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Annexin-A1 Regulates TLR-Mediated IFN-β Production through an Interaction with TANK-Binding Kinase 1

Pradeep Bist,*1 Shinla Shu,*1 Huiyin Lee,† Suruchi Arora,* Sunitha Nair,* Jyue Yuen Lim,† Jivanaah Dayalan,† Stephan Gasser,‡§ Subhra K. Biswas,† Anna-Marie Fairhurst,† and Lina H. K. Lim*‡§

TLRs play a pivotal role in the recognition of bacteria and viruses. Members of the family recognize specific pathogen sequences to trigger both MyD88 and TRIF-dependent pathways to stimulate a plethora of cells. Aberrant activation of these pathways is known to play a critical role in the development of autoimmunity and cancer. However, how these pathways are entirely regulated is not fully understood. In these studies, we have identified Annexin-A1 (ANXA1) as a novel regulator of TLR-induced IFN-β and CXCL10 production. We demonstrate that in the absence of ANXA1, mice produce significantly less IFN-β and CXCL10, and macrophages and plasmacytoid dendritic cells have a deficiency in activation following polyinosinic-polycytidylic acid administration in vivo. Furthermore, a deficiency in activation is observed in macrophages after LPS and polyinosinic-polycytidylic acid in vitro. In keeping with these findings, overexpression of ANXA1 resulted in enhanced IFN-β and IFN-stimulated responsive element promoter activity, whereas silencing of ANXA1 impaired TLR3- and TLR4-induced IFN-β and IFN-stimulated responsive element activation. In addition, we show that the C terminus of ANXA1 directly associates with TANK-binding kinase 1 to regulate IFN regulatory factor 3 translocation and phosphorylation. Our findings demonstrate that ANXA1 plays an important role in TLR activation, leading to an augmentation in the type 1 IFN antiviral cytokine response. The Journal of Immunology, 2013, 191: 4375–4382.
more, the presence of ANXA1 is necessary for survival in a murine model of LPS-induced endotoxemia, and the loss results in an increase in TNF-α, IL-6, and acute-phase proteins and decreased IL-10 production (12).

The primary mechanism by which ANXA1 is known to mediate its effects is through the formyl peptide receptor (FPR), the FPR-like 1 (FPRL1), and FPR2, the same receptor that the anti-inflammatory mediator lipoxin-A4 associates with (20, 21). The FPR family of proteins are G protein–coupled, pattern recognition receptors. Therefore, exogenous ANXA1 can act on FPRs to dampen inflammatory monocyte activation (22) and resolve inflammation (23, 24).

In the studies presented in this paper, we sort to clarify the role of Annexin-1 in modulating TRIF-mediated activation using either LPS or poly(I:C). We determined that ANXA-1 is required for optimal activation of macrophages, plasmacytoid dendritic cells (pDCs), and B cells. Furthermore, deficiency was associated with a defect in TLR-mediated IFN-β and CXCL10 production. We show that ANXA-1 associates with TBK1, a downstream signaling associated molecule of the TRIF signaling pathway possibly enhancing its activity. Furthermore, this enhancement is not dependent on the known receptor for ANXA-1, FPRL1. Collectively, these data demonstrate that ANXA1 is an important regulator of type I IFN production and identify a novel mechanism by which ANXA1 mediates its effects.

Materials and Methods

Mice

BALB/C mice (8–12 wk) were obtained from Laboratory Animal Centre (Singapore). ANXA1–/– mice on BALB/C background (backcrossed over 10 generations) were a gift from Prof. R. Flower (William Harvey Research Institute, London, U.K.) (25) and were bred in pathogen-free conditions in our animal facility. All animal work was approved by the Institutional Animal Care and Use Committee and followed National Advisory Committee for Laboratory Animals Research Guidelines (Guidelines on the Care and Use of Animals for Scientific Purposes).

In vivo administration of poly(I:C)

Wild-type (WT) and ANXA1–/– mice (6–8 wk) were injected either with PBS or 100 μg poly(I:C) i.p. Serum was obtained from mice and 4 h after injection. Mice were sacrificed by an overdose of CO2, and peritoneal lavage fluid was collected after 4 d. Macrophages were enriched by adherence onto tissue culture plates before treatment. LPS (0111:B4) or IFN-β (Promega), used according to manufacturer’s recommended protocol. Real-time RT-PCR was carried out using an Applied Biosystems 7500 Real-Time PCR System. Primers used to amplify DNA sequences were as follows: mouse IL-6, 5′-GGAGCTATGCGGTTGCACAA-3′ (forward) and 5′-TCCA CGATTCCCCAGAACA-3′ (reverse); mouse TNF-α, 5′-GGACAGTGAGCCTTATTAGG-3′ (forward) and 5′-TGTGGTTGGGAGGAAAGGG-3′ (reverse); mouse CXCL10, 5′-GGGCGCTTCGCAAGCTAAC-3′ (forward) and 5′-GCTTCTCTATGGCCCTCATT-3′ (reverse); and mouse IFN-β, 5′-GGGCGACTCAATGCTACTAT-3′ (forward) and 5′-GAGATGGCAAAAGGCTTTGAAC-3′ (reverse). Synthesized cDNA (100 ng) was added to the relevant forward and reverse primer together with 10 μl Supermix with ROX passive reference dye (Applied Biosystems) to probe for the gene of interest. Real-time PCR was run and quantified using the 7500 Real-Time PCR System (Applied Biosystems). Reactions were run in triplicate in at least three independent experiments. β-Actin was used as an internal control to normalize the variability in expression levels. Expression levels were analyzed using the 2-ΔΔCT method.

Immunofluorescence and microscopy

Cells were seeded onto sterilized glass coverslips contained in 24-well plates. They were washed with cold PBS and fixed with cold 3% paraformaldehyde in PBS for 30 min at 4°C. The fixed cells were washed and incubated with rabbit anti-IRF3 (clone FL-425; Santa Cruz Biotechnology) or mouse anti-IRF3 (Promega), used according to manufacturer’s recommended protocol. Real-time RT-PCR was carried out using an Applied Biosystems 7500 Real-Time PCR System. Primers used to amplify DNA sequences were as follows: mouse IL-6, 5′-GGAGCTATGCGGTTGCACAA-3′ (forward) and 5′-TCCA CGATTCCCCAGAACA-3′ (reverse); mouse TNF-α, 5′-GGACAGTGAGCCTTATTAGG-3′ (forward) and 5′-TGTGGTTGGGAGGAAAGGG-3′ (reverse); mouse CXCL10, 5′-GGGCGCTTCGCAAGCTAAC-3′ (forward) and 5′-GCTTCTCTATGGCCCTCATT-3′ (reverse); and mouse IFN-β, 5′-GGGCGACTCAATGCTACTAT-3′ (forward) and 5′-GAGATGGCAAAAGGCTTTGAAC-3′ (reverse). Synthesized cDNA (100 ng) was added to the relevant forward and reverse primer together with 10 μl Supermix with ROX passive reference dye (Applied Biosystems) to probe for the gene of interest. Real-time PCR was run and quantified using the 7500 Real-Time PCR System (Applied Biosystems). Reactions were run in triplicate in at least three independent experiments. β-Actin was used as an internal control to normalize the variability in expression levels. Expression levels were analyzed using the 2-ΔΔCT method.

Western blotting

Cells were scraped in the presence of lysis buffer, and supernatant was collected and stored at −80°C until evaluation by SDS-PAGE analysis. Equal amounts of protein from each sample were subjected to 10% SDS-PAGE at a constant voltage of 125 V. The proteins were transferred onto nitrocellulose membranes (Bio-Rad). Proteins were determined by Western blotting with specific Abs, and expression signals were obtained by ECL. Protein expression was normalized to β-actin levels.

Chromatin immunoprecipitation

Bone marrow–derived macrophages (1 × 10⁶) were seeded in 10-cm² dishes and stimulated with LPS or poly(I:C). Cells were fixed by formaldehyde fixation (1%), and nuclear extraction and chromatin immunoprecipitation (ChIP) were performed as described previously (12). Immunoprecipitation was carried out overnight at 4°C with 2 μg anti-IRF3 (clone FL-425; Santa Cruz Biotechnology). DNA/protein/Ab complexes were collected with protein G plus agarose for 30 min and washed in high-salt buffer followed by washes in no-salt buffer. DNA was released from the immune complex by heating overnight and extracted. The input control for the PCR was DNA from total nuclear extract. PCR was performed with total DNA (input control, 1 μl) and immunoprecipitated DNA (1 μl) using the following CXCL10 promoter–specific primers 5′-AACACTTCAGCTTTG-3′ and 5′-GTCCGTGTGGCAGTACT-3′. The length of the amplified product was 186 bp. PCR products (25 μl) were subjected to electrophoresis on 2% agarose gels.

Luciferase assay

293T or THP1 cells (1 × 10⁶ cells/well) were seeded into 12-well plates. Cells were transfected with the IFN-stimulated responsive element (ISRE) or IFN-β-Luc reporter gene plasmids (gifts from Dr. S. Akira, Osaka, Japan) using SuperFect transfection reagent (Qiagen) following the manufacturer’s protocol, along with each expression vector as indicated. The total DNA concentration was kept constant by supplementing with empty vector pcDNA3.1 pTK-Renilla (10 pg) was transfected at the same time for normalizing transfection efficiencies. Thirty-six hours after transfection, luciferase activity was determined with the dual-luciferase assay system (Promega). The values represented the average of three independent experiments.

Immunoprecipitation

Cells (1 × 10⁶) were seeded in 10-cm² dishes and transfected the following day. For transient transfection and coinmunoprecipitation experiments, transfection was performed using SuperFect transfection reagent. In the same experiment, where necessary, empty control plasmid (Flag/V5-tagged) was added to ensure that each transfection contains the same amount of DNA. After 36 h, transfected cells were lysed in 1 ml of lysis buffer. DNA was extracted using the QIAamp DNA Minikit (Qiagen) following the manufacturer’s instructions. DNA samples were subjected to electrophoresis on 2% agarose gels.
buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton, 1 mM EDTA, 1× protease and phosphatase inhibitors, and 1 mM PMSF). For each immunoprecipitation, 0.5 ml aliquot of lysate (0.2 mg) was incubated with 25 μl of a 1:1 slurry of G Plus-Sepharose (Santa Cruz Biotechnology) equilibrated in 1× lysis buffer for 3 h. The precleared lysates were further incubated with 2 μg of the indicated Ab or mouse IgG as control, 25 μl of a 1:1 slurry of G Plus-Sepharose (Santa Cruz Biotechnology) and incubated overnight. The Sepharose beads were washed with lysis buffer containing 250 mM NaCl. The precipitates were analyzed by Western blots as per the standard protocol.

**Statistical analysis**

Nonparametric Mann–Whitney U tests were used to compare two groups, and one-way nonparametric ANOVA with Dunn’s multiple comparison test were used to test multiple comparisons with normal distributions. $p < 0.05$ was considered to be statistically significant. Analyses were completed using Prism 5.0 for Windows (GraphPad Software). Results are expressed as the arithmetic mean ± SEM.

**Results**

**IFN-β and CXCL10 cytokine production are defective in ANXA1−/− macrophages**

The TLR4 ligand LPS uses both MyD88-dependent and TRIF-dependent pathways to trigger inflammatory responses involving the production of proinflammatory cytokines, whereas the TLR3 agonist poly(I:C) specifically signals through the TRIF pathway. To determine whether ANXA1 plays a role in the regulation of cytokines through MyD88 or TRIF pathways, TNF-α, IL-6, IFN-β, and CXCL10 levels were analyzed in WT and ANXA1−/− macrophages stimulated with LPS or poly(I:C). IFN-β and CXCL10 mRNA expression increased early after LPS stimulation, which was inhibited in ANXA1−/− cells (Fig. 1). Similarly, WT macrophages secreted significantly more IFN-β and CXCL10 compared with ANXA1−/− cells following stimulation with poly(I:C) (Fig. 1). This suggests that ANXA1 affects IFN-β-dependent signaling through the TRIF-dependent pathway. Furthermore, TNF-α production was enhanced in ANXA1−/− macrophages after LPS stimulation (Fig. 1C, 1F), which is consistent with earlier reports. Similarly, when cells were stimulated with the TLR9 agonist, Cpg ODN1826, which only activates the MYD88-dependent signaling pathway, TNF production in ANXA1−/− cells was enhanced (Supplemental Fig. 1A), whereas treatment with poly(I:C) did not induce significant TNF-α production (Fig. 1F), indicating that ANXA1 inhibits TNF-α production through the MyD88 pathway. Because ODN1826 only stimulates the MYD88 pathway, CXCL10 production was measured after ODN1826 stimulation to ensure that the ODN1826 was clean and free of LPS, which could activate the TRIF pathway. As expected, we observed that ODN1826 did not stimulate CXCL10 production (Supplemental Fig. 1B). Finally, IL-6 was produced to similar extents in WT and ANXA1−/− cells after treatment with LPS and ODN1826 (Supplemental Fig. 1C), suggesting that the MYD88-driven IL-6 pathway is unaffected by the deletion of ANXA1.

Collectively, these results suggest that ANXA1 interacts with both MyD88 and TRIF pathways to direct the macrophages toward augmented type I IFN responses and away from TNF-α production.
ANXA1−/− mice show a systemic defect in IFN-β production following poly(I:C) administration

Next, we examined whether ANXA1 regulates in vivo innate immune responses induced by poly(I:C). Serum cytokine levels of TNF, IL-6, IFN-β, and CXCL10 (Fig. 2) as well as IFN-α and IFN-γ (Supplemental Fig. 2A) were measured after i.p injection of poly(I:C). Maximal levels of all cytokines were detected at 4 h in both WT and ANXA1−/− mice. Consistent with our in vitro macrophage data, IFN-β and CXCL10 production were abrogated in ANXA1−/− mice (Fig. 2). Splenic leukocytes were analyzed at 24 h postinjection by flow cytometry. There were no differences in T cell, B cell, conventional DC, or pDC populations between WT and ANXA1−/− mice (Supplemental Fig. 2B). Analysis of co-stimulation markers on DCs revealed no difference in conventional DCs, but a significant inhibition in the upregulation of both CD80 and CD86 on pDCs from ANXA1−/− mice following poly(I:C) (Fig. 3A–D). Examination of the peritoneal cell infiltration and activation 24 h after poly(I:C) injection revealed no differences in the numbers of B cells, macrophages, or polymorphonuclear (PMN) cells between the two strains (Supplemental Fig. 2B). However, B cells and macrophages were less activated in ANXA1−/− mice following poly(I:C), as determined by surface CD86 expression (Fig. 3E, 3F). Analysis of peritoneal PMNs and DC revealed no difference in either CD11b or CD86 expression (Fig. 3G, 3H).

**ANXA1 is required for effective IRF3 nuclear translocation and activation**

Because our studies demonstrated that ANXA1 is required for efficient IFN-β and CXCL10 production by macrophages, we examined the upstream transcription factor required for production, IRF-3 (3). Upon stimulation, IRF3 is phosphorylated and translocates to the nucleus (3). Western blotting analysis demonstrated that LPS-induced IRF3 phosphorylation was maximal at 60 min poststimulation with a reduction in ANXA1−/− cells compared with WT (Fig. 4A). Furthermore, analysis of IRF3 recruitment to the CXCL10 promoter by ChIP analysis revealed a reduction in IRF3 binding to the CXCL10 promoter in ANXA1−/− macrophages compared with WT (Fig. 4B). In addition, LPS stimulation of macrophages resulted in IRF3 nuclear translocation in WT (Fig. 4C, Supplemental Fig. 3A) and not in ANXA1−/− cells. Moreover, confocal microscopy shows that IRF3 remained cytoplasmic 60 min poststimulation in ANXA1−/− macrophages (Fig. 4C, Supplemental Fig. 3B). Similarly, treatment of WT macrophages with poly(I:C) induced IRF3 nuclear translocation (Fig. 4D, Supplemental Fig. 4A), which was not observed in the ANXA1−/− macrophages (Fig. 4D, Supplemental Fig. 4B). STAT-1 phosphorylation was next analyzed and found to be inhibited in ANXA1−/− macrophages following LPS stimulation (Fig. 4E).

Finally, to translate this to a human system, ANXA1, or the positive control, TBK1 was silenced in human monocyte THP-1 cells and IRF3 dimerization, and activation was assessed using native PAGE. Poly(I:C) treatment induced IRF3 dimerization, which was inhibited in the presence of small interfering RNA to ANXA1 or TBK1 (Fig. 4F).

Altogether, these data demonstrate a novel regulation of IRF3 activation, dimerization, and translocation by ANXA1, which results in the modulation of IFN-β, CXCL10, and downstream targets.

**ANXA1 enhances ISRE and IFN-β promoter activity**

We then assessed the regulation of IFN-β by overexpressing ANXA1 in cultured human 293T embryonic kidney cells. Cells with transduced ANXA1 resulted in enhanced IFN-β promoter activity (Fig. 5A). Similarly, using an IRF-reporter DNA construct encoding ISRE of the ISG56 gene, overexpression of ANXA1 significantly poten-

![FIGURE 3. ANXA1−/− mice exhibit lower splenic pDC activation and peritoneal cell activation after in vivo administration of poly(I:C). WT or ANXA1−/− mice were injected with 100 μg poly(I:C) per mouse i.p. Splenic DCs (A, B) or pDCs (C, D) were stained using multicolor flow cytometry and CD80 and CD86 levels were analyzed, and mean fluorescent intensity (MFI) was measured. (E–H) Peritoneal lavage cells were obtained from mice 24 h after injection. CD86 level was examined on B cells (E) and macrophages (F) and dendritic cells (G), whereas CD11b expression was measured on PMN (H). Data are mean ± SD for n = 5–8 mice/group; *p < 0.05, **p < 0.01 versus untreated mice of the same genotype, ***p < 0.01 versus WT-treated mice.](http://www.jimmunol.org/)

**ANXA1 physically associates with TBK1**

TRIF recruits the TBK1 and IκB kinase-ι (IKKi or IKKε), which subsequently phosphorylate IRF3 (3–4). Cotransfection of ANXA1 with TRIF and TBK1 increased IFN-β promoter activity, whereas cotransfection with IKKi or IRF3 did not produce an observable change (Fig. 5E–H). This suggests that ANXA1 targets TBK1 directly. Indeed, LPS-induced TBK1 phosphorylation was reduced in ANXA1-deficient cells (Fig. 6A). Immunoprecipitation experiments demonstrated that ANXA1 is associated with TRIF under resting physiological conditions in THP-1 cells and not TRAF3 or TRAF6 (Fig. 6B). In addition, following LPS stimulation, ANXA1 associates with TBK1. Finally, we constructed structural

![ANXA1 promotes IFN-β production](http://www.jimmunol.org/)
ANXA1 mutants to determine the binding site of ANXA1, which associates with TBK1. The full-length construct of ANXA1 (1–346) demonstrated a good interaction with TBK1 (Fig. 6C). In addition, the two constructs, which included the end C terminus of ANXA1 (275–346) and (42–346), were able to bind to TBK1 with moderate efficiency despite the lack of the N terminus. However, deletion of the C terminus (1–41) showed that the N terminus does not bind to TBK1. Overall, our data demonstrate that the C terminus is important in the binding of ANXA1 to TBK1, which is not the region shown to associate with the ANXA1 receptor.

We next examined the functional impact of the truncated ANXA1 mutants on IFN-β promoter activity. In keeping with our earlier findings, overexpression of TBK1 in 293 cells induced IFN-β promoter activity, and cotransfection with the full-length ANXA1 plasmid potentiated this response (Fig. 6D). However, transfection of the N terminus mutant of ANXA1 (1–41) completely inhibited TBK1 induced IFN-β promoter activity, whereas expression of truncated ANXA1 (42–346) and the C terminus mutant of ANXA1 (275–346) partially inhibited TBK1-induced IFN-β promoter activity, suggesting that only full-length ANXA1 can potentiate TBK1 activity, whereas cleaved or truncated ANXA1 inhibits TBK1 induced IFN-β promoter activity.

**Secreted ANXA1 binding to its receptor inhibits IFN-β promoter activity**

The data described thus far indicate that ANXA1 positively regulates IFN-β promoter activity upstream of IRF3. ANXA1 can also be secreted, binding to its cognate cell surface receptor, also known as the FPRL1 to exert anti-inflammatory effects (6, 20). To determine whether endogenous ANXA1 or the stimulation of FPRL1 is responsible for the increase in IFN-β promoter activity, we transfected 293 cells, which normally do not express the FPRL1 receptor, with TBK1, together with either ANXA1 or FPRL1 plasmids. Detection using a luciferase reporter system demonstrated the expected increase of IFN-β promoter activity following ANXA1/TBK1 cotransfection (Fig. 6E). Cotransfection of TBK1 with FPRL1 did not result in a significant inhibition of luciferase activity, which was also not reversed with the addition of the FPR receptor inhibitor BOC1. However, addition of the FPRL1 receptor together with ANXA1 resulted in an inhibition of TBK1-induced IFN-β promoter activity as well as ANXA1-enhanced TBK1-induced IFN-β promoter activity. This inhibition by FPRL1 was prevented with the FPR receptor inhibitor, BOC1 peptide. Therefore ANXA1 may regulate IFN-β promoter activity via two differing mechanisms. When it is bound to FPRL1, it may inhibit IFN-β promoter activity. However, in the absence of FPRL1, it may bind to TBK1, driving IFN-β promoter activity, demonstrating a novel and differential role for ANXA1 as a signaling molecule and secreted ANXA1 as a FPRL1 ligand in the type I IFN production.

Collectively, these results show a novel function of ANXA1 in the induction of type I IFN production and activity in vitro and in vivo through the promotion of IRF3 activity in a TRIF-dependent manner.
FIGURE 5. ANXA1 modulates IFN-β and ISRE promoter activity. 293 cells were transfected with (A) IFN-β- or (B) ISRE-reporter luciferase plasmid with or without ANXA1-V5 overexpression plasmid, and luciferase activity was measured after 36 h. (C and D) THP1 cells were transfected with either IFN-β- or ISRE-reporter luciferase plasmid and specific short hairpin RNA (shRNA) targeting ANXA1. Thirty-six hours posttransfection, cells were treated with LPS or poly(I:C), and luciferase activity was measured after 8 h. (E and F) 293T cells were transiently cotransfected with IFN-β-luciferase reporter construct ANXA1-V5 plasmid, and the indicated plasmids TRIF, TBK1, IKKγ, and IRF3. Luciferase activity was measured 36 h after transfection. Results shown are representative of at least three independent experiments. *p < 0.05, **p < 0.01 versus vector control, ##p < 0.01 versus control shRNA-treated cells.

Discussion
The activation of innate immune response primarily relies on the appropriate sensing of pathogens through pattern recognition receptors such as TLR (expressed on innate immune cells like monocytes or macrophages) and activation of a signaling cascade, which orchestrates a gene expression program that shapes the immune response against pathogens (26–28). Thus, characterization of the molecular players that trigger and regulate TLR response provides vital insight into the molecular mechanism of innate immunity and their therapeutic targeting for host protection. In the current study, we demonstrate that ANXA1 acts as a key component of the TLR signaling pathway in macrophages, particularly driving the TRIF-dependent TLR cascade and the expression of IFN-inducible cytokines/chemokines involved in the innate immune response. ANXA1 has been found to have anti-inflammatory and anti-migratory effects in several models in vitro and in vivo (9, 10, 29). In contrast to its well-characterized anti-inflammatory properties, our results suggest ANXA1 as an essential mediator of TLR-induced inflammation. We demonstrate in this paper that ANXA1 can promote IFN-β production, possibly through the regulation of IRF3. Specifically, ANXA1 modulates the TRIF-dependent TLR pathway, in response to TLR4 or TLR3 stimulation. This study thus demonstrates the importance of ANXA1 in IFN-β production through the TRIF-dependent pathway.

IFN-β production is stimulated through the TRIF-dependent pathway following TLR stimulation, which is mainly induced by RNA and DNA ligands, suggesting that ANXA1 may be important in the regulation of the antiviral response. In addition, type I IFNs can be produced through the stimulation of cytoplasmic receptors for nucleic acids such as RIG-I and MDA5 (30, 31). However, in this study, we have focused on TLR stimulation, namely through TLR4 and endosomal TLR3, which stimulates the TRIF-dependent pathway (5). The TRIF-dependent pathway activates a vast range of inflammatory genes such as CXCL10. Functionally, these observations suggest an important role for ANXA1 in co-ordinating proinflammatory cytokine expression.

The regulation of MyD88-dependent genes (e.g., TNF-α) and TRIF-dependent genes (e.g., IFN-β and CXCL10) by ANXA1 was found to be distinctly different in both in vitro and in vivo experiments. Although the expression of IFN-β and CXCL10 was defective in ANXA1−/− macrophages, the expression of TNF-α was upregulated. TNF-α, IL-1, and IL-6 are known targets of the MyD88-dependent pathway (32). Yang et al. (33) have previously reported that ANXA1−/− macrophages produce higher levels of proinflammatory cytokines (IL-6, TNF-α, and ERK activation) after LPS compared with WT mice. Although our studies support the upregulated expression of TNF-α in ANXA1−/− macrophages (which is in line with the well-known anti-inflammatory role of ANXA1), our study is the first report on the role of ANXA1 in the regulation of cytokines in TRIF-dependent pathway. In addition, it was previously shown that ANXA1−/− bone marrow–derived DC exhibit increased basal expression of maturation markers, such as CD80, yet possess a diminished functional activation status after stimulation with LPS. Our study partially complements these findings where higher basal levels of CD80 expression were observed in splenic conventional DC, but after poly(I:C) stimulation in vivo, similar increases in CD80 expression compared with WT were observed. However, the activation of plasmacytid DC was markedly less in ANXA1−/− mice stimulated with poly(I:C) in vivo.

Our finding that ANXA1 is an anti-inflammatory mediator that also works in an antiviral manner is novel. However, compounds with anti-inflammatory and antiviral properties have been reported. Hydroquinone was shown to reduce proinflammatory cytokine production and recoverable plasma HIV virus in HIV-infected patients (34). Statins have also been shown to inhibit inflamma-
tory processes and suppress immune activation while also inhibiting HIV replication (35). In fact, IFN-β has been reported to have anti-inflammatory functions by inhibiting rhinovirus-induced CXCL10 and RANTES production, in addition to its antiviral properties (36).

In this context, it may be noted that ANXA1 has been classically reported as a molecule responsible for the immunosuppressive actions of GCs. However, GCs have recently been shown to inhibit IRF3 phosphorylation by targeting TBK1 activation (37), or disrupting GRIP–IRF3 binding (38), and dislocation of IRF3 from nucleus (39). This evidence clearly point toward GC in the inhibition of LPS-induced gene transcription by perturbing the TRIF pathway. Our present findings on ANXA1 as a regulator of the TRIF pathway by binding to TRIF and TBK1 provides a mechanistic basis linking GC and TRIF pathway through ANXA1. We have previously reported that ANXA1 can bind to NEMO and RIP1 (40) to enhance the activation of NF-kB. It is possible that the same regions of ANXA1 bind to TBK1 as well to regulate IRF3 activity. However, it is not apparent from our study as to how ANXA1 can shut down the same pathway under GC treatment. One possibility could be that GC treatment would induce an overproduction of ANXA1 that will sequester components of the TRIF pathway (as shown by our communoprecipitation studies of ANXA1 binding to TRIF and TBK1), making it unavailable for further activation by LPS. In addition, it may be possible that exogenous or secreted ANXA1 may have different functional effects after binding to its receptor, the FPR or FPRL1 receptor. The N-terminal region of ANXA1 has been shown to bind to the FPR, whereas our studies show that the C-terminal region of ANXA1 binds to TBK1. In addition, the N terminal is anti-inflammatory, whereas the C-terminal region of ANXA1 possesses proinflammatory properties. These differences in the full-length versus truncated or cleaved peptides of ANXA1 may explain the disparate results obtained with full-length ANXA1 as a signaling molecule regulating transcription factor activation versus secreted ANXA1 as an anti-inflammatory FPR ligand.

In summary, the present work elucidates ANXA1 as a key component of the TRIF-dependent pathway. ANXA1/−/− macrophages exhibit an aberrant activation of TBK1 that results in an inhibition of IRF3 activation and nuclear translocation, leading to abrogated expression of downstream cytokines, such as IFN-β, which is necessary for an antiviral response. However, its anti-inflammatory role is still evident in the regulation of TNF-α. The importance of ANXA1 in activating IFN-inducible cytokines/chemokines can be extended to several pathological settings such as host defense against viruses and bacteria as well as autoimmune diseases like systemic lupus erythematosus, where IFN production is a major concern (41).

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Disclosures

The authors have no financial conflicts of interest.

References

The authors have no financial conflicts of interest.
Supplemental figure legends

**Supplementary Figure 1. ANXA1<sup>-/-</sup> peritoneal macrophages exhibit higher TNF, and similar IL-6 production in response to CPG.** Thioglycolate-elicited peritoneal macrophages from WT or ANXA1<sup>-/-</sup> mice were stimulated with CPG (1μM). Supernatants were collected 24 h later for cytokine analysis by ELISA. Data represents mean ± SEM of 3 independent experiments. **p<0.01 vs indicated WT treatments.

**Supplementary Figure 2. Serum cytokine and splenic cell populations were not different in WT and ANXA1<sup>-/-</sup> mice injected with polyI:C systemically.** WT or ANXA1<sup>-/-</sup> mice were injected with 100 μg of poly(I:C) per mouse intraperitoneally. (A) Serum was obtained from mice 4h and 24h after injection for cytokine analysis. (B) Splenic cells were quantified and stained using multicolour flow cytometry. (C) Peritoneal lavage cell counts from WT and ANXA1<sup>-/-</sup> mice. Data are mean ±SEM for n=5-8 mice per group

**Supplementary Figure 3. IRF3 nuclear translocation is inhibited in ANXA1<sup>-/-</sup> macrophages after LPS treatment.** Confocal microscopy of (A) WT or (B) ANXA1<sup>-/-</sup> macrophages treated with LPS for 60 and 90 min, stained with DAPI (blue) and IRF3 (green)

**Supplementary Figure 4. IRF3 nuclear translocation is inhibited in ANXA1<sup>-/-</sup> macrophages after PolyI:C treatment.** Confocal microscopy of (A) WT or (B) ANXA1<sup>-/-</sup> macrophages treated with PolyI:C for 60 and 90 min, stained with DAPI (blue) and IRF3 (green)
Supplementary Figure 1.
Supplementary Figure 2.
Supplementary Figure 3.
Supplementary Figure 4.