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## Bruton's Tyrosine Kinase Is Required for Apoptotic Cell Uptake via Regulating the Phosphorylation and Localization of Calreticulin

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# Bruton's Tyrosine Kinase Is Required for Apoptotic Cell Uptake via Regulating the Phosphorylation and Localization of Calreticulin

Jennifer C. Byrne,\* Joan Ní Gabhann,\* Kevin B. Stacey,\* Barbara M. Coffey,\* Eoghan McCarthy,\* Warren Thomas,<sup>†</sup> and Caroline A. Jefferies\*

In addition to regulating B cell development and activation, Bruton's tyrosine kinase (Btk) functions downstream of multiple TLRs, including TLR7, to regulate innate immune responses in myeloid cells. Although critical for defense against RNA viruses such as influenza and Sendai virus, recognition of self-RNA by TLR7 also has been shown to be an important contributor to the pathophysiology of systemic lupus erythematosus. To date, the role of Btk in regulating TLR7-mediated responses is poorly understood. In the current study, we have demonstrated a hitherto undiscovered role for Btk in apoptotic cell uptake, identifying the molecular chaperone calreticulin (CRT) as a novel substrate for Btk in regulating this response. CRT together with the transmembrane receptor CD91 function at the cell membrane and regulate uptake of C1q-opsonised apoptotic cells. Our results show that Btk directly phosphorylates CRT and that in the absence of Btk, CRT fails to localize with CD91 at the cell surface and at the phagocytic cup. Critically, a blocking Ab against CRT in wild-type macrophages mimics the inability of Btk-deficient macrophages to phagocytose apoptotic cells efficiently, indicating the critical importance of Btk in regulating CRT-driven apoptotic cell uptake. Our data have revealed a novel regulatory role for Btk in mediating apoptotic cell clearance, with CRT identified as the critical component of the CRT/CD91/C1q system targeted by Btk. Given the importance of clearing apoptotic cell debris to prevent inappropriate exposure of TLRs to endogenous ligands, our results have important implications regarding the role of Btk in myeloid cell function. *The Journal of Immunology*, 2013, 190: 5207–5215.

Cell death by apoptosis is a crucial process that occurs under both normal physiologic and pathologic conditions in all tissues. It is also a critical element of inflammatory responses and in maintaining immune tolerance to self-Ags by the deletion of autoreactive T and B lymphocytes during lymphocytic development (1, 2). Rapid clearance of apoptotic cells by professional phagocytes (such as macrophages and dendritic cells) is essential so as to prevent both inflammation and recognition of autoantigens (3). Many receptors on phagocytic cells are known to recognize apoptotic cells and stimulate their uptake (3). Calreticulin (CRT) is an endoplasmic reticulum (ER) chaperone protein that ensures proper folding of proteins as well as maintaining calcium homeostasis. CRT has also been shown to localize to the cell membrane of phagocytic cells via a complex with the transmembrane protein CD91, acting as a coreceptor for the opsonin C1q, a complement component (4). Hence, CRT together with CD91 is critically important for C1q-mediated phagocytosis of opsonized pathogens and apoptotic bodies.

Bruton's tyrosine kinase (Btk) is a member of the Tec family of nonreceptor protein tyrosine kinases originally described for its role in B cell development (5). Recent studies, however, have focused on its role in regulating innate immune responses in myeloid cells, acting downstream of TLR4 and the antiviral TLRs, TLR3, -7, and -9, to regulate proinflammatory cytokine and type I IFN production (6–11). Btk has also been shown to interact with and become activated following ligation of TLR8 and -9 in the monocytic cell line THP-1 (8). Our laboratory has shown that loss of Btk profoundly affects LPS-induced cross-talk between dendritic cells and NK cells as well as the production of key inflammatory cytokines (10). Btk has also recently been demonstrated to form a complex with intracellular MHC class II via the costimulatory molecule CD40, thus facilitating a robust TLR-triggered inflammatory response in myeloid cells (12). This finding, in combination with other studies into the effect of loss of Btk on TLR signaling, increasingly supports a role for Btk as a positive regulator of TLR signaling.

Interestingly, Btk has also been shown to play a role in phagocytosis. For example, reduced Fcγ- and complement-mediated phagocytosis has been demonstrated in monocytes derived from X-linked agammaglobulemia (XLA) patients, an immunodeficiency resulting from inactivating mutations in *Btk* (13). Interestingly, XLA patients have an increased risk of developing nephritis (14, 15), potentially driven by the loss of clearance of apoptotic cell debris or immune complexes thus driving inflammation. However, the precise involvement of Btk in this process is unclear. Defects in apoptotic cell death and subsequent clearance of apoptotic bodies are also implicated in the pathogenesis of autoimmune disorders such as systemic lupus erythematosus (SLE) by virtue of the persistence of autoantigens, such as self-nucleic acids, which can trigger immune activation via recognition by the TLRs, specifically TLR7 and TLR9 (16–19). In the context of phagocytosis, TLR

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Abbreviations used in this article: BMDM, bone marrow-derived macrophage; Btk, Bruton's tyrosine kinase; CRT, calreticulin; ER, endoplasmic reticulum; ERC, endocytic recycling compartment; HO-1, heme oxygenase-1; KO, knockout; SLE, systemic lupus erythematosus; WGA, wheat germ agglutinin; WT, wild-type; XLA, X-linked agammaglobulemia.

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stimulation has been shown to differentially affect the uptake and cross-presentation of cellular Ags by monocyte-derived dendritic cells (20). Although Btk has been shown to be involved in TLR7 mediated responses in myeloid cells (11), the effect of loss of Btk on TLR7-induced changes to myeloid cells has not yet been fully explored.

We therefore set out to investigate the role of Btk in TLR7 signaling and apoptotic cell uptake in more detail. Our results indicate that the loss of Btk results in impaired cytokine production as well as reduced phagocytic uptake of apoptotic neutrophils in bone marrow-derived macrophages (BMDMs) generated from Btk-deficient mice. Pharmacological inhibition of Btk in human monocytes was also found to reduce the uptake of apoptotic cells. Critically, our study is the first, to our knowledge, to outline the interaction of Btk with the molecular chaperone CRT, and we demonstrate a novel role for Btk in regulating the uptake of apoptotic cells by myeloid cells.

## Materials and Methods

### Reagents

Imiquimod was purchased from Cayla Invivogen. L929 conditioned medium was used in BMDM derivation as a source of M-CSF. The Btk inhibitor LFM-A13 was purchased from Sigma-Aldrich.

### Mice

Btk-deficient mice (21) were housed and bred in the Biomedical Research Facility at the Royal College of Surgeons in Ireland under specific pathogen-free conditions. The mice were used at 6–10 wk of age and in accordance with ethical approval and license conditions.

### Cell culture

THP-1 cells were cultured in RPMI 1640 supplemented with 10% FCS and 10  $\mu$ g/ml gentamicin. For the generation of primary murine BMDMs, bone marrow cells were flushed aseptically from the dissected femurs of C57BL/6 mice or Btk-deficient mice, and BMDMs were derived by differentiating the bone marrow cells in DMEM supplemented with 10% L929 conditioned medium, 10% FCS, penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml) for 5–7 d. BMDMs were routinely tested for purity using the macrophage-specific Abs, anti-CD11b (BD Pharmingen), and anti-F4/80 (AbD Serotec). HEK293T cells (purchased from American Type Culture Collection) were cultured in DMEM supplemented with 10% FCS penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml). Human PBMCs were isolated from whole blood using a Ficoll gradient and cultured in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml). Experiments incorporating the use of human immune cells were performed in accordance with the Helsinki Declaration and ethical approval. All of the study participants provided written informed consent prior to enrollment.

### Two-dimensional electrophoresis

Two-dimensional gel electrophoresis was performed as previously described (22). The stained gels were scanned using a GS-800 Densitometer (Bio-Rad) and images analyzed using Progenesis SameSpots software (Nonlinear Dynamics).

### Mass spectrometry

Protein spots of interest were excised from silver-stained gels. Each gel plug was destained, reduced, alkylated, and digested overnight with trypsin (20 ng/ $\mu$ l sequencing-grade modified trypsin; Promega) at 37°C with gentle agitation. Peptides were analyzed by MALDI–tandem mass spectrometry performed on an Applied Biosystems 4800 Plus MALDI-ToF/ToF. The precursor ion masses and the masses of the daughter ions from tandem mass spectrometry experiments were scanned against the SwissProt database using Mascot Search Software incorporated in the GPS Explorer software (version 3.5).

### Cytokine multiplex assay

Macrophages were stimulated with imiquimod (20  $\mu$ g/ml) for the times indicated and cytokines in BMDM supernatant samples were simultaneously measured using a multiplex electrochemiluminescence assay (Meso Scale Discovery) and read by an Imager 2400 plate reader (Meso Scale Discovery).

### Plasmids and transient transfection

The full-length Btk construct was obtained from R. Hendriks (Department of Pulmonary Medicine, Erasmus Medical Center, Rotterdam, The Netherlands) as previously described (6). HEK293T cells were seeded at a density of  $5 \times 10^5$ /ml in a 10-cm dish and were transiently transfected with the Btk plasmid as indicated, using Metafectene (Biontex) according to the manufacturer's recommendations.

### Immunoprecipitation

Cells were lysed on ice in 1 ml Tris-based lysis buffer (50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1  $\mu$ g/ml aprotinin, leupeptin, pepstatin, 1 mM  $\text{Na}_3\text{VO}_4$ , and 1 mM KF) followed by immunoprecipitation with either anti-phosphotyrosine-conjugated agarose beads (Sigma-Aldrich) or anti-CRT Ab (Enzo Life Sciences) precoupled to protein-A Sepharose beads for 1 to 2 h at 4°C with gentle agitation. For immunoprecipitation following transient transfection of HEK293T cells, lysis was carried out using  $1 \times$  radio-immunoprecipitation lysis buffer ( $1 \times$  PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1  $\mu$ g/ml aprotinin, leupeptin, pepstatin, 1 mM  $\text{Na}_3\text{VO}_4$ , and 1 mM KF) followed by immunoprecipitation with either anti-Btk or anti-CRT precoupled to protein A-Sepharose beads. Immunoprecipitates were then analyzed by immunoblotting using appropriate Abs. To confirm the specificity of immunoprecipitation results, isotype-specific IgG-agarose was used as a negative control in all immunoprecipitation experiments.

### In vitro kinase assay

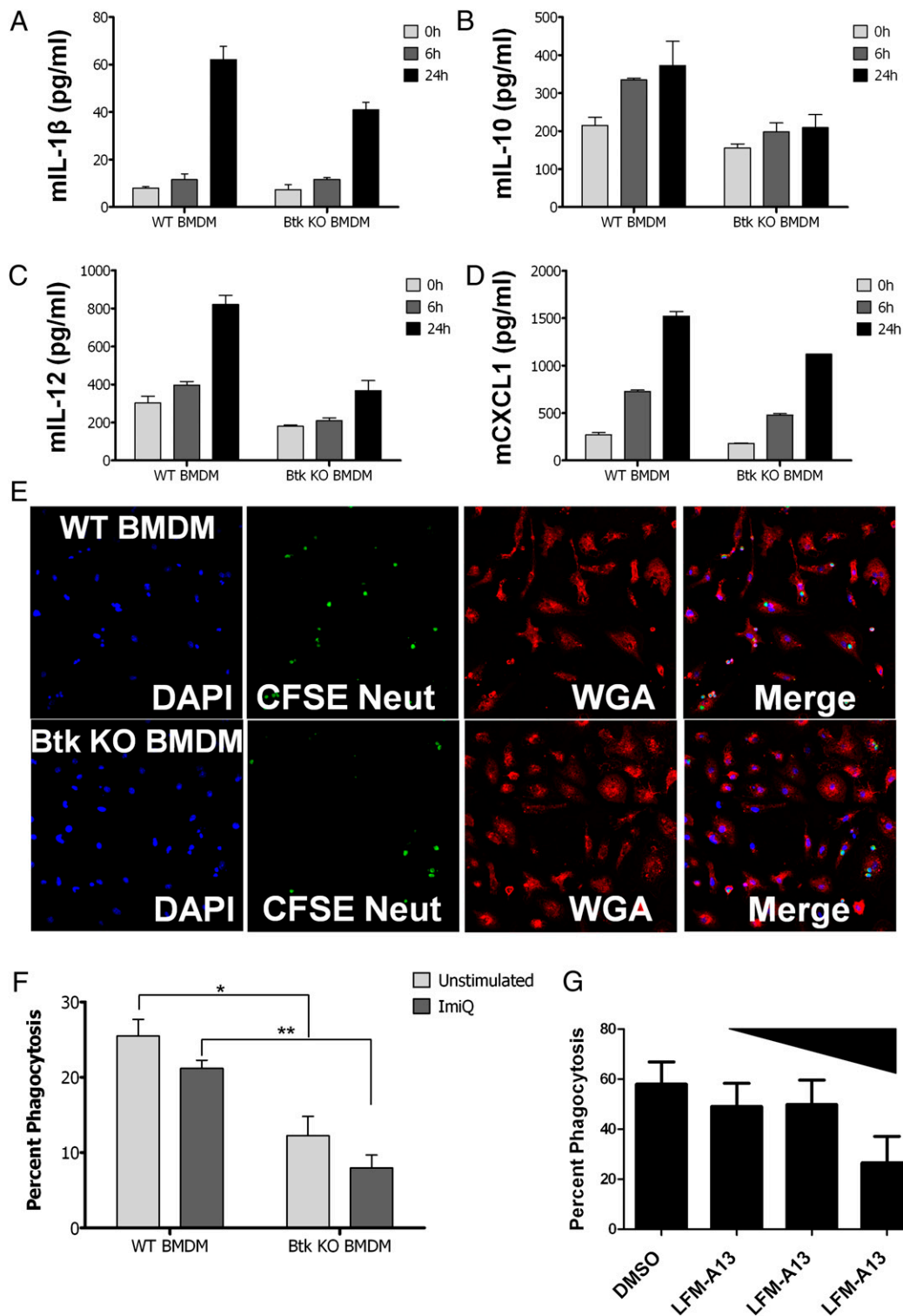
Recombinant active human Btk (200 ng; Millipore) was incubated with recombinant human CRT (1  $\mu$ g; Enzo Life Sciences) in the presence of 20  $\mu$ M ATP for 30 min at 37°C. Samples were immediately immunoblotted for tyrosine-phosphorylated residues using the monoclonal anti-phosphotyrosine Ab PT-66 (Sigma-Aldrich). Immunoprecipitation of active Btk kinase following TLR7 stimulation (20  $\mu$ g/ml) of THP-1 cells was followed by incubation with recombinant human CRT (1  $\mu$ g; Enzo Life Sciences) in the presence of 20  $\mu$ M ATP for 30 min at 37°C. Samples were immediately immunoblotted for tyrosine-phosphorylated residues using the PT-66 Ab.

### Confocal laser scanning microscopy

BMDMs were seeded onto UV-irradiated coverslips at a density of  $5 \times 10^5$  and incubated overnight at 37°C, 5%  $\text{CO}_2$  to achieve a subconfluent cell monolayer. Macrophages were stimulated with imiquimod (20  $\mu$ g/ml) as indicated, fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton-X, and blocked by incubation with FcR block (BD Pharmingen) in 1.2% fish gelatin in PBS solution for 1 h at 37°C. Incubation with CRT (Enzo Life Sciences) or CD91-specific (Abcam) primary Abs was performed for 1 h at 37°C in the presence of FcR block (BD Pharmingen). The subcellular localization of CRT or CD91 was visualized using a trirhodamine isothiocyanate- or FITC-conjugated secondary Ab, respectively (Molecular Probes). Isotype control Abs and unstained cells were used as negative controls to evaluate the nonspecific binding of target primary Ab to Ags (data not shown). Delineation of the cell surface was determined using an Alexa Fluor 633-conjugated wheat germ agglutinin (WGA; Molecular Probes). Stained cells were mounted onto microscope slides using ProLong Gold mounting solution (Molecular Probes) containing DAPI nuclear stain. Images were captured using a Zeiss LSM 510 META laser scanning microscope in multitrack configuration equipped with Plan-Neofluar 40 $\times$ /1.3 Oil DIC and Plan-Apochromat 63 $\times$ /1.4 Oil DIC objectives (Carl Zeiss). Excitation wavelengths used were 361, 488, 543, and 633 nm for DAPI, FITC, trirhodamine isothiocyanate, and Alexa Fluor 633, respectively. The images presented are representative single XY plane scan mid-sections through the cell monolayer or orthogonal XY scans combined with XZ and YZ plane scans as indicated.

### Induction of apoptosis in CFSE-labeled neutrophils

Healthy human neutrophils were isolated from whole blood by dextran sedimentation followed by density gradient centrifugation. Cells were labeled with CFSE (Invitrogen) as per manufacturer's instructions at a final concentration of 20  $\mu$ M per  $1 \times 10^6$  cells for confocal analysis or 1  $\mu$ M per  $1 \times 10^6$  cells for FACS analysis. Induction of apoptosis was carried out by exposure of CFSE-labeled neutrophils to UV irradiation at 254 nm for 15 min followed by culture in RPMI 1640 with 1% BSA for 2 to 3 h at 37°C, 5%  $\text{CO}_2$ . This approach successfully induced apoptosis of neutrophils with >80% of cells, demonstrating Annexin V–FITC positivity (TACS Annexin V Kit; Trevigen).



**FIGURE 1.** Loss of Btk affects the production of cytokines by BMDMs following TLR7 stimulation as well as the uptake of apoptotic cells. WT and Btk-deficient BMDMs were stimulated with imiquimod (20  $\mu$ g/ml) over indicated time points. Cell-free supernatants were collected and assayed for IL-1 $\beta$  (**A**), IL-10 (**B**), IL-12 (total) (**C**), and CXCL1 (**D**) using Meso Scale Discovery multiplex ELISA. Data are representative of two independent experiments (two-way ANOVA,  $p < 0.01$ ; bars represent  $\pm$  SD). (**E** and **F**) To assess the ability of BMDMs to phagocytose apoptotic cells, WT and Btk KO BMDMs were left untreated or stimulated with imiquimod (20  $\mu$ g/ml for 1 h) prior to coculture with apoptotic neutrophils (Neut; green). The cell surface of the BMDMs was delineated using WGA staining (red). The cells were then fixed and permeabilized before being visualized using a Zeiss LSM 510 META laser scanning microscope equipped with a Plan-Apochromat 40 $\times$ /1.4 DIC objective (Carl Zeiss). (**E**) Representative images of coculture of WT and Btk KO BMDMs with CFSE-labeled apoptotic neutrophils. (**F**) WT and Btk KO BMDMs were stimulated with imiquimod or left untreated prior to coculture with apoptotic neutrophils. Cells that had engulfed CFSE-labeled apoptotic neutrophils were counted, and the percentage phagocytosis was compared (unpaired  $t$  test, two tailed; bars represent  $\pm$  SEM). \* $p > 0.05$ , \*\* $p > 0.01$ . (**G**) PBMCs derived from healthy control individuals were pretreated with either DMSO vehicle control or increasing doses of the Btk inhibitor LFM-A13 prior to coculture with CFSE-labeled apoptotic neutrophils. Detection of FITC fluorescence in the monocyte gate was indicative of successful uptake ( $n = 4$ ; one-way ANOVA,  $p = 0.001$ ; bars represent  $\pm$  SD). Except where otherwise indicated, data are representative of three independent experiments. m, Murine.



### Phagocytosis assay

BMDMs were seeded onto UV-irradiated coverslips at a density of  $2.5 \times 10^5$  and incubated overnight at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . For CRT-blocking experiments, cells were pretreated with a polyclonal anti-CRT Ab (Enzo Life Sciences) at a final concentration of  $10 \mu\text{g/ml}$  for 30 min at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ , prior to washing with PBS and to culturing with CFSE-labeled human apoptotic neutrophils as previously described (23). To induce apoptotic cell uptake, adherent BMDMs were cocultured with CFSE-labeled human apoptotic neutrophils for 60 min at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . For confocal analysis, the cells were washed three times with ice-cold PBS, and delineation of the cell surface was determined using an Alexa Fluor 633-conjugated WGA (Molecular Probes). Cells were fixed and imaged as described above. The percent phagocytosis was generated by calculation of the number of BMDMs with internalized CFSE-labeled neutrophils divided by the total number of BMDMs. For investigation of apoptotic cell uptake in human cells, PBMCs were cultured either in the presence of the Btk inhibitor LFM-A13 (100, 250, and  $500 \mu\text{M}$ ) or DMSO vehicle control for 1 h at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . CFSE-labeled apoptotic neutrophils were then cocultured with PBMCs for 6 h ( $n = 4$ ). After this time, the cells were washed and subjected to FACS analysis (BD FACSCanto II; BD Biosciences). The monocyte gate was isolated from the mixed cell population based on their forward and side-scatter profiles, and the level of FITC intensity within the monocyte gate was used as a metric of apoptotic cell uptake by these cells.

### Statistical analyses

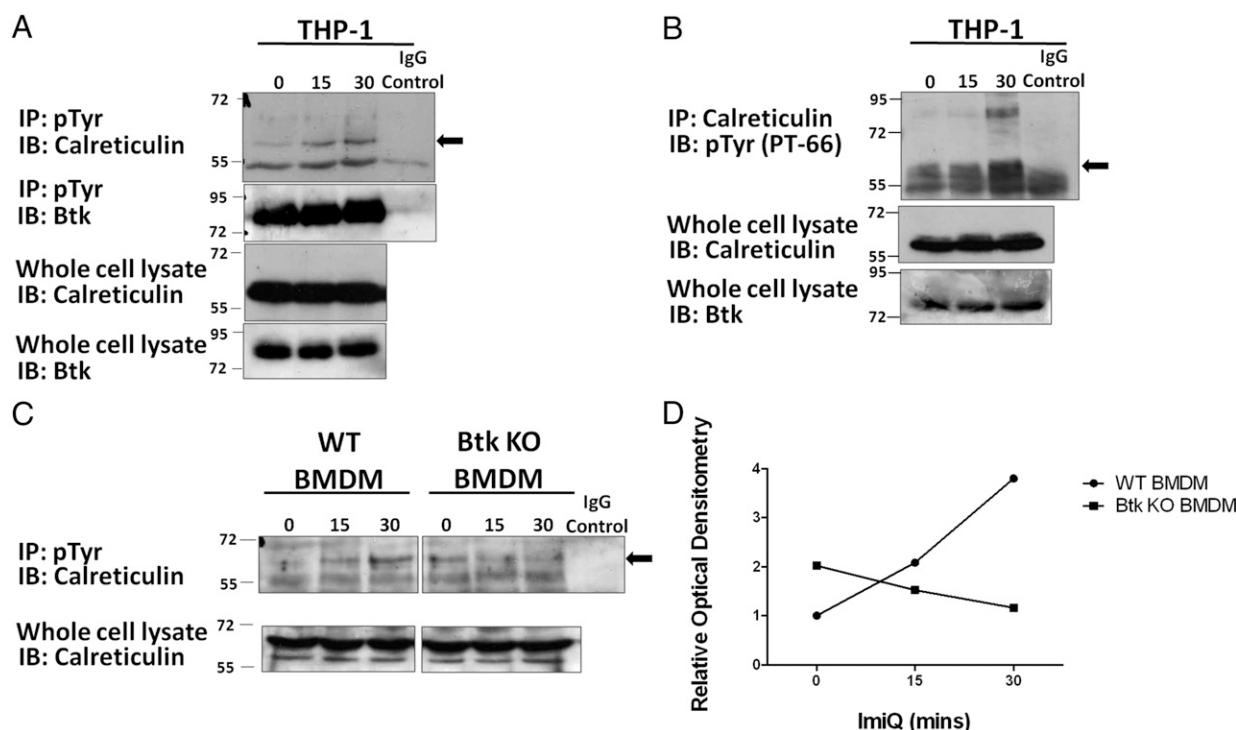
Statistical tests were carried out using GraphPad Prism (version 5.0; GraphPad) software. The  $p$  values  $<0.05$  were considered significant.

## Results

### Absence of Btk influences the production of inflammatory cytokines downstream of TLR7 stimulation as well as affecting the ability of BMDMs to uptake apoptotic neutrophils

In both murine and human cells, Btk has been shown to be a critical component of signaling pathways downstream of TLR2, -4, -7, -8,

and -9, which can positively regulate the transactivation of the p65 subunit of NF- $\kappa\text{B}$  (6–8). Using a multiplexed approach, we have assessed the production of a number of inflammatory cytokines downstream of TLR7 stimulation in BMDMs derived from wild-type (WT) and Btk-deficient animals. Overall, a statistically significant reduced production of IL- $1\beta$ , IL-10, IL-12, and CXCL1 (Fig. 1A–D; two-way ANOVA,  $p < 0.01$ ) was observed in Btk-deficient BMDMs underlining the importance of Btk in mediating TLR7-induced responses within myeloid cells. Professional APCs represent key mediators of immune response, not only through cytokine production but also through their ability to bind and phagocytose dead and foreign bodies. Given our results indicating reduced cytokine production downstream of TLR7 in the absence of Btk, we next assessed whether Btk-deficient BMDMs may have an altered capacity to uptake apoptotic cells. To evaluate this possibility, WT and Btk knockout (KO) BMDMs were cocultured with CFSE-labeled human apoptotic neutrophils over a 60-min period. Following this time, nonadherent cells were washed away with PBS, the cell membrane was delineated by WGA staining, and the cells were fixed and permeabilized. The number of BMDMs that had successfully taken up apoptotic cells was then determined using confocal microscopy (Fig. 1E, 1F). We observed a significant reduction in apoptotic cell uptake in the absence of Btk in both resting cells (unpaired  $t$  test, two-tailed  $p = 0.018$ ; Fig. 1F) and in cells pretreated with imiquimod for 1 h prior to coculture (unpaired  $t$  test, two-tailed  $p = 0.003$ ; Fig. 1F). Although there is a tendency toward a reduction in apoptotic cell uptake with TLR7 stimulation, there was no significant difference observed in either WT ( $p = 0.158$ ) or Btk KO ( $p = 0.240$ ) BMDMs. This finding is in line with a previous study of human monocyte-derived dendritic cells, which demonstrated that activation of the



**FIGURE 2.** CRT is tyrosine phosphorylated following TLR7 stimulation in both human and murine myeloid cells. THP-1 cells were stimulated with imiquimod ( $20 \mu\text{g/ml}$ ) over stated time points and an immunoprecipitation (IP) using phosphotyrosine-coupled beads followed by anti-CRT immunoblotting (IB) (A) or CRT precoupled to protein A-Sepharose beads followed by immunoblotting (B) using the PT-66 Ab, which recognizes tyrosine-phosphorylated residues, was performed. (C) WT and Btk-deficient BMDMs were stimulated with imiquimod ( $20 \mu\text{g/ml}$ ) for the indicated time points, and phosphotyrosine-containing immune complexes were immunoblotted for the presence of Btk. Data are representative of two independent experiments. (D) Relative optical densitometry of phospho-CRT indicates that there is an altered phosphorylation profile of CRT evident in the absence of Btk. In all cases, CRT is indicated by a black arrow. Except where otherwise indicated, data are representative of three independent experiments.

dendritic cells with TLR3 or TLR4 but not TLR2 or TLR7/8 ligands inhibited phagocytosis of apoptotic tumor cells (20).

To determine whether pharmacological inhibition of Btk would have similar effects on the ability of myeloid cells to clear apoptotic debris, we pretreated human PBMCs with the Btk inhibitor LFM-A13 prior to coculture with CFSE-labeled apoptotic neutrophils. Treatment with the Btk inhibitor resulted in a significant reduction in apoptotic cell clearance by monocytes in comparison with DMSO vehicle control-treated cells (one-way ANOVA,  $p = 0.001$ ; Fig. 1G). These results, together with our findings in murine macrophages, underline the importance of Btk in successful clearance of apoptotic cells by myeloid cells.

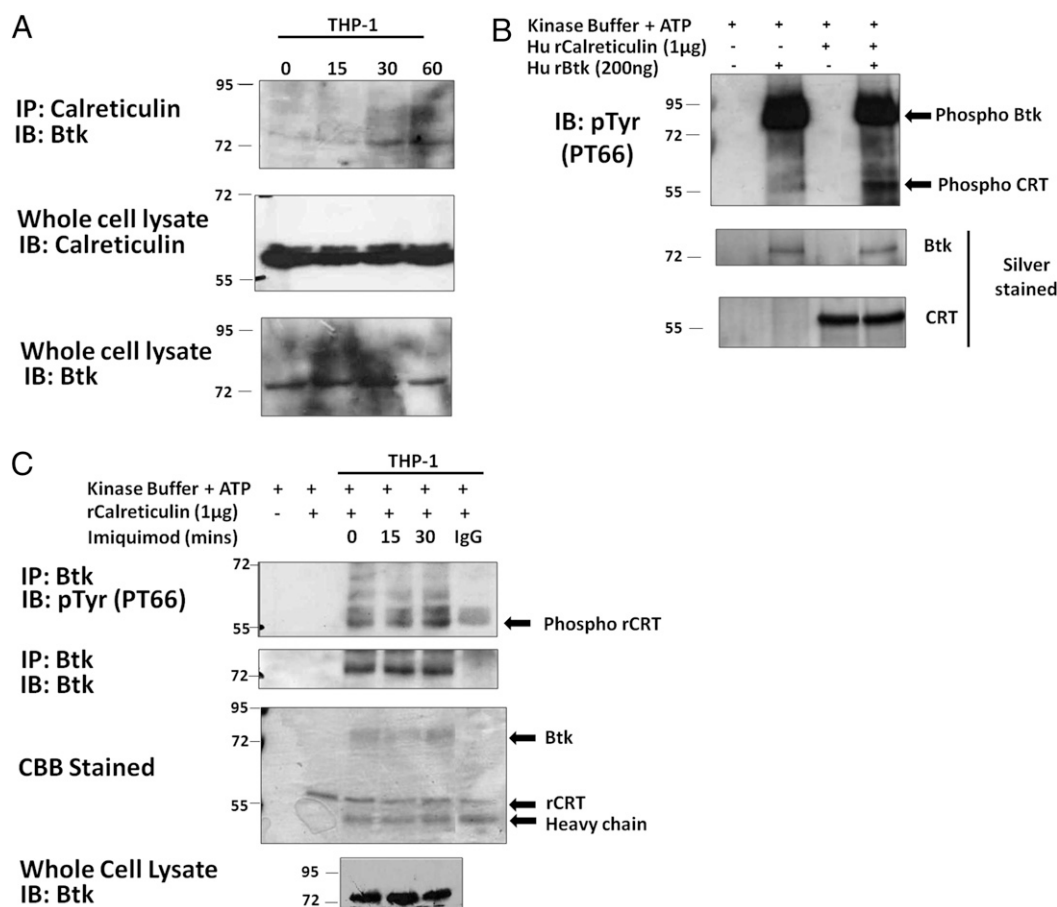
### Analysis of potential Btk substrates downstream of TLR7 stimulation

Reduced Fcγ- and complement-mediated phagocytosis has been demonstrated in monocytes derived from patients with the immunodeficiency XLA (13). Btk has been shown to be activated throughout the process of phagocytosis and can accumulate at the base of the phagocytic cup (24). Our results outlined above demonstrated a clear role for Btk in mediating apoptotic cell uptake in addition to regulating TLR7-induced cytokine responses. To identify potential substrates that Btk may be regulating to mediate phagocytosis, we carried out a two-dimensional proteomic analysis of whole-cell lysates of WT and Btk-deficient BMDMs following stimulation with imiquimod for 30 min. This study has identified the chaperone protein CRT as having altered

modification in Btk-deficient BMDMs compared with WT, suggesting CRT may be a potential substrate for Btk-mediated phosphorylation (Supplemental Fig. 1). As CRT was identified as a potential Btk substrate within this system, we next set out to determine if TLR7 could induce phosphorylation of CRT. Immunoprecipitation of either tyrosine-phosphorylated proteins or CRT from TLR7-stimulated lysates prepared from THP-1s, followed by Western blotting with either anti-CRT or anti-phosphotyrosine (PT66) as appropriate, demonstrated that CRT was inducibly phosphorylated following TLR7 stimulation (Fig. 2A, 2B, respectively). To determine the role of Btk in mediating TLR7-induced CRT phosphorylation, we compared the ability of TLR7 to induce CRT phosphorylation in WT or Btk KO BMDMs (Fig. 2C). Although CRT was inducibly phosphorylated in WT cells following TLR7 stimulation, in the absence of Btk, an altered phosphorylation profile of CRT is evident (Fig. 2C, 2D). This result may indicate that in the absence of Btk, other kinases are responsible for regulating the phosphorylation of CRT in resting cells.

### *Btk interacts with and directly phosphorylates CRT following TLR7 stimulation*

Having determined that CRT phosphorylation is altered in Btk-deficient cells, we next went on to assess whether Btk could interact with and phosphorylate CRT. Immunoprecipitation of Btk from TLR7-stimulated THP-1 cells demonstrated that Btk and CRT interact following TLR7 ligation (Fig. 3A). Reciprocal immunoprecipitation in THP-1s also indicated an interaction between

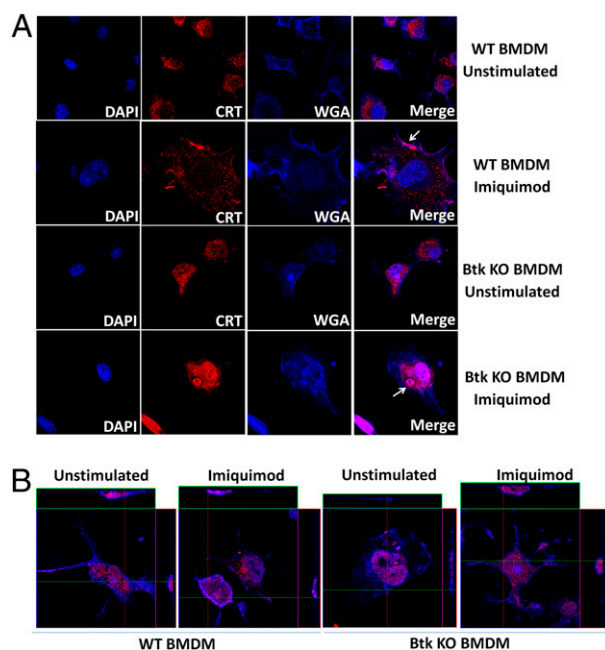


**FIGURE 3.** Btk interacts with and phosphorylates CRT downstream of TLR7 stimulation. **(A)** CRT was immunoprecipitated (IP) from THP-1 cells following TLR7 stimulation and Btk detected by immunoblotting (IB). **(B)** Recombinant active human Btk (Hu rBtk; 200 ng) was incubated with recombinant human CRT (Hu rCalreticulin; 1  $\mu$ g) in the presence of 20  $\mu$ M ATP for 30 mins at 37°C. Samples were separated by SDS-PAGE and immediately immunoblotted using PT-66 Ab. **(C)** Immunoprecipitation of Btk from THP-1 cells following imiquimod stimulation (20  $\mu$ g/ml) was followed by kinase assay using recombinant human CRT (rCalreticulin; 1  $\mu$ g). In all cases, data are representative of three independent experiments.

these proteins (Supplemental Fig. 2). To further confirm the association of Btk with CRT, Btk was overexpressed in HEK293T cells by transient transfection followed by immunoprecipitation of Btk (Supplemental Fig. 3). Although immunoprecipitation results in both primary and heterologous cells do indicate an interaction between Btk and CRT, this appears to be relatively weak or perhaps very transient. To determine if Btk phosphorylates CRT, an *in vitro* kinase assay was performed, demonstrating that recombinant active Btk directly phosphorylates recombinant CRT, indicating the CRT is indeed a substrate for Btk (Fig. 3B). Furthermore, a kinase assay was performed following isolation of active Btk from imiquimod-stimulated THP-1 cells by incubating immunoprecipitated endogenous Btk with recombinant CRT. This assay confirmed that Btk was activated following imiquimod stimulation and able to phosphorylate recombinant CRT (Fig. 3C). These studies reveal that Btk interacts with and phosphorylates CRT following TLR7 stimulation in both human and mouse myeloid cells.

*Trafficking of CRT is altered in Btk-deficient BMDMs following TLR7 stimulation, influencing the ability of these cells to uptake apoptotic neutrophils*

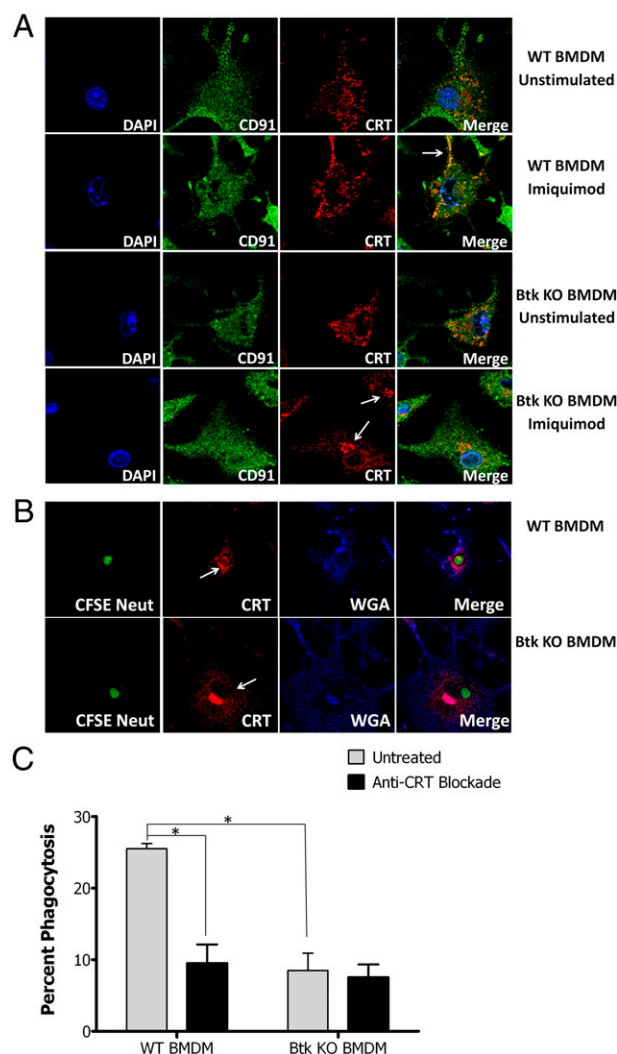
CRT is an ER-resident chaperone protein known to be involved in mediating apoptotic cell uptake (23, 25, 26). As previous studies have indicated that CRT phosphorylation status can regulate trafficking of this protein (27), we hypothesized that Btk-induced phosphorylation of CRT may regulate its trafficking in the cell and hence its activity in response to TLR7 stimulation. Confocal analysis of fixed BMDMs immunofluorescently labeled with an Ab specific for CRT demonstrated that in resting BMDMs derived from WT and Btk-deficient mice, CRT is visualized within a perinuclear structure indicative of the ER (Fig. 4A, 4B). TLR7 stimulation of WT cells results in dispersal of CRT to small ve-



**FIGURE 4.** Loss of Btk affects trafficking of CRT to the cell surface following TLR7 stimulation. **(A)** WT and Btk KO BMDMs were stimulated with imiquimod (20  $\mu$ g/ml) for 6 h and then stained for the cell surface using WGA (blue). The cells were then fixed and permeabilized before being stained for CRT (red). Images were captured using a Zeiss LSM 510 META laser scanning microscope equipped with a 63 $\times$ /1.4 Plan-Neofluar objective (Carl Zeiss). **(B)** Orthographic projection. Data are representative of three independent experiments. Images shown at original magnification  $\times 63$ .

sicular structures in the cytoplasm and translocation of CRT to the cell surface (Fig. 4A, highlighted by white arrow). In the absence of Btk however, CRT becomes sequestered in distinct cellular compartments proximal to the nucleus similar to the endocytic recycling compartment (ERC) (28). These results indicate that Btk regulates the relocation of CRT within the cell following TLR7 stimulation and that the ablation of Btk results in the dysregulation of CRT trafficking in response to extracellular stimuli.

The cell-surface receptor CD91 has been shown to complex with CRT and is involved in collectin and C1q-mediated uptake of apoptotic cells by the phagocyte (23, 25). Confocal analysis revealed that TLR7 stimulation induced a clear colocalization of



**FIGURE 5.** Absence of Btk influences colocalization of CRT with the cell-surface receptor CD91 following TLR7 stimulation and localization of CRT to the phagocytic cup. **(A)** WT and Btk KO BMDMs were stimulated with imiquimod (20  $\mu$ g/ml) for 6 h. The cells were then fixed and permeabilized before being stained for CRT (red) and CD91 (green). Images were captured using a Zeiss LSM 510 META laser scanning microscope equipped with a 63 $\times$ /1.4 Plan-Neofluar objective (Carl Zeiss). In the right panel, CRT colocalization with CD91 is indicated by the white arrow. **(B)** Confocal images from WT and Btk KO BMDMs stained with WGA (blue) and CRT (red) following coculture with human apoptotic neutrophils (green). Images shown at original magnification  $\times 63$ . **(C)** BMDMs were pretreated with anti-CRT Ab for 30 min at 37 $^{\circ}$ C, following which time CFSE-labeled neutrophils were added and apoptotic cell uptake was determined (unpaired *t* test, two tailed; bars represent  $\pm$  SEM). In all cases, data are representative of three independent experiments. \**p* < 0.01.



CRT and CD91 in WT BMDMs, particularly in the fine membrane projections (Fig. 5A, *second panel*, white arrow). In the absence of Btk however, CRT was sequestered as seen previously following TLR7 stimulation and failed to colocalize with CD91 (Fig. 5A, *fourth panel*, white arrow). This indicates that in absence of Btk abnormal trafficking of CRT leads to its reduced colocalization with CD91 at the cell membrane downstream of TLR7 stimulation. Indeed, further analysis demonstrated a reduction in staining for CRT within the phagocytic cup following coculture with apoptotic neutrophils in Btk-deficient BMDMs in comparison with WT (Fig. 5B, *second panel*, white arrow). This indicates that in the absence of Btk, there is an impairment of CRT to localize to the cell surface, leading to a reduced colocalization with CD91 and therefore a reduction in the ability to uptake apoptotic cells.

To confirm the role of CRT in the uptake of apoptotic neutrophils, we pretreated BMDMs with a polyclonal anti-CRT Ab prior to coculture with apoptotic neutrophils (Fig. 5C). Pretreatment of WT BMDMs with the anti-CRT Ab significantly reduced the ability of these cells to uptake apoptotic cells following coculture ( $p = 0.004$ ), bringing the percentage phagocytosis of the WT BMDMs in line with that of the Btk KO BMDMs. Pretreatment of the Btk KO BMDMs with the anti-CRT Ab was found to have no significant effect on uptake ( $p = 0.769$ ). Our findings point to a novel role for Btk in regulating the phagocytosis of apoptotic cells via the direct phosphorylation of CRT in response to TLR7 stimulation (outlined in Fig. 6).

## Discussion

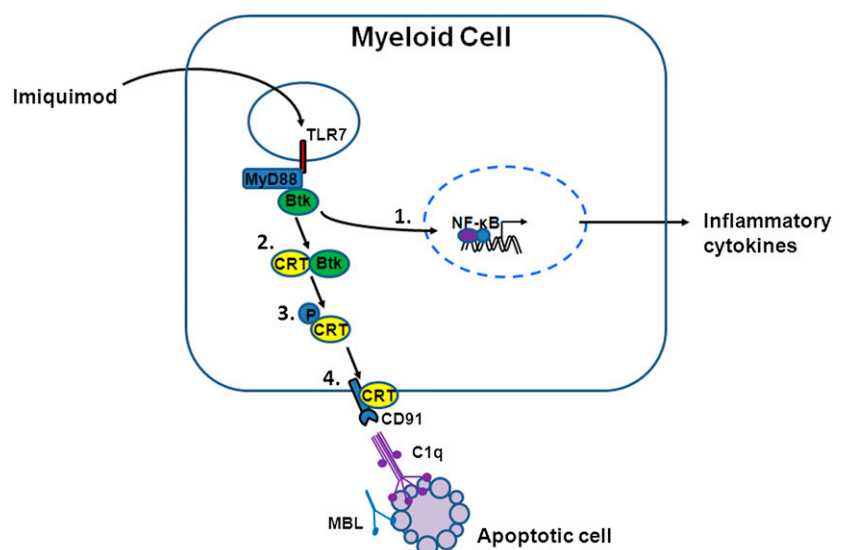
Defective clearance of apoptotic debris by myeloid cells is a hallmark trait of autoimmune conditions such as SLE (29) and has led to the suggestion that prolonged exposure to autoantigens as a result might represent an important trigger of systemic autoimmunity. Moreover, defective clearance of pathogenic immune complexes, facilitating tissue deposition, represents an important mechanism whereby tissue damage may occur. Indeed, impaired recognition of apoptotic neutrophils via a C1q/CRT/CD91-dependent pathway has been shown to be associated with SLE (30). We have demonstrated that loss of Btk results in defective CRT/CD91 complex formation as a result of defects in CRT trafficking within the cell following TLR7 stimulation (as shown in Fig. 6). In vitro studies indicate that the ability of Btk to phosphorylate CRT may be an important regulatory switch, which may underpin these mechanisms.

Our analysis of TLR7-driven cytokine responses in Btk-deficient BMDMs supports a role for Btk downstream of TLR7 and demonstrates that Btk is required for optimal cytokine production downstream of TLR7, with IL-12, IL-1 $\beta$ , and CXCL1 being markedly reduced in the absence of Btk. A role for Btk in TLR7-induced activation of macrophages has recently been shown by Vijayan et al. (11) with expression of heme oxygenase-1 (HO-1) reduced following pharmacological inhibition of Btk in macrophages. This study demonstrated that TLR-induced HO-1 expression is regulated by Btk via generation of reactive oxygen species and regulation of the transcription factor Nrf2, and it suggests that Btk is involved in complex regulation of macrophage function downstream of TLRs, with reactive oxygen species induction feeding back on the cells to limit and control inflammatory events via induction of HO-1. Although this study supports a positive role for Btk in TLR7 stimulation, the role of Btk in TLR signaling is somewhat controversial, with both a positive (6–8, 10, 31, 32) and a negative (33, 34) role for this kinase in TLR-induced cytokine activation being reported. Our results indicate that Btk is required for robust TLR7 responses with respect to cytokine production.

In addition to its role in regulating TLR-induced cytokine production, Btk is required for appropriate Fc $\gamma$ -mediated phagocytosis in macrophages (13, 24). However, the precise involvement of Btk in this process is unclear. Our results support a positive role for Btk in uptake of apoptotic cells via regulation of CRT recruitment to the phagosome and hence its ability to form a complex with CD91 and initiate apoptotic cell uptake. Ogden et al. (23) have previously demonstrated that preincubation of human monocyte-derived macrophages with anti-CD91 and anti-CRT Ab significantly inhibited the uptake of apoptotic cells. Our results would support the role of CRT together with CD91 at the surface of the phagocyte, as well as revealing the importance of Btk as a regulator of CRT modification and localization within the cell and hence influencing apoptotic cell uptake. In addition, we have shown that addition of a blocking Ab to CRT mimics loss of Btk, thus indicating the central importance of Btk in regulating CRT trafficking and function at the cell membrane of the phagocyte.

Tyrosine phosphorylation of CRT has not been explored in great detail within the literature. In a previous study by Mueller et al. (35), tyrosine phosphorylation of CRT was found to be essential for binding of the AT1 receptor mRNA in rat vascular smooth

**FIGURE 6.** Schematic model of Btk-mediated cytokine production and apoptotic cell clearance in myeloid cells. TLR7 stimulation results in activation of Btk, which plays an important role in facilitating a robust inflammatory cytokine response (1.). In addition, TLR7 stimulation was found to induce an interaction between Btk and the molecular chaperone CRT (2.). This facilitates the tyrosine phosphorylation of CRT, which is mediated through the kinase Btk (3.). Modification of CRT via Btk is important for regulation of the trafficking of CRT within myeloid cells. Trafficking of CRT to the cell surface facilitates colocalization with the cell-surface receptor CD91. Together, the CRT–CD91 complex is a crucial docking site for opsonins such as the complement component C1q and mannose-binding lectins (MBL), which coat apoptotic cells (4.). Btk-deficient cells demonstrate an alteration not only in inflammatory cytokine production downstream of TLR7 stimulation but also an impaired uptake of apoptotic debris.





muscle cells, which was mediated through Src kinase. Singh et al. (36) also demonstrated the importance of phosphorylation of CRT in simian kidney cells following rubella virus infection. CRT has also been shown to interact with and be modified by the serine kinase protein kinase C (27). Our finding of increased basal levels of tyrosine-phosphorylated CRT in Btk KO cells may indicate that in the absence of Btk, the phosphorylation of CRT can become dysregulated and increased basal levels of phospho-CRT are observed (Fig. 2C, 2D). Critically, however, TLR7 stimulation results in a dramatically different pattern of tyrosine phosphorylation in the Btk-deficient cells in comparison with WT. Interestingly, activation of protein kinase C induced not only an increase in serine phosphorylation of CRT but also a partial redistribution of CRT from the ER to the nucleus (27), indicating that modification of CRT may influence the localization and hence functionality of this protein in the cell. Our results to date suggest that Btk regulates CRT tyrosine phosphorylation downstream of TLR stimulation and that this modification is important for trafficking of CRT in macrophages. The exact mechanism of how CRT leaves the ER to reach the cell surface has yet to be elucidated. However, it is interesting to note that Btk has previously been shown to regulate the trafficking of MHC molecules via the costimulatory molecule CD40 (12). Recently, trafficking of TLRs to endocytic compartments and through the ERC has been shown to be critical for TLR function and downstream effects. For example, TLR4 has been shown to traffic from the ERC to *Escherichia coli* phagosomes, a process that required the small GTPase Rab11a (28), and also traffics from the cell membrane to endosomes to facilitate type I IFN production (37). Therefore, the central involvement of Btk in TLR-induced cytokine production and trafficking within the cell can be explained in this context and suggests perhaps a broader role for Btk in regulating trafficking within the cell.

There are a number of different receptor/ligand systems on the surface of the phagocyte that facilitate the recognition and uptake of apoptotic cells (3). CRT has long been proposed to function at the surface of phagocytic cells as a coreceptor for complement component C1q (4). CRT has also been implicated in the phagocyte-mediated recognition of other opsonins such as ficolin-3 (38), mannose-binding lectins (23), and surfactant proteins-A and -D in conjunction with the cell-surface receptor CD91 (25). Genetic studies in *Dictyostelium discoideum* (a slime mold that feeds on extracellular bacteria and therefore behaves as a professional phagocyte) have revealed that the ER pool of CRT and other ER proteins such as calnexin is required to ensure proper actin-dependent outgrowth of phagocytic cups (39). Interestingly, a proteomic assessment of phagosome-enriched proteins derived from the J774 mouse macrophage-like cell line identified CRT as being one of a number of ER-related proteins localized within the phagosome (40). Given the association of CRT with the CD91 receptor and the role of this complex in phagocytosis (23, 25) as well as our findings revealing the importance of Btk on the ability of CRT to colocalize with CD91 post TLR7 stimulation, our evidence strongly suggests that Btk influences uptake via the CRT/CD91 system. XLA patients have been shown to present with renal immune complex deposition with both membranoproliferative glomerulonephritis (14) and membranous glomerulopathy (15) being reported in patients receiving i.v. Ig. Both of these reported cases produced pathogenic immune complexes, deposition of which was thought to lead to renal dysfunction. Our results would suggest that the loss of Btk would have a significant impact on myeloid cell phagocytic capacity in XLA patients, thereby potentially contributing to the pathogenesis of this disease.

Defective clearance of apoptotic cells by monocytes is a hallmark trait of autoimmune conditions such as SLE and has led to the

suggestion that prolonged exposure to autoantigens as a result might represent an important trigger of systemic autoimmunity. Interestingly, a strong association between C1q deficiency and SLE has been demonstrated, with loss of C1q leading to accumulation of apoptotic cells, enhanced inflammation, and production of autoantibodies (41, 42). Our present study, however, suggests that although Btk may positively regulate cytokine production and Ag presentation, Btk also positively regulates apoptotic cell uptake via the CRT/CD91 receptor system. Given our findings in relation to the importance of Btk in regulation of the CRT/CD91 axis, an accumulation of apoptotic debris within Btk-deficient mice could be expected. However, given the paucity of long-term studies within the Btk KO animals, the implications of loss of Btk on apoptotic cell accumulation is unclear. In summary, we have shown that dysregulation of the TLR7 pathway through loss of Btk has a direct effect not only on the production of inflammatory cytokines but also on the phosphorylation status and subsequent localization of CRT within macrophages. Our present study suggests that Btk positively regulates cytokine production, in addition to regulating apoptotic cell uptake via the CRT/CD91 receptor system. Thus, our work demonstrates a novel role for Btk in regulating CD91-/CRT-mediated uptake of apoptotic cells.

## Disclosures

The authors have no financial conflicts of interest.

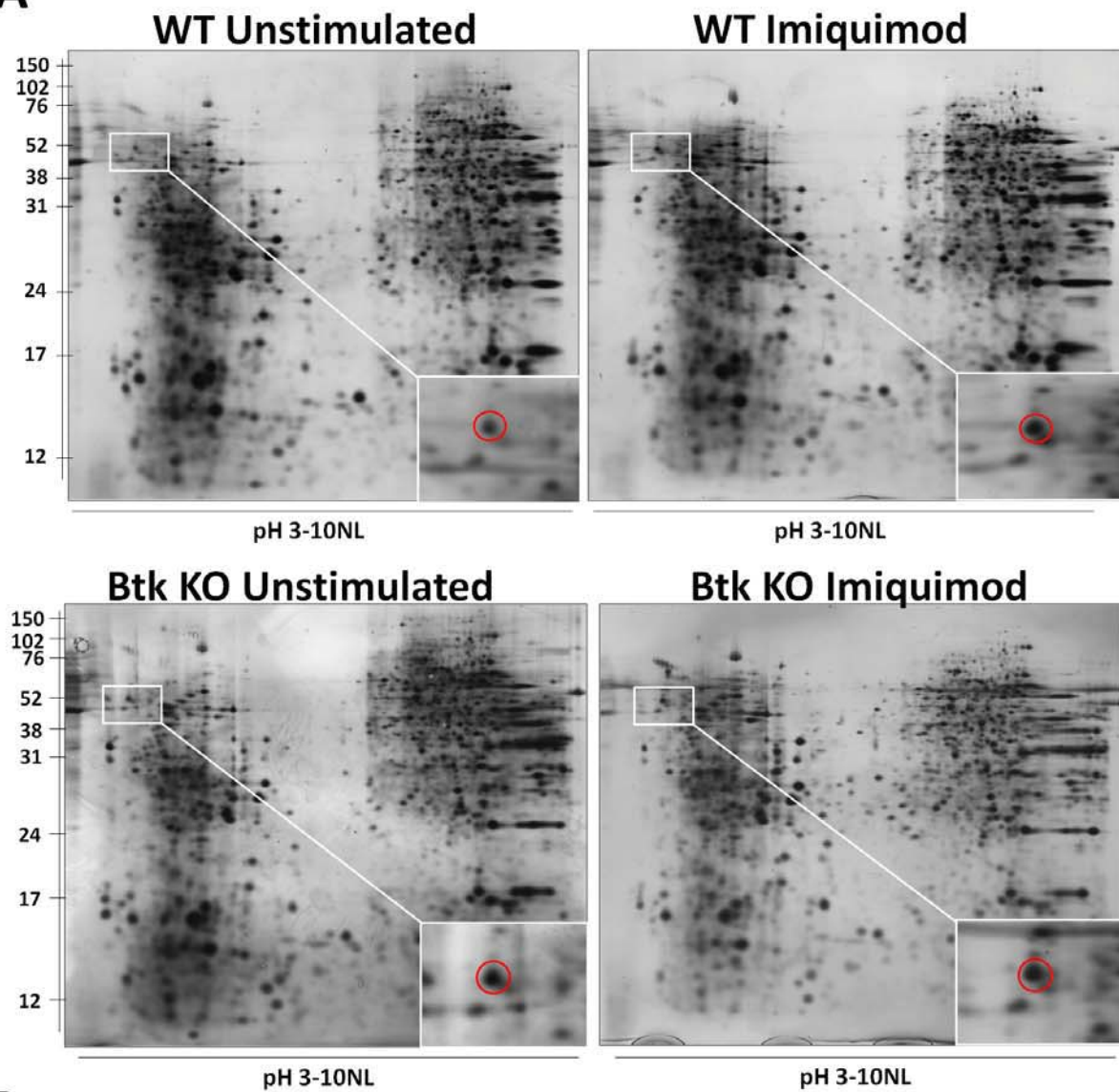
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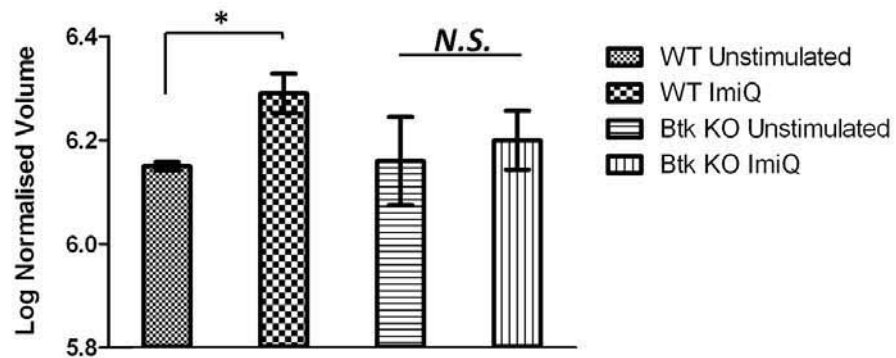
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# Supplementary Figure 1

**A**



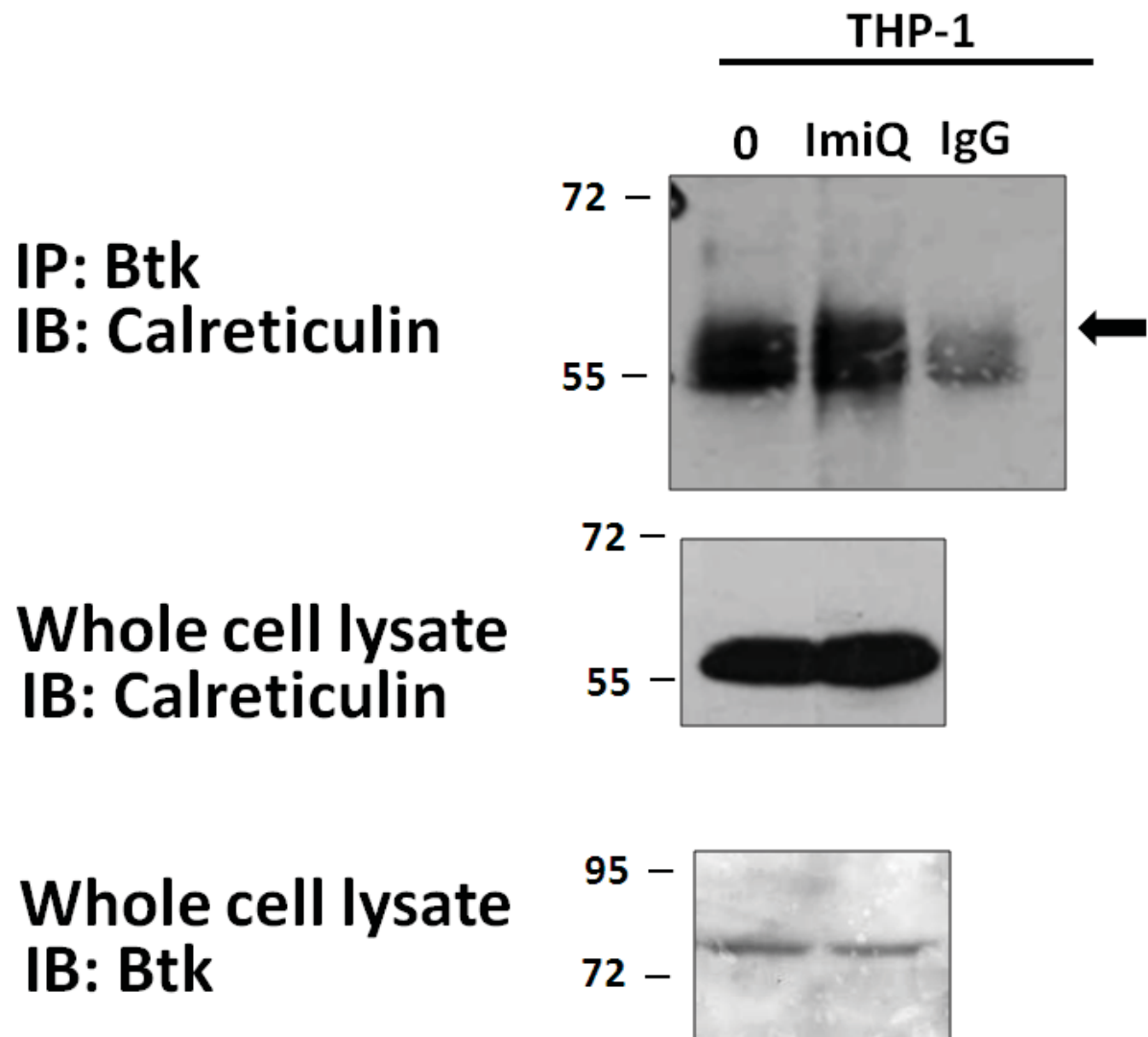
**B**



**Supplementary Figure 1: Absence of Btk influences spot intensity of calreticulin downstream of TLR7 stimulation.** *A*, WT and Btk KO BMDMs were stimulated with imiquimod (20µg/mL) for 30 minutes prior to lysis and 2D gel electrophoresis. *B*, Calreticulin (CRT) was identified as having a significant increase in spot intensity in WT BMDMs following TLR7 stimulation which was not apparent in Btk-deficient cells. Data are representative of two independent experiments carried out in duplicate (ANOVA  $p = 0.016$ , bars represent  $\pm$  S.D.).

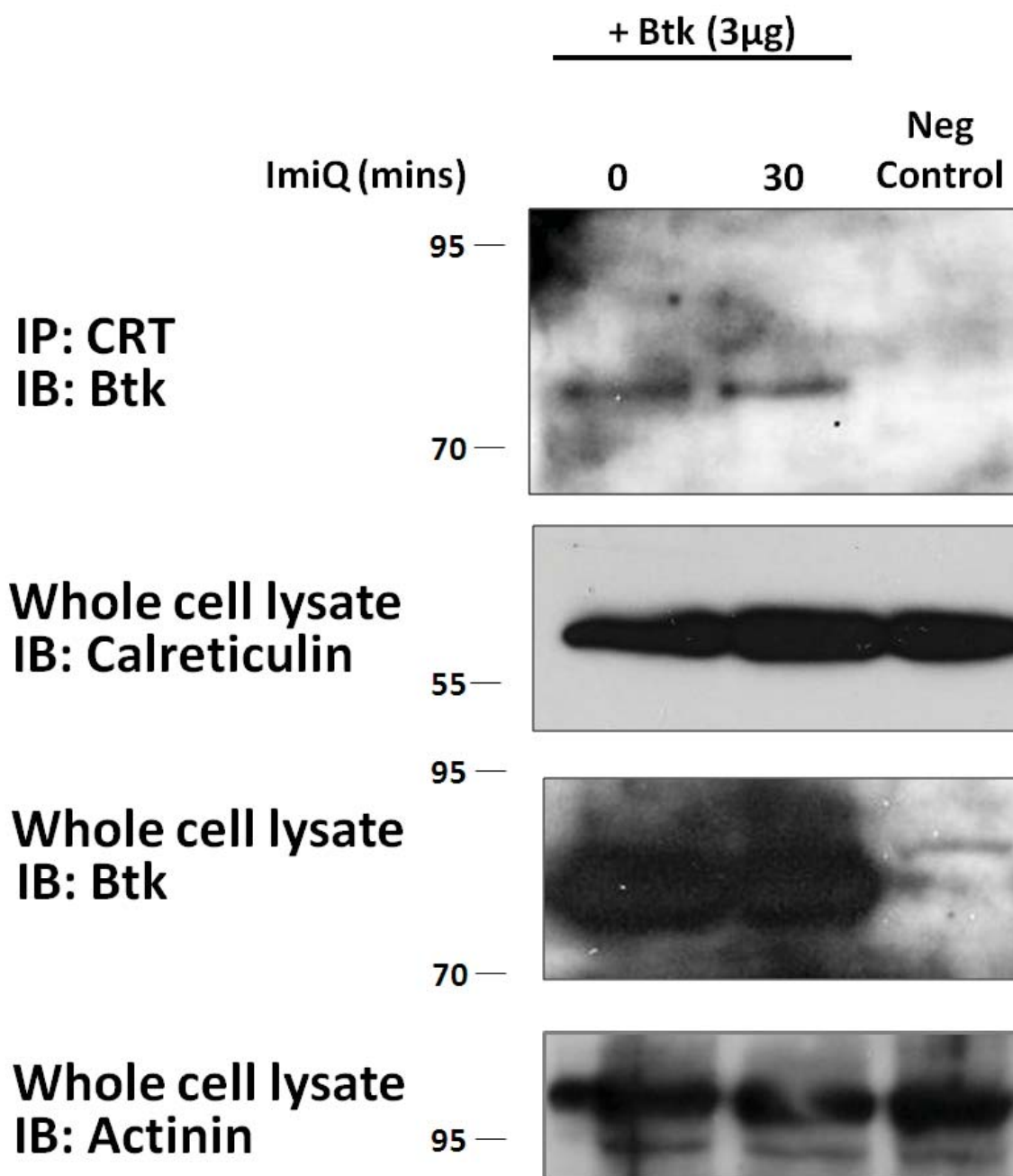


# Supplementary Figure 2



**Supplementary Figure 2: Confirmation of an interaction between Btk and calreticulin using reciprocal immunoprecipitation from THP-1 cells.** THP-1 cells were left untreated or stimulated with imiquimod (20 $\mu$ g/mL), after which time Btk-associated proteins were immunoprecipitated from lysates with an anti-Btk antibody in a pull-down experiment. An interaction between endogenous Btk and calreticulin is indicated by the black arrow. Endogenous calreticulin and Btk expression levels were analysed by immunoblotting. Data are representative of two independent experiments.

# Supplementary Figure 3



**Supplementary Figure 3: Investigation of interaction between Btk and calreticulin using overexpression in heterologous cell lines.** HEK293T cells were transiently transfected with a plasmid expressing full-length Btk (3 $\mu$ g). Twenty-four hours post transfection, the cells were stimulated with imiquimod (20 $\mu$ g/mL) and calreticulin-associated proteins were immunoprecipitated from lysates with an anti-CRT antibody in a pull-down experiment. An interaction between Btk and calreticulin was observed in cells where Btk was overexpressed but not in the negative control cells. Expression levels of Btk and CRT were analysed by immunoblotting. Data are representative of two independent experiments.