Btk regulates macrophage polarization in response to lipopolysaccharide

Ni Gabhann, J., Hams, E., Smith, S., Wynne, C., Byrne, J. C., Brennan, K., ... Jefferies, C. A. (2014). Btk regulates macrophage polarization in response to lipopolysaccharide. PloS one, 9(1), [e85834]. DOI: 10.1371/journal.pone.0085834

Published in:
PloS one

Document Version:
Publisher's PDF, also known as Version of record

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

Publisher rights
© 2014 Ni Gabhann et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

General rights
Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.
Btk Regulates Macrophage Polarization in Response to Lipopolysaccharide

Joan Ní Gabhann1, Emily Hams2, Siobhán Smith1, Claire Wynne1,3, Jennifer C. Byrne1, Kiva Brennan2, Shaun Spence6, Adrien Kissenpfennig6, James A. Johnston7, Padraic G Fallon2,4,5, Caroline A. Jefferies1,*

1 Molecular and Cellular Therapeutics and RCSI Research Institute, Royal College of Surgeons in Ireland, Dublin, Ireland, 2 Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland, 3 School of Biological Sciences, Dublin Institute of Technology, Kevin St, Dublin, Ireland, 4 Institute of Molecular Medicine, St. James’s Hospital, Trinity College Dublin, Dublin, Ireland, 5 National Children’s Research Centre, Our Lady’s Children’s Hospital Crumlin, Dublin, Ireland, 6 Centre for Infection and Immunity, School of Medicine Dentistry and Biomedical Sciences, Queen’s University, Belfast, United Kingdom, 7 Amgen Inc., Thousand Oaks, California, United States of America

Abstract

Bacterial Lipopolysaccharide (LPS) is a strong inducer of inflammation and does so by inducing polarization of macrophages to the classic inflammatory M1 population. Given the role of Btk as a critical signal transducer downstream of TLR4, we investigated its role in M1/M2 induction. In Btk deficient (Btk−/−) mice we observed markedly reduced recruitment of M1 macrophages following intraperitoneal administration of LPS. Ex vivo analysis demonstrated an impaired ability of Btk−/− macrophages to polarize into M1 macrophages, instead showing enhanced induction of immunosuppressive M2-associated markers in response to M1 polarizing stimuli, a finding accompanied by reduced phosphorylation of STAT1 and enhanced STAT6 phosphorylation. In addition to STAT activation, M1 and M2 polarizing signals modulate the expression of inflammatory genes via differential activation of transcription factors and regulatory proteins, including NF-κB and SHIP1. In keeping with a critical role for Btk in macrophage polarization, we observed reduced levels of NF-κB p65 and Akt phosphorylation, as well as reduced induction of the M1 associated marker iNOS in Btk−/− macrophages in response to M1 polarizing stimuli. Additionally enhanced expression of SHIP1, a key negative regulator of macrophage polarization, was observed in Btk−/− macrophages in response to M2 polarizing stimuli. Employing classic models of allergic M2 inflammation, treatment of Btk−/− mice with either Schistosoma mansoni eggs or chitin resulted in increased recruitment of M2 macrophages and induction of M2-associated genes. This demonstrates an enhanced M2 skew in the absence of Btk, thus promoting the development of allergic inflammation.

Citation: Ní Gabhann J, Hams E, Smith S, Wynne C, Byrne JC, et al. (2014) Btk Regulates Macrophage Polarization in Response to Lipopolysaccharide. PLoS ONE 9(1): e85834. doi:10.1371/journal.pone.0085834

Editor: Kevin Currie, Vanderbilt University Medical Center, United States of America

Received October 16, 2013; Accepted December 2, 2013; Published January 21, 2014

Copyright: © 2014 Ní Gabhann et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Health Research Board, Ireland (RP/2004/79) and P/2006/121 www.hrb.ie) and Science Foundation Ireland (Grant 08/IN.1/B2091 www.sfi.ie). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: JAJ is an employee of the company Amgen. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: cjjefferies@rcsi.ie

Introduction

Macrophages are central players in the development, progression and resolution of inflammation. Similar to the T cell paradigm of Th1 and Th2 subpopulations, macrophages polarize in response to diverse microbial and environmental signals into various sub-populations with distinct effector functions defined as classic inflammatory M1 and immunosuppressive M2 macrophages. With respect to their role in disease, increased levels of M1 macrophages are associated with autoimmune and inflammatory diseases such as lupus nephritis [1] and multiple sclerosis [2]. M2 macrophages on the other hand have been shown to play a role in promoting tumour growth [3], and in the development of allergic inflammation and airway disease through their ability to induce differentiation of Th2 cells [reviewed in [4]].

In recent years much study has gone into understanding the precise molecular mechanism regulating M1/M2 development and polarization. Several studies have implicated key transcription factors and regulatory proteins in this process, including members of the interferon regulatory factor (IRF) family, signal transducer and activator of transcription (STAT) proteins and the suppressors of cytokine signalling (SOCS) family (reviewed in [5]). M1-associated gene induction, following stimulation of macrophages with IFN-γ, LPS or TNFα, is mediated by the activation of STAT1, the p65 subunit of Nuclear factor kappa beta (NF-κB), phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinases (MAPK), resulting in enhanced production of inflammatory cytokines, chemokines and iNOS [6–9]. IL-4 and IL-13 mediate M2 macrophage polarization by inducing phosphorylation of STAT3 and STAT6 followed by nuclear translocation and M2-associated gene induction [10,11]. In keeping with the importance of STAT3 and STAT6 in driving M2 macrophage polarization, several studies have demonstrated that inhibition of these proteins promotes an M1 phenotype in macrophages [12–14]. Additionally Peroxisome proliferator-activated receptor gamma (PPAR-γ) and Krüppel-like factor 4 (KLF4) have been identified as factors that work in concert with STAT6 to promote an M2 phenotype [15,16]. Another critical modulator of macrophage polarization is the myeloid restricted Src homology-2 domain-containing inositol 5-phosphatase, (SHIP1), which is an
anti-inflammatory protein that functions to convert PI3P to 
P(3,4)P2 in order to turn off PI3K-dependent signalling and 
negatively regulate NF-kB and IRF3 activity via regulating 
complex formation and the localisation of key signalling proteins 
such as TBK1 [17–19]. Interestingly, induction of the microRNA 
mir-155 has been associated with an enhanced M1 phenotype as 
a result of SHIP1 down regulation [20]. Recently the inhibitory 
p30 subunit of NF-kB has also been shown to contribute to the 
process of tolerance and thus M2 macrophage induction by 
negatively regulating M1 macrophage polarization and IFN-β 
induction [9]. Furthermore, the suppressors of cytokine signaling 
(SOCS) proteins also contribute to macrophage polarization, with 
SOCS3 regulating M1 development while SOCS2 promotes an 
M2 phenotype [21].

Stimulation of macrophages via Toll Like receptors (TLRs) such 
as TLR4 or TLR9 has been shown to be a critical signal in driving 
macrophage polarization via activation of NF-kB or the IRF 
family members. The Tec tyrosine kinase, Bruton’s tyrosine kinase 
(Btk), is critical for LPS-induced proinflammatory cytokine 
production and IFN-γ-induced natural killer cell activation [22– 
26]. Btk interacts with TLR2, 3, 4 and 7 and in doing so mediates 
their phosphorylation and the transduction of downstream signals. 
At a molecular level Btk regulates NF-kB activation by regulating 
p65 phosphorylation downstream of multiple TLRs including 
TLR4, 7 and 9 [25–26]. In addition Btk regulates IRF3 activation 
downstream of TLR3 and hence IFN-β production in response to 
viral recognition [25]. Interestingly, IRF3 and IRF5 have been 
implicated in regulating M1 polarization and associated gene 
induction [28,29]. Thus as a critical regulator of transcription 
factors such as NF-kB and the IRFs, the possibility exists that Btk 
may regulate macrophage polarization downstream of classical 
M1 and M2 polarizing stimuli.

To fully address this possibility we examined polarisation of 
macrophages derived from Btk−/− mice, which allowed us to 
directly determine the exact contribution of Btk to this dynamic 
process. This study shows that Btk plays an important role in 
regulating LPS-driven M1 polarisation, with impaired recruitment 
of M1 macrophages and preferential polarisation towards an M2 
phenotype observed in the absence of Btk following stimulation 
with IL-4 and IL-13 or in vivo challenge with Schistosoma mansoni 
eggs, a classic model of allergic inflammation. This bias towards an 
M2 phenotype could not be recovered by treatment with an M1 
polarizing cocktail, as this too was shown to promote M2- 
associated gene induction. At a molecular level, enhanced STAT6 
and reduced NF-kB p65, Akt and STAT1 phosphorylation as well 
as altered SHIP1 induction was shown to contribute to this skew. 
These studies demonstrate a critical role for Btk in macrophage 
polarisation with Btk acting as a negative regulator of M2 
macrophage induction.

Materials and Methods
Ethics Statement
All mouse work was carried out in strict accordance with the 
requirements of Royal College of Surgeons in Ireland Ethics 
Committee with ethics approval number: REC 582. Animals were 
culled using CO2 asphyxiation and the appropriate organs and 
cells harvested.

Reagents
LPS was purchased from Casyal Invivogen (Escherichia coli 
0111:B4). Recombinant murine IFN-γ, IL-13 and IL-4 were 
purchased from ImmunoTools.

Mice
Btk deficient (Btk−/−) mice on a C57BL/6 background were a 
kid gift from Dr Rudi Hendriks [30]. Mice were housed at the 
Biomedical Research Facility at the Royal College of Surgeons in 
Ireland under specific pathogen-free conditions. Animals were 
housed with 12 hour day-night cycle with lights on at 7:30 pm in a 
temperature (22±1°C) and humidity (55±5%) controlled room. 
The animals’ health status was monitored prior to and throughout 
the experiments and all mice were free of all viral, bacterial, and 
parasitic pathogens. Prior to commencement of experiments the 
animals were separated and housed in relevant treatment groups 
in individually ventilated cages. Each treatment group consisted of 
3–4 animals and experiments were performed in triplicate. Prior to 
and during the experimental period all mice were allowed free 
access to sterile water and nutrition. All cages contained bedding 
and were enriched with mouse houses. All efforts were made to 
minimise suffering.

Intraperitoneal (i.p.) LPS Injection
Age-matched (6 to 10 weeks) wild type (WT) (n = 4) C57BL/6 
mice (Harlan Laboratories) and Btk−/− mice (n = 4) were 
administered an intraperitoneal injection (i.p.) of 1 mg/kg LPS 
dissolved in sterile saline solution. Control mice were given an i.p. 
injection of the equivalent volume of saline. Following 24 hour 
LPS treatment mice were culled by CO2 asphyxiation.

Isolation of Peritoneal Macrophages
Peritoneal cells were harvested by peritoneal lavage with of 
10 ml sterile ice cold PBS. Peritoneal cells were allowed to adhere 
to plates for 4 hours. Non-adherent cells were subsequently 
removed by washing with RPMI, and the adherent macrophages 
were refed with RPMI containing 20% calf serum and gentamicin. 
Purity of adherent cells (>95%) was determined by flow cytometry 
using F4/80 and CD11b (BD Biosciences). Macrophages were used for experiments immediately following isolation. For some experiments macrophages were treated ex 
vivo with an M1 (100 U/ml IFN-γ plus 100 ng/ml LPS) or an M2 
(10 ng/ml IL-4 plus 10 ng/ml IL-13) polarizing cocktail for the 
determined time points.

Flow Cytometric Analysis
Peritoneal macrophages were collected from WT or Btk−/− 
mice following i.p. LPS injection. Cells were stained for F4/80, 
CD11b, CD66, and IA/IE (MHCIi), using specific antibodies (BD 
Biosciences) and analysed by flow cytometry using a FACSCanToII 
flow cytometer (BD Biosciences).

Real-time PCR
Total RNA was extracted using an RNeasy kit (Qiagen) 
and reverse transcribed to cDNA using Omniscript reverse transcriptase 
(Qiagen) according to manufacturer’s recommendations. 
Quantitative real-time PCR was performed using SYBR Green 
Taq ReadyMix™ (Sigma) and the data was normalised to a β 
actin reference. Real-time PCR data was analyzed using the 
2−ΔΔct method [31].

Helminth Egg Injections
Eggs from S. mansoni–infected mice were obtained as described 
previously [32]. WT mice (n = 4) or Btk−/− mice (n = 4) were given 
an intravenous (i.v) injection of either 5,000 S. mansoni eggs or PBS.
Mice were killed on day 14 and lungs were removed. Lungs lobes 
were snap-frozen or homogenised for analysis of macrophages by 
flow cytometry or qPCR, as described previously [33].
In vivo Macrophage Activation

WT mice (n = 4) or Btk−/− mice (n = 4) were injected i.p. with approximately 800 ng Chitin (Sigma) with to induce M2 cells in the peritoneum as previously described [34]. Peritoneal cells were collected by lavage after 48 hours and gene induction was determined by qPCR.

Western Blot Analysis

WT and Btk−/− BMDMs were treated ex vivo with an M1 or M2 polarizing cocktail as indicated, Expression of STAT1(Santa Cruz Biotechnology #sc-592), STAT6 (Santa Cruz Biotechnology #sc-621), p-y-STAT1 (Cell Signaling #9171), p-y-STAT6 (Imgenex #IMG408A), Akt (Cell Signaling #9271), p-S-Akt (Cell Signaling #9272), NF-κB p65 (Santa Cruz Biotechnology #sc-372), p-S-NF-κB p65 (Cell Signaling #3036S), iNOS (Transduction Laboratories #N39120) and SHIP-1 (Santa Cruz Biotechnology #sc-6425), was determined by Western blot as described previously [23].

Statistical Analysis

Student’s unpaired t test was performed using GraphPad Prism 6.0. Results are presented as mean ± SD. Data were deemed to be significantly different at P values less than 0.05.

Results

Btk is Required for LPS-elicted Macrophage Polarization in vivo

Given the role of Btk as a central regulator of TLR4-driven pro-inflammatory cytokine production [22,23,25,26], we sought to determine whether Btk was involved in regulating in vivo M1/M2 differentiation downstream of TLR4. To date several surface antigens have been employed in order to discriminate between polarized macrophage populations. Initial studies have suggested that size and relative expression of F4/80 and CD11b can distinguish subtypes which are loosely defined as F4/80intCD11bhi M1-like macrophages and F4/80hiCD11bhi M2-like macrophages [35]. However, while this approach has utility, the authors noted that this technique did not fully address the heterogeneity of the population. It is now therefore accepted that in addition to F4/80 and CD11b the relative expression of antigen presentation molecules, co-stimulatory molecules and Ly6C can be used to more fully discern polarized macrophages, with M1 macrophages demonstrating increased expression of these antigens relative to M2 macrophages [36–39]. Taking these approaches into account, LPS was injected i.p. into WT and Btk−/− mice and 24 hours later peritoneal macrophages were profiled by flow cytometry for F4/80 and CD11b (Figure 1A), following which analysis of the later peritoneal macrophages were profiled by flow cytometry for 80hiCD11bhi cells and determined that significantly enhanced were observed compared to WT mice (Figure 1 B). Additionally F4/80hiCD11bhi M2-like macrophages was performed (Figure 1B)

LPS was injected i.p. into WT and Btk−/− mice (n = 4) with LPS alone (0.05), iNOS (Transduction Laboratories #N39120), and IFN-γ (Endogen #E1729) to induce M1 cells in WT mice (Figure 1 D). In addition, analysis of MHC Class II expression within M1 and M2 gates as defined in Figure 1A revealed that the majority of expression for this antigen was within the F4/80hiCD11bhi (M1) gated region, with WT peritoneal macrophages displaying elevated levels of MHC class II when compared to Btk-deficient peritoneal macrophages (Figure 1 E). Furthermore reduced expression of Ly6C was observed on infiltrating myeloid cells following LPS treatment in Btk−/− mice compared to WT mice (Figure 1 F). This data suggests that in the absence of Btk M2-like macrophages are preferentially recruited following exposure to LPS.

Dominant M2 Phenotype in Macrophages Lacking Btk

Given that polarized macrophage states can be modulated or reversed [41], we next asked whether Btk−/− macrophages would polarize normally in response to either M1 or M2 polarizing stimuli. Peritoneal macrophages isolated from WT and Btk−/− mice were treated ex vivo with either an M1 (LPS plus IFN-γ) or an M2 (IL-4 plus IL-13) polarizing cocktail and gene induction was determined by real time PCR. As expected, treatment with the M1 polarizing cocktail resulted in robust induction of Tnfα, Il12p40 and Ccl10 when compared to Btk−/− cells (Figure 1G). In contrast, LPS-treated WT Btk−/− macrophages had significantly reduced expression of M1-associated genes but enhanced expression of the M2-associated chemokines Ccl2, Ccl17 and Ccl22 in comparison to WT macrophages (Figure 1H). Thus not only are Btk−/− macrophages defective in their ability to induce proinflammatory cytokines in response to LPS [23–26], they appear to preferentially polarize towards anti-inflammatory M2 macrophages in response to this normally pro-inflammatory stimulus.

Altered Phosphorylation of Key Signalling Intermediaries in the Absence of Btk

We next sought to investigate the mechanism by which Btk contributes to macrophage polarization. Investigating the effects of LPS or IFN-γ on STAT1 activation we observed that tyrosine phosphorylation of STAT1 was impaired in the absence of Btk following culture of BMDMs with either LPS or IFN-γ alone (Figure 3 A). Additionally the combination of LPS and IFN-γ also induced less STAT1 phosphorylation in Btk−/− BMDMs compared to WT BMDMs (Figure 3 B). Consistent with increased
Figure 1. Impaired TLR4-mediated induction of peritoneal M1 cells in Btk<sup>−/−</sup> mice in vivo. (A–E) Peritoneal macrophages were harvested from WT or Btk<sup>−/−</sup> mice following i.p. LPS injection. (A) Representative plots demonstrate the gating strategies for macrophage discrimination where total peritoneal macrophages were defined following co-staining with CD11b APC-Cy7 and F4/80 PE-Cy7. (B–C) Macrophage subsets were further distinguished as F4/80<sup>int</sup>CD11b<sup>hi</sup> M1-like macrophages and F4/80<sup>hi</sup>CD11b<sup>hi</sup> M2-like macrophages (M1 and M2 gates, respectively). The relative percentage of M1 (B) and M2 (C) macrophages within the total macrophage gate were determined for WT or Btk<sup>−/−</sup> mice following i.p. LPS injection. (D) Expression of CD86 and MHC Class II was determined by flow cytometry. (E) MHC Class II expression was examined within the defined M1 and M2 gates following co-staining with F4/80<sup>+</sup> and CD11b<sup>+</sup>. (F) Ly6C was determined by flow cytometry. In all cases data is presented as percent increased expression above background as determined by staining with the relevant isotype control (indicated by markers in panel (D)). (G–H) Peritoneal macrophages harvested from WT or Btk<sup>−/−</sup> mice were treated ex vivo with LPS (100 ng/ml) for the indicated time course and the induction of M1- (G) and M2- (H) associated genes was determined by real time PCR (qPCR). Peritoneal macrophages were pooled after isolation, with each treatment group consisting of 3–4 animals, and all experiments were performed in triplicate. Student’s paired t test was performed comparing gene induction in Btk<sup>−/−</sup> peritoneal macrophages to WT cells at the indicated time points. Results shown are mean±SD from three independent experiments. *p<0.05.

doi:10.1371/journal.pone.0085834.g001
generation of M2 macrophages in the absence of Btk, we observed enhanced phosphorylation of STAT6 in response to IL-4 and IL-13 in the Btk−/− BMDMs compared to WT BMDMs (Figure 3 B, lower panel).

It is well established that in addition to STAT activation M1 and M2 polarizing signals modulate the expression of inflammatory genes via differential activation of transcription factors, including NF-κB, as a result of activation of key pathways including PI3K/Akt [42]. Not surprisingly given the previously identified role for Btk in regulating p65 phosphorylation and hence NF-κB activation, and the role NF-κB plays in regulating M1/M2 differentiation, we observed significantly less phosphorylation of p65 in Btk−/− BMDMs compared to WT BMDMs following LPS and IFN-γ treatment, whereas M2 polarizing conditions failed to promote p65 phosphorylation either in WT or Btk−/− BMDMs (Figure 3 C). Similarly, phospho-Akt levels were also reduced under the same conditions in Btk−/− BMDMs, and again M2-polarizing signals failed to induce Akt activation in either WT or Btk−/− cells. In addition, under the same conditions, we observed potent induction of the classical pro-inflammatory M1-associated marker iNOS in WT BMDMs when compared to Btk−/− BMDMs (Figure 3 D), with M2 polarizing signals having
Figure 3. Altered phosphorylation of key signalling intermediaries in the absence of Btk. (A) WT and Btk<sup>−/−</sup> BMDMs were treated ex vivo with LPS (100 ng/ml) or IFN-γ (100 U/ml) for 3 hours or 15 minutes, respectively, lysates were prepared and phosphorylated Y701-STAT1 levels determined by Western blot. WT and Btk<sup>−/−</sup> BMDMs were treated ex vivo with an M1 or M2 polarizing cocktail over the indicated time course, lysates were prepared and tyrosine phosphorylated STAT1 and STAT6 (B), serine phosphorylated AKT (upper panel) and NF-κB p65 (lower panel) (C) iNOS and SHIP-1 (D) levels were determined by Western blot. Results in each case are representative of three independent experiments. Densitometric analysis was performed and graphs represent phosphorylated protein levels relative to unphosphorylated proteins (B-C) or changes in total protein levels relative to β-actin (D) for WT and Btk<sup>−/−</sup> BMDMs. Student’s paired t test was performed comparing relative expression of phosphorylated or total proteins in Btk<sup>−/−</sup> BMDMs to WT BMDMs following treatment with polarizing cocktails as indicated. Results shown are mean±SD from three independent experiments. * = p≤0.05.

doi:10.1371/journal.pone.0085834.g003
no effect. These results indicate that Btk mediates M1 gene induction via promoting Akt and p65 activation. Regarding what might drive Btk−/− macrophages to become skewed towards an M2 phenotype even in the presence of M1 polarizing signals, SHIP1, as a key anti-inflammatory protein that negatively regulates PI3K-dependent signalling and subsequently activation of NF-kB and IRF3 thus negatively regulating M1 macrophage polarisation, is a potential target [17–19]. In keeping with the importance of SHIP1 in promoting M2 polarization, treatment with IL-4 and IL-13 resulted in expression of SHIP1 in both WT and Btk−/− BMDMs, however expression levels were enhanced in Btk−/− macrophages compared to WT macrophages, suggesting inappropriate regulation of SHIP1 expression and potentially activity in Btk−/− BMDMs contributes to the M2 skew observed (Figure 3 D, lower panel). Thus in the absence of Btk diminished activation of key signaling intermediaries and transcription factors and enhanced expression of the M2-promoting proteins including STAT6 and SHIP1 accounts for the inability of macrophages to effectively polarize towards an M1 phenotype.

Absence of Btk Exacerbates M2 Recruitment and Induction Following Induction of Allergic Inflammation

To date our data suggests that loss of Btk results in a predominant M2 phenotype which may potentially aggravate M2-mediated disease such as allergic inflammation. Both Schistosoma mansoni eggs and chitin drive allergic inflammation via promoting M2 induction and a Th2-mediated response [43,44]. We hypothesised that M2 induction would be exacerbated in the absence of Btk. WT and Btk−/− mice were injected i.v. with 5,000 S. mansoni eggs and 14 d later M2 macrophages were analysed in lungs by flow cytometry, with induction of M1 or M2 associated genes determined by qPCR. In keeping with our previous findings, we observed a significant (p<0.05) increase in recruitment of M2 macrophages to the lungs of Btk−/− mice compared to WT mice (Figure 4 A), accompanied by a preferential induction of M2-associated genes, Arginase 1 and Relm-α, in Btk−/− mice compared to WT mice (Figure 4 B and C).

To further address the role of Btk in in vivo generation of M1 versus M2 cells, WT and Btk−/− mice were injected i.p. with chitin following which peritoneal cells were collected by lavage and M1/M2 gene induction determined by qPCR. Similar to S. mansoni challenge, chitin treatment of Btk−/− mice resulted in enhanced induction of the M2-associated genes Arginase 1 and Relm-α, in peritoneal exudate cells compared to controls (Figure 4 D). Similar to the enhanced basal levels of M2-associated genes in the peritoneum following in vivo LPS treatment (Figure 1 G) we also observed enhanced basal levels of M2-associated genes in vivo (Figure 4 B–D). Collectively, using two separate M2 inducing in vivo mouse models, in lungs or peritoneum, there is marked polarization to M2 cells in the absence of Btk.

Discussion

This study demonstrates a novel role for Btk in determining macrophage lineage commitment, with preferential M2 macrophage induction observed in the absence of Btk as a result of altered activation of key signaling pathways and transcription factors such as NF-kB p65, Akt and proteins known to regulate macrophase polarization including SHIP1 and members of the STAT family. Furthermore we show that Btk is essentially required downstream of TLR4 and IFN-γ for optimal p65-dependent and STAT1-dependent M1 macrophage polarization (Figure 5).

To date there has been no direct evidence supporting a role for Btk in macrophage polarization and reports of Btks contribution to inflammation have been controversial. Several studies have suggested that Btk functions as a negative regulator of inflammation [45–47] while there is gathering evidence to suggest that Btk may be important in driving the inflammatory process. Studies examining APCs derived from XLA patients, who have a natural mutation in Btk, have shown that they have defects in both phagocytosis and inflammatory cytokine production following TLR stimulation [25,26,48]. Recently the potential that Btk plays a direct role in positively regulating antigen presentation and maturation of APCs was described by Lui et al. These studies found impaired TLR-driven activation of APCs due to an inability to form a MHC class II and CD40 complex in the absence of Btk [49]. At a molecular levels Btk has been shown to stabilise TNF-α mRNA via a p38-dependent pathway in X-linked immunodeficiency (Xid) mice and in XLA patients as well as being critically involved in NF-kB activation and specifically p65 phosphorylation downstream of multiple TLRs including TLR4, TLR7 and TLR9 [23–26]. More recently studies by our group have suggested that increased IL-10 production together with a significant reduction in IFN-γ production in the absence of Btk may result in an altered Th1/Th2 balance [22], potentially implicating Btk in macrophage polarization. In the current study we observed impaired activation of NF-kB p65 in the absence of Btk following TLR4 and IFN-γ treatment, further supporting a positive role for Btk in M1 macrophage polarisation. Interestingly, M2 macrophages have been shown to inhibit the expression several M1 associated chemokines including CXCL10 by modulating the activity of both NF-kB and STAT1 [50,51]. We observed impaired phosphorylation of Akt, a key MyD88-dependent signalling intermediary and inducer of M1-associated cytokines [52,53] in the absence of Btk. Studies in B cells have shown that Btk and Akt directly interact and that in the absence of Btk, Akt activation is prevented [53]. A recent study has implicated Akt as a negative regulator of Btk, phosphorylating it in order to promote 14-3-3ζ binding, a novel negative regulator of Btk signalling [54]. Thus the ability of Btk and Akt to cross-regulate each other and the involvement of Akt in regulating macrophage polarization, indicates that the inability of Btk−/− macrophages to phosphorylate Akt in response to M1 polarizing stimuli has an important contribution to the M2 skew observed in these cells.

Whilst a direct link between Btk and STAT1 in regulating macrophase activity has not been previously demonstrated, overexpression studies and studies in B cells have demonstrated that Btk and other Tec family kinases can interact with STAT family members and promote or inhibit their activation. Btk has been shown to interact directly with JAK1 and this association results in Btk phosphorylation [55]. More recently the absence of Btk was shown to result in reduced poly(I:C)-mediated activation of STAT1 [27]. Relevant to our findings, Btk has been shown to prevent STAT3 activation in B cells thus promoting apoptosis [56]. Overall our study indicates that Btk is required for optimal STAT1 activation and that in the absence of Btk, enhanced STAT6 phosphorylation is observed, in keeping with the inhibitory role for Btk regarding STAT3 activation reported by Uckun et al. [56]. Given that Btk has been implicated as key positive regulator of TLR4-mediated cytokine production and that STAT1 activation is required for optimal LPS induced activation of macrophages [57], this study demonstrates an important link between Btk activation, subsequent transcription factor phosphorylation and macrophage polarization.

Interestingly whilst exposure of Btk-deficient macrophages to M1 polarizing stimuli did not result in increased STAT6

[...]

Mathematically This study demonstrates a novel role for Btk in determining macrophage lineage commitment, with preferential M2 macrophage induction observed in the absence of Btk as a result of altered activation of key signaling pathways and transcription factors such as NF-kB p65, Akt and proteins known to regulate macrophase polarization including SHIP1 and members of the STAT family. Furthermore we show that Btk is essentially required downstream of TLR4 and IFN-γ for optimal p65-dependent and STAT1-dependent M1 macrophage polarization (Figure 5).

Discussion

This study demonstrates a novel role for Btk in determining macrophage lineage commitment, with preferential M2 macrophage induction observed in the absence of Btk as a result of altered activation of key signaling pathways and transcription factors such as NF-kB p65, Akt and proteins known to regulate macrophase polarization including SHIP1 and members of the STAT family. Furthermore we show that Btk is essentially required downstream of TLR4 and IFN-γ for optimal p65-dependent and STAT1-dependent M1 macrophage polarization (Figure 5).

To date there has been no direct evidence supporting a role for Btk in macrophage polarization and reports of Btk's contribution to inflammation have been controversial. Several studies have suggested that Btk functions as a negative regulator of inflammation [45–47] while there is gathering evidence to suggest that Btk may be important in driving the inflammatory process. Studies examining APCs derived from XLA patients, who have a natural mutation in Btk, have shown that they have defects in both phagocytosis and inflammatory cytokine production following TLR stimulation [25,26,48]. Recently the potential that Btk plays a direct role in positively regulating antigen presentation and maturation of APCs was described by Lui et al. These studies found impaired TLR-driven activation of APCs due to an inability to form a MHC class II and CD40 complex in the absence of Btk [49]. At a molecular levels Btk has been shown to stabilise TNF-α mRNA via a p38-dependent pathway in X-linked immunodeficiency (Xid) mice and in XLA patients as well as being critically involved in NF-kB activation and specifically p65 phosphorylation downstream of multiple TLRs including TLR4, TLR7 and TLR9 [23–26]. More recently studies by our group have suggested that increased IL-10 production together with a significant reduction in IFN-γ production in the absence of Btk may result in an altered Th1/Th2 balance [22], potentially implicating Btk in macrophage polarization. In the current study we observed impaired activation of NF-kB p65 in the absence of Btk following TLR4 and IFN-γ treatment, further supporting a positive role for Btk in M1 macrophage polarisation. Interestingly, M2 macrophages have been shown to inhibit the expression several M1 associated chemokines including CXCL10 by modulating the activity of both NF-kB and STAT1 [50,51]. We observed impaired phosphorylation of Akt, a key MyD88-dependent signalling intermediary and inducer of M1-associated cytokines [52,53] in the absence of Btk. Studies in B cells have shown that Btk and Akt directly interact and that in the absence of Btk, Akt activation is prevented [53]. A recent study has implicated Akt as a negative regulator of Btk, phosphorylating it in order to promote 14-3-3ζ binding, a novel negative regulator of Btk signalling [54]. Thus the ability of Btk and Akt to cross-regulate each other and the involvement of Akt in regulating macrophage polarization, indicates that the inability of Btk−/− macrophages to phosphorylate Akt in response to M1 polarizing stimuli has an important contribution to the M2 skew observed in these cells.

Whilst a direct link between Btk and STAT1 in regulating macrophase activity has not been previously demonstrated, overexpression studies and studies in B cells have demonstrated that Btk and other Tec family kinases can interact with STAT family members and promote or inhibit their activation. Btk has been shown to interact directly with JAK1 and this association results in Btk phosphorylation [55]. More recently the absence of Btk was shown to result in reduced poly(I:C)-mediated activation of STAT1 [27]. Relevant to our findings, Btk has been shown to prevent STAT3 activation in B cells thus promoting apoptosis [56]. Overall our study indicates that Btk is required for optimal STAT1 activation and that in the absence of Btk, enhanced STAT6 phosphorylation is observed, in keeping with the inhibitory role for Btk regarding STAT3 activation reported by Uckun et al. [56]. Given that Btk has been implicated as key positive regulator of TLR4-mediated cytokine production and that STAT1 activation is required for optimal LPS induced activation of macrophages [57], this study demonstrates an important link between Btk activation, subsequent transcription factor phosphorylation and macrophage polarization.

Interestingly whilst exposure of Btk-deficient macrophages to M1 polarizing stimuli did not result in increased STAT6
phosphorylation or SHIP1 induction, we did however observe significantly impaired activation of key M1 promoting signalling molecules, such as NF-κB p65, STAT1 and AKT, as previously published [6–9]. Together our data suggests that not only is Btk critical for the induction of signals driving M1 polarization but that it also functions as a negative regulator of M2-polarizing signalling pathways, as evidenced by the hyper-phosphorylation of STAT6 and increased induction of SHIP1 we observed following IL-4 and IL-13 treatment in the absence of Btk. The possibility therefore exists that much like SHIP1 [58–60] and Akt [61,62], Btk may have a dual function in mediating macrophage polarization. This role is supported by studies demonstrating that Btk phosphorylates the key TLR4 adaptor Mal, resulting in SOCS1-mediated ubiquitination and degradation of Mal and hence negative regulation of TLR4-dependent pathways [7]. Additionally unpublished observations in our group demonstrate that SHIP1 and Btk interact and that this interaction regulates the phosphorylation and presumably the activity of SHIP1 in an as yet undetermined manner. Thus the lack of Btk contributes not only to the expression of SHIP1 but may also play a role in regulating its activity.

Thus our data suggests that loss of Btk results in a predominant M2 phenotype which may potentially exacerbate Th2 mediated disease such as allergic asthma. Asthma is traditionally thought of as a disease mediated by an imbalance between Th1/Th2/Th17 cells, however it is becoming more apparent that alveolar macrophages play an important role in directing disease outcome [2,4]. Indeed the enhanced production of M2-associated chemokines by alveolar macrophages is thought to contribute to small airway and peripheral lung inflammation observed in asthma patients [63]. Our data in two models of allergic disease demonstrate that a lack of Btk exacerbates M2 polarization, indicating the critical role of this protein in ensuring a balanced response and strongly suggests that lack of a proper M1/M2 balance in the lung in the absence of Btk may exacerbate allergic inflammation. Indeed a study of allergic airway inflammation in mice reports increased IgE responses an exaggerated airway inflammation in the absence of Btk [64]. Clinically, most likely due to absent circulating immunoglobulin and a role for Btk in mast cell degranulation, reports of allergy in XLA patients are rare [65,66]. Despite the rarity of allergic reactions, pulmonary complications such as decreased lung function and increased thickening have been observed in XLA patients [67]. Given the known role of macrophages in driving airway disease, it is tempting to propose that an M1/M2 imbalance may be driving this response.

Btk inhibitors are currently in trial for a number of conditions including B cell malignancies and inflammatory autoimmune conditions [68,69]. Whilst undoubtedly Btk inhibitors contribute to reduced production of pro-inflammatory cytokines by macrophages, Btk inhibition also affects apoptotic cell uptake, in addition to its role in regulating macrophage polarization as demonstrated

Figure 4. Increased in vivo M2 macrophage generation in the absence of Btk. (A–C) WT and Btk−/− mice were injected i.v. with 5,000 live S.mansoni eggs. (A) The percentage of pulmonary M2 macrophages was evaluated by flow cytometry using F4/80 and CD11b co-staining. (B–C) Induction of M2-associated genes was determined by qPCR. (D) WT and Btk−/− mice were injected i.p. with approximately 800 ng Chitin. Peritoneal cells were collected by lavage after 48 hours and gene induction of M2-associated genes was determined by qPCR. In all cases peritoneal macrophages were pooled after isolation, treatment groups consisted of 3–4 animals, and experiments were performed in triplicate. Student’s paired t test was performed comparing gene induction following in vivo exposure to S.mansoni eggs or Chitin as indicated in WT and Btk−/− peritoneal macrophages. Results shown are mean±SD from three independent experiments. * = p<0.05.

doi:10.1371/journal.pone.0085834.g004
here [70]. Given the emerging role for macrophages in allergy, asthma, cancer and certain autoimmune conditions such as systemic lupus erythematosus, our findings suggest that manipulation of Btk activity may have unwanted effects in certain disease settings and indicates the need for more extensive analysis of the role of Btk in macrophages in inflammatory disease.

References
1. Orme J, Mohan C (2012) Macrophage subpopulations in systemic lupus erythematosus. Discov Med 13: 151–158.
2. Shechter R, Schwartz M (2013) Harnessing monocyte-derived macrophages to control central nervous system pathologies: no longer ‘if’ but ‘how’. J Pathol 229: 332–346.
3. Hao NB, Lu MH, Fan YH, Cao YL, Zhang ZR, et al. (2012) Macrophages in tumor microenvironments and the progression of tumors. Clin Dev Immunol 2012: 948098.
4. Dasgupta P, Keegan AD (2012) Contribution of alternatively activated macrophages to allergic lung inflammation: a tale of mice and men. J Inmate Immun 4: 478–483.

Figure 5. Proposed transcriptional regulation of macrophage polarization in the absence of Btk. (A) This study has demonstrated that in response to LPS and IFN-γ Btk contributes to M1 polarizing of myeloid cells via promoting the phosphorylation of Akt and subsequently the p65 subunit of NF-κB, in addition to enhancing phosphorylation of STAT1. (B) In the absence of Btk exposure of myeloid cells to LPS and IFN-γ results in the preferential induction of M2 associated genes and preferentially recruit M2 cells in vivo. Previous studies in Btk−/− mice have observed increased levels IL-10 systemically following LPS treatment. IL-10 is known to activate STAT3 and there is some evidence to suggest that STAT3 may also play a role in promoting M2 macrophage polarization. Thus in the absence of Btk, in response to M1 polarizing stimuli increased IL-10 production together with reduced phosphorylation of key signaling intermediaries, combined with activation of p50 the inhibitory subunit of NF-κB could potentially account for the observed preferential skew towards an M2 phenotype. Additionally this study has shown that in response to IL-4 and IL-13 Btk−/− cells demonstrate an increased capacity to polarize towards an M2 phenotype as a result of enhanced STAT6 phosphorylation and increased SHIP1 expression.
doi:10.1371/journal.pone.0085834.g005

Author Contributions
Conceived and designed the experiments: JNG EH SS CW JCB SS KB AK PGF JAJ CAJ. Performed the experiments: JNG EH SS CW JCB SS KB. Analyzed the data: JNG EH SS CW JCB SS KB. Contributed reagents/materials/analysis tools: JNG EH SS CW JCB SS KB AK PGF JAJ CAJ. Wrote the paper: JNG EH SS CW JCB SS KB AK PGF JAJ CAJ.
5. Sica, A, Mantovani A (2012) Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 122: 787–795.
6. Yu H, Pardoll D, Jove R (2009) STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 9: 786–809.
7. Miettinen M, Salmi A, Doyle SL, Grant P, Ford GA et al. (2006) Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. Nat Immunol 7: 148–155.
8. Pelegrin P, Surprenant A (2009) Dynamics of macrophage polarization reveal new mechanisms to inhibit IL-1β release through pyrophosphates. EMBO J 28: 2114–2127.
9. Porta C, Rimoldi M, Raes G, Blys L, Ghezzi P, et al. (2009) Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB. Proc Natl Acad Sci U.S.A. 106: 14978–14983.
10. Mantovani A, Sica A (2010) Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Curr Opin Immunol 22: 231–237.
11. Pauleau AL, Rutschman R, Lang R, Persin A, Watowich SS, et al. (2004) Enhancer-mediated control of macrophage-specific arginase 1 expression. J Immunol 172: 7503–7537.
12. Kortylewski M, Kijjavi M, Wang T, Wei S, Zhang S, et al. (2005) Inhibiting Stat3 signaling in the hematopoietic system elicits multicellular antigenic tumor immunity. Nat Med 11: 1314–1321.
13. Ostrand-Rosenberg S, Grusby MJ, Clemmons VK (2006) Cutting edge: STAT6-deficient mice have enhanced tumor immunity to primary and metastatic mammary carcinoma. J Immunol 165: 6013–6019.
14. Sinha P, Clements VK, Ostrand-Rosenberg S (2005) Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitates the rejection of established metastatic disease. J Immunol 174: 636–645.
15. Ogedaa JR, Ricardo-Gonzalez RR, Goroth MH, Morel CR, Subramanian V, et al. (2007) Macrophage-specific PPARγ controls alternative activation and improves resistance to cancer. Nature 447: 1116–1120.
16. Liao X, Sharma N, Kapadia F, Zhou G, Lu Y, et al. (2011) Kruppel-like factor 4 and enhanced TLR3-dependent IFN-beta production. J Immunol 184: 2314–2320.
17. Sly LM, Rauh MJ, Ho V, Pereira C, Sham A, et al. (2005) SHIP represses the inositol phosphatase SHIP is essential for endotoxin tolerance. Immunity 21: 229–239.
18. An H, Xu H, Zhang M, Zhou J, Feng T, et al. (2005) Src homology 2 domain-containing inositol phosphatase 1 (SHIP1) negatively regulates TLR4-mediated LPS response primarily through a phosphatase activity and PI-3K independent mechanism. Blood 105: 4605–4609.
19. Gabehann JN, Higgs R, Brennan K, Thomas W, Damen JE, et al. (2010) Absence of SHIP-1 results in constitutive phosphorylation of tank-binding kinase 1 and enhanced TLR3-dependent IFN-beta production. J Immunol 184: 2314–2320.
20. O’Connell RM, Chaudhuri AA, Rao DS, Baltimore D (2009) Inositol 1,4,5-trisphosphate 3 kinase mediates Toll-like receptor signaling. J Exp Med 198: 2114–2127.
21. Spence S, Fitzsimons A, Boyd CR, Kessler J, Fitzgerald D, et al. (2013) Akt1 and Akt2 protein kinases differentially contribute to macrophage polarization and improves nuclear factor kappaB. Proc Natl Acad Sci U S A 110: 14978–14983.
22. Zhang F, Zhang T, Shi C, Zhan X, Shu S, et al. (2013) Akt1 and Akt2 promote IL-10 production in alternatively polarized M2 macrophages. J Biol Chem 288: 2114–2127.
23. Ramalingam TR, Reiman RM, Wynan TA (2005) Exploiting worm and allergy models to understand Th2 cytokine biology. Curr Opin Allergy Clin Immunol 5: 392–398.
24. Reese TI, Liang HE, Tager AM, Luster AD, Van Rooijen N, et al. (2007) Chlamydia induces accumulation in tissue of innate immune cells associated with allergy. Nature 447: 92–96.
25. Perez de Diego R, Lopez-Granados E, Pozo M, Rodriguez C, Sabina P, et al. (2006) Bruton’s tyrosine kinase is not essential for LPS-induced activation of human monocytes. J Immunol 177: 1465–1473.
26. Gagliardi MC, Finocchi A, Orlandi P, Carrella C, Cancrini C, et al. (2006) Bruton’s tyrosine kinase defect in dendritic cells from X-linked agammaglobulinemia patients does not influence their differentiation, maturation and antigen-presenting cell function. Clin Exp Immunol 144: 113–122.
27. Jourouqui H, Geng L, Torner GA, Vinekar K, Feng D, et al. (2008) Monocyte twins with a microdeletion syndrome involving BTK, DD1P, and two other genes: evidence of intact dendritic cell development and TLR responses. Eur J Pediatr 167: 927–931.
28. Amoras AL, da Silva MT, Zoller RL, Kanegane H, Miyazaki T, et al. (2007) Expression of Fc gamma and complement receptors in monocytes of X-linked agammaglobulinemia and common variable immunodeficiency patients. Clin Exp Immunol 150: 422–428.
29. Liu X, Zhan Z, Li D, Xu L, Ma F, et al. (2011) Intracellular MHC class II molecules promote TLR-triggered innate immune responses by maintaining activation of the kinase Btk. Nat Immunol 12: 416–424.
30. Li Q, Verma IM (2002) NF-kappaB regulation in the immune system. Nat Rev Immunol 2: 725–734.
31. Hu X, Li WP, Meng C, Iurashiv LB (2003) Inhibition of IFN-gamma signaling by glucocorticoids. J Immunol 170: 4033–4038.
32. Li X, Tupper JC, Bamumn WS, Rhode JK, Rhodes CJ, et al. (2003) Phosphoinositide 5 kinase mediates Toll-like receptor 4-induced activation of NF-kappaB in endothelial cells. Infect Immun 71: 4414–4420.
33. Kitaura J, Arai K, Maeda-Yamamoto M, Kawakami Y, Kimura U, et al. (2000) Akt-dependent cytokine production in mast cells. J Exp Med 192: 729–740.
34. Mohamed DK, Noer BF, Hussain A, Gustafsson MO, Mohamed AJ, et al. (2013) Dual Phosphorylation of Btk by Akt/Protein Kinase B Provides Docking for 14-3-3-Sigma, Regulates Shuttling, and Alternates Both Tonic and Induced Signaling in B Cells. Mol Cell Biol 33: 3214–3226.
35. Takahashi-Tenaka M, Hiba M, Fujitani Y, Fukada T, Yamaguchi T, et al. (1997) Tce tyrosine kinase links the cytokine receptors to PI-3 kinase probably through Jak3. Oncogene 14: 2273–2282.
36. Urban F, Ober Z, Vassilev A (2007) Bruton’s tyrosine kinase prevents activation of the anti-apoptotic transcription factor STAT3 and promotes apoptosis in neoplastic B-cells and B-cell precursors exposed to oxidative stress. Br J Haematol 136: 574–389.
37. Ohmori Y, Hamilton TA (2001) Requirement for STAT1 in LPS-induced gene expression in macrophages. J Leukoc Biol 69: 596–604.
38. Raai MJ, Ho V, Pereira C, Sharma A, Sly LM, et al. (2005) SHIP represses the generation of alternatively activated macrophages. Immunity 23: 361–374.
39. Antonutti-Fabrak M, Krautzsch M, Wimmers J, Zhang A, et al. (2010) SHIP is required for dendritic cell maturation. J Immunol 184: 2085–2101.
40. Weiser SB, McLaren KW, Vognaicr N, van Netten-Thomas CJ, Antov A, et al. (2011) Alternative activation of macrophages by IL-4 requires SHIP derepression. Eur J Immunol 41: 1743–1753.
41. Arranz A, Douski C, Vergadi E, Martínez de la Torre Y, Vaprožik K, et al. (2012) Akt1 and Akt2 protein kinases differentially contribute to macrophage polarization. Proc Natl Acad Sci U S A 109: 5917–5922.
62. Xu F, Kang Y, Zhang H, Piao Z, Yin H, et al. (2013) Akt1-Mediated Regulation of Macrophage Polarization in a Murine Model of Staphylococcus aureus Pulmonary Infection. J Infect Dis 208: 528–538.

63. Taha RA, Minshall EM, Miotto D, Shimbara A, Luster A, et al. (1999) Eotaxin and monocyte chemotactic protein-4 mRNA expression in small airways of asthmatic and nonasthmatic individuals. J Allergy Clin Immunol 103: 476–483.

64. Kawakami Y, Inagaki N, Salek-Ardakani S, Kitaura J, Tanaka H, et al. (2006) Regulation of dendritic cell maturation and function by Bruton’s tyrosine kinase via IL-10 and Stat3. Proc Natl Acad Sci U S A 103: 153–158.

65. Hata D, Kawakami Y, Inagaki N, Lantz CS, Kitamura T, et al. (1998) Involvement of Bruton’s tyrosine kinase in FcεRI-dependent mast cell degranulation and cytokine production. J Exp Med 187: 1235–1247.

66. Shabestari MS, Rezaei N (2008) Asthma and allergic rhinitis in a patient with BTK deficiency. J Investig Allergol Clin Immunol 18: 300–304.

67. Costa-Carvalho BT, Wandahen GF, Pulici G, Aranda CS, Sole D (2011) Pulmonary complications in patients with antibody deficiency. Allergol Immunopathol (Madr) 39: 129–132.

68. D’Cruz OJ, Uckun FM (2013) Novel Bruton’s tyrosine kinase inhibitors currently in development. Onco Targets Ther 6: 161–176.

69. Honigberg LA, Smith AM, Sirisawad M, Verner E, Loury D, et al. (2010) The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. Proc Natl Acad Sci U S A 107: 13075–13080.

70. Byrne JC, Ní Gabhann J, Stacey K, Coffey BM, McCarthy E, et al. (2013) Bruton’s Tyrosine Kinase Is Required for Apoptotic Cell Uptake via Regulating the Phosphorylation and Localization of Calreticulin. J Immunol In press.