P-Rex1 and Vav1 Cooperate in the Regulation of Formyl-Methionyl-Leucyl-Phenylalanine–Dependent Neutrophil Responses

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GPCR stimulation. Vav activation is largely mediated through Src and Syk family protein tyrosine kinases (17–19). Work carried out with COS^{phox} cells (which stably express the components of the neutrophil NADPH oxidase) suggests that, upon GPCR stimulation, Vav1 may be activated further by interacting with p67^{phox} (20). Interestingly, overexpression of P-Rex1, but not Vav1, is sufficient to elicit a GPCR-dependent ROS response in this system (21).

Of the P-Rex family, only P-Rex1 is expressed in neutrophils (16, 22). P-Rex1$^{−/−}$ neutrophils have a partial reduction in GPCR-dependent Rac2 activation, whereas Rac1 activation is near normal (23). P-Rex1 deficiency leads to a strong defect in GPCR-dependent ROS production in LPS-primed neutrophils, but the response is less affected in unprimed or TNF-primed cells (23). Chemotaxis of isolated neutrophils is only slightly reduced, with a mild defect in cell speed but normal polarization and directionality, although recruitment of neutrophils to inflammatory sites in vivo is substantially impaired (23, 24). Hence, P-Rex1 controls a subset of Rac-dependent neutrophil functions.

The predominant isoforms of the Vav family in mouse neutrophils are Vav1 and Vav3 (25). Similarly to P-Rex1, Vav1-deficient neutrophils have reduced GPCR-dependent ROS formation and a minor defect in chemotaxis (26). Integrin-dependent sustained adhesion and spreading are largely normal in cells deficient in Vav1 or Vav3 (25), but FcR-dependent phagocytosis and ROS production are impaired in Vav3$^{−/−}$ neutrophils (10). Neutrophils deficient in both isoforms (Vav1$^{−/−}$/Vav3$^{−/−}$) have defects in integrin-dependent sustained adhesion and spreading, as well as some (but not all) forms of complement-mediated phagocytosis, although their ability to chemotax toward GPCR ligands is unaffected (25, 27). FcR-dependent phagocytosis and ROS production are abolished in Vav1$^{−/−}$/Vav3$^{−/−}$ cells (10). Neutrophils

Expression of Mac-1 integrin

The expression of the integrin Mac-1 ($\alpha_M\beta_2$, CD11b/CD18) on the surface of neutrophils was assessed using total bone marrow cells. Cells were flushed from bone marrow with endotoxin-free HBSS (containing 15 mM HEPES and 0.25% fatty acid-free BSA, but not Ca^{2+} or Mg^{2+} [pH 7.4] at room temperature), then suspended in buffer A at 5×10^7 cells/ml. Two hundred microliters of cells was incubated with or without 20 ng/ml TNF or 50 ng/ml GM-CSF for 30 min at either 0 C or 37 C, then stained with both Gr1-FITC Ab and Cy5 anti-mouse CD11b Ab (catalog no. 19-0112; eBioscience, San Diego, CA) for 20 min at 0 C. Neutrophils were identified from their forward scatter and FITC-stained Gr1 properties using FACSCalibur. The median intensity of Cy5 fluorescence within each sample, determined using FlowJo software (Tree Star, Ashland, OR), was used to assess the expression of Mac-1 on the surface of these neutrophils.

To determine total Mac-1 expression in isolated neutrophils, cells were pretreated with the protease inhibitor diisopropyl fluorophosphate (6 mM) for 10 min at room temperature, and total cell lysates were separated by SDS-PAGE. Western blots for CD11b (catalog no. ab75476; Abcam, Cambridge, U.K.) were performed, and the signal was detected by ECL (GE Healthcare). Coomassie staining of blots was employed to assess loading.

Rac activation

Neutrophils at 13×10^7 cells/ml were preincubated in buffer B, then stimulated for 0–15 s with 10 nM fMLF (Sigma-Aldrich). The reaction was stopped by addition of 5 volumes of ice-cold lysis buffer, and GTP-Rac was isolated from lysates by Pak-CRIB domain pull-down assay as described (23). Two percent of the total lysate was used as a control for Rac1 and Rac2 expression levels. Western blots were performed with monoclonal Rac1 and polyclonal Rac2 Abs (catalog no. 05-389 and 07-604, respectively; both from Upstate Biotechnology [now Millipore]). Blots were then scanned and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis

Data were analyzed by one-sample t test, with Bonferroni correction; ANOVA with Dunnett post hoc test; or independent t test as indicated in figure legends. Statistical significance is designated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Results

P-Rex and Vav family Rac-GEFs have both been implicated in neutrophil GPCR signaling. In this project, we assessed the roles of the P-Rex and Vav families in GPCR-dependent neutrophil function to determine potential redundancy or cooperation between them. Previous studies have shown that, of the P-Rex family, P-Rex1 is highly expressed in neutrophils, whereas P-Rex2 expression is undetectable, suggesting that P-Rex1 is the only P-Rex family member in these cells (22, 31). All three Vav isoforms are expressed, however Vav2 is 30-fold less abundant than Vav1 and 120-fold less abundant than Vav3 (25); hence, Vav1 and Vav3 are the major isoforms in mouse neutrophils. In this study, we generated mouse strains with homozygous deletions of the predominant isoforms from each family, namely P-Rex1 plus either Vav1 or Vav3 (P1V1 and P1V3). Their neutrophil responses were compared with those from mice lacking the entire P-Rex family (P1 or P12), the entire Vav family (V123), or Rac2.

Western blotting of neutrophil lysates showed that Vav1 expression was unaffected by P-Rex1 deficiency (Supplemental Fig. 1A, 1B), and P-Rex1 expression was normal in V123 cells (Supplemental Fig. 1C). Hence, the loss of one Rac-GEF family was not simply compensated by an overexpression of the other. Similarly, P-Rex1 and Vav1 expression were unaffected by Rac2 deficiency (Supplemental Fig. 1D), and the levels of Rac1 and Rac2 were normal in GEF-deficient neutrophils (Supplemental Fig. 1A–1C). Western blots for Vav3 were unsuccessful due to insufficient sensitivity or specificity of available Vav3 Abs. Expression of P-Rex2 remained undetectable in neutrophils from all strains (data not shown). Furthermore, we assessed the function of neutrophils from P1 and P12 mice in a range of experiments and found them to be comparable (data not shown). Hence, P-Rex2 is unlikely to play a role in neutrophil function, which allowed us to use either P1 or P12 mice to assess the role of the P-Rex family in our experiments.

Blood cell development in P1V1 and P1V3 mice

First, we assessed the impact of GEF deficiency on blood cell development. In peripheral blood, P1V1 mice had twice as many myeloid cells than did WT mice, which could largely be accounted for by elevated levels of granulocytes and eosinophils (Supplemental Fig. 2A–2C). P1V3 mice showed a similar increase in myeloid cells (mainly monocytes, granulocytes, and basophils) and also exhibited a 60% increase in the number of circulating lymphoid cells (Supplemental Fig. 2A–2C). For each genotype, the proportion of myeloid cells was near normal in bone marrow, except for P1V1 mice, where they represented 20% of bone marrow WBCs, compared with 12% in WT mice (Supplemental Fig. 2D).

P1V1 mice had a trend for decreased total thymocyte numbers (Supplemental Fig. 2E), however splenocyte numbers were normal (Supplemental Fig. 2F). The number of thymocytes was significantly reduced at the CD4+CD8+ double-positive and CD4 and CD8 single-positive stages (Supplemental Fig. 2G). CD3+CD4+ and CD3+CD8+ splenic T cells were also depleted, possibly due to the lack of cells exiting the thymus (Supplemental Fig. 2H), whereas spleen B cells (including marginal zone B cells) were normal (not shown). In P1V3 mice, thymic and splenic lymphocyte populations were unaffected (Supplemental Fig. 2E–2H).

Overall, the effect of P1V1 or P1V3 deletion on bone marrow or circulating blood cells was relatively mild (within 2-fold of WT), 40931505.3925 0m 1_01 Tf 0.715 0 Td 835.

Hence, P-Rex1 and Vav1 cooperate in the control of this response. Vav3 is not capable of taking over the role of Vav1 in this co-operation.

When LPS-primed, fMLF-stimulated ROS production in P1V1 neutrophils was reduced by 65% ($p = 0.01$), and by 50% in P1V3 ($p = 3.3 \times 10^{2.5}$

defects were observed in the adhesion of P1V3, P1, or V123 cells (Fig. 4Aii). Therefore, whereas neither P-Rex1 nor the Vav family alone is essential, P-Rex1 and Vav1 synergize in the regulation of GPCR-stimulated adhesion to the integrin ligand pRGD. As with fMLF-stimulated ROS formation, Vav3 could not take over the role of Vav1 in this cooperation. We obtained similar results when adhesion to glass was measured (data not shown).

The Vav family controls spreading of neutrophils

Once neutrophils are firmly adhered onto a surface, they undergo Rac-dependent spreading. Unstimulated WT neutrophils spread on pRGD-coated coverslips with a mean surface area of 70 μm², as assessed by image analysis of Gr1-FITC–stained cells. P1 and P1V3 cells spread normally (Fig. 4Bi); however, the surface area of V123 neutrophils was, on average, 60% smaller than that of WT cells (45 μm², p = 0.0004, Fig. 4Bi, 4Biii). Cell area was also significantly reduced in P1V1 and Rac2 neutrophils (to 55 μm², p = 0.002 and 0.003, respectively; Fig. 4Bi). From these data, it can be concluded that P-Rex1 is not involved in spreading on pRGD, and there is no obvious cooperation between the P-Rex and Vav families in this response. Instead, spreading on pRGD appears to be dependent on the Vav family alone (predominantly Vav1), in agreement with previous reports that the Vav family controls integrin-dependent spreading (25).

The addition of 1.5 mM fMLF stimulated an increase in the spreading of cells from all strains. The mean area of P1V1 and Rac2 cells increased to 100 μm², compared with 120 μm² for WT cells, whereas V123 cells spread significantly less (80 μm², p = 0.005, Fig. 4Bii). Hence, to some extent, fMLF stimulation can overcome the spreading defect caused by Vav deficiency, possibly by stimulating an upregulation of integrins on the cell surface.

P1V1 and Rac2-deficient neutrophils show reduced Mac-1 integrin expression on the cell surface

The defects we observed in fMLF-stimulated adhesion and che-motaxis suggested that P1V1 cells might show changes in their adhesion receptors. To investigate this, we measured the expression of Mac-1 integrin (the major neutrophil adhesion receptor) on the cell surface by flow cytometry. We found that Mac-1 surface expression on freshly isolated P1V1 cells (preserved by incubation at 0 C) was significantly reduced, whereas it was normal in all other genotypes (Fig. 5A). Under conditions that allowed integrin trafficking (unprimed cells, incubated at 37 C), Mac-1 surface expression tended to be lower on both P1V1 and Rac2 neutrophils (Fig. 5B). This pattern was also evident when integrin upregula-

that, together, they constitute a major driving force for Rac-dependent signaling downstream of fMLFRs. They also suggest that Vav3 cannot take over the role of Vav1 in this signaling pathway.

Whereas P-Rex1 preferred Rac2 as its in vivo substrate (23), peak activation of Rac1 was reduced by 50%, and Rac2 by 30%, in V123 cells (Fig. 6A). This indicates that the Vav family is also capable of activating Rac in response to fMLF, and it may have a preference for Rac1 over Rac2 in vivo. The time course of Rac1 and Rac2 activation was similar in all genotypes, with the peak after 5–10 s of fMLF stimulation, although the response seemed slightly more sustained in V123 cells than in the other strains (Fig. 6B). At all time points tested, fMLF-stimulated Rac1 and Rac2 activation was lowest in P1V1 cells (Fig. 6C).

Rac isoforms could be particularly important for neutrophil responses that require both Rac1 and Rac2, for example, chemotaxis, which involves Rac1 for directionality and Rac2 for movement per se (6, 12), or phagocytosis, during which Rac1 and Rac2 have been shown to have differential membrane localization (37, 38). We are hopeful that, in the future, it might be possible to use fluorescence resonance energy transfer (FRET) imaging technology to visualize the activation of different Rac pools by the P-Rex and Vav families, provided these pools are spatially sufficiently separate.

Previous studies suggested that neither Vav1$^{2/2}$ nor Vav1$^{2/2}$/Vav3$^{2/2}$ neutrophils have defects in fMLF-stimulated Rac activation (25, 26); however, we found that activation of Rac1 and Rac2 in V123 cells was reduced by 50 and 30%, respectively. This discrepancy may reflect the additional deficiency of Vav2 but, as there is low expression of Vav2 in neutrophils (25), it is more likely due to different assay conditions, notably the time points tested. GPCR-dependent Rac activation in neutrophils is rapid and transient (peaks at around 10 s), but the assays available to measure Rac activity in vivo do not allow for extensive time courses. Just as FRET technology is required for visualization of Rac activity in spatial terms, it would also be useful for better temporal resolution and quantification of in vivo Rac activity. Such FRET-based assays are currently available in transfected cell systems (39) but are unfeasible for use with primary neutrophils.

In most instances, P-Rex1 cooperated with Vav1 rather than Vav3, despite Vav3 being 4-fold more abundant than Vav1 in mouse neutrophils (25). The mechanism of this selectivity is unknown, but it may reflect subtle differences in the intracellular localization of Vav1 and Vav3 or their interaction with distinct sets of binding partners. In fMLF-dependent chemotaxis, P-Rex1 could cooperate with either Vav1 or Vav3, despite Rac activation being more severely impaired in P1V1 than in P1V3 cells. This could mean that different Rac pools are activated by Vav1 and Vav3, with the Vav3-dependent pool being more important in chemotaxis than in other responses. Alternatively, Vav3 might play a GEF activity-independent role in chemotaxis. Some GEF activity-independent roles have been described for the Vav family in other cell types; for example, a GEF-dead version of Vav1 can mediate TCR-induced calcium flux and cellular polarization in T cells (40).

Whereas fMLF-dependent responses were controlled by P-Rex1 and Vav1, particle-induced ROS production and cell spreading were solely dependent on the Vav family, and LPS-primed ROS production required both GEF families. This shows that different GEFs couple to different types of receptor, as expected. However, an unexpected level of complexity was observed when comparing chemotaxis in response to different GPCR stimuli. fMLF-dependent migration was impaired in P1V1 and P1V3 neutrophils, but C5a-stimulated migration was normal (although both were dependent on Rac2). Hence, different GEFs control fMLF-dependent

or C5a-dependent chemotaxis. Perhaps, the fMLF and C5a recep-