Spleen Tyrosine Kinase (Syk)-dependent Calcium Signals Mediate Efficient CpG-induced Exocytosis of Tumor Necrosis Factor α (TNFα) in Innate Immune Cells

Sheila Rao 1,‡§, Xiaohong Liu 6, Bruce D. Freedman 6, and Edward M. Behrens 1‡§

From the 1Division of Pediatric Rheumatology, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, the 6Immunology Graduate Group, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and the 1Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Pattern recognition receptors expressed by cells of the innate immune system initiate the immune response upon recognition of microbial products. Activation of pattern recognition receptors result in the production and release of proinflammatory cytokines, including TNFα and IL-6. Because these cytokines promote disparate effector cell responses, understanding the signaling pathways involved in their regulation is critical for directing the immune response. Using macrophages and dendritic cells deficient in spleen tyrosine kinase (Syk), we identified a novel pathway by which TNFα trafficking and secretion are regulated by Syk following stimulation with CpG DNA. In the absence of PLCγ2, a Syk substrate, or the calcium-responsive kinase calcium calmodulin kinase II, CpG-induced TNFα secretion was impaired. Forced calcium mobilization rescued the TNFα secretion defect in Syk-deficient cells. In contrast to its effect on TNFα, Syk deficiency did not affect IL-6 secretion, suggesting that Syk-dependent signals participate in differential sorting of cytokines, thus tailoring the cytokine response. Our data report a novel pathway for TNFα regulation and provide insight into non-transcriptional mechanisms for shaping cytokine responses.

Pattern recognition receptors (PRRs)2, including Toll-like receptors (TLRs), triggered by pathogen-associated molecular patterns (PAMPs) activate a variety of signaling events, leading to initiation of the immune response (1). This response includes up-regulation of costimulatory molecules on innate immune cells, production of antiviral molecules and proinflammatory cytokines, and activation of antigen-specific immunity. Although TLR activation is crucial for host defense, excessive TLR signaling can lead to inflammatory and autoimmune disease (2). TNFα is a potent proinflammatory cytokine produced following PAMP recognition by PRRs that is crucial for the clearance of certain pathogens, including Listeria monocytogenes and Mycobacterium tuberculosis, but whose activity has also been linked to pathogenesis of inflammatory disease (3, 4). How this balance between beneficial and detrimental effects of TNFα is determined represents a significant gap in our understanding of PRR signaling. Gaining an understanding of the signaling events that contribute to TNFα production and release is critical for identifying possible targets for directing the immune response downstream of PAMP recognition.

Syk is a protein tyrosine kinase that regulates many cellular processes, including B cell antigen receptor (immunoglobulin) and integrin signaling, inflammasome activation, phagocytosis, and reactive oxygen species production (5). Syk signaling is also important for cytokine release downstream of PAMP recognition. However, the mechanism by which Syk regulates this process is unresolved. Conflicting studies suggest that Syk acts downstream of receptors either to promote cytokine production (6–9) or to limit cytokine production (10–12). The various approaches employed to study Syk may be contributing to the contradictory functions ascribed to this enzyme. Embryonic deletion of Syk results in perinatal lethality because of severe vascular abnormalities. Hence, mice with germ line deletion of the gene cannot be used for analysis (13–15). Although the use of radiation chimeras circumvents perinatal lethality, this produces developmental abnormalities, including blocks in B cell maturation that may confound the interpretation of the effects of Syk deletion in innate immune cells. Other studies have relied on pharmacological inhibition of Syk that are likely complicated by off-target effects of these drugs (16, 17).
Recognizing the limitations of these approaches, we have used complementary methodologies in primary cells and in a model cell line to resolve the role of Syk in signaling downstream of one critical PRR, TLR9, which responds to CpG DNA. Utilizing genetic deletion selectively in DCs and genetic knockdown in a macrophage cell line, we observed that Syk deficiency results in impaired CpG-induced exocytosis of TNFα but not IL-6. Syk-deficient DCs and macrophages exhibited defective calcium signaling in response to CpG, which was responsible for the defect in TNFα secretion. Our data suggest a novel mechanism for TNFα exocytosis involving a Syk-PLCβ-1/CaMKII pathway downstream of PAMP signaling and provide insight into how particular cytokine responses are generated post-translationally.

**EXPERIMENTAL PROCEDURES**

*Mice*—Syk<sup>lox/lox</sup>CD11c Cre<sup>+</sup> and Syk<sup>lox/lox</sup> CD11c Cre-negative mice were housed in our Association for Assessment and Accreditation of Laboratory Animal Care-certified animal facility. Mice used in experiments were between 7 and 10 weeks of age. All experiments were performed with approval of the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee.

**Antibodies and Reagents**—The following Western blot antibodies were purchased from Cell Signaling Technology, Inc.: TNFα (catalog no. 3707), phospho-ERK (clone D13.14.4E), total ERK (clone L34F12), phospho-p38 (clone 28B10), phospho-CaMKII (catalog no. 3361), pan-CaMKII (clone D11A10), NF-κB p65 (clone C22B4), PLCγ2 (catalog no. 3872), and IκBα (clone 44D4).

The following Western blot antibodies were purchased from Santa Cruz Biotechnology, Inc.: β-actin (clone C-11), Syk (clone N-19), and MHC class II (clone M5/114). Secondary antibodies (mouse, goat, rat, and rabbit IgG) were purchased from Licor. Antibodies used for flow cytometry from BD Biosciences. Antibodies (mouse, goat, rat, and rabbit IgG) were purchased from Santa Cruz Biotechnology, Inc.: phospho-p38 (clone 28B10), phospho-CaMKII (catalog no. 3361), pan-CaMKII (clone D11A10), NF-κB p65 (clone C22B4), PLCγ2 (catalog no. 3872), and IκBα (clone 44D4).

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Ionomycin (Molecular Probes) was used at 1 μg/ml. TAPI-0 (20 μM, EMD Millipore) was used to inhibit TACE activity and thus prevent cleavage of surface TNFα.

**Cell Culture and Lentiviral Transduction**—The mouse RAW264.7 macrophage cell line was cultured in DMEM (Invitrogen) containing antibiotics (penicillin, streptomycin, and glutamine), fetal bovine serum, and GM-CSF (3.3 ng/ml, Peprotech). On day 8, cells were stimulated with CpG for various assays.

**Preparation of Splenic DCs**—Control and Syk flox mice were injected subcutaneously with 5 million B16-F103L cells. This melanoma cell line is genetically engineered to secrete Flt3L. Fourteen days post-injection, mice were sacrificed, and spleens were harvested and pooled. Spleen cells were cultured for 8 days in Iscove's modified Dulbecco's medium (Invitrogen) containing antibiotics (penicillin, streptomycin, and glutamine), fetal bovine serum, and GM-CSF (3.3 ng/ml, Peprotech). On day 8, cells were stimulated with CpG for various assays.

**Cytokine Secretion Measurements**—After stimulation of cultured cells with CpG, supernatants were collected and centrifuged for 10 min at 13,200 rpm at 4 ºC degrees. Cytokine concentrations were measured in the supernatants using TNFα and IL-6 ELISA kits (BD OptEIA, BD Biosciences) according to the instructions of the manufacturer. For detection of serum TNFα from CpG-injected control and Syk flox mice, blood was collected from the cheek vein and centrifuged for 20 min at 13,200 rpm. Serum was tested using an ELISA kit.

**Flow Cytometry**—For detection of surface TNFα, cultured cells stimulated with CpG and TAPI-0 were harvested, pelleted, and resuspended in FACs buffer (PBS containing 2% fetal bovine serum and 0.01% sodium azide). Cells were then resuspended sequentially in 4% paraformaldehyde (20 min) and Pe-Cy7-conjugated rat anti-TNF antibody (BD Biosciences). For detection of intracellular TNFα, cultured cells were stimulated with CpG in the presence of brefeldin A, which prevents transport of newly synthesized proteins from the endoplasmic reticulum. After harvesting, cells were fixed and permeabilized for 20 min with 1× PermWash (BD Biosciences) and stained with Pe-Cy7 or AF700-conjugated rat anti-TNF antibody (BD Biosciences). All steps were performed at 4 ºC. Cells were analyzed on an LSR II (BD Biosciences) using FACS Diva software, and data were analyzed using FlowJo software (TreeStar).

**Western Blotting and TACE Activity Assay**—For preparation of whole cell lysate, cells were washed in cold PBS and lysed in 1% Nonidet P-40 buffer containing 5 mM NaCl, 1 mM Tris (pH 7.4), complete protease inhibitor mixture (Sigma), 100 mM NaF, 100 mM Na vanadate, and 100 mM phenylmethanesulfonylfluoride. Lysates were centrifuged for 10 min at 13,200 rpm and stored at −20 ºC. For preparation of membrane lysates, cells were washed in cold PBS and resuspended in PBS containing protease and phosphatase inhibitors. Cells underwent 5 cycles of freeze/thaw lysis and were frozen in an ethanol/dry ice bath and thawed in a 37 ºC degree water bath. Lysates were centrifuged at 20,000 × g for 15 min. Pellets (membrane fraction) were
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washed in PBS, centrifuged again, and resuspended in 1% Nonidet P-40 lysis buffer. To obtain nuclear lysates to investigate nuclear translocation of NF-κB, CpG-stimulated cells were lysed using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific) according to the instructions of the manufacturer. All lysates were boiled and separated on SDS-PAGE (4% Bis-Tris gel, Invitrogen) and analyzed by Western blotting. Licor analysis software was used to analyze blots developed on Odyssey.

Membrane lysates were also plated on a black 96-well plate for the Sensolyte 520 TACE activity assay (Anaspec) according to the instructions of the manufacturer. Briefly, a peptide substrate, the fluorescence of which was quenched in the intact peptide, was added to lysates. In the presence of TACE, the peptide was cleaved, and fluorescence of the cleaved substrate, which was directly proportional to the amount of TACE activity, was measured.

*Preparation of RNA for Real-time PCR*—RNA was isolated from cultured cells using the RNeasy kit (Qiagen) according to the instructions of the manufacturer, and cDNA was synthesized using the SuperScript III kit (Invitrogen) from 1 μg of RNA. Quantitative PCR was performed on the cDNA using TaqMan gene expression assays (Applied Biosystems) with probes for mouse TNFα (Mn00443258_m1), mouse TLR9 (Mn00446193_m1), and mouse GAPDH (Mn99999915_g1). Samples were run and analyzed using a Sequence Detection System 7500 PCR machine from Applied Biosystems. TNFα message levels and TLR9 message levels were normalized to that of GAPDH.

*Intracellular Calcium Measurements*—RAW cells were loaded with fura-2 AM (3 μM, Invitrogen) for 45 min at room temperature, and intracellular calcium was detected in response to CpG by digital imaging as described previously (18). The fluorescence emission ratio of fura-2 was detected at 510 nm in a solution containing no calcium to establish a baseline reading. CpG was added before the cells were returned to a bath solution containing 155 mM NaCl, 4.5mK KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM glucose, 10 mM HEPES (pH 7.4). Cells were then stimulated with ATP (10 μM) and ionomycin.

*Statistical Analysis*—Graphs and statistical tests were generated and analyzed using Prism Version 5.0. In general, Student’s unpaired t test was used to compare groups. To analyze membrane TNFα data (Fig. 4, Western blot analysis), membrane TNFα was normalized to MHC class II for each cell type. The relative amount of membrane TNFα was computed by dividing normalized TNFα of the Syk-deficient cells to that of the control cells. The resulting ratio was compared with the value of 1, representing no change using a one-sample t test. Similarly, the MFI of surface TNFα was compared between Syk-deficient and control cells, and the resulting ratio was analyzed against the value of 1.

For studies involving calcium signaling (Fig. 7), pCaMKII was normalized to total ERK for each cell genotype. Data were analyzed on the basis of fold induction of normalized pCaMKII from time 0 and on the basis of percentage of ionomycin-stimulated signal of normalized pCamKII.

**RESULTS**

**Syk Deficiency Results in Impaired Secretion of CpG-induced TNFα**—To study the role of Syk in DCs in vivo, we crossed mice expressing Cre recombinase under the control of the CD11c promoter to mice with loxP sites flanking Syk (19), resulting in the conditional deletion of Syk in CD11c+ DCs (Syk flox). Western analysis of splenic DCs and BMDCs from Syk flox mice showed near complete deletion of Syk protein (supplemental Fig. S1). To assess the role of Syk in cytokine response, Syk flox and control (Syk flox, Cre-negative) mice were injected with CpG. Serum from CpG-injected Syk flox mice contained markedly less TNFα compared with that of control mice (Fig. 1A), demonstrating an in vivo role for Syk in the regulation of CpG-induced TNFα.

We next sought to investigate the contribution of CD11c+ cells to TNFα production and secretion. Culture supernatants from splenic DCs and BMDCs isolated from Syk flox mice contained a 2-fold reduction in CpG-induced TNFα compared with supernatants from cells isolated from control mice (Fig. 1, B and C). We confirmed this phenotype using shRNA knockdown of Syk in the RAW macrophage cell line. Syk-deficient RAW cells also showed a decreased level of TNFα in the supernatant following CpG stimulation compared with cells transduced with empty vector (control) (Fig. 1D and supplemental Fig. S1) or to RAW cells transduced with an irrelevant shRNA sequence that did not result in effective knockdown of Syk (supplemental Fig. S1). In contrast to the reduction in TNFα, Syk-deficient splenic DCs and BMDCs secreted IL-6 normally in response to CpG (Fig. 1, B and C), indicating that Syk deficiency does not result in global dysfunction of the CpG signaling pathway. Together, these data suggest that Syk selectively regulates TNFα but not IL-6 levels.

We speculated that Syk regulates TNFα levels by promoting its production or secretion. However, it is possible that Syk regulates TNFα consumption or biological activity, as has been seen in other cellular systems (20). To test the possibility that Syk deficiency might alter consumption of secreted TNFα, CpG-stimulated or unstimulated control and Syk-deficient RAW cells were exposed to a fixed amount of exogenous TNFα in the presence of a TACE inhibitor, which prevents cleavage and secretion of synthesized, endogenous TNFα into the supernatant. We measured no difference in TNFα levels in culture supernatants from cells replete or deficient in Syk (supplemental Fig. S2), suggesting that the defect observed from Syk-deficient cells is not due to increased cellular consumption of TNFα.

Soluble TNF receptor cleaved from the cell surface can bind to soluble TNFα, thereby acting as a TNFα antagonist (21). To rule out the possibility that Syk deficiency promotes the production of soluble proteins binding secreted TNFα and, thus, preventing its detection by ELISA, we examined TNFα levels in supernatants that were boiled and denatured to disrupt protein-protein interactions, thus allowing for detection of all secreted TNFα. Western blot analysis of denatured supernatants from CpG-treated RAW cells revealed a decrease in secreted TNFα in the absence of Syk, arguing against selective
production of an interfering TNFα binding protein in the context of Syk deficiency (supplemental Fig. S2).

Canonical Signaling Downstream of TLR9 Is Intact in the Absence of Syk—Syk is known to be activated downstream of CpG stimulation (8, 17). To determine the role of canonical TLR9 signaling in Syk-dependent regulation of TNFα, we investigated signaling events downstream of TLR9 in control and Syk-deficient cells stimulated with CpG. Control and Syk-deficient cells expressed similar levels of TLR9 (Fig. 2A), and CpG-induced phosphorylation of the MAP kinases ERK and p38 occurred similarly in the presence and absence of Syk (B). IκBα, a cytoplasmic protein whose interaction with NF-κB blocks its nuclear translocation, was similarly degraded after 30 min of CpG stimulation in control and Syk-deficient RAW cells and BMDCs (Fig. 2C), thus allowing nuclear translocation of NF-κB (D). These data demonstrate that canonical signaling downstream of TLR9 is intact in the absence of Syk.

MAP Kinase and NF-κB Activity Can Regulate TNFα Transcription (22–24). Consistent with the observation that activation of these signaling intermediates was intact, TNFα mRNA levels were also similar in control and Syk-deficient cells following CpG stimulation (Fig. 3A). Moreover, Syk-deficient RAW cells, splenic DCs, and BMDCs produced TNFα protein similar to control cells following CpG stimulation in the presence of brefeldin A, which prevents exocytosis (Fig. 3B). Taken together, these data demonstrate that, in response to CpG, TNFα mRNA and protein generation occurs independently of Syk.

TNFα Trafficking to the Cell Surface Is Impaired in the Absence of Syk—We next considered whether Syk might be playing a role in the exocytosis and subsequent secretion of synthesized TNFα. To investigate this possibility, control and Syk-deficient RAW cells were stimulated with CpG, and membrane lysates were measured by Western blot analysis and ELISA. Both measurements revealed elevated levels of TNFα protein in membranes of Syk-deficient cells compared with control cells (Figs. 4, A and B). Because the membrane preparations included intracellular and plasma membranes, this approach cannot distinguish TNFα retention in organelles within the cell from TNFα residing at the cell surface. It is known that prior to secretion, TNFα translated at the endoplasmic reticulum membrane traffics through the Golgi apparatus.
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and the endosomal compartment before reaching the plasma membrane (25, 26). At the plasma membrane, membrane TNFα is cleaved by TACE, allowing soluble TNFα to be secreted from the cell (27). TNFα retention inside the cell would reflect a trafficking defect, whereas increased cytokine at the plasma membrane would suggest a defect in TACE activity. To distinguish between these possibilities, we assayed TACE activity in isolated membrane lysates and found no difference in TACE activity between the control and Syk-deficient cells, suggesting that both constitutive and CpG-induced TACE activity is intact in the absence of Syk (Fig. 4C).

To investigate the role of Syk in TNFα trafficking to the plasma membrane, RAW cells and BMDCs were stimulated with CpG alone or with CpG in the presence of a TACE inhibitor (TNFα protease inhibitor, TAPI). TAPI allows TNFα to accumulate at the plasma membrane by blocking its cleavage and secretion, thus enabling us to measure trafficking of total TNFα to the cell surface, as reported previously (28, 29). We observed similar amounts of surface TNFα on Syk-sufficient and Syk-deficient cells after CpG stimulation in the presence of TACE activity when TNFα was able to be cleaved and subsequently secreted (Fig. 4D, CpG). However, in the presence of TAPI, significantly less TNFα accumulated at the surface of the Syk-deficient RAW cells and BMDCs compared with control cells (Fig. 4D, CpG/TAPI). The decrease in surface TNFα in the absence of Syk was almost 2-fold, consistent with the 2-fold reduction in TNFα secretion (Fig. 1) and the 2-fold increase in TNFα membrane protein (Fig. 4, A and B). Taken together, these data demonstrate that CpG-induced TNFα fails to traffic efficiently to the cell surface in the absence of Syk.

Calcium Signaling Is Impaired in the Absence of Syk in Response to CpG—Known downstream effectors of Syk include PLCγ (30) and cytosolic calcium (31, 32), and calcium signaling has been implicated in synaptic vesicle trafficking and exocytosis in neurons (33). We therefore sought to determine whether Syk-dependent mobilization of intracellular calcium regulates TNFα secretion and accounts for the defect observed in Syk-deficient cells. Control RAW cells exhibited a rise in intracellular calcium levels in response to CpG in the presence of extracellular calcium (Fig. 5A, 8 min). In contrast, Syk-deficient RAW cells failed to mobilize calcium upon CpG stimulation under the same conditions. This defect in calcium was selective, however, as cells lacking Syk were able to mobilize calcium in response to ATP, a ligand for G-protein-coupled purinergic receptors that results in Syk-independent calcium flux (34), and to pharmacological release of calcium from internal stores with ionomycin. These data demonstrate that CpG-induced calcium mobilization is defective in the absence of Syk.

To test whether the increase in intracellular calcium we observed following CpG stimulation was important for TNFα secretion, we treated RAW cells with BAPTA-AM, an intracellular calcium chelator. Stimulation of BAPTA-treated cells with CpG failed to elicit secretion of TNFα (Fig. 5B), suggesting that the Syk-dependent calcium response regulates TNFα secretion. To test this further, we designed a gain-of-function experiment, reasoning that pharmacological mobilization of calcium should rescue the TNFα secretion defect in the Syk-deficient cells. Indeed, addition of ionomycin and CpG increased the total amount of TNFα secreted into the supernatants from the Syk-deficient RAW cells to control levels (Fig. 5C), suggesting that calcium signaling is important for efficient TNFα regulation and that Syk is mediating this process. These results indicate that CpG mediated Syk-dependent calcium signals regulate efficient trafficking of TNFα to the plasma membrane for its secretion from the cell.
PLCγ2 is a known target of Syk kinase activity, and its activation results in calcium release from the endoplasmic reticulum. We therefore, reasoned that suppression of PLCγ2 should result in a similar phenotype as suppression of Syk. To test this hypothesis, we gene-silenced PLCγ2 using shRNA in RAW cells and found that PLCγ2-deficient cells phenocopied Syk-deficient cells. TNFα protein was produced normally, but its secretion was impaired with a similar magnitude as Syk deficiency following CpG stimulation (Fig. 6). Moreover, TNFα secretion was rescued upon addition of ionomycin. These data are consistent with PLCγ2 acting as the downstream effector of Syk and being required for the optimal calcium-induced exocytosis of TNFα.

We next sought to identify the target of calcium that regulates TNFα secretion in macrophages and DCs. We focused on CaMKII, a serine threonine protein kinase whose phosphorylation has been reported following TLR stimulation (35). Binding of CaMKII to calcium induces a structural rearrangement, resulting in its autophosphorylation (36), a necessary step for activation of its downstream targets. In neurons, CaMKII has been reported to participate in the phosphorylation of molecules involved in exocytosis (37, 38). Consistent with CaMKII being the target of CpG-induced Syk and PLCγ2-dependent calcium signaling, we found that Syk-deficient BMDCs exhibited defective CpG-induced CaMKII phosphorylation (Fig. 7A).

We next looked more directly at the role of CaMKII in TNFα secretion by suppressing its expression in RAW cells. We found that CaMKII-deficient cells, like those lacking Syk or PLCγ2, generated normal amounts of TNFα protein but secreted less cytokine in response to CpG (Fig. 7, B and C). This secretion defect was not rescued by ionomycin, which is consistent with the notion that CaMKII is downstream of calcium mobilization. Thus, we have identified Syk-PLCγ2 dependent activation of calcium-induced CaMKII signaling as a novel pathway for the regulation of TNFα secretion (Fig. 8). This suggests that, in addition to activating the transcription and translation of cytokines, CpG provides an active signal to promote the trafficking and secretion of TNFα.

**DISCUSSION**

Here we demonstrate that, in response to CpG, Syk is required for optimal trafficking of TNFα to the plasma membrane prior to secretion in macrophages and DCs. Mice harboring a DC-specific deletion of Syk showed a substantial reduction of serum TNFα levels following CpG injection. *In vitro* in cells lacking Syk, CpG stimulation resulted in activation of the canonical TLR9 signaling cascade, resulting in normal increases in TNFα message and protein but diminished secretion of TNFα. Syk deficiency did not result in a global defect in cytokine secretion, however, as IL-6 secretion was normal. Syk-deficient cells displayed significantly less TNFα at the cell surface compared with Syk-sufficient cells in response to CpG, consistent with the observation that total membrane TNFα levels were higher in the absence of Syk and that TACE activity was unaffected by Syk deletion. Together, these data suggest that Syk is important for trafficking of membrane-bound TNFα to the cell surface. This appears to involve Syk dependent activation of PLCγ2 and calcium mobilization downstream of CpG, resulting in phosphorylation of CaMKII. Collectively, these data demonstrate a novel mechanism for the differential secretion of cytokines downstream of CpG and suggest that CpG induces an active signal for cytokine secretion independent of production.

PRRs, including TLRs and Nod-like receptors, expressed by cells of the innate immune system can shape the immune response by bridging innate and adaptive immunity. Upon pathogen recognition, these receptors signal for the production of cytokines and for the up-regulation of costimulatory molecules, both of which recruit and activate cells of the adaptive immune system. The type of cytokine released plays a key role in determining the appropriate effector cell response. TNFα is a potent proinflammatory cytokine. Although its signaling is important for clearance of certain pathogens, including *L. monocytogenes* and *M. tuberculosis* (3, 4), dysregulated TNFα production and signaling can lead to inflammatory dis-
Therefore, to maintain immune homeostasis, TNFα production and release must be tightly regulated. Indeed, TNFα transcription and translation are controlled by several factors (22–24, 40). Additionally, posttranslational mechanisms of TNFα regulation have also been reported. Among these are cleavage of TNFα prior to secretion at the plasma

ease (39). Therefore, to maintain immune homeostasis, TNFα production and release must be tightly regulated. Indeed, TNFα transcription and translation are controlled by several
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FIGURE 7. CaMKII is important for CpG-induced TNFα secretion downstream of Syk. A, BMDCs were stimulated with CpG or ionomycin (Iono) at various times, and lysates were harvested and analyzed by Western blot analysis for phospho-CaMKII. For quantification, pCamKII was normalized to total ERK, and that ratio, at each time of stimulation, was compared with the signal at base line (left panel). Normalized pCamKII from CpG-stimulated cells was also compared with the maximum signal achieved with ionomycin stimulation (right panel). The blot is representative of three independent experiments. B, RAW cells transduced with CaMKII shRNA delete CaMKII protein (Western blot analysis). CaMKII control cells were transduced with an irrelevant shRNA sequence. CaMKII-deficient RAW cells were stimulated with CpG or with and ionomycin for TNFα detection by ELISA. C, RAW cells were stimulated with CpG in the presence of brefeldin A for detection of intracellular TNFα by flow cytometry (center panel). ELISA data are representative of four independent experiments performed in triplicate. Fold induction of intracellular TNFα with CpG was calculated on the basis of median fluorescence intensity. The bar graph (B, right panel) represents summary data from three independent experiments. Data are mean ± S.E.; *, p < 0.05. Untrx, untreated. MFI, median fluorescence intensity.

FIGURE 8. Upon CpG stimulation, the canonical TLR9 machinery activates NF-κB, resulting in Syk-independent transcription of proinflammatory cytokines like IL-6 and TNFα. These cytokines are packaged into secretory vesicles after synthesis. CpG also activates a Syk-dependent signaling cascade, resulting in calcium release through PLCγ2. Calcium binds to CaMKII, leading to its autophosphorylation, a step necessary for activation of its downstream targets. This signaling pathway is important for the trafficking of intracellular TNFα, but not IL-6, to the plasma membrane. Thus, Syk activation downstream of CpG stimulation results in differential trafficking of cytokines.

IL-6 is a proinflammatory cytokine whose actions differ from that of TNFα. Although both cytokines can recruit other cells of the immune system to the site of injury, such as monocytes, and promote inflammation, IL-6 can also influence the differentiation of T helper 17 cells (42, 43). Therefore, differential sorting and secretion of these cytokines is essential for tailoring the immune response to appropriately combat specific pathogens. Indeed, IL-6 and TNFα segregate to different locations within the recycling endosome during exocytosis in macrophages, and these cytokines are delivered to different sites at the plasma membrane prior to secretion (29, 41). We find that Syk-deficient cells secrete IL-6 normally in response to CpG, which demonstrates that Syk is responsible for differential secretion of TNFα, perhaps at the level of endosomal sorting or plasma membrane sorting. Similarly, T cells can also differentially regulate cytokine secretion at the immunological synapse by polarizing certain cytokines at specific sites at the plasma membrane or by promoting more generalized secretion (44). Therefore, understanding the pathways involved in exocytosis of various cytokines and chemokines will provide insight into targeting specific effector molecules to treat inflammatory disease.

All TLRs signal through the transcription factor NF-κB, which leads to transcription of a variety of cytokines and chemokines (45). However, the release of such cytokines occurs differently upon stimulation of different receptors. For example, the amount of secreted TNFα varies upon TLR3, TLR4, or TLR9 ligation (46, 47), even though these TLRs all activate NF-κB similarly. Indeed, our data demonstrate normal NF-κB activation but defective TNFα secretion following CpG stimulation in the absence of Syk, whereas IL-6 secretion was unaffected. Differential activation of trafficking molecules downstream of PAMP recognition may be a useful strategy for cells to membrane by TACE (27) and regulation of its transport within the cell (25, 26, 28, 41). Here we report a novel mechanism of TNFα regulation by way of exocytosis activated by PAMPs through the protein tyrosine kinase Syk in macrophages and DCs. This finding may have implications for additional immune cell types such as neutrophils, which were shown to have defective TNFα secretion in response to opsonized bacteria upon Syk deletion (9). Similar to our data, these neutrophils generated TNFα protein normally, suggesting that Syk is also important for efficient exocytosis of TNFα downstream of PAMP signaling in this lineage.
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affect the qualitative cytokine response of receptors and, therefore, tailor the immune response. Furthermore, an active signal distinct from that of NF-κB-induced transcription is critical for differentially sorting cytokines for appropriate release.

Syk is a protein tyrosine kinase whose activation relies on its recruitment to phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs). These motifs are present in receptors like the B-cell receptor and Fc receptor, both of which signal through Syk. Syk phosphorylation has been reported in response to TLR ligation (8, 11, 17, 48), but these receptors do not contain immunoreceptor tyrosine-based activation motifs or associate with known immunoreceptor tyrosine-based activation motif-bearing adaptor molecules. Indeed, Syk phosphorylation following stimulation with ligands for TLR3, TLR4, or TLR9 was dependent on the integrin CD11b, suggesting that Syk activation is indirect of TLR9 (11). Furthermore, calcium mobilization occurred in B cells deficient in TLR9 following CpG stimulation and relied on a scavenger receptor (49). Therefore, it is possible that Syk-dependent exocytosis of TNFα occurs independently of TLR9 and, instead, relies on a currently unknown receptor for CpG recognition. This seems plausible, given that canonical TLR9 signaling occurs normally in the absence of Syk.

Once activated, PLCγ cleaves phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol trisphosphate. In turn, inositol trisphosphate binds to its receptors located on the endoplasmic reticulum membrane, and these receptors serve as calcium channels and thus allow an influx of calcium from endoplasmic reticulum stores. PLCγ2-deficient DCs cannot mobilize calcium in response to the TLR4 ligand LPS (50). We found that knockdown of PLCγ2, a Syk binding partner, resulted in decreased TNFα secretion, which was rescued by ionomycin. Knockdown of the calcium-responsive kinase CaMKII also resulted in decreased CpG-induced TNFα secretion. Similarly, Syk-deficient cells showed defective CpG-induced calcium mobilization and CaMKII phosphorylation. When CaMKII phosphorylation was rescued by ionomycin, TNFα secretion was restored in Syk-deficient and PLCγ2-deficient cells to control levels. These data demonstrate that a Syk-dependent pathway signaling through a PLCγ-calcium-CaMKII axis is responsible for optimal TNFα secretion downstream of CpG activation.

Calcium signaling is a well-established pathway for exocytosis, particularly for the transport of synaptic vesicles in neurons (33). For example, intracellular membrane fusion events necessary for cytokine secretion involve the assembly of soluble NSF attachment protein receptors (SNAREs), the activation of which has been linked to calcium mobilization. CaMKII was reported to phosphorylate proteins important for exocytosis in neurons, including the SNARE-interacting molecule synaptotagmin, which initiates membrane fusion events (37, 38). In macrophages, SNAREs, including VAMP3, Syntaxin6, and Vti1B, were up-regulated following LPS stimulation, and VAMP3 colocalized with TNFα in recycling endosomes (26, 41). It is plausible, therefore, that Syk is orchestrating a calcium-dependent signaling event that triggers exocytosis by way of synaptotagmin and SNARE activation downstream of CpG stimulation. IL-6 trafficking and exocytosis were also reported to be dependent on the SNARE machinery, but it is possible that IL-6 relies on a different set of CaMKII-independent trafficking intermediates for its secretion. Therefore, in addition to signaling for activation of NF-κB and cytokine transcription, CpG must also provide distinct signals for activation of the trafficking machinery to ensure exocytosis and secretion of cytokines. This provides a useful way for tightly coordinating the differential secretion of potent proinflammatory cytokines to adequately direct the effector cell response. Furthermore, because similar trafficking intermediates are employed for cytokine release downstream of PRRs in innate immune cells and for synaptic vesicle exocytosis in neuronal cells (51), understanding the signaling pathways that regulate these processes have broad implications for release of cellular contents in different tissues throughout the body.

In summary, we have demonstrated a novel pathway involving a PLCγ2-calcium-CaMKII axis downstream of Syk important for the exocytosis of TNFα, but not IL-6, following CpG stimulation. These findings establish Syk as an important intermediate for the differential release of cytokines downstream of PAMP activation to elicit an appropriate cytokine response. The regulation of exocytosis by PAMPs marks a new area to explore in the control of cytokine release by PRRs. There likely exist other biochemical mediators activated by PRRs that regulate the secretion of cytokines other than TNFα. Understanding in which vesicular compartments these cytokines reside and which pathways regulate their secretion will provide greater insight into how particular cytokine responses are generated.

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