a syringe pump<sup>[14,15]</sup>. Interactions of neutrophils with monolayer were allowed by stopping the flow at different times, after which the flow was carried out again at a shear stress of 1.2 dyn/cm<sup>2</sup>. The adherent cells could then be counted by using an inverted microscope (Olympus Optical, Tokyo, Japan) equipped with a camera (Panasonic, Yokohama, Japan) connected to a VCR and a TV monitor. Each data represented the percentage of the number of remaining cells visible when flow was re-infused to that of all halted cells after flow was stopped in 10 fields of view under a  $\times 10$  objective.

For antibody blocking experiments, neutrophils (or antibody-engaged neutrophils) were treated with TS1/18 at a concentration of 10  $\mu$ g/mL at 4 °C for 15 min preceding being infused on the monolayer. In other experiments the P-selectin/ICAM-1 monolayer was blocked with 9E1, the mAb recognizing the extra-cellular domain of recombinant human P-selectin, at a concentration of 10  $\mu$ g/mL at 4 °C for 15 min to block the engagement of PSGL-1 with P-selectin.

For inhibition experiments, the isolated neutrophils were treated with 20  $\mu$ mol/L cytochalasin B at 4 °C for 30 min preceding the engagement of PSGL-1 and the interaction with the ICAM-1 (or P-selectin/ICAM-1) monolayer. The control cells were treated with the equivalent volume of dimethyl

sulfoxide (DMSO).

**Fluorescence assay** To examine the expression of  $\beta_2$ -integrins on the surface of neutrophils, cells ( $1 \times 10^6/\text{mL}$ ) were treated with (or without) PL2 (10  $\mu\text{g}/\text{mL}$ ) at 37 °C for 30 min, then washed with PBS twice before being fixed with 4% paraformaldehyde for 20 min at room temperature. The expression of CD18 was visualized by staining with FITC-conjugated  $\beta_2$ -integrins antibody IB4 (10  $\mu\text{g}/\text{mL}$ ) for 30 min. The levels of surface expression of  $\beta_2$ -integrins were detected with Fluorescence Reader Gemini EM (Molecular Device Corporation, Sunnyvale, CA, USA). The well was set with an equal number of cells, without antibody treatment and FITC-labeled CD18 antibody IB4 staining as blank, the data of all sample wells were normalized by the value of blank. The data indicate the OD values of the detector's absorbance of the fluorescence that samples emit, and each data represents the mean $\pm$ SD of three independent experiments and the significant difference from the control was determined by ANOVA ( $P < 0.01$ ).

**Results**

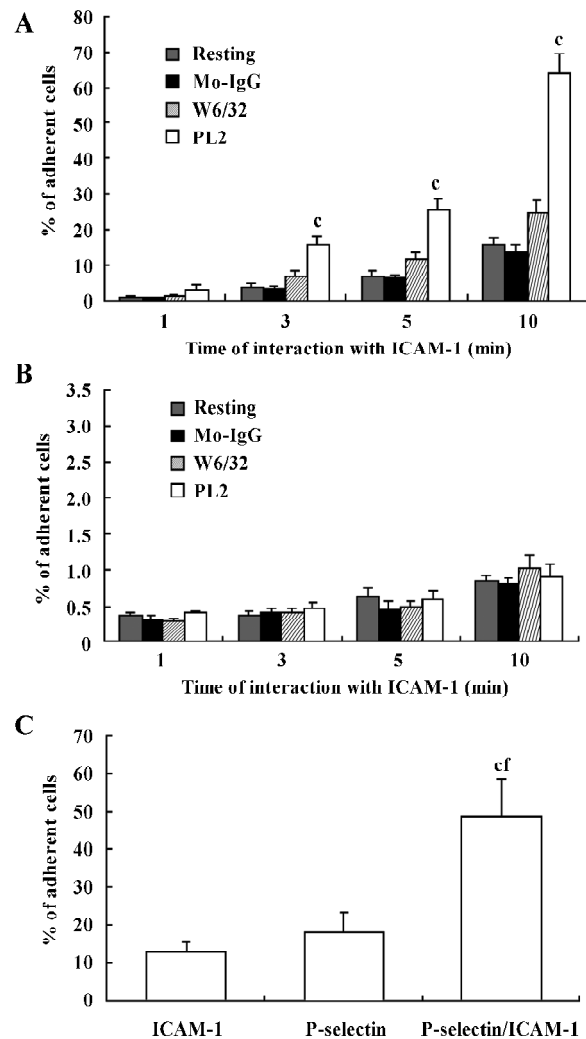
**Engagement of PSGL-1 enhanced the adhesion of neutrophils to recombinant ICAM-1** We set up an *in vitro* flow system to investigate the contribution of signals transduced by PSGL-1 to the activation of neutrophils and the mechanism by which the interaction of PSGL-1 with P-selectin enhances the adhesion of neutrophils to ICAM-1 expressed on the surface of activated endothelial cells during inflammation.

First, recombinant ICAM-1 was coated as a monolayer on the petri dish bottom. After engagement with (or without) antibodies, the neutrophils were infused in the flow system at a shear stress of 1.2  $\text{dyn}/\text{cm}^2$ . The adhesion of neutrophils to the recombinant ICAM-1 was allowed for different times. The percentage of adherent cells for those engaged with PL2 was significantly higher compared with that of resting, Mo-IgG-treated or W6/32-treated neutrophils (Figure 1A). The extreme lack of adhesion for those differently treated neutrophils to BSA-coated petri dishes implied the specificity of the interaction of engagement-activated neutrophils to the ICAM-1 monolayer (Figure 1B).

Second, the isolated neutrophils were infused by flow on the petri dish bottom, which was coated with P-selectin, ICAM-1, or both P-selectin and ICAM-1 as a monolayer. The neutrophils were infused in the flow system and the adhesion of neutrophils to the monolayer was allowed for 10 min. As shown in Figure 1C, the percentage of neutrophils adherent to P-selectin/ICAM-1 monolayer was significantly increased compared with that of neutrophils adherent to

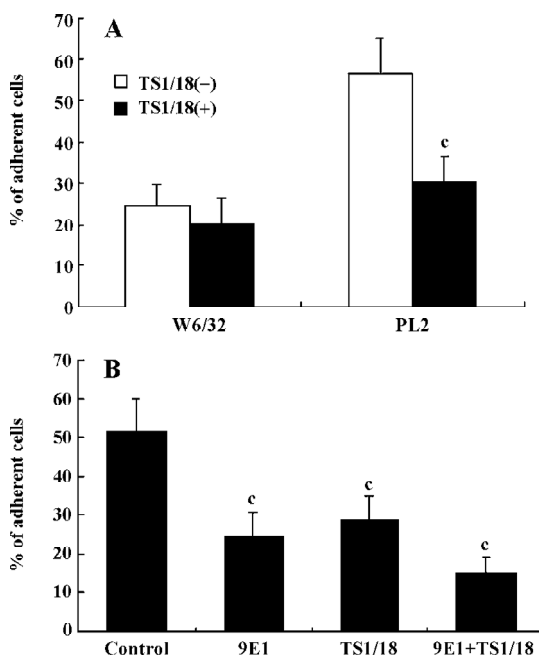
ICAM-1 or P-selectin monolayer alone, which suggested that the presence of P-selectin enhances the interaction of neutrophils with the recombinant ICAM-1 (Figure 1C).

**$\beta_2$ -Integrins were involved in the adhesion of PSGL-1-engaged neutrophils to recombinant ICAM-1** The leukocyte-specific  $\beta_2$ -integrins regulate the migration of leuko-



**Figure 1.** The engagement of PSGL-1 enhanced the adhesion of neutrophils to recombinant ICAM-1. A, The percentage of adherent neutrophils on the recombinant ICAM-1 monolayer in differently treated groups after interaction for time intervals as indicated. B, The percentage of adherent neutrophils on the BSA monolayer in differently treated groups and after interaction for time intervals as indicated. C, The percentage of adherent cells after the interaction of isolated neutrophils with different recombinant protein monolayers (ICAM-1, P-selectin or P-selectin/ICAM-1) for 10 min. Each data point represents the mean $\pm$ SD of three independent experiments and the significant difference from the control was determined by ANOVA (A,B) <sup>c</sup> $P < 0.01$  vs Resting; (C) <sup>c</sup> $P < 0.01$  vs ICAM-1. <sup>f</sup> $P < 0.01$  vs P-selectin.

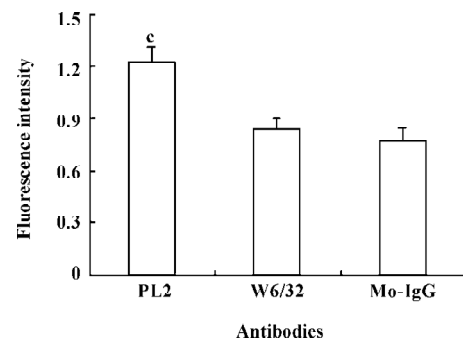
cytes and the initiation of immune responses through binding to ICAM-1, -2 or -3<sup>[16,17]</sup>. In order to investigate the role of  $\beta_2$ -integrins in the adhesion of PSGL-1-engaged neutrophils to the recombinant ICAM-1 monolayer, we performed antibody-blocking experiments by using antibodies TS1/18 and 9E1, respectively, against CD18 and P-selectin (both antibodies recognize the extra-cellular ligand-binding domains of CD18 and P-selectin). The results showed that the increased percentage of adherent neutrophils induced by PL2 antibody-cross-linking was abolished by the incubation of neutrophils with TS1/18, the mAb against CD18 (Figure 2A). Moreover, if the P-selectin/ICAM-1 monolayer and neutrophils were respectively pre-incubated with 9E1 and TS1/18 alone, or pre-incubated with both 9E1 and TS1/18 prior to the neutrophils being infused in the flow system, the percentage of the adherent neutrophils decreased in different degrees compared with that of the control. Especially when the neutrophils interacted with the monolayer were blocked with both 9E1 and TS1/18, the percentage of the adherent neutrophils dropped more than 70% compared with that of



**Figure 2.**  $\beta_2$ -Integrins were involved in the adhesion of PSGL-1-engaged neutrophils to recombinant ICAM-1. A, The percentage of adherent cells on the recombinant ICAM-1 monolayer in the consequence of the blocking of TS1/18 to the neutrophils engaged with PL2 or control antibody. B, The percentage of adherent cells on the recombinant P-selectin/ICAM-1 monolayer in different groups, in which the isolated neutrophils or the monolayer were blocked with indicated antibodies. Each data point represents the mean $\pm$ SD of three independent experiments and the significant difference from the control was determined by ANOVA ( $^cP<0.01$  vs control).

the control (from 51.36% to 15.08%) (Figure 2B). The results from the antibody-blocking experiments suggested that  $\beta_2$ -integrins were involved in the increased adhesion of neutrophils to the recombinant ICAM-1 caused by the engagement of PSGL-1.

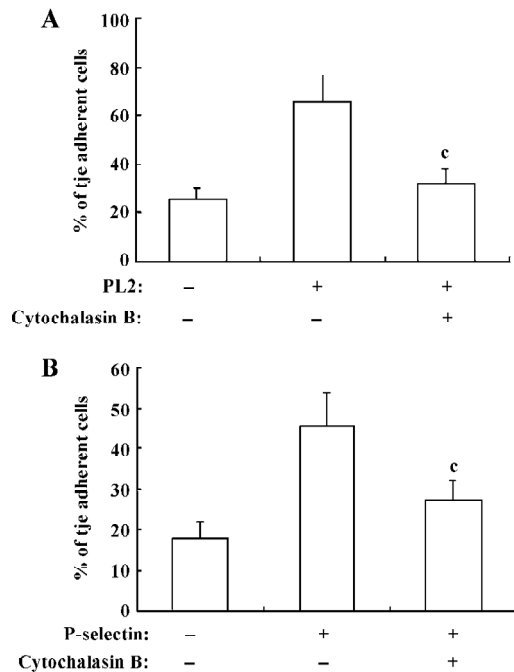
To further understand the contribution of  $\beta_2$ -integrins to the adhesion of activated neutrophils to the recombinant ICAM-1, we investigated the alteration of  $\beta_2$ -integrin expression. The neutrophils were incubated with PL2 or control IgG at 37 °C for 30 min, and then fixed and labeled with FITC-conjugated antibody IB4. The result showed that the engagement of PSGL-1 induced an increased expression of  $\beta_2$ -integrins on the surface of activated neutrophils (Figure 3).



**Figure 3.** The engagement of PSGL-1 induced the increased expression of  $\beta_2$ -integrins on the surface of activated neutrophils. The data represent the OD values of the detector's absorbance of the fluorescence that the samples emitted and each data represent the mean $\pm$ SD of three independent experiments and the significant difference between PL2-treated group and W6/32- or Mo-IgG-treated group was determined by ANOVA ( $^cP<0.01$ ).

**$\beta_2$ -Integrin-involved adhesion of neutrophils to recombinant ICAM-1 depends on the dynamics of F-actin-based cytoskeleton** Because the cytoskeleton often plays an important role in the transduction of extra-cellular signals, we further explored the significance of the dynamics of the cytoskeleton to the PSGL-1-initiated and  $\beta_2$ -integrin-involved adhesion of neutrophils to the recombinant ICAM-1. We pre-incubated the isolated neutrophils with cytochalasin B, the cells were then engaged with mAb and infused to the ICAM-1 monolayer, or the inhibitor-treated cells were infused to the P-selectin/ICAM-1 monolayer. Consequently, the adhesion of PSGL-1-engaged neutrophils to recombinant ICAM-1 was significantly suppressed, suggesting that the PSGL-1-initiated adhesion of neutrophils to the recombinant ICAM-1 depend on the dynamics of the cytoskeleton (Figure 4A and 4B). The exposure of neutrophils to cytochalasin B

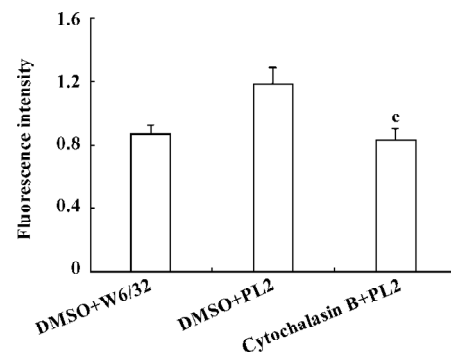
also resulted in a dramatic blocking of the increased expression of  $\beta_2$ -integrins (Figure 5), implying the significance of the dynamics of the F-actin-based cytoskeleton to the  $\beta_2$ -integrin-mediated adhesion of neutrophils to ICAM-1.



**Figure 4.** The effects of cytochalasin B on the adhesion of PSGL-1-engaged neutrophils to recombinant ICAM-1. A, The percentage of adherent cells on the recombinant ICAM-1 monolayer in different groups, in which neutrophils were engaged with PL2 or control mAb W6/32 and inhibited with or without cytochalasin B. B, The percentage of adherent cells in different groups, in which the isolated neutrophils were allowed to interact with the monolayer in the presence or absence of P-selectin and inhibited with or without cytochalasin B. Each data point represents the mean±SD of three independent experiments and in the presence of the engagement of PSGL-1, the significant difference between groups with or without cytochalasin B was determined by ANOVA (<sup>c</sup>*P*<0.01).

### Discussion

During the rolling adhesion of leukocytes on the activated endothelial cells, PSGL-1 functions not only as an anchor to arrest the leukocytes through the interaction with P-selectin, but also as a signal receptor to transduce the extra-cellular signals and participate in the activation of leukocytes<sup>[18, 19]</sup>. ICAM-1 is an Ig-like cell adhesion molecule expressed by several cell types, including leukocytes and endothelial cells after stimulation with cytokines such as IL-1, TNF- $\alpha$  or IFN- $\gamma$ <sup>[20]</sup>. Within the vascular system, ICAM-1 expressed on the surface of activated endothelial cells interacts with integrins expressed by leukocytes to achieve the



**Figure 5.** Cytochalasin B blocked the increase of  $\beta_2$ -integrins expression caused by the engagement of PSGL-1. Prior to the neutrophils being engaged with PL2 or control mAb W6/32, the cells were pre-incubated with or without cytochalasin B. The  $\beta_2$ -integrin expression of different groups was detected with a Fluorescence Microplate Reader. The well was set with an equal number of cells, without antibody treatment and FITC-labeled CD18 antibody IB4 staining as blank, the data of all sample wells were normalized by the value of blank. The data represent the OD values of the detector's absorbance of the fluorescence that that samples emitted and each data represent the mean±SD of three independent experiments. <sup>c</sup>*P*<0.01 vs DMSO+PL2.

firm adhesion of leukocytes on the endothelium.

CD18 integrins are leukocyte-specific, and it is still controversial as to what mechanism CD18 integrins rely on to enhance the adhesion of activated leukocytes to ICAM-1. It may be the extroversion of the  $\beta_2$ -integrin from the intracellular vesicles<sup>[21]</sup>, increased affinity of  $\beta_2$ -integrin due to the conformational change of extra-cellular structure, *de novo* synthesis or simply the cluster of the molecules on the surface of leukocytes<sup>[17]</sup>.

In the present study, we addressed the implication of leukocyte-specific  $\beta_2$ -integrins in the adhesion of neutrophils to endothelial cells induced by the engagement of PSGL-1 with P-selectin during inflammation. We established an *in vitro* adhesion system by using a parallel-plate flow chamber. The incubation with TS1/18 abolished the increased adhesion of neutrophils caused by the engagement of PSGL-1. Meanwhile, fluorescence assay revealed that the engagement of PSGL-1 led to an increase of  $\beta_2$ -integrin expression on the activated neutrophils, which suggested the involvement of CD18 integrins in the adhesion process. Additionally, the pre-incubation of neutrophils with cytochalasin B not only significantly decreased the percentage of the adherent cells, but also blocked an increase in the level of CD18 on the PSGL-1-engaged neutrophils, which suggested that the dynamics of cytoskeleton is essential for the  $\beta_2$ -integrin-mediated adhesion of PSGL-1-engaged neutrophils to ICAM-1.

It is well known that PSGL-1-transduced signals can enhance  $\beta_2$ -integrin-mediated adhesion of activated neutrophils to ICAM-1. Our present study revealed that increased expression of  $\beta_2$ -integrin contributed to this adhesion enhancement. Our previous study demonstrated engagement of PSGL-1 with antibodies led to the association of PSGL-1 with F-actin-based cytoskeleton<sup>[12]</sup>, and the present study further elaborated that PSGL-1-transduced signals depended on the dynamic of F-actin-based cytoskeleton to increase the expression of CD18 integrin and  $\beta_2$ -integrin-mediated adhesion.

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