TIPE2 protein serves as a negative regulator of phagocytosis and oxidative burst during infection

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Phagocytosis and oxidative burst are two major effector arms of innate immunity. Although it is known that both are activated by Toll-like receptors (TLRs) and Rac GTPases, how their strengths are controlled in quiescent and TLR-activated cells is not clear. We report here that TIPE2 (TNFAIPBL2) serves as a negative regulator of innate immunity by linking TLRs to Rac. TLRs control the expression levels of TIPE2, which in turn dictates the strengths of phagocytosis and oxidative burst by binding to and blocking Rac GTPases. Consequently, TIPE2 knockout cells have enhanced phagocytic and bacterial activities and TIPE2 knockout mice are resistant to bacterial infection. Thus, TIPE2 sets the strengths of phagocytosis and oxidative burst and may be targeted to effectively control infections.

Results and Discussion

Phagocytosis and oxidative burst (or respiratory burst) are two fundamental effector mechanisms of innate immunity that work in concert to eliminate infectious microbes (1, 2). Phagocytosis allows the phagocytes of the immune system (monocytes and granulocytes) to engulf infectious microbes and to contain them in a special vacuole called a phagosome. Oxidative burst in turn injects the vacuole reactive oxygen species (ROS) (e.g., superoxide radical and hydrogen peroxide) that kill the microbes. Deficiency in either of these innate immune mechanisms leads to immune deficiency and uncontrolled infections (3–6).

Both phagocytosis and oxidative burst are controlled by the Rac proteins of the Ras small GTPase superfamily (1–4). There are three mammalian Rac GTPases, which are designated as Rac1, Rac2, and Rac3. Small GTPases are enzymes that hydrolyze GTP. They are active when bound to GTP and inactive when bound to GDP and serve as molecular “on-and-off” switches of signaling pathways that control a wide variety of cellular processes including growth, motility, vesicle trafficking, and death (7). Rac GTPases control phagocytosis by promoting actin polymerization through their effector proteins such as p21-activated kinases (PAKs), WASP family Verprolin homology domain-containing protein (WAVE), and IQ motif containing GTPase-activating protein-1 (IQGAP1) (1). Rac GTPases also mediate ROS production by binding and activating the NADPH oxidase complex through the p67(Phox) protein (1). Rac GTPase deficiency in mice and humans leads to an immune-deficient syndrome, which is characterized by defective phagocytosis and oxidative burst, recurrent infection, and granulomas (3–6).

Although quiescent phagocytes are capable of phagocytosis and ROS production, their levels are low. Toll-like receptor (TLR) activation or microbial infection significantly up-regulates these innate immune processes (8–11). However, the mechanisms whereby microbes promote them are not well understood. TIPE2, or tumor necrosis factor-α-induced protein 8 (TNFAIP8)-like 2 (TNFAIP8L2), is a member of the TNFAIP8 family, which is preferentially expressed in hematopoietic cells (12–18). It is significantly down-regulated in patients with infectious or autoimmune disorders (15, 19). The mammalian TNFAIP8 family consists of four members: TNFAIP8, TIPE1, TIPE2, and TIPE3, whose functions are largely unknown (14, 20). We recently generated TIPE2-deficient mice and discovered that TIPE2 plays a crucial role in immune homeostasis (14). We report here that TIPE2 controls innate immunity by targeting the Rac GTPases.

TIPE2 Binds to Rac GTPases Through Their C-Terminal CAAX Motif. To identify TIPE2-interacting proteins, we performed large-scale coimmunoprecipitation and mass spectrometry screenings using RAW 264.7 cells. Among the peptides identified, 16 were shared by the small GTPases Rac1 and Rac2, members of the Rho family. Both Rac1 and Rac2 are expressed in neutrophils and macrophages. They share 92% identity in primary sequences and perform crucial functions in innate immune responses (2).

To determine whether TIPE2 indeed interacts with the Rac GTPases in mammalian cells, we undertook four complementary approaches. First, we expressed Flag-tagged ‘TIPE2 and Myc-tagged Rac1 or Rac2 in 293T cells and, by coimmunoprecipitation (co-IP) analyses, we found that TIPE2 interacted with both Rac1 and Rac2 (Fig. 2A). Second, we determined whether endogenous TIPE2 interacts with endogenous Rac. Murine RAW 264.7 macrophage extracts were used to immunoprecipitate Rac-
binding proteins with anti-Rac1/2/3 polyclonal antibodies. Upon blotting with anti-TIPE2 antibody, a strong TIPE2 signal was detected in the precipitates (Fig. 2A), indicating that TIPE2 is constitutively associated with Rac proteins in nonstimulated immune cells. By contrast, in the same RAW cells, we did not detect any interaction between endogenous TIPE2 and several other GTPases, including Cdc42, RhoA, RalA, and HRas, suggesting that the TIPE2–Rac interaction is specific. Third, we tested whether TIPE2 and Rac might directly interact with each other. TIPE2 and Rac1 proteins were synthesized separately, using an in vitro transcription–translation system. After mixing them together, we found that TIPE2 physically associated with Rac1, indicating that the two proteins might directly bind to each other (Fig. 2C and D). Finally, using both GDP and GTP forms of Rac1 (22, 23), we showed that TIPE2–Rac interaction was not affected by the Rac1-activating status; i.e., TIPE2 binds to both GDP and GTP forms of Rac1 (Fig. 2E).

To map the region within Rac that is responsible for TIPE2 interaction, we generated full-length Rac1 (amino acids 1–192) and a series of deletion constructs in frame with an HA tag (Fig. 3A). Among five deletion Rac1 mutants we generated, two (with amino acids 48–123 deletion and amino acids 136–161 deletion) were unstable in cells and could not be further studied. By contrast, Rac1 mutants without the N terminus (amino acids 1–47), the insert region (amino acids 124–135), or the C terminus (amino acids 162–192) were stable and therefore were used in this study. By co-IP, we found that the C terminus, but not the N terminus or the insert region, was required for TIPE2 interaction (Fig. 3B).

The C terminus of Rac contains a polybasic region (PBR) and a CAAX motif. To determine which of these elements is involved in TIPE2 binding, we made two additional Rac1 mutants, one without the PBR and one without the CAAX motif. By co-IP, we found that the CAAX motif, but not the PBR, was required for TIPE2 interaction (Fig. 3C). The cysteine residue, Cys-189, in the C-terminal CAAX motif is crucial for the posttranslational modification and function of Rac (23). Using a point mutant form of Rac1, Rac1C189S, in which Cys-189 is replaced by serine, we found that TIPE2 binding required Cys-189 (Fig. 3D).

**TIPE2 Inhibits Rac Membrane Translocation, Rac Activation, and Downstream Rac Signaling.** The hydrophobic C-terminal region of Rac is responsible for targeting and anchoring Rac to the plasma membrane. Our finding that TIPE2 binds to the C terminus of Rac suggests that it may regulate membrane translocation of Rac. To test this possibility, we transiently expressed increasing amounts of TIPE2 in 293T cells. At 8 h after transfection, TIPE2 did not induce a significant amount of cell death (Fig. S1), but reduced the membrane-bound, not the cytosolic, endogenous Rac in a dose-dependent manner (Fig. 3E). The TIPE2 N-terminal lysine or arginine residues, Lys-15, Lys-16, and Arg-24, are important for TIPE2–Rac1 interaction because replacing them with glutamine or alanine markedly reduced the Rac1 binding (Fig. 3F). By contrast, Lys-20 mutation did not affect the binding. As a consequence, the TIPE2 K15/16Q and R24A mutants were not as effective as wild-type TIPE2 in inhibiting Rac membrane translocation (Fig. 3G). As stated above, LPS markedly reduced TIPE2 levels in macrophages. This reduction was associated with increased Rac1 membrane translocation in LPS-treated cells (Fig. 3H), indicating that TIPE2 regulates Rac membrane translocation.

Because plasma membrane translocation of small GTPases is required for their activation, these results indicate that TIPE2 may regulate Rac activation. To test this hypothesis, we undertook three complementary approaches. First, using confocal microscopy and an antibody that recognized GTP-Rac but not GDP-Rac, we examined the levels of membrane-bound GTP-Rac in 293T cells that did or did not receive TIPE2 transfection. We found that 293T cells exhibited prominent endogenous GTP-Rac staining on the plasma membrane, which was significantly blocked in cells transfected with

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Fig. 1. TLR stimulation and bacterial infection markedly diminish TIPE2 expression. (A) Wild-type (WT) bone marrow-derived macrophages (BMDMs) were stimulated with lipopolysaccharide (100 ng/mL), Poly(I:C) (10 μg/mL), and Zymosan A (100 μg/mL) for 2 h. TIPE2, IL-6, TNFα, and IFNγ mRNA levels were determined by real-time PCR. (B and C) WT BMDMs (B) or RAW 264.7 cells (C) were infected with *Escherichia coli* or *Listeria monocytogenes* at a multiplicity of infection (MOI) of 10 for 2 h. The mRNA levels of indicated genes were determined by real-time PCR. Error bars represent the SDs of the means. *P < 0.05, **P < 0.01.

Fig. 2. TIPE2 interacts with Rac via the C-terminal region (amino acids 124–192) of Rac1. **A** WT BMDMs were treated with LPS (100 μg/mL) and Zymosan A (100 μg/mL) or Poly(I:C) (10 μg/mL) for 2 h. TIPE2, IL-6, TNFα, and IFNγ mRNA levels were determined by real-time PCR. Error bars represent the SDs of the means. **B** WT BMDMs were infected with or without the indicated bacteria for 8 or 16 h. TIPE2 protein levels were analyzed by Western blot. **C** WT BMDMs were infected with or without the indicated bacteria for 8 or 16 h. TIPE2 protein levels were analyzed by Western blot. Statistics were performed on pooled data from three independent experiments. Error bars represent the SDs of the means. *P < 0.05, **P < 0.01.

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were synthesized in vitro, using the TNT transcription system and analyzed by SDS/PAGE and Western blotting. (E) Lysate was immunoprecipitated with anti-Myc or Ig control and then analyzed by SDS/PAGE and Western blotting. (F) Lysate was immunoprecipitated with anti-Flag or control Ig and analyzed by SDS/PAGE and Western blotting.

In 293T cells, we found that constitutively active Rac expression regulated Rac activity through protein expression or degradation. (C) [35S]Methionine-labeled TIPE2 and unlabelled Myc-tagged Rac1 were synthesized in vitro, using the TNT transcription-translation system. The mixture was immunoprecipitated with anti-Myc or Ig control and then analyzed by SDS/PAGE and Western blotting. (Upper) Autoradiograph of the immunoprecipitates on SDS-PAGE; (Lower) anti-Myc immunoblotting result. (D) In vitro synthesized [35S]Methionine-labeled Rac1 and Flag-tagged TIPE2 mixture were immunoprecipitated with anti-Flag or control Ig and analyzed as in C. (E) 293T cells were transiently transfected with Flag-TIPE2, Myc-tagged Rac1-17N, or Rac1-61L. Eighteen hours later, lysates of Myc-Rac1-17N- and Myc-Rac1-61L-transfected cells were loaded with GDP and GTP, respectively, and mixed with lysates of Flag-TIPE2-transfected cells. After immunoprecipitation with anti-Flag or control Ig, the samples were subjected to Western blotting with antibodies for the indicated antigens. The experiments were repeated at least three times with similar results.

TIPE2, but not in cells transfected with a control GFP plasmid (Fig. 4A). Second, using a GST pull-down assay that specifically recognized active GTP-bound Rac, we tested the TIPE2 effect in 293T cells that did or did not express a constitutive active form of HRas (HRas12V). We found that TIPE2 transfection decreased the Rac-GTP levels in both cell types (Fig. 4B). Third, we tested the effect of TIPE2 deficiency on Rac activation in macrophages. We found that resting TIPE2-deficient murine macrophages exhibited an increase in active Rac levels over wild-type controls. The difference was more dramatic upon the activation of the Rac pathway with fibronectin (Fig. 4C). The total Rac levels were not affected by TIPE2 overexpression or deficiency, indicating that TIPE2 may not regulate Rac activity through protein expression or degradation.

A number of studies have demonstrated that Rac can activate both the c-Jun N-terminal kinase (JNK) and the PAK pathways (24). In 293T cells, we found that constitutively active Rac expression did not induce JNK and PAK activation as reflected by increased phosphorylation; this effect was blocked by TIPE2 in a dose-dependent manner (Fig. 4D). Active Rac1 can induce the assembly of a filamentous (F)-actin structure through PAK-dependent and -independent signals (24). Because polymerization of F-actin can be induced by LPS, we next examined the effect of TIPE2 deficiency on LPS-induced F-actin polymerization. We found that in TIPE2-deficient BMDMs, the F-actin polymerization rate was significantly enhanced compared with that in wild-type cells (Fig. 4E). A similar phenotype was observed in TIPE2-deficient splenocytes (21).

**TIPE2 Inhibits the Basal Level of Phagocytosis Through Rac, and TIPE2 Down-Regulation Contributes to TLR-Induced Augmentation of Phagocytosis**. Rac proteins are key molecular switches of phagocytosis (2). To determine the roles of TIPE2 in phagocytosis, we used both TIPE2 loss-of-function and gain-of-function approaches. Thus, macrophages were first generated from WT and Tipe2-deficient murine bone marrow. They showed no differences in surface marker expression or adhesion phenotype between WT and Tipe2-deficient murine bone marrow. They showed no differences in surface marker expression or adhesion phenotype. However, when stimulated with LPS, TIPE2-deficient macrophages exhibited an increase in phagocytosis (21).

Figure 2. TIPE2 interacts with Rac GTPase. (A) 293T cells were transfected with pRKS vector or expression plasmids for Flag-tagged TIPE2 and Myc-tagged Rac1 or Rac2 as indicated. Eighteen hours later, cell lysates were prepared and immunoprecipitated with anti-Flag, anti-Myc, or control Ig. The precipitates and cell lysates were analyzed by SDS/PAGE and Western blotting. (B) Total cell lysates of RAW 264.7 cells were immunoprecipitated with rabbit polyclonal anti-Rac1/2/3 or control rabbit Ig. The immunoprecipitates and cell lysates were then blotted with anti-TIPE2 or anti-Myc antibodies. (C) Total cell lysates of RAW 264.7 cells were immunoprecipitated with anti-Flag or control Ig, and then analyzed by SDS/PAGE and Western blotting. The experiments were repeated at least three times with similar results.

Figure 3. TIPE2 binding to Rac requires the C-terminal CAAX motif, and TIPE2 inhibits Rac membrane translocation. (A) Schematic diagram of the Rac1 protein. (B–D) Lysates of 293T cells transiently transfected with HA-Rac1 constructs and Flag-TIPE2 construct were immunoprecipitated with anti-Flag and subjected to Western blotting. (E) 293T cells were transiently transfected with increasing amounts of TIPE2 plasmids for 8 h. Cell lysates were separated into membrane and cytosolic fractions. Equal amounts of cytosolic and membrane materials (3 μg) were separated by SDS/PAGE and immunoblotted for integrin β1, β-actin, and Rac. (F) Lysates of 293T cells transiently transfected with or without HA-Rac1 construct and Flag-TIPE2 constructs were immunoprecipitated and subjected to Western blotting. (G) 293T cells were transiently transfected with or without the indicated TIPE2 plasmids. Equal amounts of cytosolic and membrane protein fractions were subjected to Western blotting. The experiments were repeated at least three times with similar results.
expression (Fig. S2). They were then fed with apoptotic thymocytes from GFP transgenic mice. Thirty minutes later, the engulfment of GFP-positive cells was measured by flow cytometry. Remarkably, Tipe2 deficiency increased the basal phagocytosis rate by approximately twofold (Fig. S4). Moreover, the mean fluorescence intensity of Tipe2-deficient GFP-positive cells was also significantly increased. Addition of the actin polymerization inhibitor, cytochalasin B, markedly reduced phagocytosis in both WT and Tipe2−/− groups, indicating that actin remodeling is required and that Tipe2 may regulate internalization rather than adhesion (Fig. S4). The increased phagocytic activity of Tipe2-deficient cells was not restricted to apoptotic cells, because Tipe2-deficient macrophages were significantly more active in engulfing fluorescently labeled 2-μm beads (Fig. 5B) and live or dead bacteria (Fig. 5C). The increase in phagocytosis of Tipe2-deficient cells was not affected by the culture media used [with or without 10% (vol/vol) fetal bovine sera or freshly prepared murine sera that contain different amounts of Ig and/or complements], indicating that the Tipe2 effect on phagocytosis may not be dependent on Fc or complement receptor use (Fig. S3). In contrast to phagocytosis, endocytosis of fluorescence-labeled dextran was not affected by Tipe2 deficiency (Fig. S4).

To directly test the effect of Tipe2 on phagocytosis, we overexpressed Tipe2 in WT and Tipe2−/− macrophages. In Tipe2−/− cells, ectopic Tipe2 reduced phagocytosis by more than fourfold, whereas in WT cells that constitutively expressed high levels of
TIPE2 it had a much smaller effect (Fig. 5D and Fig. S5). Importantly, ectopic TIPE2 completely eliminated the augmented phagocytic activity of Tipe2−/− cells compared with WT cells.

To determine whether TIPE2 regulates phagocytosis through Rac, we manipulated the Rac activity using Rac mutants and chemical blockers. Expression of a constitutively active Rac (61L) dramatically increased phagocytosis in both WT and Tipe2−/− macrophages and abolished the difference between them. On the other hand, dominant-negative Rac1 (17N) significantly reduced phagocytosis in Tipe2−/− cells and eliminated the difference between WT and Tipe2−/− cells (Fig. 5E and Fig. S6). Similarly, the Rac1 antagonist NSC23766 depressed the ability of macrophages to uptake apoptotic cells and reduced the difference between WT and Tipe2−/− groups in a dose-dependent manner (Fig. 5F).

The basal level of phagocytosis is significantly increased upon TLR activation (8, 9). Because TLRs down-regulate TIPE2 expression, we asked whether TLRs augment phagocytosis through TIPE2. WT and Tipe2−/− macrophages were therefore stimulated with LPS and their phagocytic ability was tested. As reported, LPS stimulation enhanced phagocytosis of WT cells in a time-dependent manner. However, this enhancement was significantly reduced in Tipe2−/− cells (Fig. 5G and Fig. S7). These results indicate that TIPE2 down-regulation contributes to the TLR-induced increase in phagocytosis.

**TIPE2 Inhibits Oxidative Burst, and Its Down-Regulation Contributes to TLR-Induced Augmentation of Bactericidal Activity.** Because Rac proteins are crucial for assembling the NADPH complex and for producing ROS, we next investigated whether TIPE2 regulates ROS production and bacterial killing. We found that Tipe2−/− macrophages exhibited enhanced bacterial clearance within 2 h of bacterial infection. They killed L. monocytogenes and E. coli more efficiently than WT macrophages. Similar differences were observed between Tipe2−/− and WT neutrophils (Fig. 6A).

We then assessed ROS production in bone marrow neutrophils of WT and Tipe2−/− mice, using a horseradish peroxidase (HRP)-dependent chemiluminescence assay. N-formyl-methionyl-leucyl-phe- nylalanine (fMLP), a bacterial peptide, and L. monocytogenes were used to induce ROS production. Compared with WT cells, Tipe2−/− neutrophils exhibited significantly enhanced ROS production after...
stimulation (Fig. 6B). Intracellular ROS levels were then measured in the dichlorofluorescin diacetate (DCFDA) assay (Fig. 6C). GM-CSF priming and fMLP stimulation triggered significantly more ROS production in Tipe2−/− cells than in WT cells. Similarly, when cells were challenged with Listeria, more ROS were detected in Tipe2−/− neutrophils than in WT neutrophils. Intracellular ROS production was also tested by luminol-dependent luminescence assay in the absence of HRP. Again, significant differences in the ROS production between Tipe2−/− and WT neutrophils were observed (Fig. S8).

TLR activation increases the bactericidal activity of macrophages (25, 26). We next investigated whether TIPE2 down-regulation contributes to LPS-induced enhancement of bacterial killing. We found that LPS stimulation significantly enhanced the killing ability of WT macrophages. By contrast, the LPS effect was markedly reduced in Tipe2−/− cells, indicating that TIPE2 is involved in the LPS-induced regulation of bacterial killing (Fig. 6D).

**TIPE2-Deficient Mice Are Resistant to Bacterial Infection.** In vitro, down-regulation of TIPE2 is associated with increased phagocytosis and bacterial killing. To assess the in vivo relevance of these findings, we infected Tipe2−/− and WT mice with a lethal dose of L. monocytogenes (Fig. 7A). More than 80% of WT mice succumbed within 7 d after infection, whereas all Tipe2−/− mice survived (P < 0.01). The increased mortality of WT mice was associated with enhanced bacterial growth, tissue damage, and cytokine production. At day 3 postinfection, the Listeria titer in the liver and spleen of WT mice was significantly higher than that in Tipe2−/− mice (Fig. 7B). The blood alanine transaminase (ALT) and aspartate transaminase (AST) levels are indicators of hepatic injury. After Listeria infection, the blood ALT and AST levels were significantly higher in WT than in Tipe2−/− mice (Fig. 7C). Fewer serum inflammatory cytokines were detected in Tipe2−/− mice compared with WT mice (Fig. 7D). Additionally, we also challenged the Tipe2−/− and WT mice with Staphylococcus aureus. Consistent with the Listeria experiment, Tipe2−/− mice survived for a longer time and carried significantly fewer bacteria in their liver and spleen (Fig. 7E and F). Taken together, these results demonstrate that TIPE2 inhibits immunity to bacteria.

**Methods.**

**Mice.** C57BL/6 mice that carry a Tipe2 gene null mutation were generated by backcrossing Tipe2−/− 129 mice to C57BL/6 (86) mice for 12 generations.

**Methods.** The following methods are described in SI Methods: plasmid constructs; cell culture and transfection; macrophage generation and neutrophil isolation; gene transfer in primary macrophages; microbial strains and infection; real-time quantitative PCR; protein extraction, cell subcellular fractionation, and immunoblotting; cell lysate preparation, protein in vitro translation, and immunoprecipitation; loading of Rac1 with GDP and GTP; immunofluorescence and confocal microscopy; PBD pull-down assay; F-actin determination; phagocytosis assay; determination of bactericidal activity; detection of ROS; ELISA; and statistical analyses.

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Supporting Information

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SI Discussion

Innate immunity is essential for host defense but its dysregulation can have devastating consequences, which include unresolving inflammation, sepsis, and autoimmune disorders (1, 2). Resting phagocytes are at a quiescent state whose phagocytic and bacterialidal activities are low. Upon encountering microbial products, these activities are significantly up-regulated (3–6). The mechanisms of this adaptation of the innate immune response are not well understood. Results reported here indicate that TIPE2 (TNFAIP8L2) serves as a key regulator of this process. TIPE2 is constitutively expressed at high levels in immune cells; it binds to Rac GTPases to prevent their activation and to maintain the quiescent phenotype of phagocytes. Exposure of microbes markedly down-regulates TIPE2 levels, which sets free the Rac GTPases; the free activated Rac GTPases in turn initiate their downstream effector signals, leading to enhanced innate immune responses (including phagocytosis and oxidative burst). Thus, TIPE2 serves as a negative regulator of innate immunity.

Toll-like receptors (TLRs) may enhance phagocytosis through multiple mechanisms (3–6). In addition to the TIPE2-dependent mechanism reported here, they may do so by up-regulating membrane receptors required for engulfment, intracellular signals required for cytokine remodeling, and cytokines that promote phagocytosis, and so on (3–7). However, our finding that TIPE2 deficiency alone significantly altered the basal and induced levels of phagocytoses indicates that the TIPE2-dependent mechanism is essential for controlling phagocytosis. The quiescent state of phagocytes may not be maintained in the absence of TIPE2. Therefore, TIPE2-dependent regulation of innate immunity is crucial for host defense and may be targeted to promote or dampen antimicrobial immune responses.

Rac proteins are members of the Rho family of small GTPases. They are activated by guanine nucleotide exchange factors (GEFs), e.g., Dock180, and inactivated by GTPase-activating proteins (GAPs), e.g., RacGAP (8). Additionally, they can also be regulated by Rho GDP-dissociation inhibitors (RhoGDIs), which include RhoGDIα, RhoGDIβ, and RhoGDIγ (9). RhoGDIs can inhibit Rac activation by extracting prenylated GDP:Rac-GTP from membranes and by preventing the exchange of GTP for GDP. Paradoxically, RhoGDIs also prevent the degradation of Rac proteins, thereby enhancing their activities (9–11). The roles of RhoGDIs in phagocytosis and oxidative burst are not clear because mice deficient in them do not exhibit notable abnormalities in these processes (12–14). Results reported here indicate that TIPE2 represents a unique class of Rac inhibitors, distinct from the aforementioned regulators. Although TIPE2 exhibits Rac inhibitory activity similar to that of RhoGDIs, it is not required for maintaining Rac protein stability, a well-known function of RhoGDIs (12–14). On the other hand, TIPE2, but not RhoGDIs, is essential for controlling innate immunity. However, TIPE2 may use mechanisms similar to those of RhoGDIs to inhibit Rac function. It binds to the GDP and GTP forms of Rac and prevents their membrane translocation and function. Therefore, because of the potential functional overlap between TIPE2 and RhoGDIs, it is plausible that combined deficiency in both classes of Rac inhibitors may lead to more dramatic abnormalities.

Rac proteins are over 90% identical in their sequences. They differ primarily in the C-terminal polybasic region. For example, this region in Rac1 is composed of six basic amino acids (i.e., KKKRRK), whereas in Rac2, three of these are replaced by neutral amino acids (i.e., PQQKR). Our finding that polybasic region (PBR) deletion did not affect TIPE2-Rac binding, but Cys-189 mutation did, explains why TIPE2 is able to interact with more than one Rac protein. Cys-189 is subjected to posttranslational modifications such as prenylation and carboxymethylation, which are essential for Rac membrane translocation and activation. Our finding that TIPE2 binds to this site may explain the inhibitory effect of TIPE2 on Rac. However, among the five deletional Rac1 mutants we generated, two (with amino acids 48–123 deletion and amino acids 136–161 deletion, respectively) were unstable in cells and therefore were not further studied. Whether these corresponding regions are involved in TIPE2-Rac interaction or the inhibitory function of TIPE2 is unclear.

Our previous studies showed that TIPE2 knockout 129 mice were hypersensitive to septic shock (15). A sharp decline in the survival rate and a significant increase in seral inflammatory cytokines were observed in lipopolysaccharide (LPS)-treated TIPE2 knockout mice. In this study, TIPE2 knockout C57BL/6 (B6) mice exhibited resistance to Listeria monocytogenes challenge. Unlike LPS, the endotoxin of Gram-negative bacteria, L. monocytogenes is an intracellular bacterium, which can invade various types of cells, including epithelial cells, hepatocytes, endothelial cells, fibroblasts, and macrophages. After entering host cells, L. monocytogenes is trapped temporarily in the phagosome. If the bacterium cannot be killed immediately, it will escape from the phagosome and enter the cytoplasm, where it undergoes rapid replication. Enhanced phagocytosis and reactive oxygen species (ROS) production in TIPE2 knockout mice may prevent the escape of the bacterium from the phagosome, thus inhibiting its propagation in the cytoplasm and the spreading to other cells. This action may explain why TIPE2 knockout mice have decreased bacterial load, reduced inflammatory responses, and diminished death rate.

To date, three binding partners (caspase-8, RGL, and Rac) have been identified for TIPE2. We do not have evidence that TIPE2, Rac, RGL, and caspase-8 are in a single complex. The interesting thing is that these binding partners control similar downstream signal pathways and therefore their regulation by TIPE2 may lead to similar outcomes. For example, AKT and the c-Jun N-terminal kinase (JNK) pathways may be regulated by all of the known TIPE2-binding proteins. By targeting several signaling pathways that play related roles, TIPE2 may more effectively control complex cellular processes such as phagocytosis and survival than by targeting only a single pathway.

SI Methods

Mice. Wild-type B6 and C57BL/6-Tg(UBC-GFP)30Scha/J mice were obtained from the Jackson Laboratory. C57BL/6 mice that carry a Tipe2 gene null mutation were generated by backcrossing Tipe2−/− 129 mice (15) to B6 mice for 12 generations. All animal procedures were preapproved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Plasmid Constructs. Full-length TIPE2 was generated from the cDNA clone by PCR and cloned in frame with an N-terminal Flag into vector pRK5. pEGFP-TIPE2 was generated by cloning an amplified TIPE2 fragment into vector pEGFP-N3. Human wild-type Rac1, Rac1 T17N, Rac1 O61L, and Rac2 cDNAs were obtained from Addgene and subcloned into pRK5 with Myc or HA tag at the N terminus. The mutant Rac1 (C189S), TIPE2 (K15/160), TIPE2 (K20A), and TIPE2 (R24A) were generated by PCR-based site-directed mutagenesis. Truncated forms of Rac1 lacking the N-terminal amino acids 1–47 and C-terminal amino acids 162–192 or 189–192 were generated from the cDNA clone by PCR with appropriate primers and cloned in-frame with an N-terminal HA tag.
into vector pRK5. Human HRas G12V cDNA was obtained from Addgene. cDNAs encoding TIFE2, wild-type Rac1, Rac1 T17N and Rac1 Q61L, and HRas G12V were subcloned into the murine stem cell virus (MSCV)-based retroviral vector that coexpresses the truncated human nerve growth factor receptor (NGFR).

**Cell Culture and Transfection.** The murine RAW264.7 macrophages (ATCC) and HEK293T cells (ATCC) were cultured in DMEM containing 10% (vol/vol) heat-inactivated FBS, 2mM l-glutamine, and 100 units/mL penicillin/streptomycin (D10). Transfections were performed using Lipofectamine LTX according to the manufacturer’s instructions (Invitrogen).

**Macrophage Generation and Neutrophil Isolation.** To generate bone marrow-derived macrophages (BMDMs), bone marrow cells were harvested from mice and cultured for 7 d in 30% t-929 cell culture supernatant and 70% D10. Cells were washed twice with cold DPBS and collected with 5 mM EDTA in DPBS. After centrifugation, they were resuspended in D10 and rested for 24 h before functional assays. BMDMs were >95% CD11b+ and F4/80+ as determined by flow cytometry. Morphologically mature neutrophils were purified from murine bone marrow by Percoll gradient centrifugation as previously described (16). Briefly, bone marrow cells were harvested from mice using neutrophil isolation buffer (1x HBSS without Ca2+ and Mg2+ containing 0.25% BSA). After RBC lysis, cells were layered on a three-step Percoll gradient (81%, 62%, 55%). Following centrifugation at 1,200 × g for 30 min at room temperature, cells at the 81%-62% interface were removed and washed once with the isolation buffer before being used in the experiment. Neutrophil viability was >95% according to the results from trypan blue staining. Purity was typically ~75–85% as assessed by flow cytometry based on the forward and side scatter and high Gr1 staining.

**Gene Transfer in Primary Macrophages.** To produce retroviruses, packaging cells (293T) were cultured in 6-cm culture dishes with D10 and transfected with NGFR MSCV-based retroviral vector containing the gene of interest along with pVSVG and pCFp sequences, using the CalPhos mammalian transfection kit (Clontech). The transfection medium was replaced with fresh medium 6 h posttransfection. Forty-eight hours later, the culture medium containing recombinant retroviruses was harvested and filtered (0.45 μm). Bone marrow cells were isolated from WT and Tipe2+/− mice and cultured in 70% D10 and 30% t-929 cell culture supernatant. The cells were infected with retroviruses at day 2 and day 3 and allowed to differentiate into BMDMs. The infection efficiency was ~10% as determined by FACS analysis (for NGFR* cells) at day 7 of the culture.

**Microbial Strains and Infection.** Wild-type and hly+ mutant *L. monocytogenes* (10403s) were provided by H. Shen and Y. Paterson (University of Pennsylvania). *L. monocytogenes*, *Escherichia coli* (strain DH5α), and *Staphylococcus aureus* (ATCC 29213) were grown at 37 °C in brain-heart-infusion medium (Becton Dickinson), Luria–Bertani medium (Fisher), and Columbia medium with 2% NaCl, respectively. For all assays, midlog-phase bacteria were used. Bacterial in vitro infection was assayed in 12-well plates. A total of $5 \times 10^{5}$ BMDMs or 2 × 10^{5} Raw264.7 cells were seeded in each well, followed by culturing overnight in DMEM with 10% FBS. Cells were infected with *L. monocytogenes* or *E. coli* at multiplicity of infection (MOI) of 5 or 10. Synchronous infection conditions and an enhanced bacterium–host cell interaction were achieved by a 2-min centrifugation (500 × g). The end of the centrifugation was considered the starting point of infection. Fifteen minutes after the inoculation, cells were washed three times with PBS, and fresh medium containing gentamycin (30 μg/mL) was added. At various time points, cells were washed and lysed with buffer for RNA or protein extraction.

For in vivo bacterial infection, *L. monocytogenes* was grown in brain-heart-infusion medium until the absorbance at 600 nm reached 0.1 of optical density. *S. aureus* was grown in Columbia media with 2% NaCl. Six- to 7-wk-old Tipe2+/+ and Tipe2−/− mice were infected i.v. with $2 \times 10^{3} L. monocytogenes$ in 200 μL PBS or $2 \times 6 \times 10^{3} S. aureus$ in 200 μL saline. For measurement of the bacterial burden in liver and spleen, mice were killed 24 or 72 h after inoculation, organs were homogenized in 0.1% Triton in PBS, and serial dilutions of the homogenate were plated on brain-heart-infusion agar plates or Columbia Agar with 5% sheep blood plates (Becton Dickinson). The colonies were counted 24 h later. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using the Infinity ALT or AST liquid stable reagent (Thermo).

**Real-Time Quantitative PCR.** Total RNA was isolated using RNeasy Kits (Qiagen) primed with random hexamer oligoconucleotides and reverse transcribed using Invitrogen reverse transcriptase II. Real-time quantitative PCR was carried out in an Applied Biosystems 7500 system, using Power SYBR Green PCR Master Mix (Applied Biosystems). Relative levels of gene expression were determined with GAPDH as the control. Quantitact primers for mouse GAPDH, TIFE2, IL-6, IFNβ1, and TNFa were purchased from Qiagen.

**Protein Extraction, Cell Subcellular Fractionation, and Immunoblotting.** Whole-cell lysate was prepared by suspending the cells in cell lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 100 μM Na3VO4, 5 mM EDTA, 1 mM PMSF) supplemented with 1× complete protease inhibitors mixture (Roche). 293T-cell membrane protein and cytoplasmic protein were prepared using a Subcellular Protein Fractionation Kit (Pierce) according to the manufacturer’s instructions. A Qprotecme Cell Compartment Kit (QIAGEN) was used to prepare cytosolic and membrane protein from BMDMs. Protein concentration was determined by BCA assay (Pierce). Equal quantities of proteins were separated by SDS/PAGE, transferred to a nitrocellulose membrane, and blotted with specific antibodies. The membrane was developed using Pierce SuperSignal reagent. Antibodies used were as follows: rabbit anti-TIFE2 (provided by Y. Fan, Shandong University, Jinan, China), mouse anti-β-actin and HRP-conjugated mouse anti-Flag (Sigma-Aldrich); HRP-conjugated mouse anti-Myc, HRP-conjugated mouse anti-HA, rabbit anti-Rac1/2/3, rabbit anti-integrin β1, rabbit anti-phospho-PAK1 (Ser144)/PAK2 (Ser141), rabbit anti-PAK1/2/3 antibody, rabbit anti-phospho SAPK/JNK (Thr183/Tyr185), mouse anti-JNK, and rabbit anti-RhoGDI (Cell Signaling); and HRP-conjugated anti-mouse or anti-rabbit Ig (GE Healthcare).

**Cell Lysate Preparation, Protein in Vitro Translation, and Immuno-precipitation.** Raw cells or 293T were lysed in immunoprecipitation (IP) buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 0.5% Nonidet P-40, 100 μM Na3VO4, 1 mM PMSF) supplemented with 1× complete protease inhibitors mixture (Roche). Extracts were assayed for protein content, using the BCA protein assay kit (Pierce) after clarification by high-speed centrifugation at 4 °C. In some experiments, in vitro translated proteins were used for immunoprecipitation. 35S-labeled or unlabeled proteins were synthesized by using the TNT quickcoupled transcription/translation system from Promega according to the manufacturer’s instructions. Immunoprecipitation was performed using Dynabeads protein G (Invitrogen). In brief, 1.5 mg protein-G Dynabeads was coated with 5 μg specific antibodies or Ig control for 1 h at room temperature with rotation. After removing unbound antibody, the bead–antibody complex was incubated with 500 μL cell lysate or in vitro translated protein mixture (1:4 diluted in IP buffer) for 4 h at 4 °C with rotation. The captured Dynabead/Ab/Agn complex was washed four times with PBS and boiled in 2× Laemmliri buffer. The eluted proteins were
fractionated by SDS/PAGE and detected by autoradiography or Western blot.

**Loading of Rac1 with GDP and GTP.** The 293T cells were transiently transfected with Myc-tagged Rac1-17N or Rac1-61L for 18 h. Cells were lysed in cell lysis buffer (50 mM Tris, pH 7.5, 10 mM EDTA, 0.2 M NaCl, 0.5% Nonidet P-40, and 1x protease inhibitors mixture) (Roche). Totals of 1 mM GDP and 0.2 mM GTP (Enzo Life Sciences) were loaded to Rac1-17N- and Rac1-61L-transfected cell extracts, respectively. After 20 min incubation at 30 °C, samples were placed on ice immediately and MgCl₂ was added to a final concentration of 10 mM to stop nucleotide exchange.

**Immunofluorescence and Confocal Microscopy.** The 293T cells were grown on two- or four-well chamber slides (Lab-Tec) and transfected with GFP-tagged TIPE2 or GFP control plasmids. Eight hours after transfection, cells were fixed in 2% paraformaldehyde and permeabilized in 0.3% Triton X-100. The cells were then blocked with 4% BSA and stained with mouse anti-GFP-Rac mAb (NewEast), followed by Alexa Fluor 555-conjugated anti-mouse IgG (Invitrogen). The slides were mounted in ProLong Gold antifade reagent with DAPI (Molecular Probes). Fluorescence images were captured with a laser confocal microscope (Zeiss LSM 510META NLO microscope) at 63x magnifications.

**PBD Pull-Down Assay.** The 293T cells were transfected with 1.25 μg of plasmids encoding TIPE2 or HRas G12V. Empty vector was added so that the total amount of DNA used per well of the six-well plate was 2.5 μg. Eight hours after transfection, cells were washed in PBS and lysed in PBD lysis buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 0.2 M NaCl, 0.5% Nonidet P-40, and 1x protease inhibitors mixture) (Roche). The lysate was incubated with 20 μg of p21-activated kinase (PAK)-GST protein beads (Cytoskeleton) for 30 min at 4 °C. After washing, protein on beads and in total cell lysates was subjected to Western blot to determine the levels of active Rac. To measure Rac activity in mouse macrophages, wild-type or Tipe2⁺/⁺ BMDMs were collected with 5 mM EDTA-PBS. After washing, cells were held in suspension for 1 h in 0.5% BSA and permeabilized in 0.3% Triton X-100. The cells were then fixed with 3.7% formaldehyde and labeled with Alexa Fluor 488-conjugated anti-GFP antibody. Confocal microscopy was used to visualize the localization of GFP in the cells.

**F-Actin Determination.** F-actin content was determined by NBD-phallacidin labeling. Briefly, after LPS (100 ng/mL; Sigma-Aldrich) treatment, the cells were fixed with 3.7% formaldehyde for 15 min at room temperature and permeabilized with 0.5% Triton X-100 for 5 min. F-actin was then stained with 50 units/mL NBD-phallacidin (Molecular Probes) for 1 h at room temperature. After washing, F-actin-bound NBD-phallacidin was extracted with methanol for 1 h. Extracts were centrifuged and measured using a fluorescence plate reader with excitation and emission wavelengths set at 465 nm and 535 nm, respectively.

**Phagocytosis Assay.** To prepare apoptotic cells for phagocytosis assay, GFP-positive thymocytes were harvested from 3- to 4-wk-old C57BL/6-Tg(UBC-GFP)30Scha/J mice, and apoptosis was induced by incubation at 37 °C in 5% CO₂ for 5 h in the presence of 5 μM dexamethasone (Sigma-Aldrich). After dexamethasone treatment, cells were washed three times with PBS and resuspended in DMEM with 2% FBS. This treatment routinely yielded over 70% apoptosis thymocytes as measured by annexin V staining. Phagocytosis assay was performed in a 12-well nontissue culture-treated plate (BD Falcon). A total of 4 × 10⁶ BMDMs were seeded in each well, followed by culturing overnight in DMEM with 2% FBS. GFP⁺ apoptotic thymocytes were then added at a ratio of 5:1, and centrifugation was performed at 500 × g for 2 min to synchronize binding and internalization. After 30 min of incubation at 37 °C, plates were rapidly washed two times with ice-cold PBS, and cells collected with 5 mM EDTA-PBS. Cells were then fixed with 2% paraformaldehyde in PBS, stained with APC-conjugated anti-F4/80, and analyzed by flow cytometry. Gates were set for macrophages in FSC/SSC dot plots. Experiments using fluorescent latex beads (2 μm; Sigma-Aldrich) were performed in a similar fashion. For bacterial phagocytosis assay, L. monocytogenes were washed twice with sterile PBS and incubated (at 1 × 10⁶/mL bacteria) in 2 μM carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes) for 30-30 min under constant shaking at 37 °C. CFSE-labeled bacteria were washed three times with PBS before being used. Live or heat-killed bacteria (70 °C for 60 min) were used to measure phagocytosis, bacterial killing, DCFH, and ROS were analyzed by flow cytometry. In brief, 2.5 × 10⁶ cells and 2.5 × 10⁶ bacteria were mixed with 1 mL balanced salt solution (BSS) containing 5% NMS in polypropylene snap-cap tubes (12 × 75 mm). The tubes were rotated end-over-end for 20 min at 37 °C and then centrifuged at 250 × g for 8 min at 4 °C. Free bacteria were removed by three washes with 2 mL of ice-cold BSS and one additional wash with 1 mL 30% sucrose. The cells were resuspended in 1.0 mL of BBS/5% NMS. A 0.1-M sample was removed for enumeration of the baseline infection rate for each sample. The remaining 0.9-mL samples were further incubated for another 120 min at 37 °C and then bacterial colonies quantitated. Macrophages were lysed in sterilized Millipore water (pH 8.0), and water at pH 11 was used to lyse neutrophils. Bacterial activity was calculated by comparing cfus before and after the 120-min incubation. The killing rate is calculated as follows: 100 × (cfus before incubation − cfus after incubation)/cfus before incubation.

**Detection of ROS.** ROS production was measured by a luminal-dependent chemiluminescence assay in the absence and presence of exogenously added horseradish peroxidase (HRP) (for intracellular and total ROS, respectively). To measure total ROS production after N-Formyl-Met-Leu-Phe (fMLP) stimulation, prewarmed fMLP (5 μM; Sigma-Aldrich) was added with luminol (100 μM; Sigma-Aldrich) and HRP (20 units/mL; Sigma-Aldrich) at the same time and measurement started immediately. ROS production was monitored every 10 s for 5 min with a luminometer. In the bacteria stimulation assay, cells were incubated with luminol in the absence or presence of HRP for 10 min at 37 °C, followed by stimulation with L. monocytogenes (MOI = 10). ROS production was monitored every 10 min for 2 h.

Intracellular ROS was also detected using 2′,7′-dichlorofluorescein diacetate (DCFDA) (Sigma-Aldrich). Neutrophils were primed with 10 ng/mL GM-CSF (R&D Systems) for 30 min at 37 °C and then incubated with the fluorogenic probe DCFDA for 30 min. Cells were then treated with HBSS or 1 μM fMLP for 15 min at 37 °C. ROS was determined on the basis of the fluorescence intensity by flow cytometry. ROS was measured in the absence or presence of HRP for 10 min at 37 °C, followed by stimulation with L. monocytogenes (MOI = 10). ROS production was monitored every 10 min for 2 h.

**ELISA.** Sera were collected from Type2⁺/⁺ and Type2⁻/⁻ mice 24 h after Listeria infection and kept at −80 °C. Antibodies used in ELISA were purchased from BD Pharmingen and eBioscience, including purified and biotinylated rat anti-mouse IL-6 and IFNγ. Quantitative ELISA was performed using paired mAbs specific for corresponding cytokines according to the manufacturer’s recommendations.

**Statistical Analyses.** The differences in mRNA, cytokines, phagocytosis, bacterial killing, DCFH, and ROS were analyzed by two-tailed Student’s t test. The differences in survival rate were
analyzed by a Mann–Whitney U test. Statistical analyses were performed on pooled data, and statistics and error bars shown are for independent experiments and not for replicates within a single experiment.


Fig. S1. Cell death in TIPE2-overexpressing 293T cells. 293T cells were transfected with the indicated amounts of Flag-TIPE2 construct. Cell death was assessed by trypan blue staining. Data shown are mean±SEM (n = 4) of three independent experiments. *P < 0.05; **P < 0.01.

Fig. S2. The phenotypes of BMDMs. BMDMs from WT and Tipe2−/− mice were stained with anti-mouse CD11b, F4/80, and Ly6G and then analyzed by flow cytometry.
**Fig. S3.** Phagocytosis in different media. WT and *Type2*−/− BMDMs were subjected to the phagocytosis assay as in Fig. 5A, in (Top) DMEM without sera (DMEM), (Middle) DMEM with 10% heat-inactivated FBS (10% FBS), and (Bottom) DMEM with 5% fresh normal mouse sera (5% NMS).

**Fig. S4.** Endocytosis of dextrans. BMDMs from WT and *Type2*−/− mice were incubated with or without 1 mg/mL FITC-labeled dextran (Invitrogen) in PBS containing 1% BSA, for 30 min at 4 °C or 37 °C. After the incubation, cells were washed five times and analyzed by flow cytometry.
**Fig. S5.** TIPE2 inhibits phagocytosis. BMDMs were treated and tested as in Fig. 5D. (A) Each number represents the percentage of NGFR⁺ cells in macrophages. (B) Each number represents the percentage of GFP⁺ cells in NGFR⁺ macrophages. Results are representative of three independent experiments.

**Fig. S6.** TIPE2 inhibits phagocytosis through Rac. BMDMs were treated and tested as in Fig. 5E. (A) Each number represents the percentage of NGFR⁺ cells in macrophages. (B) Each number represents the percentage of GFP⁺ cells in NGFR⁺ macrophages. Results are representative of three independent experiments.
Fig. S7. LPS-induced increase in phagocytosis is diminished in TIPE2-deficient cells. BMDMs were treated and tested as in Fig. 5G. Each number represents the percentage of GFP* cells in the macrophages.

Fig. S8. Oxidative burst in neutrophils. Bone marrow neutrophils from wild-type and Tipe2−/− mice were analyzed for Listeria-induced ROS production, using the Luminol assay. The assay was performed in the presence (A) and absence (B) of exogenously added HRP to measure total and intracellular ROS, respectively. RU, relative units.