Phosphatase Wip1 Negatively Regulates Neutrophil Migration and Inflammation

Bo Sun, Xuelian Hu, Guangwei Liu, Bo Ma, Yamei Xu, Tao Yang, Jianfeng Shi, Fan Yang, Hongran Li, Lianfeng Zhang and Yong Zhao

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Supplementary Material

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Neutrophils are critically involved in host defense and tissue damage. Intrinsic signal mechanisms controlling neutrophil activities are poorly defined. We found that the expression of wild-type p53-induced phosphatase 1 (Wip1) in mouse and human neutrophils was downregulated quickly after neutrophil activation through JNK-microRNA-16 pathway. Importantly, the Wip1 expression level was negatively correlated with inflammatory cytokine productions of neutrophils in sepsis patients. Wip1-deficient mice displayed increased bactericidal activities to Staphylococcus aureus and were hypersensitive to LPS-induced acute lung damage with increased neutrophil infiltration and inflammation. Mechanism studies showed that the enhanced inflammatory activity of neutrophils caused by Wip1 deficiency was mediated by p38 MAPK-STAT1 and NF-κB pathways. The increased migration ability of Wip1KO neutrophils was mediated by the decreased CXCR2 internalization and desensitization, which was directly regulated by p38 MAPK activity. Thus, our findings identify a previously unrecognized function of Wip1 as an intrinsic negative regulator for neutrophil proinflammatory cytokine production and migration through multiple signal pathways. The Journal of Immunology, 2014, 192: 1184–1195.
tively masters neutrophil proinflammatory cytokine production and migration.

Materials and Methods

Mice and human blood samples

C57BL/6 (B6) and CD45.1+ B6 mice were purchased from Beijing University Experimental Animal Center (Beijing, China). Wip1KO and p53+/− mice were provided by the Key Laboratory of Human Diseases Comparative Medicine (Ministry of Public Health, Beijing, China). Wip1KO mice have been previously described (12, 17, 18) and backcrossed to the B6 genetic background in our laboratory. Wip1KO and p53+/− bone marrow cells (BMCs) from either WT or Wip1KO mice into lethally irradiated syngeneic mice (19, 20). Experimental protocols were approved by the Animal Ethics Committee of the Institute of Zoology (Beijing, China).

Patients with bacterial infection were enrolled in Dongzhimen Hospital, Beijing University of Traditional Chinese Medicine (Beijing, China) from May 2012 to September 2012. The blood samples and clinical data were collected within the first 24 h of admission and analyzed for each patient to calculate the APACHE II scores with the approval of the ethics committee of the hospital. The basic clinical information of infected patients was briefly summarized in Table I. Blood samples from age- and sex-matched healthy individuals were used as controls.

mAbs and reagents

For flow cytometry (FCM) analysis of surface markers, cells were stained with Abs in PBS containing 0.1% (w/v) BSA and 0.1% Na2. The Abs were purchased from eBioscience: anti-CD45R/B220 (RA3-6B2), anti-CD11b (M1/70), anti–Gr-1 (RB6-8C5), anti-CD45.1 (A20), and anti-CD45.2 (53-6.7) (104). The following Abs were purchased from BD Biosciences: anti-CXCR2 (clone 242216), anti-CD18a and anti-CXCR2 were measured by FCM. Briefly, neutrophils (0.5 × 10⁶) were incubated with anti-CD16/CD32 mAbs (clone 2.4G2), followed by 30 min of incubation at 4°C with fluorescent-labeled Abs: anti-CXCR2, anti-CD18, and anti-Gr-1 mAbs. The cells were then washed and analyzed by FCM using CellQuest software. Cell numbers of various populations were calculated by multiplication of the total cell number by the percentages of each individual population from the same mouse.

To determine the internalization of CXCR2, the neutrophils were treated for different time with CXCL1 or IL-8. The surface expression of CD18 and CXCR2 was measured by FCM. Briefly, neutrophils (0.5 × 10⁶) were incubated with anti-CD16/CD32 mAbs (clone 2.4G2), followed by 30 min of incubation at 4°C with fluorescent-labeled Abs: anti-CXCR2, anti-CD18, and anti-Gr-1 mAbs. The cells were then washed and analyzed by FCM using CellQuest software. Neutrophils were identified by their light scattering properties and expression of Gr-1 in granulocytes cells. The expression of CXCR2 was determined in this population.

Table I. The basic information of patients with bacterial infection

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<th>Sputum Culture</th>
<th>Urine Culture</th>
<th>Blood Culture</th>
<th>Temperature (˚C)</th>
<th>WBC (×10⁹/l)</th>
<th>CRP (mg/l)</th>
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CRP, C-reactive protein (mg/L); G, gram staining; Temperature, body temperature; WBC, WBC count.
Isolation of neutrophils

Neutrophils from bone marrow or peripheral blood were isolated on a Percoll gradient (16). The enriched neutrophil fraction was recovered at the interface of 65 and 72% Percoll. The purity of the enriched neutrophils was >80%. In some cases, neutrophils were sorted by a high-speed cell sorter (MoFlo; Beckman Coulter, Pasadena, CA) using anti-Gr1/CD11b mAbs. The purity of the sorted neutrophils was usually >98%.

RNA and protein analyses

Frozen tissue samples were homogenized in ice-cold lysis buffer containing 10 mM HEPES, 2 mM EDTA (pH 8), 5 mM DTT, 1 mM Pefabloc, and one tablet of mixture of proteinase inhibitors (Roche). Real-time PCR was performed with probe sets from Applied Biosystems and normalized against the endogenous control gene HPRT (19). The primers used in the current study were summarized in Table II. Immunoblot was performed with the following mAbs: Wip1 (H-300), p-S6 (Ser235/236), p-AKT (Ser473), p-AKT (Thr308), p-STAT1 (Ser727), p-ERK (Thr202/Tyr204), and β-actin (Sigma-Aldrich) were purchased from Cell Signaling Technology or Santa Cruz Biotechnology (21).

Analysis of neutrophil degranulation

As previously described with minor modifications (22), neutrophils at a concentration of 2–3 x 10^6 cells/ml were plated on wells in the presence or absence of 100 ng/ml TNF-α. After 1 h of incubation at 37°C, cells were spun, and the supernatants were removed for measurement of release of matrix metalloproteinase 9 (MMP-9), elastase, lactoferrin, and myeloperoxidase (MPO). Elastase and MPO release was measured with the Enz-Check Elastase Assay Kit and an MPO activity assay kit (E-120556 and E33856; Invitrogen), renaturation in 2.5% Triton X-100 buffer, and development of zymography buffer (200 mM NaCl, 5 mM CaCl2, and 50 mM Tris) overnight. Gels were stained with Coomassie blue dye and quantified or with probe sets from Applied Biosystems and normalized against the standard curve. The amounts of lactoferrin were detected using the standard curve.

In vitro bactericidal activity by neutrophils

Bone marrow neutrophils were sorted as described above. S. aureus was grown overnight at 37°C, washed in PBS, and counted. Suspension of S. aureus containing a bacterial concentration corresponding to 10^8 CFU was incubated with or without 5 x 10^5 neutrophils in flat-bottom 96-well plates (Costar) in a total of 200 µl RPMI 1640 medium at 37°C in 5% CO_2 for 3 h. For phagocytosis experiments, neutrophils were collected and blocked with anti-mouse FcγR mAb (clone 2.4G2) and stained with anti-Ly6G-FITC (eBioscience). After washing with cold PBS three times, the percentage of Ly6G^+ cells with phagocytosis in the gated Ly6G^+ cells were determined by FCM. For S. aureus survival experiments, some wells were treated with 0.01% Triton X-100 in water for 5 min to lyse neutrophils and then washed twice with 100 µl PBS. Surviving S. aureus CFUs were determined, and the survival was calculated as (S. aureus number incubated with neutrophils) divided by (S. aureus number incubated without neutrophils).

In vivo bactericidal activity by neutrophils

WT or Wip1KO mice were injected with 5 ml air in the central back region on day 0. On day 3, the air pouches were reinflated with 3 ml sterile air. On day 6, S. aureus was grown to a 5 x 10^8 CFU and then washed and diluted in sterile PBS to a 2 x 10^5 CFU. Air pouches were injected with 0.5 ml bacterial culture. At the indicated time points, the infected mice were sacrificed, and the air pouches were lavaged with HBSS. Cells were counted and stained with anti-mouse CD11b-PE and anti-mouse Ly6G-FITC and propidium iodide for analysis by FCM. The amount of Ly6G^+ Ly6G^+ cells on live (PI^-) neutrophils was counted. The live bacteria from the air pouch were plated to quantify the number of live CFUs, normalized to the initial number of bacteria put into the air pouch. In addition, supernatants from the air-pouch lavage were used to measure MPO, elastase, lactoferrin, and MMP-9 release, as described above. Some neutrophils were collected for real-time PCR analysis of MPO, elastase, lactoferrin, and MMP-9.

Table II. Primer sequences used for real-time PCR assays

<table>
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<tr>
<th>Gene Name</th>
<th>Primer Sequences (5'-3')</th>
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| Mouse HPRT | Forward: AGTACGCCCCAAAATGTGTTAAAG  
Reverse: CTTAGGCTTTGTATTTTGGTCCTTTC |
| Human HPRT | Forward: CAAGGATGTTGATGAAGAAACAGACA  
Reverse: ATGATGGCTCGGTCGTGATGTCG |
| Mouse Wip1 | Forward: CTGACTGATAGCCCTACTTACAACA  
Reverse: GAGAAGCCATTACCTGAGGAACA |
| Human Wip1 | Forward: TTGTGAGTGAGTCGAGGTCGT  
Reverse: TGGGAAAACACAGTGGTGACAG |
| Mouse IL-1β | Forward: CCATCAGAGGCAAGAGGGAA  
Reverse: ATACAGCACCTACCTCACAAC |
| Human IL-1β | Forward: ATAGGCACACTCTAGCCT  
Reverse: ATTGGCCCTGAAGAGGAGAA |
| Mouse IL-6 | Forward: GCAGTGGCAATCTGAGTTAGT  
Reverse: AAGGACCTGTGGCTTTGCTTTC |
| Human IL-6 | Forward: CTCCTGGCTCAAGGCTCCTCT  
Reverse: CCAGTGCCTCTCTGGCTCTTTC |
| Mouse TNF-α | Forward: GAGTGAACAGGCTGTAGCC  
Reverse: CTCCTGGTAATGAGATAGC |
| Human TNF-α | Forward: CAAGGCTCTTCTCTTGAAGAATGG  
Reverse: GCGAGACCCCACTCTAGAAGAGA |
| Mouse Elastase | Forward: GGGGGCCAAACAGACCCAG  
Reverse: GCAAACTCACCCACAGG |
| Mouse MPO | Forward: TCCCACTCAGCAAGGCTCT  
Reverse: TAAGACGACGCAAATCGAG |
| Mouse lactoferrin | Forward: ACCGCAGGCTGGAAACTC  
Reverse: CACCCCTCTCCTCACCAATACAC |
| Mouse MMP9 | Forward: ATAGGAAGAGGCCATACAGG  
Reverse: GTGTACACCCCAATTTGACG |
| Mouse CXCL1 | Forward: GCACCCAAACAGCAAGTATACATGAG  
Reverse: AGAAGCCAGGCTTCACAGGA |
| Mouse CXCL2 | Forward: GCCCAGACAGAATCTACAGG  
Reverse: CTCCCTCCTTACGAGTCAGTTA |
Induction of sterile peritonitis

Mice were injected i.p. with 1.5 ml of thioglycollate broth (TG; Sigma-70157) or PBS as a control. After 6 h, peritoneal exudate cells were harvested by peritoneal lavage with 20 ml cold PBS as described previously (23).

LPS-induced acute lung damage

Experimental ALD was induced by i.p. injection of LPS (5 μg/g body weight) as described previously (24). Mice were sacrificed 2 h after LPS injection, and lungs were removed for further analysis.

Histological analyses

Lung tissue was fixed in 4% paraformaldehyde, embedded in paraffin, cut into 5-μm sections, and subsequently stained with H&E for histological analyses, as described previously (25).

Transwell assays

Chemotaxis was evaluated according to the procedure described previously (26). A 24-well microchemotaxis plate (Corning) in which the chambers were separated by polyvinylpyrrolidone-free polycarbonate membrane (5-μm pore size) was used. DMEM containing 0.01% BSA (control group) or CXCL1 (25 nM or as the indicated doses) was placed in the lower chamber. Purified Wip1KO and WT mouse bone marrow neutrophil suspensions (5 x 10^7 cells in 500 μl) were placed in the upper chamber. Chambers were incubated at 37°C with 5% CO2 for 2 h. The results are expressed as the mean number of neutrophils per well and are representative of triplicate measurements. In some assays, cells were pretreated with inhibitors for 30 min as indicated.

Neutrophil migration assay

Mouse bone marrow neutrophils were plated onto mice Fn-coated assay dishes and allowed to adhere for 15 min for steady-state adhesion. Cell migration was assayed under ×10 phase microscopy (Nikon TE300) and allowed to migrate for the desired time after stimulated with FMLP (1 μM). Images were captured using a CCD camera (Hamamatsu) with Metamorph software (Molecular Devices) and then analyzed by ImageJ as described previously (27).

Immunoprecipitation

Lysates were prepared using the following lysis buffer (pH 8): 50 mM Tris-HCl and 1% Triton X-100, with a protease inhibitor mixture (AEBSF, pepstatin A, E-64, bestatin, leupeptin, and aprotenin) from Sigma-Aldrich (28). The concentration of total protein was measured by bichinchoninic acid analysis (Pierce, Rockford, IL). To determine the direct interaction of CXCR2 with p38 MAPK, lysates of 293T cells transfected with eGFPN1-CXCR2 plasmid and lysates of mouse bone marrow PMNs pretreated with or without anisomycin for 30 min were incubated with 3 μg/ml of the first Ab overnight at 4°C and followed with the addition of Wip1 inhibitor in a dose-dependent manner.

TNF-α, IL-6, and IL-1β decreased Wip1 expression as well (p < 0.001; Fig. 1A). Consistently, Wip1 protein expression in isolated human neutrophils was downregulated in sepsis patients compared with healthy individuals (Fig. 1B). Neutrophils, as one of the first cell types to arrive at infection sites, produce an array of inflammatory cytokines and chemokines to mediate the cross-talk between innate and adaptive immunity (reviewed in Ref. 2). Surprisingly, the inflammatory cytokine expression including TNF-α, IL-6, and IL-1β was negatively correlated with Wip1 expression in the isolated neutrophils of sepsis patients as detected by real-time PCR (Fig. 1C). In contrast, Wip1 mRNA and protein expression in mouse neutrophils was quickly and gradually downregulated when these cells were stimulated with LPS in dose- and time-dependent manners (Fig. 1D–F). Other inflammatory cytokines such as TNF-α, IFN-γ, and IL-8 decreased Wip1 expression as well (p < 0.001; Fig. 1G). These results indicate that the downregulation of Wip1 expression in neutrophils might be specific to inflammatory response.

It is known that the upregulation of Wip1 expression can be induced by p53, p38 MAPK, and E2F1 (29). However, we know much less about the downregulation pathways for Wip1 expression, except of one recent report showing that microRNA-16 directly inhibits Wip1 expression in tumor cells (30). To identify the signal pathways to downregulate Wip1 transcription, we used chemical inhibitors to specifically block the intracellular signal molecules downstream of TLR4. Inhibiting p38 MAPK and ERK failed to rescue LPS-caused downregulation of Wip1, but inhibiting JNK and NF-κB significantly reversed LPS-reduced Wip1 expression (Fig. 1H). Meanwhile, the expression of microRNA-16, which has been identified to inhibit Wip1 expression by directly targeting 3’-untranslated region of Wip1 in tumor cells (30), was remarkably upregulated during neutrophil activation. Inhibition of JNK and NF-κB by a specific chemical inhibitor significantly blocked LPS-induced microRNA-16 expression (p < 0.01; Fig. 1J). In addition, blocking JNK and NF-κB also partially reversed the downregulation of Wip1 and upregulation of microRNA-16 caused by TNF-α or IFN-γ treatment (data not shown). Thus, TLR4 ligands and inflammatory cytokines downregulate Wip1 expression through upregulation of microRNA-16 by JNK and NF-κB activation in neutrophils.

The downregulated expression of Wip1 during the early stage of neutrophil activation as well as the negative correlation between Wip1 and inflammatory cytokines expression in neutrophils prompted us to hypothesize that Wip1 might be an important intrinsic regulator for neutrophil function. To address whether Wip1 is involved in neutrophil inflammatory response, we treated mouse neutrophils with Wip1 inhibitor (2,5-bis-(2-thienylidene)cyclopentanone, number 529578; Merck) (31). Inhibition of Wip1 significantly enhanced TNF-α, IL-1β, and IL-6 mRNA expression as determined by real-time PCR (p < 0.01; Fig. 1K). The LPS-induced IL-1β, IL-6, and TNF-α protein expression in mouse neutrophils was also increased with the addition of Wip1 inhibitor in a dose-dependent manner (Fig. 1L). Thus, Wip1 negatively controls neutrophil inflammatory activation.

Wip1 is a key molecule suppressing neutrophil-mediated acute inflammation

To further determine the effects of Wip1 in neutrophil functions, we employed Wip1-deficient mouse models. Wip1KO neutrophils produced significantly more inflammatory cytokines including...
TNF-α, IL-12, IL-6, and IL-1β than WT neutrophils after the treatment with LPS in vitro (Fig. 2A). To test the hyperresponse of Wip1KO neutrophils in a more acute inflammatory setting, the LPS-induced acute lung damage (ALD) model was used. As shown in Fig. 2, Wip1KO mice showed more severe innate immune-mediated ALD than WT mice after LPS challenge for as short as 2 h, determined by pathological changes (Fig. 2B), increased ratio of wet to dry lung weight (Fig. 2C), significantly higher MPO activity in the lungs (Fig. 2D), the increased bronchoalveolar lavage (BAL) cell number (Fig. 2E) and higher percentage of PMNs in BAL cells (Fig. 2F) in Wip1KO mice. To characterize immune cell populations involved in mediating ALD, we next analyzed the infiltrating inflammatory cells in lungs. There were similar percentages and absolute numbers of PMNs in BAL cells in LPS-untreated Wip1KO mice and WT mice, though the levels in both mice were low (Fig. 2F, data not shown). Impressively, there were markedly higher percentages and absolute cell numbers of CD11b+Ly6G+ or CD11b+Ly6C- infiltrating neutrophil-like cells in BAL cells of Wip1KO mice compared with those in WT mice (Fig. 2F, Supplemental Fig. 1A). The cell numbers of other immune subsets including macrophages, dendritic cells, NK cells, B cells, and T cells were similar in WT and Wip1KO mice after LPS treatment. However, the percentages of these immune cells in BAL cells in LPS-treated Wip1KO mice were decreased (Supplemental Fig. 1A, 1B), possibly caused by the increased percentage of neutrophils in BAL cells in Wip1KO mice. Furthermore, we compared cytokine mRNA expression in lung samples from Wip1KO and WT mice with or without LPS treatment (Fig. 2F, Supplemental Fig. 1A). The results represent one of three independent experiments performed. Data in (A) were analyzed by Student t test. Other data were analyzed by one-way or two-way ANOVA analysis using SPSS software. **p < 0.01, ***p < 0.001 compared with the control group or the indicated group.
without LPS treatment by real-time PCR. Only basal levels of these molecules could be detected in both mice without LPS challenge. However, after LPS treatment, the expression of inflammatory cytokines such as TNF-α, IL-6, and IL-1β was greater in Wip1KO mice than in WT mice at all tested time points (**p < 0.01; Fig. 2G).

To define whether Wip1 intrinsically controls neutrophil acute inflammation response, we performed ALD assays in full bone marrow chimera mice established by the transfer of WT or Wip1KO mouse BMCs into lethally irradiated WT mice. Mice were treated with LPS 12 wk after bone marrow transfer as reported previously (16). WT recipients who received Wip1KO BMCs showed more severe lung tissue damage and significant histological inflammatory cell infiltration than WT mice who received WT BMCs by 2 h after LPS treatment (Fig. 2H–J). Consistently, FCM analysis displayed that neutrophils were the most abundant cells in the pathological lungs (Fig. 2K). Thus, Wip1 deficiency in hematopoietic cells exacerbates innate cell-mediated ALD. In contrast, the expression of CXCR2 on neutrophils in the lungs of Wip1KO mice was higher than those in WT mice after LPS treatment (Supplemental Fig. 1C). The expression of CXCR2 on neutrophils in the lungs of Wip1KO mice was higher than those in WT mice after LPS treatment (Supplemental Fig. 1C).

To further determine the altered inflammatory function of Wip1-deficient neutrophils, we used sterile TG-induced peritonitis, which served as a well-established in vivo model to study neutrophil migration and inflammation in the early stage (32). By 6 h after injection of 1 ml 3% TG into WT and Wip1KO mice, peritoneal cells were harvested and analyzed. A significantly higher percentage and cell number of recruited neutrophils were observed in the peritoneal exudate cells of Wip1KO mice compared with WT control (***p < 0.001; Fig. 3A, 3B). These recruited neutrophils in Wip1-deficient mice expressed slightly higher levels of CXCR2, CD80, CD18, and MHC II molecules after TG induction (Fig. 3C), although neutrophils isolated from untreated WT and Wip1KO mice expressed identical surface molecules such as Ly6G, CD11b, TLR2, TLR4, MHC II, CD80, CD86, CD18, and CD62L (data not shown). Importantly, after TG injection, recruited Wip1KO neutrophils expressed significantly higher inflammatory cytokines including TNF-α, IL-12, IL-6, and IL-1β as determined by real-time PCR and intracellular staining FCM assays (Fig. 3D, 3E).
Altogether, these results demonstrate that Wip1 deficiency significantly enhances local neutrophil infiltration and promotes neutrophil-mediated acute inflammation in vivo. Wip1 is a repressor of neutrophil function against bacteria. Neutrophils play a key role against bacterial infections. We observed the ability of Wip1KO neutrophils to phagocytize and kill S. aureus, whose infection is one of the leading causes of mortality among infectious diseases (33). As shown in Fig. 4, Wip1KO neutrophils showed identical phagocytosis of S. aureus and subsequent killing ability for S. aureus in vitro (Fig. 4A–C). Furthermore, we performed the in vivo functional assay using a nonlethal skin abscess model of S. aureus infection in which S. aureus clearance is mediated predominantly by neutrophils within the first 48 h of infection (34). As predicted, by 6 h postinfection, neutrophils represented the most prominent cell type within the infiltrates (Fig. 4D). Notably, Wip1KO neutrophils were recruited to the infected air pouch more efficiently than WT control (p, 0.001; Fig. 4D–F). Few other immune cells including macrophages, T cells, dendritic cells, and B cells were presented by 12 h postinfection (Fig. 4E). Wip1KO mice showed almost complete clearance of the bacteria by 12 h, whereas the S. aureus remained high in WT mice (p < 0.001; Fig. 4F). Moreover, the mRNA expression of granule proteins, including elastase, lactoferrin, MMP9, and MPO, was significantly higher in the infected local neutrophils of Wip1KO mice than in WT mice (p < 0.001; Fig. 4H), which is nicely correlated with the enhanced degranulation response of Wip1KO neutrophils (Fig. 4I). Taken together, our results indicate that Wip1 acts as an important negative modulator of neutrophil ability against bacterial infection in vivo.

Wip1 negatively controls neutrophil function through multiple signal pathways. Wip1KO neutrophils freshly isolated from peritonitis models showed significantly higher phosphorylation of p38 MAPK, STAT1, STAT3, and p65 than WT controls (Fig. 5A). Consistently, after treatment with LPS or TNF-α, neutrophils from Wip1KO mice showed normal phosphorylation of ERK but more phosphorylation of p38 MAPK, STAT1, STAT3, and p65 than WT neutrophils (Fig. 5B, data not shown). p65 expression was higher in the nuclei of Wip1KO neutrophils after LPS stimulation compared with WT cells (Fig. 5C). The increased NF-κB activity is easily expected to be one of the key reasons for the increased neutrophil activity in Wip1KO neutrophils, based on current literatures (35). In addition, some reports have found that p38 MAPK, ERK, and STAT pathways could promote the neutrophil functional activities as well (36–39).

To see whether the hyperactivity of Wip1KO neutrophils is mediated by these pathways, we treated neutrophils with pharmacological specific inhibitors during neutrophil activation. Inhibition of p38 MAPK and STAT1 by SB203580 and MTA, respectively, significantly rescued the Wip1-mediated hyperactivity of neutrophils to TNF-α in terms of elastase, lactoferrin, MMP9, and MPO expression, but inhibition of ERK by U0126 failed to do so (p, 0.01; Fig. 5D, 5E), whereas these inhibitors efficiently blocked the corresponding pathways (data not shown). These results indicate that p38 MAPK-STAT1 and NF-κB pathways are at least partially in-

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**FIGURE 3.** Increased neutrophil recruitment to 3% TG-induced acute peritonitis site in Wip1KO mice. WT and Wip1KO mice were injected with 1 ml 3% TG i.p., and peritoneal cells were collected and characterized by FCM after 6 h. Representative FCM staining was shown (A), and the summarized cell numbers in peritoneal cavity were presented (B). Wip1 deficiency significantly recruits more neutrophils in peritoneal cavity in acute sterile peritonitis (bar, mean ± SD n = 5). The expression levels of cell surface molecules including TLR2, TLR4, CXCR2, CD80, CD86, CD62L, CD18, and MHC II on the gated CD11b+/Ly6G+ neutrophils assayed by FCM (C). The mRNA (D) and protein (E) expression of inflammatory cytokines, including TNF-α, IL-12, IL-6, and IL-1β in WT and Wip1KO neutrophils, were detected by real-time PCR and FCM. Data represent one of three independent experiments with similar results. Data were analyzed by two-way ANOVA analysis using SPSS software. ***p < 0.001 compared with the control group.
involved in Wip1-mediated regulation of neutrophil inflammatory function.

p53 is an important regulating target for Wip1 (40). To exclude the possible involvement of p53 in the hyperactivity of Wip1−/− neutrophils, we observed the function of neutrophils from Wip1−/− p53−/− mice. P53 deficiency did not cause significant changes for enzyme release of neutrophils and also failed to rescue Wip1 deficiency–induced functional alteration of neutrophils (Supplemental Fig. 2). Thus, p53 is very unlikely involved in Wip1-mediated regulation on neutrophil function.

Wip1 intrinsically controls neutrophil migration through p38 MAPK–mediated decreasing CXCR2 internalization

The chemokine CXCL-1 and CXCL-2 expression in the lung tissues was identical in Wip1KO and WT mice by 2 h after LPS treatment (Supplemental Fig. 3). After observing that neutrophil infiltration into the inflammation sites was significantly enhanced in S. aureus–infected Wip1−/− p53−/− mice. P53 deficiency did not cause significant changes for enzyme release of neutrophils and also failed to rescue Wip1 deficiency–induced functional alteration of neutrophils (Supplemental Fig. 2). Thus, p53 is very unlikely involved in Wip1-mediated regulation on neutrophil function.

CXCR2 expression. The expression of CXCR2 on neutrophils was increased during its maturation, but Wip1KO immature and mature neutrophils expressed higher levels of CXCR2 than WT cells (Supplemental Fig. 4A, 4B). In contrast, CXCR4 expression on Wip1KO neutrophils was decreased (data not shown). Thus, increased CXCR2 expression may contribute to enhanced migration of Wip1KO neutrophils to CXCL1. In contrast, it is known that CXCL1 induces CXCR2 desensitization by promoting CXCR2 internalization (41). The decreased expression of CXCR2 on neutrophils induced by CXCL1 and LPS significantly was prevented in Wip1KO neutrophils (Fig. 6F, Supplemental Fig. 4C, 4D). To clarify the molecular mechanism by which Wip1 deficiency prevents CXCR2 internalization on neutrophils, we studied the role of p38 MAPK, which was hyperactivated in Wip1KO neutrophils, in the CXCR2 internalization. The pretreatment of neutrophils with p38 MAPK inhibitor enhanced CXCL1-induced CXCR2 internalization in Wip1KO neutrophils (Fig. 6G), whereas p38 MAPK activator reduced CXCL1-induced CXCR2 internalization in WT neutrophils (Fig. 6H). Consistent with the CXCR2 expression, inhibition of p38 MAPK significantly blocked...
neutrophil migration in the Transwell assays, whereas inhibition of ERK, JNK, and STAT1 failed to significantly reverse the enhanced migration of Wip1KO neutrophils (Fig. 6I). To see whether the inhibitory effects of p38 MAPK on CXCR2 internalization is a direct action or not, we detected the interaction of p38 MAPK and CXCR2 using immunoprecipitation (IP) assays. Overexpressed CXCR2-GFP had direct interaction with p38 MAPK in 293T cells as determined by IP assays (Fig. 6J). Importantly, activated p38 MAPK by its activator anisomycin enhanced the direct interaction with CXCR2 in primary neutrophils as detected by IP assays (Fig. 6K). Thus, Wip1KO neutrophils are resistant to the CXCR2 desensitization caused by CXCL1 through the enhanced direct inhibiting effects of p38 MAPK on the internalization of CXCR2.

Discussion

In the current study, we found that Wip1 expression in neutrophils was downregulated during bacterial infection and its expression levels were negatively correlated with the inflammatory cytokine productions of neutrophils in clinical sepsis patients. TLR ligand- and cytokines-activated JNK-microRNA-16 pathway contributes to the infection-reduced Wip1 expression in neutrophils. With Wip1KO mouse models, we provide evidence that Wip1 negatively modulates neutrophil immune response including migration and releasing inflammatory/chemokine factors to pathological sites in an intrinsic manner. Therefore, this study identified one previously unknown regulating pathway for neutrophil defense response.

Neutrophils play a fundamental role in the innate immune response against pathogens through phagocytosis and production of reactive oxygen species and lytic enzymes with potent antimicrobial activity (3). Wip1KO neutrophils had similar phagocytosis ability against Gram-positive S. aureus in vitro as WT neutrophils. However, our previous studies showed that Wip1KO neutrophils had enhanced phagocytosis ability to Gram-negative E. coli in vitro (16). The reasons for the inconsistency are not clear now. The different bacteria strains like Gram-positive and -negative stains used in these studies may be one of the reasons for the different results. However, Wip1-deficient mice showed the decreased bacteria survival and significantly increased infiltrated neutrophils in the infection sites. The increased neutrophil infiltration might contribute to the increased resistance to S. aureus infection of Wip1KO mice in this model. Neutrophils in Wip1-deficient mice produced more cytokines (IL-6, TNF-α, IL-1β, and so on) and granule proteins (elastase, lactoferrin, MPO, and MMP9) after LPS stimulation. In line with this hyperactivity of neutrophils in vitro, Wip1-deficient mice were more susceptible to neutrophils-mediated acute inflammation in ALD and TG-induced peritonitis models. NF-κB, p38 MAPK, and STAT1 but not ERK, p53, and JNK were markedly enhanced in Wip1-deficient neutrophils. Inhibiting p38 MAPK by SB203580, a specific pharmacological inhibitor of p38α and p38β, and STAT1 by MTA significantly reversed the hyperactivity of Wip1-deficient neutrophils when stimulated with LPS or TNF-α. These results are
trophils showed similar function as WT cells. Wip1
same host. Data are one representative of three independent experiments. (C) The migratory motility of sorted neutrophils from WT and Wip1KO mice in migration assays with fMLP in vitro were summarized. (D) Sorted WT and Wip1KO CD11b+Ly6G+ neutrophils were labeled with CFSE and PKH, respectively, and were then i.v. injected to syngeneic mice for the in vivo recruiting assays. (E) More Wip1KO neutrophils were detected in the TG-injected peritoneal cavity than WT cells in the same host. Data are one representative of three independent experiments. (F) Wip1KO neutrophils had slower CXCR2 internalization than WT cells after CXCL1 stimulation. (G) Inhibiting p38 MAPK activity by 5 μM SB203580 significantly increased CXCR2 internalization of Wip1KO neutrophils. (H) Activation of p38 MAPK decreased CXCR2 internalization of WT neutrophils during CXCL1 stimulation. (I) Inhibiting p38 MAPK activity significantly blocked both WT and Wip1KO neutrophil migration in Transwell assays. A total of 10 μM U0126 (ERK inhibitor), SP600125 (JNK inhibitor), 5 μM SB203580 (p38 inhibitor), and 10 μM MTA (STAT1 inhibitor) were used in this assay. Data are shown as mean ± SD (three samples per group) and represent one of two independent experiments. (J) The direct interaction of p38 MAPK with the overexpressed CXCR2 in 293T cells as determined by co-IP assays. (K) Activation of p38 MAPK by its activator anisomycin increased the direct interaction of p38 MAPK with CXCR2 in neutrophils as determined by co-IP assays. One representative of two independent experiments is shown. Data in (A, I) were analyzed by two-way ANOVA analysis. Data in (C, E) were analyzed by independent samples t test using SPSS software. ***p < 0.001 compared with WT control.

parallel well with previous reports showing the critical role of p38 MAPK-STAT1 and NF-κB signaling pathways in innate immunity (12, 42, 43). It is reported that the activated NF-κB by TNF-α could directly interact with the promoter region of the human MMP-9 gene (44). p38 MAPK is closely involved in the expression of MMP-9, MPO, superoxide, and elastase in neutrophils or B-CLL cells (45–48). Thus, we conclude that the enhanced function of Wip1-deficient neutrophils may be mainly mediated by the increased p38 MAPK-STAT1 and NF-κB pathways.

It is demonstrated that the activity of p53, which plays a central role in preserving genomic integrity, is one of the key target molecules attenuated by Wip1 in many different type cells (40, 49). However, we failed to detect alteration of p53 activity in Wip1-deficient neutrophils of mice (16). Importantly, p53−/− neutrophils showed similar function as WT cells. Wip1−/−/p53−/− mice displayed identical function of neutrophils in bone marrow and peripheral blood as Wip1KO mice. Therefore, Wip1 negatively controls neutrophils via a p53-independent mechanism. It is sharply distinct from the positive regulatory role of Wip1 in T cell development in the thymus, which is dependent on the presence of p53 (50) and its oncogene activity (10). The opposite effects of Wip1 on neutrophils and other cells may be due to the distinct expression pattern of Wip1 and the different roles of p53 and p38 MAPK pathways in these cells (18).

As early as the initiation of infection and inflammatory response, neutrophil migration to the challenge sites is essential and results mainly from the release of neutrophil chemoattractant factors by resident cells, including CXC chemokines such as CXCL1 and inflammatory mediators like TNF-α (51). The lung tissues expressed similar levels of CXCL1 and CXCL2 in WT and Wip1KO mice or in WT→WT and Wip1KO→WT full chimera mice in the early stage of LPS stimulation. These results suggest that locally increased chemokines in Wip1KO mice may not be the key reason for the enhanced neutrophil infiltration in the lungs after LPS injection. Significantly enhanced neutrophil infiltration into the inflammation sites was observed in Wip1KO mouse models and/or Wip1KO bone marrow–reconstituted WT mouse models of S. aureus infection, LPS-induced ALD, and TG-induced peritonitis. In parallel, significantly increased CXCR2 expression and impeded CXCR2 internalization in Wip1-deficient neutrophils were observed when stimulated with chemokine CXCL1, relative to WT cells. Therefore, the resistance to CXCR2 internalization and desensitization of neutrophils may be one of the major reasons for the increased neutrophils in the inflammatory sites in Wip1KO mice.

An important feature of G protein–coupled receptors, including chemokine receptors, is their rapid internalization from plasma membrane into endosomal cell compartments and subsequently their desensitization after stimulation. CXCR2 is downregulated
after stimulation with CXCL1 or CXCL8 (41). This event may be responsible for the downregulation of chemokine effects like chemotaxis (52). It has been reported that receptor activation induces an increase in the G protein–coupled receptor kinase, which in turn phosphorylates G protein–coupled receptors to signal receptor internalization and desensitization (53, 54). In contrast, p38 MAPK activity directly inhibits G protein–coupled receptor IMLP internalization (54, 55). Wip1-deficient neutrophils showed poor CXCR2 internalization and desensitization as evaluated by the membrane expression after CXCL1 stimulation. Our preliminary study showed similar p-G protein–coupled receptor kinase levels in Wip1KO and WT neutrophils after CXCR2 ligand, CXCL1, treatment as determined by Western blot assays (data not shown). However, p-p38 MAPK activity in Wip1KO neutrophils was significantly higher than in WT neutrophils. Inhibition of p38 MAPK significantly rescued the decreased CXCR2 internalization of Wip1KO neutrophils and the enhanced migration of these cells as evaluated in Transwell assays. Biochemical and functional assays showed that activated p38 MAPK could interact with CXCR2 directly and activation of p38 MAPK inhibited CXCR2 internalization in neutrophils. These data collectively suggest that the enhanced p38 MAPK activity caused by Wip1 deficiency likely contributes to the resistance to CXCL1–induced CXCR2 internalization and desensitization and subsequently the enhanced migratory ability of Wip1KO neutrophils.

In summary, the expression of phosphatase Wip1 was regulated dynamically during neutrophil activation. Our data support a model in which Wip1 acts as a key negative fine-turner of the migration and function of granulocytic cells mainly by NF-kB and p38 MAPK-mediated activation of p16(Ink4a)-p19(Arf) pathway. Mice deficient for the wild-type p53-induced phosphatase gene (Wip1) exhibit defects in reproductive organs, immune function, and cell cycle control. Mol. Cell. Biol. 22: 1094–1103.

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Disclosures

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References


