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P-selectin primes leukocyte integrin activation during inflammation

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Selectins mediate leukocyte rolling and prime leukocytes for integrin-mediated leukocyte adhesion. However, neither the *in vivo* importance of nor the signaling pathway by which selectin-mediated integrin activation occurs has been determined. We report here that P-selectin-deficient mice manifested impaired leukocyte adhesion, which was 'rescued' by soluble P-selectin. Mechanistically, the cytoplasmic domain of P-selectin glycoprotein ligand 1 formed a constitutive complex with Nef-associated factor 1. After binding of P-selectin, Src kinases phosphorylated Nef-associated factor 1, which recruited the phosphoinositide-3-OH kinase p85-p110δ heterodimer and resulted in activation of leukocyte integrins. Inhibition of this signal-transduction pathway diminished the adhesion of leukocytes to capillary venules and suppressed peritoneal infiltration of leukocytes. Our data demonstrate the functional importance of this newly identified signaling pathway mediated by P-selectin glycoprotein ligand 1.

The recruitment of leukocytes to a site of infection or tissue injury entails a cascade of cellular adhesive events, including tethering, rolling, adhesion, diapedesis, transmigration and chemotaxis[1–7]. Selectins mediate leukocyte tethering and rolling, thereby bringing circulating leukocytes into proximity with extracellular stimuli such as cytokines, chemokines and chemoattractants that are displayed on or released from activated endothelium. By transducing signals through G_i-type heterotrimeric G protein–coupled receptors, these extracellular stimuli activate integrins, which mediate adhesion of leukocytes.

The selectin family of cell adhesion molecules includes the following[1,2,4–6]: L-selectin (CD62L), which is constitutively expressed on most leukocytes, E-selectin (CD62E), which is expressed after cytokine stimulation exclusively on endothelial cells, and P-selectin (CD62P), which is stored on membranes of endothelial Weibel-Palade bodies and platelet α-granules. After inflammatory and thrombogenic challenges, P-selectin rapidly translocates to the cell surface and binds to P-selectin glycoprotein ligand 1 (PSGL-1), which mediates leukocyte tethering and rolling of leukocytes on stimulated endothelial cells, as well as heterotypic aggregation between activated platelets and leukocytes. It is generally believed that PSGL-1 functions as a principal leukocyte ligand for P-selectin and that all three selectins interact with PSGL-1 (refs. 4–6). Extensive studies of mice lacking selectins or PSGL-1 have shown that the interactions of P-, E- and L-selectins with PSGL-1 are essential for leukocyte tethering and rolling *in vivo*[7,8].

Firm adhesion of leukocytes to the endothelium is mediated by integrins, a large family of transmembrane heterodimeric adhesion receptors expressed on the cell surface of eukaryotic cells. Integrins are essential for cell-cell and cell-matrix interactions as well as cellular communication. The β_2 subfamily of integrins, often called 'leukocyte integrins', consists of four members whose four distinct α subunits (α_M, α_L, α_X and α_D) combine with a common β_2 subunit[1,4,9,10]. Prominent among the β_2-integrins expressed on neutrophils are $\alpha_M\beta_2$ (also called CD11b and CD18, or Mac-1) and $\alpha_L\beta_2$ (also called CD11a and CD18, or LFA-1); $\alpha_M\beta_2$ binds to many ligands, including intercellular cell adhesion molecule 1 (ICAM-1), whereas the ligand repertoire of $\alpha_L\beta_2$ is more restricted[9]. The recognition of ligands by integrins is a malleable event governed by conformational changes, clustering at the cell surface and alterations in subcellular localization[1,11].

Members of the phosphoinositide-3-OH kinase (PI(3)K) family are important in inflammatory processes[12]. Members of the PI(3)K family of heterodimeric enzymes consist of a regulatory subunit (p85α, p85β or p55γ) and a catalytic subunit (p110α, p110β, p110γ and p110δ)[13]. Notably, the regulatory subunit of p85 contains an Src homology 2 domain that recognizes phosphorylated tyrosine residue in the 'YXXM' motif (where 'X' is any amino acid). After recruitment to the inner leaflet of the plasma membrane, PI(3)K phosphorylates phosphatidylinositol-(4,5)-bisphosphate to yield phosphatidylinositol-(3,4,5)-trisphosphate, which activates signaling pathways, including the kinase Akt, glycogen synthase kinase 3β and Tec family

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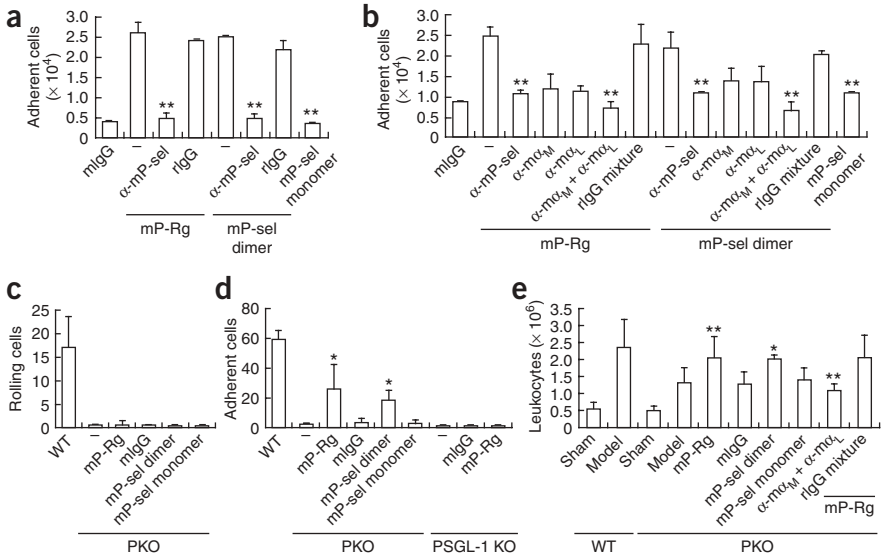

Figure 1 Soluble P-selectin restores the adhesion-dependent infiltration of leukocytes in P-selectin-deficient mice. (**a**,**b**) *In vitro* adhesion of mouse neutrophils incubated with mouse IgG (mIgG) and mP-Rg or soluble mP-sel monomer or dimer in the presence (α-) or absence of specific antibodies (horizontal axes) or rat isotype control antibody (rIgG), then transferred to wells coated with mouse fibrinogen (**a**) or ICAM-1 (**b**). **, $P < 0.01$. (**c**,**d**) *In vivo* rolling (**c**) and adhesion (**d**) of leukocytes from wild-type (WT), P-selectin-deficient (PKO) and PSGL-1-deficient (PSGL-1 KO) mice left untreated or injected intravenously with mouse IgG and mP-Rg or mP-sel monomer or dimer, monitored in the capillary venules of the cremaster muscle and recorded by intravital microscopy. *, $P < 0.05$. (**a**–**d**), buffer alone. (**e**) Infiltration of leukocytes from wild-type and P-selectin-deficient mice stimulated by intraperitoneal injection of saline (Sham; $n = 5$ mice) or thioglycollate (Model; $n = 5$ mice) without pretreatment ($n = 5$ mice) or after pretreatment with mP-Rg ($n = 16$ mice), mouse IgG ($n = 12$ mice) or mP-sel monomer ($n = 6$ mice) or dimer ($n = 8$ mice), or with mP-Rg plus blocking antibodies specific for α_M and α_L integrins (α-mα_M + α-mα_L; $n = 9$ mice) or rat IgG ($n = 10$ mice). **, $P < 0.01$; *, $P < 0.05$. Data are the mean ± s.d. of triplicate measurements in five (**a**,**b**) or three (**c**,**d**) separate experiments or the mean ± s.d. of three experiments (**e**).

tyrosine kinases, that regulate cell adhesion, migration, growth, differentiation, proliferation, apoptosis, metabolism and intracellular trafficking.

Selectin-mediated tethering and rolling slows circulating leukocytes, thus allowing arrest after *in situ* interaction of cell surface integrins and extracellular stimuli[1–6]. It has been reported that crosslinking of leukocyte PSGL-1 by P-selectin induces an intermediate state of integrin activation[14–17]. By acting cooperatively with extracellular stimuli such as interleukin 8 (IL-8) and platelet-activating factor (PAF), P-selectin induces full integrin activation to achieve maximum leukocyte adhesion. However, neither the biological importance of this phenomenon during inflammation nor the molecular mechanism by which selectins transactivate integrins is understood. Here we sought to assess the functional importance of P-selectin-induced integrin transactivation *in vivo* and to elucidate the PSGL-1 signaling mechanism responsible for integrin activation. We found that leukocyte adhesion was impaired in P-selectin-deficient mice. In addition, we identified a function for Nef-associated factor 1 (Naf1), which bound constitutively to the cytoplasmic domain of PSGL-1, in transmitting selectin-induced integrin-activating signals.

RESULTS

'Rescuing' impaired leukocyte adhesion

To investigate the functional importance of PSGL-1 signaling *in vivo*, we constructed, expressed and purified soluble mouse P-selectin (mP-sel) and mouse P-selectin receptor globulin (mP-Rg), which included the lectin domain, the epidermal growth factor domain and the first four and part of the fifth complement-like repeat domains of mP-sel fused with the heavy chain of mouse immunoglobulin G (IgG; **Supplementary Fig. 1** online). As isolated, mP-sel was a mixture of monomers and dimers; we used gel-filtration chromatography to separate these forms. As $\alpha_M\beta_2$ mediates the adhesion of neutrophils to fibrinogen, whereas $\alpha_M\beta_2$ and $\alpha_L\beta_2$ both mediate the adhesion of neutrophils to ICAM-1 (refs. 1,3,4,9), we examined the effects of mP-Rg and mP-sel on the adhesion of mouse neutrophils to those substrates. Consistent with existing data[14], dimeric but not monomeric mP-sel enhanced adhesion of mouse neutrophils to immobilized mouse fibrinogen (**Fig. 1a**) and mouse ICAM-1 (**Fig. 1b**). We found that mP-Rg also elicited adhesion of mouse neutrophils to mouse fibrinogen (**Fig. 1a**) and mouse ICAM-1 (**Fig. 1b**). Preincubation of mP-Rg or the mP-sel dimer with RB40.34 (a rat leukocyte adhesion–blocking antibody specific for mouse P-selectin) blocked adhesion of mouse neutrophils to mouse fibrinogen (**Fig. 1a**) and mouse ICAM-1 (**Fig. 1b**), but preincubation with control rat IgG did not. Notably, M1/70 (a rat leukocyte adhesion–blocking antibody specific for mouse α_M) or M17/4 (a rat leukocyte adhesion–blocking antibody specific for mouse α_L) only partially inhibited adhesion of mouse neutrophils to mouse ICAM-1, but a combination of M1/70 and M17/4 completely abolished adhesion to mouse ICAM-1 (**Fig. 1b**). These results are fully consistent with results obtained with human leukocytes[14–19], attesting to the phenotypic fidelity of mouse neutrophils for investigating the PSGL-1 signal-transduction pathway involved in the transactivation of integrins such as $\alpha_M\beta_2$ and $\alpha_L\beta_2$.

Using intravital microscopy, we examined leukocyte rolling versus adhesion in P-selectin-deficient mice. After surgical exteriorization of the cremaster muscle, leukocytes rolled in the capillary venules of wild-type mice but not those of P-selectin-deficient mice (**Fig. 1c**). Leukocytes adhered avidly to the capillary venules of wild-type but not P-selectin-deficient mice (**Fig. 1d**). Administration of mP-Rg or the mP-sel dimer partially restored adhesion of leukocytes in P-selectin-deficient but not PSGL1-deficient mice, but administration of mouse IgG or mP-sel monomer did not (**Fig. 1d**). Thus, only soluble P-selectin reagents that crosslink PSGL-1 and presumably induce integrin-mediated adhesion of neutrophils[14] can restore leukocyte adhesion in P-selectin-deficient mice. We noted the failure of leukocytes to adhere to the capillary venules of P-selectin-deficient mice after surgical injury alone; in contrast, robust adhesion of leukocytes was apparent in P-selectin-deficient mice pretreated with tumor necrosis factor for several hours and in cremaster muscles treated with a calcium ionophore (A23187) for a few minutes (data not shown). These reagents directly activate leukocyte integrins. As activated integrins are known to mediate leukocyte rolling to certain extent[20,21], our data should not be extrapolated to suggest that soluble

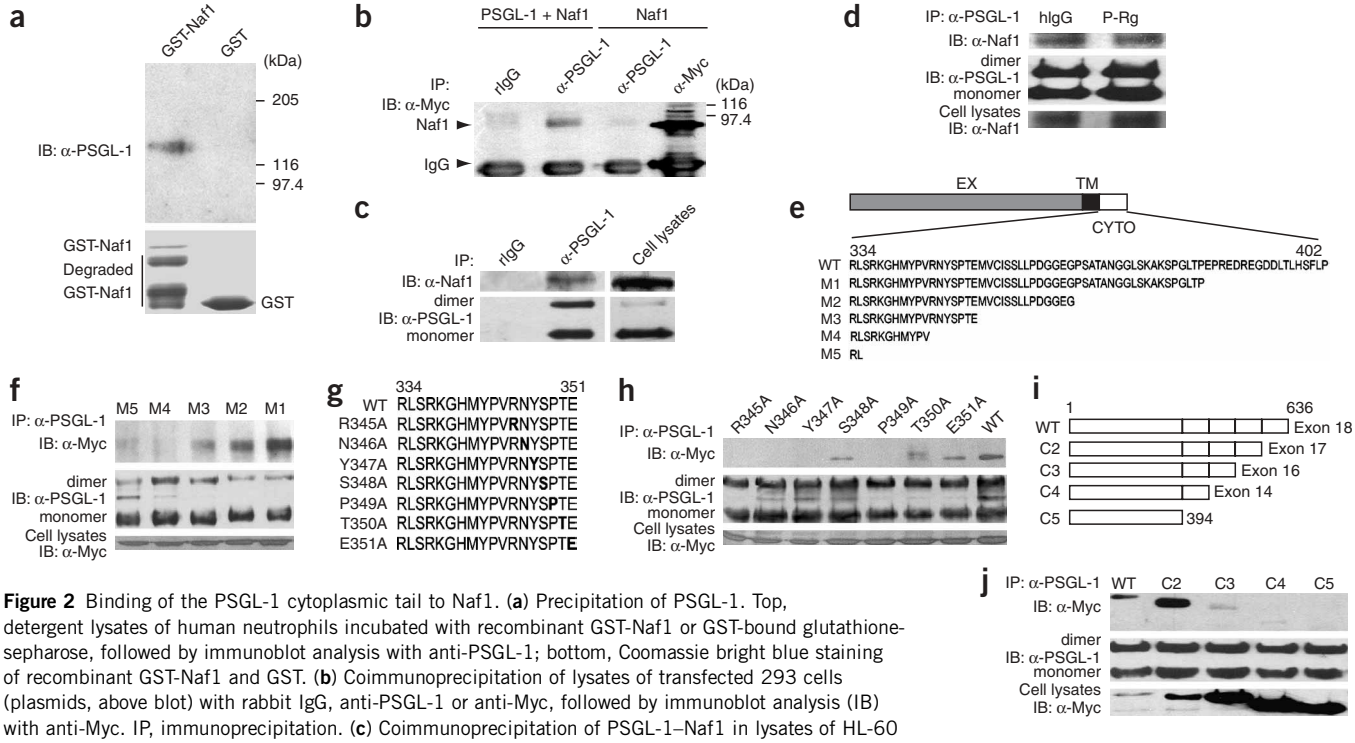

Figure 2 Binding of the PSGL-1 cytoplasmic tail to Naf1. (**a**) Precipitation of PSGL-1. Top, detergent lysates of human neutrophils incubated with recombinant GST-Naf1 or GST-bound glutathione-sepharose, followed by immunoblot analysis with anti-PSGL-1; bottom, Coomassie bright blue staining of recombinant GST-Naf1 and GST. (**b**) Coimmunoprecipitation of lysates of transfected 293 cells (plasmids, above blot) with rabbit IgG, anti-PSGL-1 or anti-Myc, followed by immunoblot analysis (IB) with anti-Myc. IP, immunoprecipitation. (**c**) Coimmunoprecipitation of PSGL-1–Naf1 in lysates of HL-60 cells with rabbit IgG or anti-PSGL-1, followed by immunoblot analysis (antibodies, left margin). (**d**) Effect of P-selectin on the PSGL-1–Naf1 interaction in HL-60 cells incubated with human IgG (hIgG) or P-Rg; lysates precipitated with anti-PSGL-1 were analyzed by immunoblot (antibodies, left margin). (**e**) Amino acid sequences of the cytoplasmic domain of human PSGL-1 and deletion mutants. EX, extracellular portion; TM, transmembrane domain; CYTO, cytoplasmic tail. (**f**) PSGL-1 mutagenesis analysis of 293 cells cotransfected with the PSGL-1 mutants in **e**; Myc-Naf1 was precipitated from lysates with anti-PSGL-1, followed by immunoblot analysis (antibodies, left margin). (**g**) Site-specific mutagenesis of the PSGL-1 cytoplasmic domain. Bold, substituted residues. (**h**) Binding of the PSGL-1 mutants in **g** to Naf1, analyzed as described in **f**. (**i**) Domain structure of Naf1 and its deletion mutants. (**j**) Binding of the Naf1 mutants in **i** to PSGL-1, analyzed as described in **f**. Data are representative of three independent experiments.

P-selectin arrests leukocytes instantaneously (that is, no leukocyte tethering and rolling before firm adhesion) in P-selectin-deficient mice. Fewer leukocytes infiltrated the peritoneum of P-selectin-deficient mice at 2 h after intraperitoneal injection of thioglycollate (**Fig. 1e**). Analogously, mP-Rg and the mP-sel dimer, but not mouse IgG or the mP-sel monomer, restored peritoneal accumulation of leukocytes in P-selectin-deficient mice; this infiltration was suppressed by blockade of $\alpha_M\beta_2$ and $\alpha_L\beta_2$. Our results collectively indicate the functional importance of P-selectin in mediating not only leukocyte rolling but also β_2-integrin-dependent leukocyte adhesion *in vivo*.

PSGL-1–Naf1 complex

To elucidate the signaling pathway through which PSGL-1 alters leukocyte adhesion, we used the cytoplasmic domain of human PSGL-1 as the 'bait' in a yeast two-hybrid screen of a human leukocyte cDNA library. From this screen, we identified a fragment of Naf1α[22] (called 'Naf1' here; also known as 'A20 binding inhibitor-1 of NF-κB'[23] or 'virion-associated nuclear-shuttling protein'[24]) as a binding partner of the cytoplasmic tail of PSGL-1 (**Supplementary Fig. 2a** online). We used PCR to amplify full-length cDNA encoding human Naf1 and generated a rabbit Naf1-specific polyclonal antibody that recognized endogenous Naf1 from human neutrophils and human promyeloid HL-60 cells (**Supplementary Fig. 2b**).

To confirm the interaction of PSGL-1 with Naf1, we used precipitation and coimmunoprecipitation experiments with glutathione *S*-transferase (GST) fusion proteins. GST-Naf1 but not GST alone precipitated PSGL-1 from detergent lysates of human neutrophils (**Fig. 2a**). Similarly, Myc-tagged Naf1 (Myc-Naf1) bound to wild-type PSGL-1 in transfected human embryonic kidney 293 cells (**Fig. 2b**), and endogenous PSGL-1 bound to endogenous Naf1 in HL-60 cells (**Fig. 2c**). Notably, Naf1 bound PSGL-1 in a constitutive way in coimmunoprecipitation experiments with antibody to PSGL-1 (anti-PSGL-1; ref. 25); crosslinking of PSGL-1 by P-Rg was apparently not required for formation of the PSGL-1–Naf1 complex in HL-60 cells (**Fig. 2d**).

To identify the domains involved in the PSGL-1–Naf1 interaction, we constructed five deletion mutants (M1–M5) in which the cytoplasmic domain of PSGL-1 was truncated 'stepwise' from the carboxyl terminus (**Fig. 2e**). In 293 cells cotransfected with plasmids encoding the PSGL-1 deletion mutants and wild-type Myc-Naf1, Naf1 bound avidly to M1, moderately to M2 and poorly to M3 deletion mutants (**Fig. 2f**). Naf1 failed to bind to M4 and M5 deletion mutants of PSGL-1.

Because Naf1 bound to the M3 but not the M4 deletion mutant of PSGL-1, we did alanine-scanning mutagenesis of full-length PSGL-1 for the seven amino acid residues in the M3 mutant (**Fig. 2g**). In 293 cells cotransfected with plasmids encoding the PSGL-1 site-specific mutants and wild-type Myc-Naf1, Naf1 bound to the S348A, T350A and E351A mutants of PSGL-1 but not to the R345A, N346A, Y347A and P349A mutants of PSGL-1 (**Fig. 2h**). Thus, at least four amino acid residues, Arg345, Asn346, Tyr347 and Pro349, in the cytoplasmic domain of PSGL-1 were obligatory for Naf1 recognition. Notably, the Naf1-binding sites in the PSGL-1 cytoplasmic domain were apparently distinct from the residues critical for the recognition of ezrin, radixin and moesin[26].

We also constructed four deletion mutants of Naf1 (C2–C5; **Fig. 2i**). We found that 293 cells cotransfected with plasmids encoding

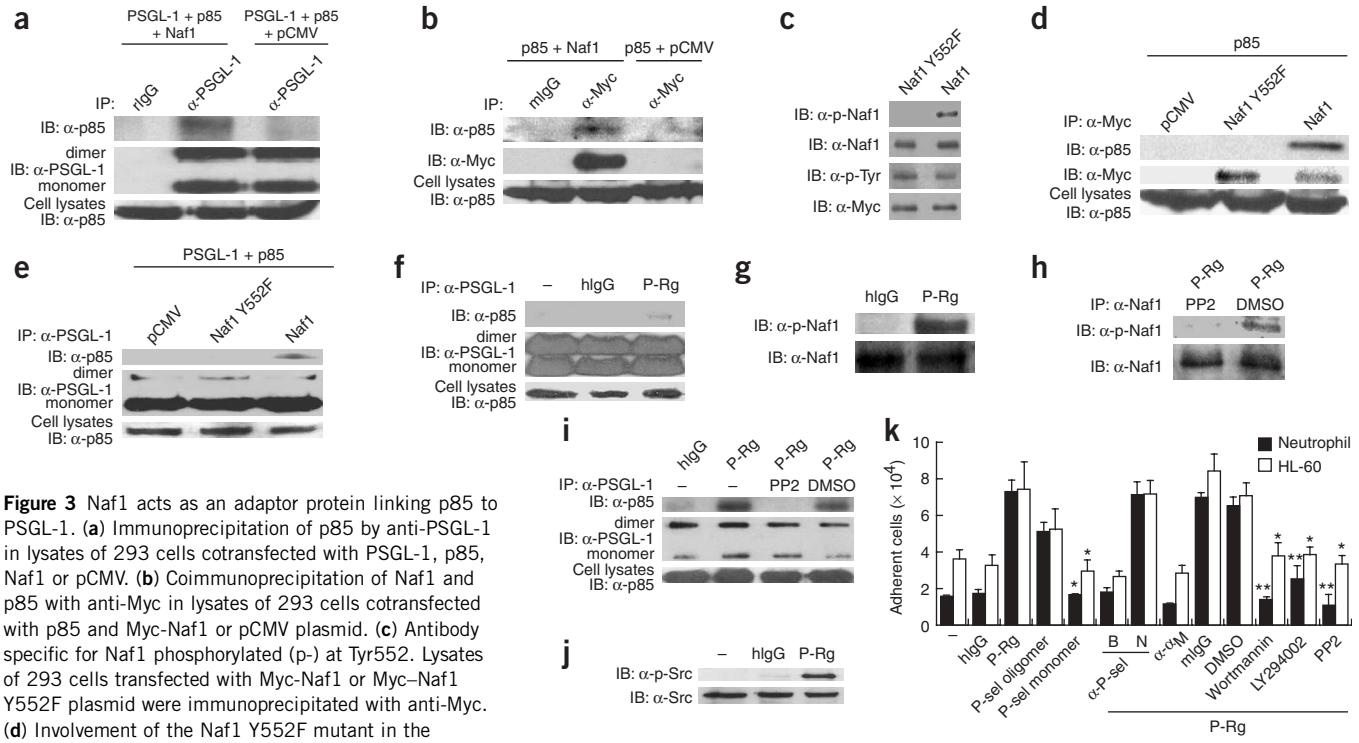

Figure 3 Naf1 acts as an adaptor protein linking p85 to PSGL-1. (**a**) Immunoprecipitation of p85 by anti-PSGL-1 in lysates of 293 cells cotransfected with PSGL-1, p85, Naf1 or pCMV. (**b**) Coimmunoprecipitation of Naf1 and p85 with anti-Myc in lysates of 293 cells cotransfected with p85 and Myc-Naf1 or pCMV plasmid. (**c**) Antibody specific for Naf1 phosphorylated (p-) at Tyr552. Lysates of 293 cells transfected with Myc-Naf1 or Myc–Naf1 Y552F plasmid were immunoprecipitated with anti-Myc. (**d**) Involvement of the Naf1 Y552F mutant in the precipitation of p85 in lysates of 293 cells cotransfected with p85 and Myc-Naf1, Myc–Naf1 Y552F or pCMV plasmid plasmid. (**e**) Involvement of the Naf1 Y552F mutant in the precipitation of p85 with anti-PSGL-1 in lysates of 293 cells cotransfected with PSGL-1 plus p85, and Myc-Naf1, Myc–Naf1 Y552F or pCMV plasmid. (**f**) Precipitation of p85 in human neutrophils preincubated with buffer (–), human IgG or P-Rg. (**g**) Naf1 phosphorylation in human neutrophils incubated with human IgG or P-Rg. (**h**) Inhibition of Naf1 phosphorylation in human neutrophils incubated with P-Rg in the presence of PP2 or dimethyl sulfoxide (DMSO); lysates were immunoprecipitated with anti-Naf1. (**i**) Inhibition of the precipitation of p85 by anti-PSGL-1 in human neutrophils incubated with human IgG or P-Rg in the absence (–) or presence of PP2 or dimethyl sulfoxide; lysates were precipitated with anti-PSGL-1. (**j**) Src kinase activation in human neutrophils incubated with buffer (–), human IgG or P-Rg. In **a**–**j**, lysates or immunoprecipitates were analyzed by immunoblot (antibodies, left margin). (**k**) Inhibition of leukocyte adhesion in human neutrophils and HL-60 cells incubated with buffer (–), human IgG, P-Rg or P-selectin oligomer (platelet P-selectin) or monomer, in the presence or absence of dimethyl sulfoxide, PP2, wortmannin or LY294002, before being transferred to wells coated with immobilized fibrinogen. For antibody inhibition experiments, P-Rg was preincubated with blocking (B) or nonblocking (N) anti-P-selectin F(ab′)$_2$, whereas neutrophils were preincubated with anti-α_M or its isotype control mouse IgG. Results are mean ± s.d. *, $P < 0.05$; **, $P < 0.01$. Data are representative of three independent experiments.

wild-type PSGL-1 and wild-type Myc-Naf1 or the Myc-tagged Naf1 deletion mutants, PSGL-1 bound avidly to wild-type Myc-Naf1 and the C2 mutant but poorly to the C3 mutant (**Fig. 2j**). PSGL-1 failed to bind to the C4 and C5 mutants. These data identified the specific segments and amino acid residues of PSGL-1 and Naf1 required for formation of the PSGL-1–Naf1 complex in mammalian cells.

Recruitment of PI(3)K to Naf1

While searching for a function for the PSGL-1–Naf1 complex, we recognized a motif ('YPPM') in Naf1 as a potential binding motif for the p85 subunit of PI(3)K ('YXXM', in which phosphorylated 'Y' is required for p85 binding and 'X' is any amino acid residue)[13]. Therefore, we hypothesized that Naf1 might act as an adaptor protein linking the cytoplasmic tail of PSGL-1 to PI(3)K. To test that hypothesis, we assessed protein interactions in 293 cells cotransfected with plasmids encoding PSGL-1, p85 and/or Myc-Naf1 or with an empty cytomegalovirus vector (pCMV). PSGL-1 bound to p85 in the presence of Myc-Naf1 (**Fig. 3a**). Notably, anti-PSGL-1 also precipitated a small amount of p85 in the absence of Myc-Naf1 (**Fig. 3a**), perhaps because of the presence of endogenous Naf1 in 293 cells (data not shown). In 293 cells cotransfected with plasmids encoding p85 and Myc-Naf1 or pCMV, anti-Myc precipitated a small amount of p85 (**Fig. 3b**), consistent with the finding that only a small fraction of transfected Naf1 (less than 20%) was phosphorylated on Tyr552 before P-selectin engagement (**Fig. 3c**). To determine whether the YPPM motif in Naf1 is essential for p85 recognition, we constructed a Naf1 mutant in which the tyrosine at position 552 was substituted with phenylalanine. Introduction of this Y552F substitution abolished the interaction between Naf1 and p85 (**Fig. 3d**) as well as the link between the cytoplasmic tail of PSGL-1 and p85 (**Fig. 3e**).

To determine whether P-selectin induces formation of the endogenous PSGL-1–Naf1–p85 complex, we incubated human neutrophils with buffer, human IgG or P-Rg and measured precipitation of PSGL-1 together with p85. We detected interactions of PSGL-1 and p85 only in neutrophils treated with P-Rg (**Fig. 3f**). Notably, only a minor population (less than 10%) of p85 was present in the PSGL-1–Naf1 complex, consistent with the presence of a single docking site on Naf1 for PI(3)K[22–24] and the many PSGL-1-independent functions of PI(3)K in leukocytes[12].

Next we hypothesized that the binding of P-selectin to PSGL-1 triggers phosphorylation of Naf1 at Tyr552. To test that idea, we synthesized two peptides corresponding to amino acids 543–562 of Naf1 in which Tyr552 was phosphorylated or not. We used these two peptides to generate a monoclonal antibody (3B8) that specifically recognized phosphorylated Tyr552 (**Fig. 3c**) and a monoclonal antibody (5C4) that recognized both phosphorylated and unphosphorylated Tyr552 (**Fig. 3f**). Treatment with P-Rg induced phosphorylation of Tyr552 (**Fig. 3g**).

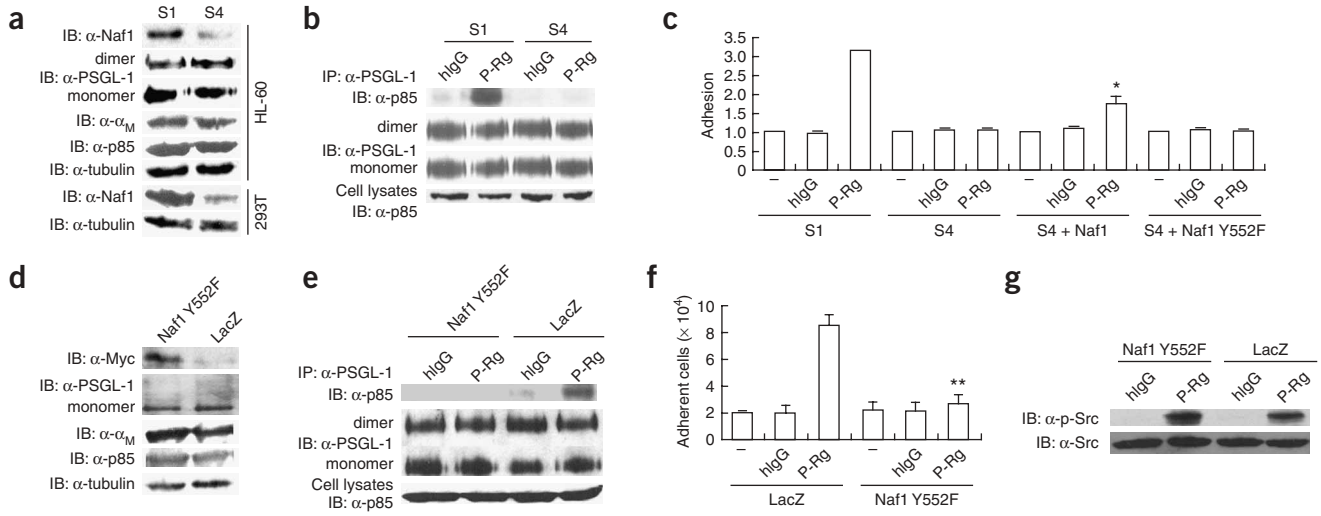

Figure 4 Obligatory requirement for Naf1 in P-selectin-induced integrin activation. (**a**) Immunoblot analysis of protein expression in HL-60 cells (top) and 293T cells (bottom) transfected with Naf1-inefficient siRNA (S1) or Naf1-efficient siRNA (S4). (**b**) Analysis of p85 precipitation after transfection of HL-60 cells with Naf1-inefficient or Naf1-efficient siRNA (assessed as described in **Fig. 3a**). (**c**) Adhesion of HL-60 cells transfected with Naf1-inefficient or Naf1-efficient siRNA in the presence or absence of Naf1–yellow fluorescent protein constructs (below graph) on fibrinogen-coated wells, presented as adherent cells treated with human IgG or P-Rg relative to adherent cells treated with buffer (–), set as 1. *, $P < 0.05$, versus S4-transfected cells treated with P-Rg. (**d**) Immunoblot analysis of protein expression in lysates of HL-60 cells infected with LacZ or Myc–Naf1 Y552F lentivirus. (**e**) Analysis of the p85–PSGL-1 interaction in HL-60 cells infected with LacZ or Myc–Naf1 Y552F lentivirus (assessed as described in **Fig. 3a**). (**f**) Adhesion of HL-60 cells infected with LacZ or Myc–Naf1 Y552F lentivirus, then infected cells were incubated with buffer (–), human IgG or P-Rg before transfer to wells coated with fibrinogen. **, $P < 0.01$, versus LacZ-infected cells treated with P-Rg. (**g**) Immunoblot analysis of Src phosphorylation in HL-60 cells infected with LacZ or Myc–Naf1 Y552F lentivirus. Data are representative of three experiments or are the mean ± s.d. of triplicate measurements in five separate experiments (**c**,**f**).

While searching for tyrosine kinase(s) responsible for the phosphorylation of Naf1 at Tyr552, we noted that PP2, an inhibitor of Src kinases, inhibited P-selectin-induced phosphorylation of Tyr552 (**Fig. 3h**) and immunoprecipitation p85 together with PSGL-1 (**Fig. 3i**). We next tested whether P-selectin stimulation triggered activation of Src kinases and whether PP2 inhibited activation of $\alpha_M\beta_2$ induced by P-selectin. Preincubation of human neutrophils with P-Rg, but not preincubation with buffer or human IgG, induced tyrosine-phosphorylation of Src family kinases, as shown by immunoblot analysis with an antibody specific for phosphorylated Tyr416 of Src kinase (**Fig. 3j**); this antibody reportedly cross-reacts with the phosphorylated tyrosine residues of the Lyn and Hck Src kinases but not Fgr Src kinases, which are present in human neutrophils[27].

In addition, treatment with P-Rg and human platelet P-selectin increased the adhesion of human neutrophils and HL-60 cells to fibrinogen, whereas buffer, human IgG and the P-selectin monomer had no such effect (**Fig. 3k**). Preincubation of P-Rg with antibodies that block P-selectin or α_M, or with the chemical inhibitors of Src kinases or PI(3)K, abrogated the adhesion of neutrophils and HL-60 cells to fibrinogen. These data collectively indicate that the binding of P-selectin to PSGL-1 activates Src kinases, which phosphorylate Naf1 at Tyr552 and that this process results in recruitment of PI(3)K to the PSGL-1–Naf1 complex and transactivation of integrins, including $\alpha_M\beta_2$.

Naf1 in integrin activation

To evaluate the function of Naf1 in recruiting and/or activating PI(3)K during P-selectin-induced integrin transactivation, we used small interfering RNA (siRNA) to silence Naf1 expression in HL-60 and 293 cells (**Fig. 4a**). Expression of PSGL-1, α_M, p85 and α-tubulin was not altered by Naf1-efficient or Naf1-inefficient siRNA oligonucleotides. Although incomplete, siRNA-mediated knockdown of Naf1 expression was sufficient to prevent the immunoprecipitation of p85 together with PSGL-1 in P-Rg-treated HL-60 cells (**Fig. 4b**) as well as the P-selectin-induced adhesion of HL-60 cells to immobilized fibrinogen (**Fig. 4c**). Transfection with Naf1-efficient siRNA did not alter the expression of α_M (**Supplementary Fig. 3a** online) or PSGL-1 (**Supplementary Fig. 3b**) on the surface of HL-60 cells and did not affect the binding of P-selectin to PSGL-1 (**Supplementary Fig. 3c**). Cotransfection with a plasmid encoding yellow fluorescent protein–tagged wild-type Naf1 but not cotransfection with a plasmid encoding Y552F Naf1 (**Supplementary Fig. 4** online) allowed partial restoration of P-Rg-induced adhesion.

Given that P-selectin triggered phosphorylation of Naf1 at Tyr552 (**Fig. 3g**) and that conservative substitution of Tyr552 abolished the interaction between Naf1 and p85 (**Fig. 3c**,**d**), we speculated that Naf1 Y552F might act as a 'dominant negative' construct to inhibit P-selectin-induced recruitment of p85 to Naf1 and P-selectin-induced activation of $\alpha_M\beta_2$. We infected HL-60 cells with lentivirus encoding Myc–Naf1 Y552F or LacZ (control). Expression of PSGL-1, α_M, p85 and α-tubulin was not altered by retroviral expression of Myc–Naf1 Y552F (**Fig. 4d**), but expression of Myc–Naf1 Y552F diminished P-Rg-induced interaction of p85 with PSGL-1 (**Fig. 4e**). Notably, in contrast to the LacZ lentivirus, the Myc–Naf1 Y552F lentivirus also attenuated P-selectin-triggered adhesion of HL-60 cells to fibrinogen (**Fig. 4f**). However, overexpression of Myc–Naf1 Y552F did not affect P-selectin-induced phosphorylation of Src at Tyr416 (**Fig. 4g**), cell surface expression of α_M (**Supplementary Fig. 5a** online) or PSGL-1 (**Supplementary Fig. 5b**), or the interaction between PSGL-1 and P-selectin (**Supplementary Fig. 5c**). These findings indicate that Naf1, acting as an adaptor protein that links

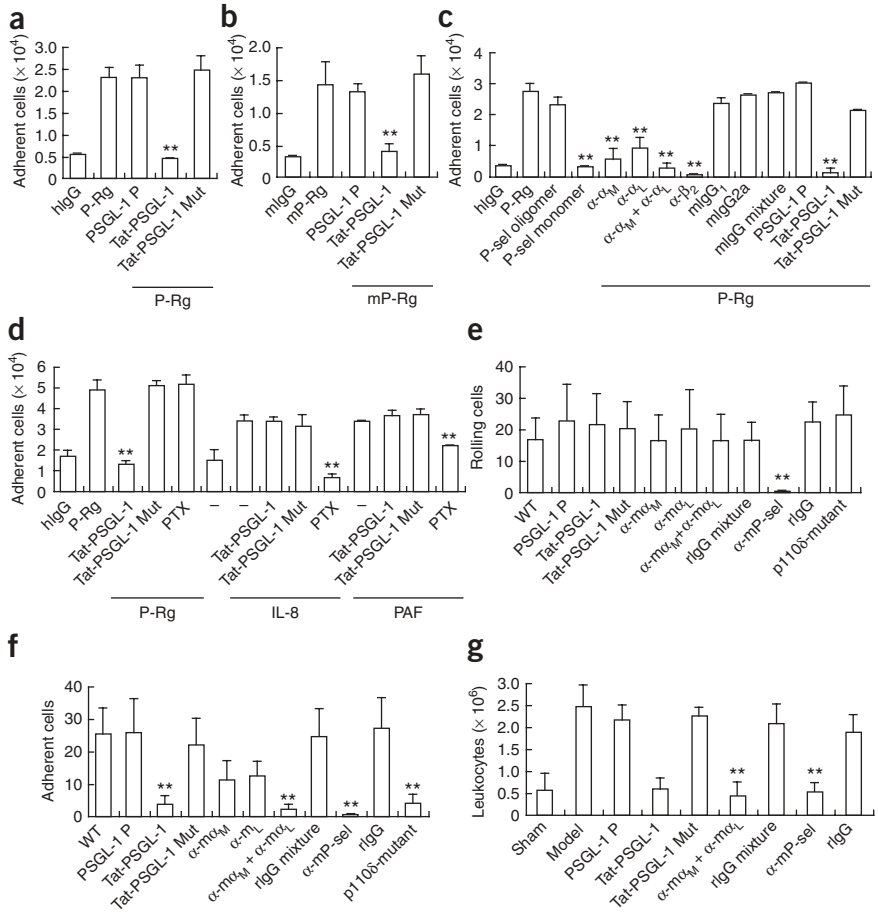

Figure 5 Requirement for the PSGL-1–Naf1 complex in P-selectin-induced integrin activation. (**a**,**b**) Inhibition of the adhesion to fibrinogen of human neutrophils (**a**) and mouse neutrophils (**b**) incubated with human IgG and P-Rg (**a**) or mouse IgG and mP-Rg (**b**) in the presence of PSGL-1 P, Tat–PSGL-1 or Tat–PSGL-1 Mut, before transfer to fibrinogen-coated wells. (**c**) Inhibition of the adhesion to ICAM-1 of human neutrophils incubated with human IgG and P-Rg or P-sel oligomer or monomer, in the presence of PSGL-1 P, Tat–PSGL-1 or Tat–PSGL-1 Mut, before transfer to wells containing immobilized ICAM-1. Specificity control, neutrophils preincubated with anti-α_M, anti-α_L, anti-α_M plus α_L or anti-β_2, and their control mouse IgG. (**d**) Adhesion to fibrinogen of human neutrophils incubated with Tat–PSGL-1 or PTX, followed by P-selectin, IL-8 or PAF, before transfer to fibrinogen-coated wells. (**e**,**f**) Leukocyte rolling (**e**) and adhesion (**f**) in the capillary vessels of cremaster muscles of mice intravenously injected with PSGL-1 P, Tat–PSGL-1, Tat–PSGL-1 Mut or various antibodies (horizontal axis), monitored and recorded by intravital microscopy. (**g**) Leukocyte infiltration in mice ($n = 7$ per group) injected intraperitoneally with saline (Sham) or thioglycollate (Model), with or without PSGL-1 P, Tat–PSGL-1, Tat–PSGL-1 Mut or antibodies (horizontal axis). **, $P < 0.01$. Data are the mean ± s.d. of two to five separate experiments.

the p85 subunit of PI(3)K to the cytoplasmic tail of PSGL-1, has a principal function in regulation of P-selectin-induced, $\alpha_M\beta_2$-mediated leukocyte adhesion.

PSGL-1–Naf1 in integrin activation

To demonstrate the functional importance of PSGL-1–Naf1 interactions *in vivo*, we expressed and purified polypeptides corresponding to the amino acid sequence of the cytoplasmic tails (**Fig. 2f**) of human PSGL-1 or the R345A, N346A, Y347A and P349A mutants of human PSGL-1 fused with the Tat sequence of human immunodeficiency virus, which facilitates incorporation of fusion polypeptides into mammalian cells[28] (resulting in Tat–PSGL-1 or Tat–PSGL-1 Mut, respectively; **Supplementary Fig. 6a** online). In contrast to incubation with the fluorescein isothiocyanate–conjugated polypeptide of the cytoplasmic tail of wild-type human PSGL-1 without the Tat sequence (PSGL-1 P), incubation with fluorescein isothiocyanate–conjugated Tat–PSGL-1 and Tat–PSGL-1 Mut resulted in visible fluorescence in the cytoplasm of mouse neutrophils, confirming Tat-mediated uptake of PSGL-1 polypeptides (**Supplementary Fig. 6b**). We obtained similar results in neutrophils isolated from mice intravenously injected with these fluorescein isothiocyanate–conjugated polypeptides (**Supplementary Fig. 6c**) and in human neutrophils incubated with these fluorescein isothiocyanate–conjugated polypeptides *in vitro* (data not shown). PSGL-1 P and Tat–PSGL-1 but not Tat–PSGL-1 Mut precipitated endogenous Naf1 from lysates of human neutrophils (**Supplementary Fig. 6d**) and mouse neutrophils (**Supplementary Fig. 6e**). Preincubation of the human neutrophil lysates with Tat–PSGL-1 and PSGL-1 P but not preincubation with Tat–PSGL-1 Mut also inhibited the immunoprecipitation of endogenous PSGL-1 together with Naf1 (**Supplementary Fig. 6f**) and P-selectin-induced phosphorylation of Naf1 at Tyr552 (**Supplementary Fig. 6g**). These data demonstrate that Tat–PSGL-1 was incorporated into the cytoplasm of neutrophils, where it acted as a competitive inhibitor of PSGL-1–Naf1 interactions. In contrast, PSGL-1 P was not incorporated into the cytoplasm but it did interact with Naf1 in cell lysates. Even though Tat–PSGL-1 Mut was incorporated into the cytoplasm, it failed to recognize Naf1 and thus did not interrupt PSGL-1–Naf1 interactions.

Next we tested whether Tat–PSGL-1 diminished P-selectin-induced activation of β_2-integrins *in vitro* and *in vivo*. P-Rg induced the adhesion of human neutrophils to fibrinogen (**Fig. 5a**) and elicited the adhesion of mouse neutrophils to mouse fibrinogen (**Fig. 5b**). Notably, Tat–PSGL-1, but neither PSGL-1 P nor Tat–PSGL-1 Mut, completely abolished the P-selectin-induced adhesion of human and mouse neutrophils to fibrinogen (**Fig. 5a**,**b**). However, Tat–PSGL-1 did not affect the rolling velocity or the number of rolling human neutrophils (**Supplementary Fig. 7a**,**b** online) or mouse neutrophils (**Supplementary Fig. 7c**,**d**) on immobilized P-Rg or mP-Rg *in vitro*. These polypeptides also did not interfere with the cell surface expression of α_M (**Supplementary Fig. 8a** online) or PSGL-1 (**Supplementary Fig. 8b**) and did not suppress the binding of P-selectin to PSGL-1 (**Supplementary Fig. 8c**).

Because P-selectin triggered $\alpha_M\beta_2$- and $\alpha_L\beta_2$-mediated adhesion of neutrophils to ICAM-1, we examined the effect of Tat–PSGL-1 on the P-selectin-induced activity of $\alpha_L\beta_2$ in human neutrophils. P-Rg and the P-sel dimer increased the adhesion of human neutrophils to immobilized ICAM-1 (**Fig. 5c**). Preincubation with blocking antibodies specific for α_M, α_L or β_2 diminished neutrophil adhesion. Notably, simultaneous blockade of α_M and α_L completely abolished

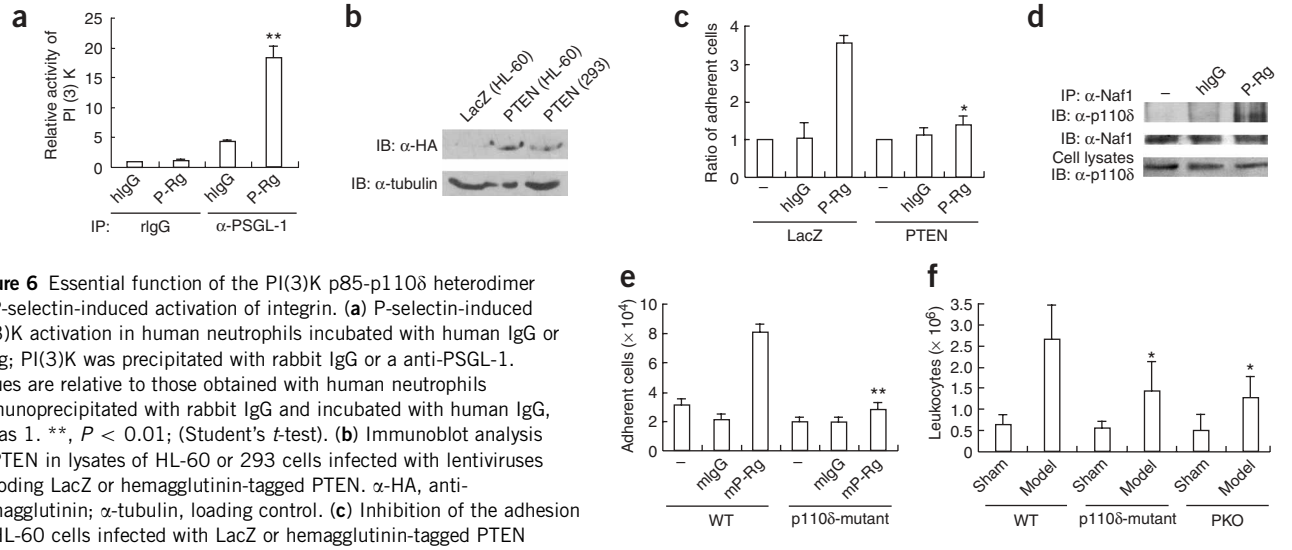

Figure 6 Essential function of the PI(3)K p85-p110δ heterodimer in P-selectin-induced activation of integrin. (**a**) P-selectin-induced PI(3)K activation in human neutrophils incubated with human IgG or P-Rg; PI(3)K was precipitated with rabbit IgG or a anti-PSGL-1. Values are relative to those obtained with human neutrophils immunoprecipitated with rabbit IgG and incubated with human IgG, set as 1. **, $P < 0.01$; (Student's *t*-test). (**b**) Immunoblot analysis of PTEN in lysates of HL-60 or 293 cells infected with lentiviruses encoding LacZ or hemagglutinin-tagged PTEN. α-HA, anti-hemagglutinin; α-tubulin, loading control. (**c**) Inhibition of the adhesion of HL-60 cells infected with LacZ or hemagglutinin-tagged PTEN lentivirus and incubated with buffer (–), human IgG or P-Rg before transfer to wells containing immobilized fibrinogen. Data are presented as the ratio of adherent cells incubated in the various experimental conditions to adherent cells incubated with buffer alone. *, $P < 0.05$, versus LacZ-infected cells treated with P-Rg. (**d**) Precipitation of p110δ by anti-Naf1 in lysates of human neutrophils incubated with buffer (–), human IgG or P-Rg, analyzed by immunoblot. (**e**) Adhesion to mouse fibrinogen of wild-type or p110δ-mutant neutrophils preincubated with buffer (–), mouse IgG or mP-Rg and then added to wells containing immobilized mouse fibrinogen. **, $P < 0.01$, versus mP-Rg-treated wild-type cells. (**f**) Peritoneal infiltration of leukocytes from wild-type and p110δ-mutant mice ($n = 7$ per group) as well as P-selectin-deficient mice ($n = 5$ per group) injected intraperitoneally with saline (Sham) or thioglycollate (Model). *, $P < 0.05$ versus wild-type injected with thioglycollate. Data are representative of duplicate (**a**) or triplicate (**c**,**e**) measurements in three (**a**,**c**) or five (**e**) separate experiments or the mean ± s.d. of two separate experiments (**f**).

the adhesion of human neutrophils to ICAM-1. Tat–PSGL-1, but not PSGL-1 P or Tat–PSGL-1 Mut, considerably reduced the P-selectin-induced adhesion of neutrophils to ICAM-1, indicating that the PSGL-1 signal-transduction pathway functions as a common mechanism for the transactivation of various integrins, including $\alpha_M\beta_2$ and $\alpha_L\beta_2$, in human neutrophils. Consistent with the published finding that anti-PSGL-1 augments the adhesion of human neutrophils to fibrinogen[14], preincubation with anti-PSGL-1 enhanced the adhesion of human neutrophils to fibrinogen and to ICAM-1 (**Supplementary Fig. 9** online). Notably, although our results support the idea that both integrins are involved, because of potential preactivation during neutrophil isolation[29–31], the mechanism for mediating neutrophil adhesion to adhesive substrates *in vitro* could possibly involve $\alpha_M\beta_2$ or $\alpha_L\beta_2$ or both.

As Tat–PSGL-1 inhibited P-selectin-induced integrin activation (**Fig. 5a–c**) and pertussis toxin (PTX), an inhibitor of G protein–coupled receptor signaling, inhibits integrin activation elicited by IL-8 and PAF[32,33], we tested the effects of Tat–PSGL-1 and PTX on P-selectin- versus IL-8- and PAF-triggered neutrophil adhesion to fibrinogen. P-Rg, IL-8 and PAF all increased the adhesion of human neutrophils to immobilized fibrinogen relative to the adhesion obtained with human IgG or buffer alone (**Fig. 5d**). As expected, PTX suppressed IL-8- and PAF-induced adhesion of neutrophils to fibrinogen, and Tat–PSGL-1 suppressed P-selectin-induced neutrophil adhesion. However, PTX failed to abrogate P-selectin-induced neutrophil adhesion, whereas Tat–PSGL-1 failed to abrogate IL-8- and PAF-induced neutrophil adhesion. Our findings thus indicate that the signaling pathway for P-selectin-mediated integrin activation is independent of G protein–coupled receptors and thus is mechanistically distinct from that induced by IL-8 and PAF.

We next investigated the effect of PSGL-1 signaling blockade on leukocyte rolling versus adhesion *in vivo*. After surgical exteriorization of mouse cremaster muscle, leukocyte rolling and adherence to the capillary venules of wild-type mice could be readily assessed (**Fig. 5e**,**f**). Intravenous injection of polypeptides of PSGL-1 P, Tat–PSGL-1 or Tat–PSGL-1 Mut or antibodies blocking $\alpha_M\beta_2$ and/or $\alpha_L\beta_2$ did not substantially affect leukocyte rolling (**Fig. 5e**). In contrast, Tat–PSGL-1, but not PSGL-1 P or Tat–PSGL-1 Mut, abolished leukocyte adhesion to capillary venules; adhesion was also partially abolished by blockade of either $\alpha_M\beta_2$ or $\alpha_L\beta_2$ and was completely abolished by inhibition of both integrins (**Fig. 5f**). Consistent with our results obtained with P-selectin-deficient mice (**Fig. 1c**,**d**), blockade of mouse P-selectin abolished both rolling and adhesion of leukocytes (**Fig. 5e**,**f**). Our results suggest that the leukocyte rolling and adhesion caused by surgical manipulation depended on P-selectin, thus providing direct evidence of an essential function for PSGL-1 signaling in integrin-mediated leukocyte adhesion in mice.

To assess the importance of the PSGL-1–Naf1 complex in the pathology of inflammation, we implemented a mouse model of acute peritonitis, which is mainly dependent on P-selectin[7]. Intraperitoneal injection of thioglycollate induced an increase of more than fourfold in the peritoneal recruitment of leukocytes in BALB/c mice at 2 h relative to that obtained with saline (**Fig. 5g**). Tat–PSGL-1 substantially diminished the peritoneal accumulation of leukocytes, whereas PSGL-1 P and Tat–PSGL-1 Mut had no such effect. The peritoneal recruitment of leukocytes was completely inhibited by blockade of both $\alpha_M\beta_2$ and $\alpha_L\beta_2$ or P-selectin. Tat–PSGL-1 did not interfere with leukocyte rolling (**Supplementary Fig. 10a** online), but Tat–PSGL-1, not PSGL-1 P or Tat–PSGL-1 Mut, suppressed leukocyte adhesion (**Supplementary Fig. 10b**) and peritoneal infiltration of leukocytes (**Supplementary Fig. 10c**) after stimulation with tumor necrosis factor. In addition, caution should be exercised in concluding that the effects of Tat–PSGL-1 depended solely on Naf1, as Tat–PSGL-1 may interfere with interaction of the PSGL-1 cytoplasmic tail with other cellular proteins; however, the data obtained with Tat–PSGL-1 Mut as control would challenge this possibility. These observations

collectively establish the importance of a PSGL-1-induced signal-transduction pathway in the transactivation of integrins in host defense and innate immunity.

The PI(3)K p85-p110δ heterodimer

To directly demonstrate P-selectin-induced recruitment and/or activation of PI(3)K, we measured the kinase activity of PI(3)K in human neutrophils treated with P-Rg. Treatment with P-Rg enhanced the enzyme activity of PI(3)K that coimmunoprecipitated with PSGL-1 (**Fig. 6a**). These results confirm that P-selectin-induced recruitment of PI(3)K to the PSGL-1–Naf1 complex in the inner leaflet of the cell surface membrane activates PI(3)K in human neutrophils.

As P-selectin activates $\alpha_M\beta_2$ (ref. 14), we predicted that P-selectin-induced recruitment and/or activation of PI(3)K would result in $\alpha_M\beta_2$ activation. To test that hypothesis, we used the PI(3)K-specific chemical inhibitors wortmannin and LY294002. Both compounds substantially reduced P-selectin-induced, $\alpha_M\beta_2$-mediated adhesion of human neutrophils and HL-60 cells to fibrinogen (**Fig. 3k**). To verify those findings, we used a lentiviral approach to express the phosphatase PTEN, which dephosphorylates phosphatidylinositol-(3,4,5)-trisphosphate at the D3 position to form phosphatidylinositol-(4,5)-bisphosphate[34]. We hypothesized that overexpression of PTEN would result in depletion of the cytoplasmic pool of phosphatidylinositol-(3,4,5)-trisphosphate, resulting in the suppression of P-selectin-induced transactivation of integrins. HL-60 and 293 cells infected with lentivirus encoding hemagglutinin-tagged PTEN had less P-selectin-induced adhesion to fibrinogen than did cells infected with control lentivirus encoding LacZ (**Fig. 6b**,**c**). Infection of HL-60 cells with the hemagglutinin-tagged PTEN lentivirus did not perturb the cell surface expression of α_M (**Supplementary Fig. 5a**) or PSGL-1 (**Supplementary Fig. 5b**) and did not suppress the binding of P-selectin to HL-60 cells (**Supplementary Fig. 5c**). These findings collectively indicate that PI(3)K is critical in the P-selectin-triggered activation of $\alpha_M\beta_2$.

Next we determined whether PSGL-1 binds to p110δ and whether P-selectin induces $\alpha_M\beta_2$ activation in neutrophils isolated from mice expressing a loss-of-function p110δ mutant[35]. PSGL-1 precipitated together with p110δ from detergent lysates of human neutrophils stimulated with P-Rg but not those stimulated with buffer or human IgG (**Fig. 6d**). We found that mP-Rg induced adhesion of wild-type but not p110δ-mutant neutrophils to mouse fibrinogen (**Fig. 6e**). Wild-type and p110δ-mutant neutrophils had similar surface expression α_M (**Supplementary Fig. 11a** online) and PSGL-1 (**Supplementary Fig. 11b**) and similar binding of PSGL-1 to P-selectin (**Supplementary Fig. 11c**). The p110δ-mutant neutrophils also adhered to fibrinogen after treatment with phorbol 12-myristate 13-acetate (data not shown). Notably, leukocytes rolled avidly on but adhered poorly to the capillary venules of p110δ-mutant mice (**Fig. 5e**,**f**). Furthermore, compared with wild-type mice, p110δ-mutant mice, like P-selectin-deficient mice, showed impaired peritoneal accumulation of neutrophils (**Fig. 6f**). Notably, there was an apparent discrepancy between the partial reduction in the peritoneal infiltration of leukocytes in P-selectin-deficient C57BL/6 mice (**Figs. 1e** and **6f**) and the complete inhibition of the peritoneal deposition of leukocytes by P-selectin blockade in BALB/c mice (**Fig. 5g**). This inconsistency was probably due to differences in the genetic backgrounds of C57BL/6 mice and BALB/c mice, as peritoneal accumulation of leukocytes was only partially attenuated by P-selectin blockade in C57BL/6 mice (data not shown). These observations suggest involvement of p110δ in PSGL-1 signaling for the intracellular activation of integrins. These data collectively identify a signaling pathway emanating from the cytoplasmic tail of PSGL-1 that culminates in integrin activation and leukocyte adhesion (**Supplementary Fig. 12** online).

DISCUSSION

Here we have documented the biological importance of leukocyte priming by selectins for the transactivation of integrins, thereby providing direct evidence for functions of selectins not only as cell adhesion receptors but also as cell signaling molecules. Furthermore, we have characterized a previously unknown signaling pathway 'downstream' of PSGL-1 required for the modulation of selectin-induced, integrin-mediated adhesion of circulating leukocytes to activated vascular endothelium in inflamed tissues. Signaling through PSGL-1 depended on the constitutive association of the cytoplasmic tail of PSGL-1 with Naf1. The binding of P-selectin to PSGL-1 activated Src kinases, which in turn phosphorylated Naf1. Phosphorylated Naf1 recruited the p85-p110δ heterodimer and perhaps other heterodimers of PI(3)K, which ultimately resulted in 'inside-out' activation of leukocyte integrins $\alpha_M\beta_2$ and $\alpha_L\beta_2$.

Given the published data[14–19], we propose that in the presence of extracellular stimuli such as cytokines, chemokines and chemoattractants, crosslinking of PSGL-1 by P-selectin primes, by intracellular signaling, integrin activation. P-selectin stimulation, together with cytokines, chemokines and chemoattractants, acts synergistically to achieve full integrin activation. Surgical exteriorization of the cremaster muscle and thioglycollate-induced acute peritonitis may stimulate the production of cytokines, chemokines and chemoattractants. In the absence of extracellular stimuli (such as in uninflamed dermal microvessels[36,37]), P-selectin alone is insufficient for full integrin activation. Furthermore, in the absence of P-selectin-mediated PSGL-1 signaling (such as in P-selectin-deficient mice or p110δ-mutant mice or after treatment with Tat–PSGL-1), integrins were not fully activated by extracellular stimuli of cytokines, chemokines and chemoattractants alone. That hypothesis is supported by the findings that specific blockade of PSGL-1–Naf1 interactions or suppression of p110δ activity inhibited P-selectin-induced integrin activation *in vitro* and *in vivo* and blocked peritoneal infiltration of leukocytes in a mouse model of acute peritonitis. The presence of a high concentration of serum P-selectin in the circulation in inflammatory and autoimmune diseases[38] and evidence of the formation of dimers and/or oligomers of native and circulating P-selectin from platelets and endothelial cells[39,40] further indicate the existence of this mode of PSGL-1 engagement during integrin activation *in vivo*.

Given that selectin-mediated leukocyte rolling occurs before integrin-mediated leukocyte adhesion[1–6,9], selectins apparently act on leukocytes early in the sequence of events that coordinate optimal activation of integrins. In this context, our finding that P-selectin is fundamentally involved in the initial stage of leukocyte adhesion for the eventual accumulation of leukocytes at sites of inflammation demonstrates the biological importance of selectin-mediated priming of leukocytes for integrin activation *in vivo*. Our data demonstrating that the Tat–PSGL-1 polypeptide abolished leukocyte adhesion and attenuated peritoneal deposition of leukocytes lend strong support for the idea that engagement of PSGL-1 by P-selectin initiates a signaling cascade leading to integrin activation. Similar to PSGL-1-mediated transactivation of integrins, CD44 has been shown to associate with $\alpha_4\beta_1$ and to directly link rolling to adhesion in T lymphocytes[41].

Among the Src family of protein tyrosine kinases in mammalian cells, Fgr, Lyn and Hck have prominent functions in neutrophil responses[42]. Notably, P-selectin activates β_2-integrins by a mechanism dependent on Lyn and Hck[43]. The requirement for Fgr, Lyn and Hck

in E-selectin-induced activation of β_2-integrins has been demonstrated genetically with polymorphonuclear cells isolated from mice lacking Fgr, Lyn and Hck[44]. The necessary presence of Fgr and Hck for the activation of β_2-integrins in sustained adhesion[45] further indicates the potential functional importance of selectin-induced activation of Src kinases in integrin-mediated leukocyte adhesion. We have shown here that the binding of P-selectin to PSGL-1 activated Src kinases, probably Lyn and Hck, in human neutrophils. Activated Src kinases phosphorylated Naf1, which recruited and consequently activated PI(3)K. PP2, an inhibitor of Src kinases, prevented Naf1 phosphorylation and immunoprecipitation of p85 together with PSGL-1, and inhibited $\alpha_M\beta_2$ activation. These findings reinforce the idea of a central function for Src kinases in PSGL-1 signaling for integrin transactivation. Our findings collectively indicate that PSGL-1 signaling may function as a chief pathway for integrin-mediated firm adhesion in the regulation of leukocyte recruitment and emphasize many molecular targets for therapeutic intervention in leukocyte recruitment in situations of pathogenic inflammation.

METHODS

Mice. C57BL/6J mice, P-selectin-deficient mice (B6.129S7-*Selp*^{tm1Bay}/J)[46] and PSGL-1-deficient mice (B6.Cg-*Selpl*^{tm1Fur}/J) were from the Jackson Laboratory. The p110δ-mutant mice (*p110δ*$^{D910A/D910A}$) have been described[35]. Mice were maintained in specific pathogen–free animal rooms at the Shanghai Laboratory Animal Company and the animal core facility of the University of Minnesota Medical School. Mouse experiments were approved by the institutional animal committees of the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences and University of Minnesota.

Reagents. P-Rg was constructed and prepared as described[47]. Platelet P-selectin, which is biophysically characterized as an oligomer[44], was isolated from detergent lysates of 'outdated' (beyond the expiration date for clinical use) human platelets by affinity chromatography with monoclonal antibody PS1 (ref. 47). Soluble human P-sel, which is characterized as a monomer[48], and human and mouse ICAM-1 and IL-8 were from R&D Systems. The 293 cells (CRL-1573), 293T cells (CRL-11268) and HL-60 cells (CCL-240) were from American Type Culture Collection and were maintained at 37 °C in the presence of 5% CO_2 in DMEM or RPMI 1640 medium (Gibco BRL) supplemented with heat-inactivated newborn bovine calf serum, 4 mM L-glutamine, 100 units/ml of penicillin and 100 μg/ml of streptomycin. Rabbit polyclonal antibody specific for human PSGL-1 and preimmune rabbit IgG were prepared as described[25]. G1 F(ab′)$_2$ (IgG1 specific for and blocking P-selectin) and PS1 F(ab′)$_2$ (IgG1 specific for but not blocking P-selectin) were prepared and characterized as described[47,49]. KPL-1 (IgG1 specific for PSGL-1) and CBRM1/5 (antibody to human α_M) were from eBioscience. M1/70 (IgG2b to mouse α_M), M17/4 (IgG2a to mouse α_L), RB40.34 (IgG1 to mouse P-selectin) and rat IgG were from BD Pharmingen. The 38 antibody to human α_L was from Axxora. Hybridoma cell lines 44a (antibody to α_M), OKM1 (antibody to α_M), IB4 (antibody to β_2) and 9E10 (antibody to c-Myc) were from American Type Culture Collection, and their respective IgG molecules were purified from mouse ascites fluid by protein A chromatography. Anti-p85 (06-195) was from Upstate Biotechnology. Anti-α-tubulin (B5-1-2), human (I2511) and mouse (I5381) IgG molecules, and PAF were from Sigma.

cDNA cloning. Full-length cDNA encoding human Naf1 was isolated by PCR with the Human Leukocyte Matchmaker cDNA Library as the template and forward primer 5′-TTACGGATCCATGGAAGGGAGAGGACCGTAC-3′ and reverse primer 5′-CGCCAAGCTTAAATGACACAATCTGGTCTCACTG-3′. The PCR product was digested with *Bam*HI and *Hin*dIII and was ligated into those same sites in pCMV-Tag 3B (Stratagene) to allow expression of Myc-tagged Naf1 in mammalian cells. Its identity as sequence encoding human Naf1-α was verified by nucleotide sequencing.

Cell adhesion assay. The use of human blood was approved by the Institutional Review Board of the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences and University of Minnesota. Neutrophils were isolated from the peripheral venous blood of healthy volunteers as described[14]. Mouse neutrophils were isolated as described[50]. Human and mouse neutrophils or HL-60 cells were incubated for 30 min at 37 °C with 10 μg/ml of human IgG or P-Rg, human P-selectin monomer or platelet P-selectin, mouse IgG or mP-Rg, mP-sel monomer or dimer, 100 ng/ml of PAF and 150 nM IL-8 in the presence or absence of dimethyl sulfoxide (solvent control), 25 nM PP2, 150 nM wortmannin, 10 μM LY294002, 200 ng/ml of PTX (all inhibitors from Calbiochem) and 2.5 μM PSGL-1 P, Tat–PSGL-1 or Tat–PSGL-1 Mut. Cells were then transferred to 96-well cell culture plates coated with immobilized fibrinogen (Enzyme Research Laboratories), mouse fibrinogen (Sigma), or human and mouse ICAM-1 (R&D Systems; all at a concentration of 10 μg/ml; 100 μl/well). The compound concentrations used were the minimum amounts that maximally inhibited the P-selectin-induced adhesion of neutrophils to immobilized fibrinogen, according to our dose-course experiments (data not shown). Antibody-inhibition experiments were done as described[14]. Cell adhesion assays were done as described[14,47,49].

GST precipitation assay. A fragment encoding Naf1 amino acids 394–629 was inserted into the pGEX-4T-2 vector (GE Healthcare Life Sciences) for generation of the GST-Naf1 fusion protein, which was expressed and purified according to the manufacturer's protocols. Neutrophils were isolated from the peripheral blood of healthy volunteers as described[14] and were lysed for 1 h in ice-cold CHAPS buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% (wt/vol) BSA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml of aprotinin, 2 μg/ml of leupeptin, 1 μg/ml of pepstatin A and 3% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate hydrate). After centrifugation for 10 min at 4 °C and 12,000*g*, supernatants (1×10^7 neutrophils/aliquot) were incubated with 10 μl glutathione-Sepharose beads (GE Healthcare Life Sciences) that had been preloaded for 4 h at 4 °C with equal amounts of GST or GST-Naf1. After being washed three times with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.05% (vol/vol) Triton X-100, bound proteins were eluted by being boiled in reducing SDS sample-loading buffer. After electrophoresis, proteins were stained with Coomassie bright blue or were transferred to polyvinylidene fluoride membrane (Immobilon-P; Millipore) for immunoblot analysis with the appropriate antibodies.

Mutagenesis, transfection, coimmunoprecipitation and immunoblot analysis. The PSGL-1 or Naf1 deletion and site-specific mutants were generated with the MutantBEST Kit (TaKaRa Biotechnology). All mutations were verified by nucleotide sequencing. For detection of the association of PSGL-1 with Naf1 in mammalian cells, 293 cells were cotransfected by calcium phosphate precipitation with plasmids encoding PSGL-1 and Myc-tagged wild-type or mutant Naf1. Then, 2 d later, transfectants were washed with ice-cold PBS, were detached and were lysed for 1 h at 4 °C in ice-cold lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml of aprotinin, 2 μg/ml of leupeptin, 1 μg/ml of pepstatin A, 1 mM sodium orthovanadate, 20 mM NaF, 1% (vol/vol) Triton X-100 and 10% (vol/vol) glycerol). After centrifugation for 10 min at 12,000*g*, each aliquot of supernatant was incubated with protein A–Sepharose beads (GE Healthcare Life Sciences) that had been incubated for 4 h at 4 °C with 2 μg of the appropriate antibody. After being washed three times, bound proteins were eluted by being boiled in reducing SDS sample-loading buffer and were analyzed by SDS-PAGE and immunoblot with appropriate antibodies followed by the appropriate horseradish peroxidase–conjugated secondary antibodies and chemiluminescence detection.

Generation of antibodies. For the generation of monoclonal antibodies specific for Naf1 phosphorylated at Tyr552, two peptides were synthesized containing amino acid residues 543–562 of Naf1: HLCGAYPYAUPPM PAMVPHH (where 'U' indicates phosphorylated tyrosine) and HLCGAYP YAYPPMPAMVPHH. These peptides were fused to keyhole limpet hemocyanin or ovalbumin; keyhole limpet hemocyanin–conjugated peptides were used as antigens to immunize mice, and ovalbumin-conjugated peptides were used as substrates for the screening of hybridoma supernatants based on enzyme-linked immunosorbent assay[47,49].

siRNA transfection. HL-60 cells were transfected with 1 μM annealed double-stranded siRNA oligonucleotides (S1, 5′-GGAGAAUUCCCGGCUGAAGTT-3′

and 3′-TTCCUCUUAAGGGCCGACUUC-5′; S4, 5′-GCUUUUGGAAGAGUCCCAGTT-3′, 3′-CTCGAAAACCUUCUCAGGGUC-5′; Ambion), in the presence or absence of plasmids encoding Naf1, with the Nucleofector Kit V (Axama) according to the manufacturer's protocol. Cotransfected cells were cultured for 24 h before use. Alternatively, 293 cells were transfected for 48 h with 100 nM annealed double-stranded siRNA oligonucleotides oligonucleotides with Lipofectamine 2000 (Invitrogen).

Generation of lentiviruses. Sequences encoding Myc–Naf1 Y552F, hemagglutinin-tagged PTEN or LacZ were inserted into the pLenti6-V5-Dest vector (ViraPower Lentiviral Expression System; Invitrogen); vectors were transfected together into 293FT cells with the ViraPower packaging mix (Invitrogen) to generate the respective lentiviruses. Viral stocks were made and these were used to infect HL-60 cells according to the manufacturer's protocols.

Measurement of PI(3)K activity. Human neutrophils were 'starved' for 2 h in M199 medium containing 2% (vol/vol) FCS and then were stimulated for 10 min at 37 °C with 10 μg/ml of human IgG or P-Rg. PI(3)K was coprecipitated from neutrophil lysates with rabbit IgG or anti-PSGL-1 as described above. PI(3)K activity was measured *in vitro* with a competitive enzyme-linked immunosorbent assay format (Echelon Biosciences) according to the manufacturer's instructions.

Statistical analysis. Quantitative data are expressed as mean ± s.d. Statistical significance was determined by analysis of variance followed by the Bonferroni post-hoc test for multiple comparisons, or by Student's *t*-test where specifically noted. For all tests, *P* values of less than 0.05 and 0.01 were considered statistically significant and very significant, respectively.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

H.-B.W., J.-T.W., L.Z., Z.H.G., W.-L.X. and T.X. did the research and analyzed the data; Y.H., X.Z., E.F.P., M.C. and J.-G.G. designed the research; and J.-G.G. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/natureimmunology/.

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