

Bruton tyrosine kinase mediates *TLR9*-dependent human dendritic cell activation

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Background: Bruton tyrosine kinase (BTK) plays an essential role in various biologic functions of different cell types. Mutations in BTK lead to X-linked agammaglobulinemia (XLA) in humans. BTK was recently linked to the innate immune system, in particular, the Toll-like receptor (TLR) pathway; however, the TLR9 pathway has never been studied in dendritic cells (DCs) of patients with XLA.

Objective: The aim of this study was to investigate the role of BTK in human DC activation upon TLR9 stimulation.

Methods: DCs of patients with XLA and healthy donors were stimulated via TLR4/9 and evaluated for cell activation and cytokine production.

Results: We showed that BTK plays an essential role in DC responses to unmethylated CpG oligodeoxynucleotide: although responses to lipopolysaccharide/TLR4 induce normal DC activation in terms of upregulation of specific markers (CD86, CD83, CD80, HLA-DR), the CpG/TLR9 pathway is completely impaired in patients with XLA. Furthermore, cytokine production upon TLR9 activation in patients with XLA is radically impaired in terms of IL-6, IL-12, TNF- α , and IL-10 production. Interestingly, BTK mediated STAT1/3 upregulation in a TLR9-dependent manner. The important role of BTK in human DC activation was confirmed after incubation of healthy DCs with ibrutinib, the specific BTK inhibitor, which resulted in impairment of TLR9 responses as seen in patients with XLA. **Conclusion:** Analysis of these data suggests that BTK regulates human DC responses upon TLR9 engagement in terms of activation, cytokine production, and STAT1/3 upregulation. These findings may be of important significance for better understanding and managing different clinical conditions, such as agammaglobulinemia and lymphoid malignancies. (*J Allergy Clin Immunol* 2014;■■■:■■■-■■■.)

Key words: BTK, dendritic cells, TLR9, STAT1, STAT3

X-linked agammaglobulinemia (XLA) is considered the prototype of humoral immunodeficiencies and is characterized by

Abbreviations used

BTK: Bruton tyrosine kinase
CpG: Unmethylated CpG oligodeoxynucleotide
DC: Dendritic cell
LPS: Lipopolysaccharide
STAT: Signal transducer and activator of transcription
TLR: Toll-like receptor
XLA: X-linked agammaglobulinemia

markedly reduced levels of serum antibodies and the absence of circulating B cells in the presence of normal T-cell numbers and function. As a result, affected patients have an increased susceptibility to a variety of encapsulated bacteria and enteroviruses, microorganisms for which antibodies play an especially critical role in host defense. In 1993, 2 groups reported that XLA resulted from mutations in a cytoplasmic tyrosine kinase called Bruton tyrosine kinase (BTK),^{1,2} which highlights the essential role of this molecule in B-cell differentiation through the pre-B-cell receptor and B-cell receptor signaling. BTK is a member of a family of cytoplasmic tyrosine kinases, called TEC kinases, which include TEC, ITK, RLK, and BMX.³⁻⁷ These enzymes are predominantly expressed in hematopoietic cells; in fact, most cell lineages contain more than 1 family member (eg, neutrophils and macrophages express BTK, ITK, and TEC kinases; mast cells express BTK, ITK, RLK, and TEC kinases). This finding extended the concept that a defect of BTK could affect, although at different extents, various cell lineages, such as monocytes, neutrophils, macrophages, dendritic cell (DC), natural killer cells and others,⁸⁻¹¹ which suggests that its biologic role is broader and more complex than originally thought. BTK, therefore, may act as a link between adaptive and innate immunity, although with certain differences between the human and the animal models.¹²

Along this line, whereas neutrophils of patients with a BTK deficiency were reported to have normal function,¹³ the mouse model (xid mice) showed evidence of an important role of BTK for the development and function of neutrophils.⁸ Recently, BTK also was reported to negatively regulate the production of reactive oxygen species and stimulation-induced apoptosis in human neutrophils.¹⁴ BTK also was found to be essential for natural killer cells in terms of activation and cytotoxic activity¹¹ in a BTK/TLR3-dependent manner. Furthermore, TLR8-dependent production of IL-6 and TNF- α by DCs of patients with XLA was significantly impaired.¹⁵ BTK was recently shown to interact with TLR9 and to play an important role both in the TLR9 signaling in the monocytic cell line THP1 and in the CpG-induced IL-6 production from peripheral blood mononuclear cells of patients with XLA.¹⁶ However, the TLR9 pathway in DCs from patients with XLA has not been investigated yet. In the present study, we showed that BTK plays an essential role in human DC

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The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement no 201549 (EURO-PADnet HEALTH-F2-2008-201549). The research that led to these results also received funding from the "Fondazione C. Golgi," Brescia.

Disclosure of potential conflicts of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication July 4, 2013; revised December 20, 2013; accepted for publication December 31, 2013.

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0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2013.12.1085>

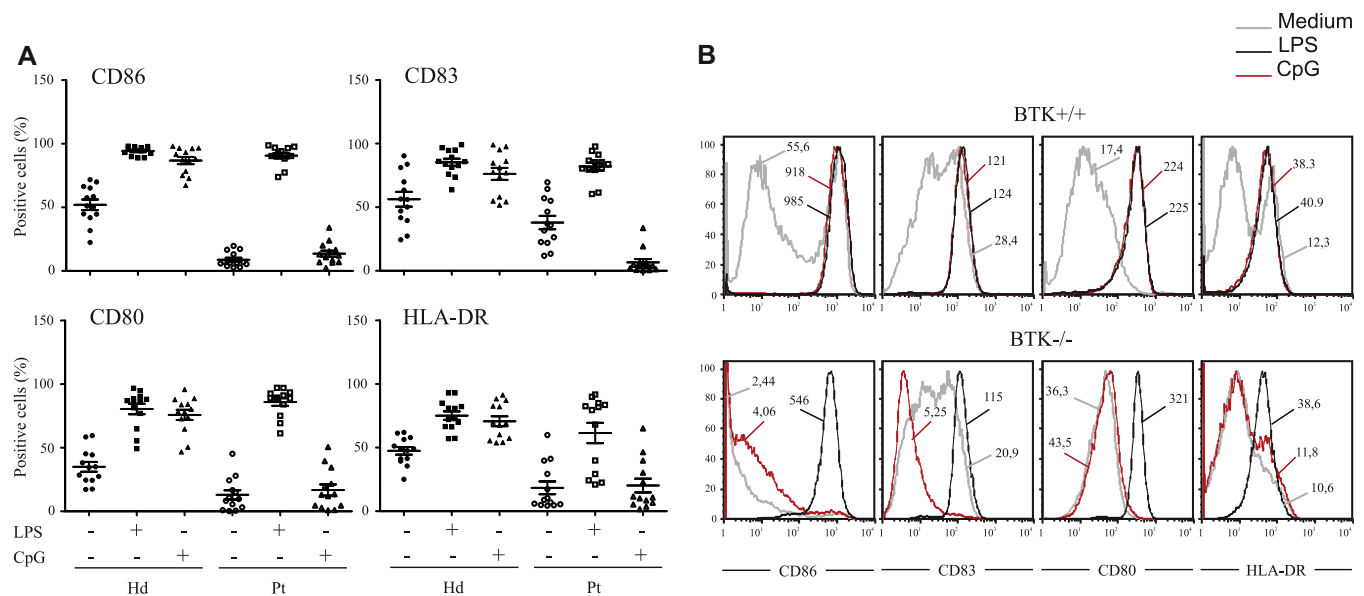


FIG 1. Human DCs activation upon LPS or CpG stimulation in the presence or absence of BTK. **A**, Summarized data for upregulation of CD86, CD83, CD80, and HLA-DR upon LPS or CpG stimulation in human DCs from healthy donors (Hd) ($n = 13$) and patients with XLA (Pt) ($n = 13$). **B**, Histograms that depict the mean fluorescence intensity of CD86, CD83, CD80, and HLA-DR upon LPS (black) and CpG (red) stimulation in DCs from a healthy control (BTK^{+/+}) (upper panel) and an XLA patient (BTK^{-/-}) (lower panel). Cells cultured in medium alone were used as control (gray) (representative experiment).

responses to CpG. In fact, although XLA DCs respond normally to LPS/TLR4 signalling in terms of upregulation of activation markers and cytokine production, the CpG/TLR9 signalling cascade is severely impaired. Interestingly, we also show that BTK is essential for STAT1/3 upregulation in a TLR9-dependent manner. The important role of BTK in human DC activation was further confirmed after incubation of healthy DCs with the specific BTK inhibitor (ibrutinib), which results in impairment of TLR9 responses as seen in patients with XLA.

METHODS

Patient samples

Thirteen patients affected with molecularly proven XLA regularly followed up at the Pediatrics Clinic, University of Brescia, were enrolled in this study. The type of BTK mutations, residual peripheral B-cell percentages and relevant clinical features of the patients with XLA included in this study are described in Table E1 (in this article's Online Repository at www.jacionline.org). Thirteen age- and sex-matched healthy controls also were enrolled. Approval from the institutional review board was obtained. Informed consent was obtained from all patients or patients' parents, depending on age.

Cell culture

Peripheral blood samples were processed, and monocytes were separated by positive selection by using CD14 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol. Monocytes were cultured in 24-well plates at the concentration of 5×10^5 cells/well with RPMI supplemented with 10% FCS (EuroClone Life Science, Milan, Italy), penicillin-streptomycin (EuroClone Life Science), and L-glutamine (EuroClone Life Science), and were stimulated with GM-CSF (50 U/mL) and IL-4 (20 ng/mL) for 4 to 5 days to obtain DCs. DCs were stimulated with LPS (100 ng/mL) or CpG ODN 2216 (5 μ M) for 24 hours. Flow cytometry analysis was then performed for the expression of cell activation markers (see below). DCs from healthy controls were incubated with ibrutinib (Selleck Chemicals, Houston, Tex), the irreversible BTK inhibitor, at a concentration of

10 μ M for 30 minutes followed by LPS or CpG stimulation performed as described above. The expression of cell-activation markers then was evaluated by cytometric analysis (see below), and the cytokine induction by assessment of the levels of cytokine-specific mRNAs.

Flow cytometry

Monocyte purity upon positive selection was evaluated by cell incubation with the CD14 monoclonal-labeled antibody (FITC mouse anti-human CD14) (BD Biosciences, San Jose, Calif) (>90% CD14 positive cells). DCs were incubated with monoclonal-labeled antibodies (FITC mouse anti-human CD14, PE mouse anti-human CD86, PE mouse anti-human CD80, PerCP-Cy 5.5 mouse anti-human CD83, PerCP-Cy 5.5 mouse anti-human HLA-DR) (BD Biosciences). After 20 minutes of incubation at 4°C, the cells were washed twice with PBS 1% FBS at 1600 rpm for 5 minutes and then samples were acquired on a BD 3-color flow cytometer (BD FACSCalibur, BD Biosciences). Flow cytometric analysis was performed by using the FlowJo software (Tree Star Inc, Ashland, Ore).

Real-time quantitative PCR

Total RNA prepared by using RNeasy mini kit (QIAGEN Inc, Valencia, Calif) was reversed transcribed with a High-Capacity RNA-to-cDNA Kit (Life Technologies, Carlsbad, Calif) by using oligo-dT primers according to the manufacturer's protocol. Quantitative PCR was performed by using iQ SYBR Green Supermix and CFX96 Touch (Bio-Rad, Hercules, Calif) according to the manufacturer's protocol. All the probes were designed by using ProbeFinder Software (Roche Applied Science, Basel, Switzerland), a free Web-based tool based on the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, Mass). The sequences of IL-6, IL-12, TNF- α , IL-10, STAT1, STAT3, and GAPDH primers are summarized in Table E2 (in this article's Online Repository at www.jacionline.org).

Statistical analysis

The 2-tailed unpaired Student *t* tests were used for the analysis of real-time experiments. Data are expressed as means ($\pm$ SE) of experiments performed in

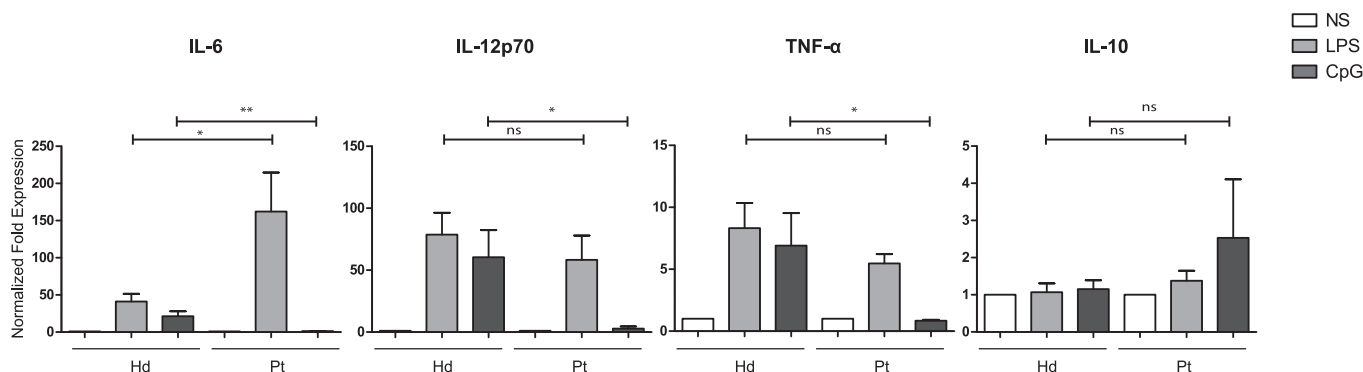


FIG 2. Human DCs cytokine induction upon LPS or CpG stimulation in the presence or absence of BTK. Cytokine mRNA levels for IL6, IL-12, TNF- α , and IL-10 upon LPS or CpG stimulation from human DCs from healthy donors (Hd) ($n = 10$) and patients with XLA (Pt) ($n = 10$). Bar graphs show mean values of 2 (+ SD) technical replicates. P values were calculated by using the unpaired Student t test (* $P = .01-.05$; ** $P = .01-.001$). ns, Not significant.

duplicate. The values of $P = .01-.05$ were considered significant (*), $P = .01-.001$ were considered very significant (**), and $P < .001$ was considered highly significant (***).

RESULTS

Impaired DC activation in patients with XLA

Immature DCs of 13 patients with XLA and 13 healthy controls were stimulated with LPS or CpG. Upon LPS stimulation, XLA DCs responded similarly to healthy controls as defined by the upregulation of activation markers CD86, CD83, CD80, and HLA-DR; on the contrary, upon CpG stimulation, XLA DCs failed to upregulate the same activation markers when compared with healthy controls (Fig 1, A). A representative experiment of DCs responses to LPS and CpG stimulation in terms of upregulation of CD86, CD83, CD80, and HLA-DR of an patient with XLA and a healthy control is shown in Fig 1, B (histograms) and in Fig E1 (density plot) (in this article's Online Repository at www.jacionline.org). These data underline the important role of BTK in the TLR9 signaling cascade in human DCs.

Impaired cytokine induction in XLA DCs

Because DC activation after CpG stimulation is impaired in the absence of BTK, we decided to evaluate whether a similar impairment would be observed in terms of cytokine induction. DCs were incubated for 24 hours with LPS or CpG, and then were evaluated for specific cytokine mRNA expression. After LPS stimulation, DCs of patients with XLA ($n = 10$) and healthy controls ($n = 10$) responded with IL-6, IL-12, TNF- α , and IL-10 induction, although at a different extent. On the contrary, after CpG stimulation, DC production of IL-6, IL-12, and TNF- α was severely reduced in patients with XLA compared with normal controls (Fig 2), whereas IL-10 production was slightly increased, which highlights the important role of BTK in DC responses upon TLR9 stimulation in terms of cytokine induction.

STAT1/3 upregulation upon TLR engagement in DCs of patients with XLA

DCs are known to be potent antigen-presenting cells in antiviral host defense, a process in which signal transducers and activators

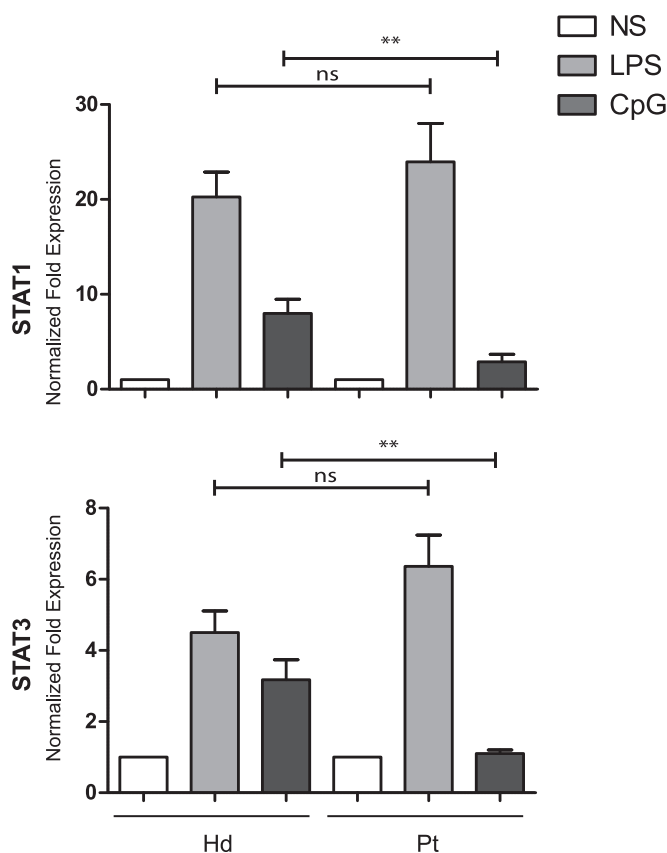


FIG 3. STAT1/3 upregulation in human DCs upon LPS or CpG stimulation in the presence or absence of BTK. STAT1 and STAT3 mRNA levels were measured in DCs from healthy donors ($n = 10$) and patients with XLA ($n = 10$) (BTK $^{-/-}$). Bar graphs show values of 2 (+ SD) technical replicates. P values were calculated by using unpaired Student t test (** $P = .01-.001$). ns, Not significant.

of transcription (STAT) family members, such as STAT1, play a critical role. Therefore, when considering the novel data presented here on the role of BTK in DC activation, we decided to evaluate if BTK $^{-/-}$ DC responses also were impaired at the mRNA level for specific signaling transducer molecules involved

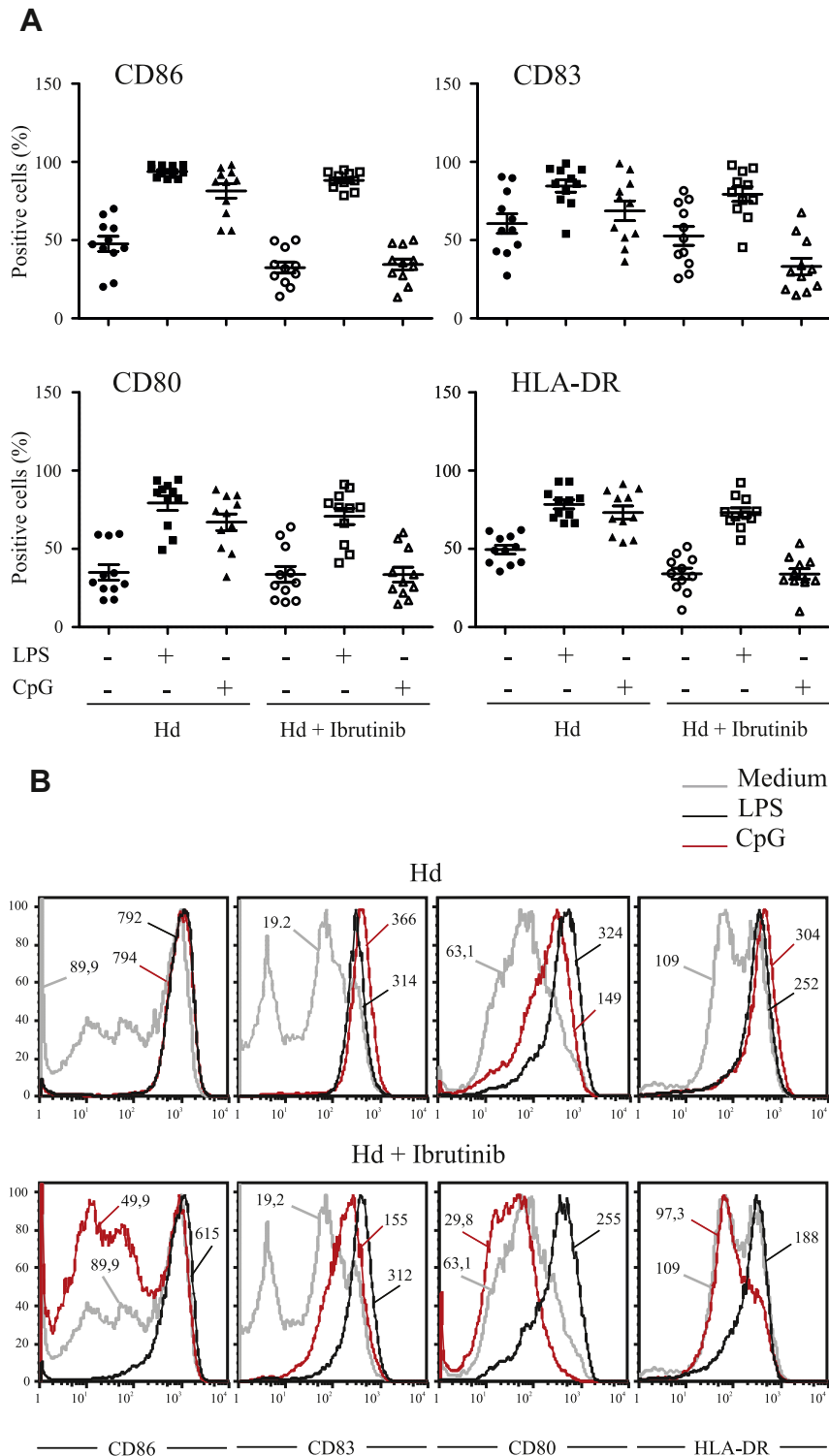


FIG 4. Human DC activation upon LPS or CpG stimulation in the presence or blockade of BTK. **A**, Summarized data for upregulation of CD86, CD83, CD80, and HLA-DR upon LPS or CpG stimulation in human DCs from 11 healthy donors (*Hd*) in the presence or absence of the specific BTK inhibitor ibrutinib. **B**, Representative histograms, depicting the mean fluorescence intensity of CD86, CD83, CD80, and HLA-DR expression upon LPS (black) and CpG (red) stimulation in DCs from healthy donors in the presence (*Hd* + ibrutinib) or absence (*Hd*) of specific BTK inhibitor. Cells cultured in medium alone were used as control (gray).

in viral infections, that is, *STAT1*. Stimulation of healthy DCs with either CpG or LPS induced upregulation of *STAT1* mRNA. On the contrary, although LPS stimulation of BTK^{-/-} DCs induced a

strong upregulation of *STAT1* mRNA, CpG stimulation did not, which underlies that BTK is required for *STAT1* upregulation upon TLR9 engagement (Fig 3, upper panel). To evaluate if this

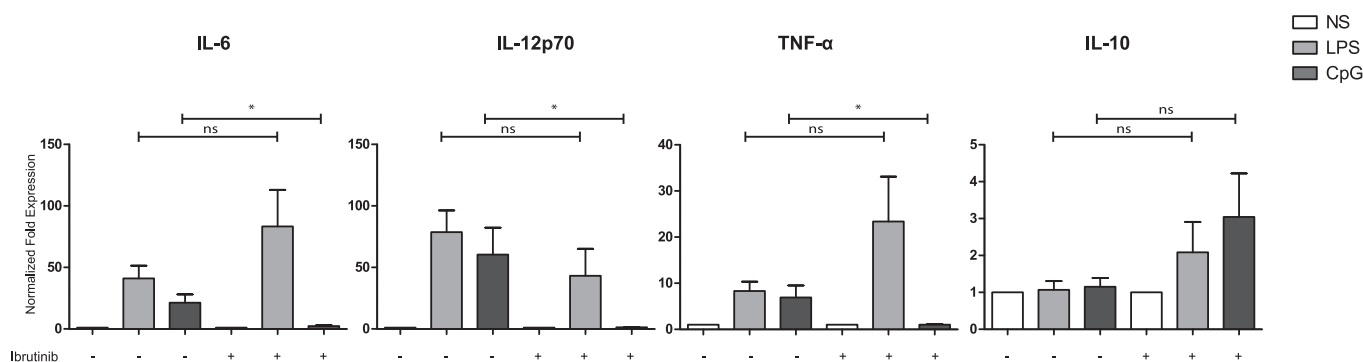


FIG 5. Human DCs cytokine induction upon LPS or CpG stimulation in the presence or blockade of BTK. Normalized levels of IL-6, IL-12, TNF-α, and IL-10 mRNA from healthy human DCs (n = 10) upon LPS or CpG stimulation in the presence or absence of ibuprofen. Data are expressed as normalized fold expression levels with respect to cells not stimulated. Bar graphs show values of 2 (+ SD) technical replicates. P values were calculated by using unpaired Student t test (*P = .01-.05). ns, Not significant.

effect was *STAT1* specific, *STAT3* upregulation also was evaluated: *STAT3* upregulation upon CpG stimulation resembles that of *STAT1* (Fig 3, lower panel).

In vitro inhibition of BTK in healthy DCs recapitulates XLA-DCs

To further validate the role of BTK on DC activation, we decided to evaluate the effect of ibuprofen, a drug that irreversibly inhibits BTK, on the activation of DCs of normal subjects. Thus, immature DCs of 11 healthy donors were cultured with ibuprofen for 30 minutes before LPS or CpG stimulation. Treatment with ibuprofen did not affect DC responses to LPS as defined by upregulation of activation markers; on the contrary, ibuprofen treatment severely impaired DC responses to CpG in terms of the upregulation of the same activation markers (Fig 4, A). A representative experiment of DC responses to LPS and CpG stimulation upon BTK inhibition in terms of upregulation of activation markers from a healthy control is shown in Fig 4, B (histograms) and in Fig E2 (density plot) (in this article's Online Repository at www.jacionline.org).

When considering the results on healthy DC activation upon BTK inhibition, we further decided to evaluate the effect of BTK inhibition on cytokine induction upon LPS or CpG stimulation. Cytokine induction was evaluated by quantitative real-time PCR, and data were normalized to not stimulated DCs. DC responses upon LPS stimulation resulted in IL-6 and TNF-α mRNA induction in agreement with already published data,^{16,17} whereas IL-12 was moderately influenced. DC responses to CpG, on the contrary, were severely reduced after BTK inhibition in terms of IL-6, IL-12, and TNF-α mRNA induction (Fig 5). However, the IL-10 mRNA induction was enhanced upon BTK inhibition, similarly to DCs of patients with XLA. These data provide further evidence for the important role of BTK in the TLR9 signaling cascade in human DCs for cytokine production. Furthermore, although ibuprofen-treated DCs of 10 healthy subjects stimulated with LPS showed upregulation of *STAT1* mRNA similar to that observed when using ibuprofen-untreated DCs, *STAT1* mRNA upregulation was severely reduced in ibuprofen-treated DCs upon CpG stimulation (Fig 6, upper panel). *STAT3* upregulation upon CpG stimulation in ibuprofen-treated DCs resembles that of *STAT1* (Fig 6, lower panel).

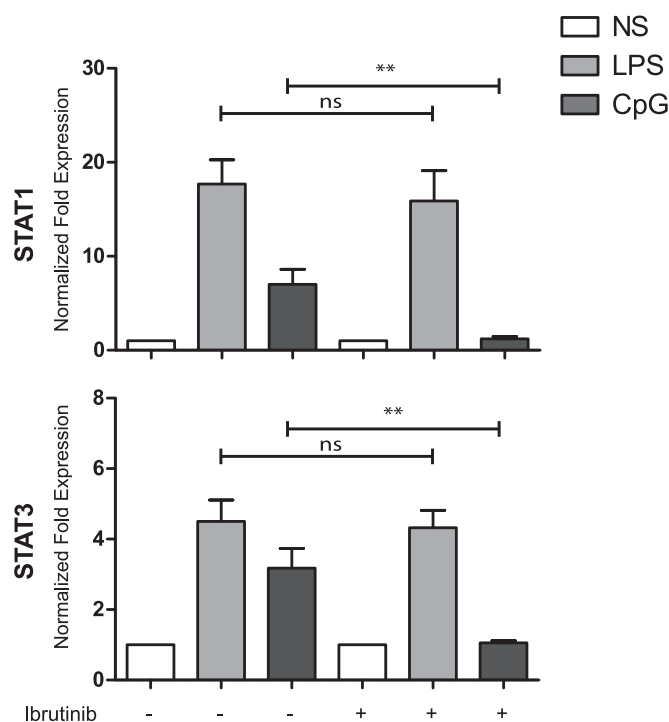


FIG 6. STAT1/3 upregulation in human DCs upon LPS or CpG stimulation in the presence or blockade of BTK. DCs from 10 healthy donors were stimulated with LPS or CpG in the presence or absence of ibuprofen. *STAT1* and *STAT3* mRNA levels were measured and normalized to cells not stimulated. Each datum represents the mean of 2 (+ SD) independent repeat. P values were calculated by using unpaired Student t test (**P = .01-.001). ns, Not significant.

DISCUSSION

BTK has been largely studied in B cells as an essential downstream signaling component of the pre-BCR and BCR cascade.^{4,18,19} Analysis of recent findings have suggested that the biologic role of BTK is not limited to B cells but is broader and involves different cell types, such as monocytes, macrophages, natural killer cells, neutrophils, and others.^{8-10,13-15} Therefore, it is likely that BTK may function as a bridge between adaptive and innate immunity. The role of the TLR family members in the innate arm of immunity, however, is largely under

investigation. Among the TLR family members, TLR9 is expressed primarily on antigen-presenting cells and plays an essential role in the specific cellular response to CpG DNA because TLR9^{-/-} mice are completely unresponsive to CpG DNA, whereas TLR2^{-/-} and TLR4^{-/-} mice respond normally.¹⁷ However, the mechanism by which TLR9 is activated remains largely elusive, and the proteins involved in this activation process are only partially known. In the present study, when using XLA as an “experimentum naturae” model, we provide the first evidence that BTK is involved in the TLR9 signaling cascade in human DCs. CpG stimulation in DCs of patients with XLA failed to upregulate the expression of activation markers such as CD86, CD83, CD80, and HLA-DR, whereas DC responses to LPS stimulation in patients with XLA were normal in terms of upregulation of activation markers. This is the first evidence that BTK is essential for human DC activation, which is severely impaired in patients with XLA upon TLR9 engagement. Furthermore, TLR9 engagement in XLA DCs failed to induce proinflammatory cytokine induction such as IL-6, IL-12, TNF- α , whereas IL-10 induction was enhanced in the absence of BTK. However, DCs responses to LPS stimulation in patients with XLA resulted in accordance with already published data.^{16,17} Therefore, it is evident that the role of BTK in TLR9 signaling in human DCs is not limited to cell activation but involves important biologic functions of human DCs, for example, cytokine production. Analysis of these results suggests that patients with XLA present a broader immune dysfunction than originally suspected, rather than a pure B-cell defect.

Interestingly, we demonstrated that BTK mediates a novel regulatory function by inducing *STAT1/3* expression upon TLR9 engagement in human DCs. Given that APCs, such as B cells and DCs are essential for the initiation of the immune response against bacterial and viral infections, our novel observation that DCs of patients with XLA cannot upregulate *STAT1*, which is known to be involved in antiviral responses, upon TLR9 engagement suggests a potential susceptibility of these patients to enteroviral infections. Indeed, our observation might account for the observed differences in the development of enteroviral infections between patients with XLA and those with autosomal recessive agammaglobulinemia. *STAT3*, another member of the STAT family, showed a similar pattern of induction, which suggests that BTK may have broader regulatory effects on STAT family members.

Furthermore, the role of BTK in the TLR9-signaling cascade was emphasized by the inhibitory effect of ibrutinib on CpG-mediated DC maturation. BTK blockade with ibrutinib had no negative effect on the upregulation of DC activation markers or cytokine production upon TLR4 activation in healthy donors. DC responses upon TLR9 engagement after BTK blockade with ibrutinib recapitulate XLA DC responses, which underscores the essential role for BTK in human DC activation. Given that BTK inhibition has recently been introduced in the treatment of B-cell malignancies for blocking the BCR signaling cascade,¹⁸⁻²⁴ our results suggest that the inhibitory effect of ibrutinib is not restricted to B cells but also affects DCs.

When taken together, our results suggest that BTK is an important component of the TLR9 signaling cascade in human DCs activation. Furthermore, BTK mediates a novel regulatory function by controlling STATs, such as *STAT1* and *STAT3* upregulation in DCs. BTK congenital deficiency or BTK pharmaceutical inhibition results both in severe impairment of human DC

activation upon TLR9 engagement. These findings should be taken into consideration in current clinical management of pathologies such as agammaglobulinemia and B-cell lymphoid malignancies.

We thank Fondazione Camillo Golgi, and Associazione Immunodeficienze Primitiva, Brescia, Italy. We also thank the patients, the patients' families, and the nurses for all their efforts.

Key messages

- DCs of patients with XLA show profound defects upon TLR9 engagement in terms of activation and cytokine production.
- *STAT1/3* upregulation in human DCs upon TLR9 engagement is BTK dependent.
- Ibrutinib, the BTK inhibitor, impairs healthy DC responses upon TLR9 engagement.

REFERENCES

1. Tsukada S, Saffran DC, Rawlings DJ, Parolini O, Allen RC, Klisak I, et al. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell* 1993;72:279-90.
2. Vetrie D, Vořechovský I, Sideras P, Holland J, Davies A, Flinter F, et al. The gene involved in X-linked agammaglobulinemia is a member of the Src family of protein-tyrosine kinases. *Nature* 1993;361:226-33.
3. Hussain A, Yu L, Faryal R, Mohammad DK, Mohamed AJ, Smith CI. TEC family kinases in health and disease—loss-of-function of BTK and ITK and the gain-of-function fusions ITK-SYK and BTK-SYK. *FEBS J* 2011;278:2001-10.
4. Horwood NJ, Urbaniak AM, Danks L. Tec family kinases in inflammation and disease. *Int Rev Immunol* 2012;31:87-103.
5. Mohamed AJ, Yu L, Bäcksjö CM, Vargas L, Faryal R, Aints A, et al. Bruton's tyrosine kinase (Btk): function, regulation, and transformation with special emphasis on the PH domain. *Immunol Rev* 2009;228:58-73.
6. Gomez-Rodriguez J, Readinger JA, Vioritto IC, Mueller KL, Houghtling RA, Schwartzberg PL. Tec kinases, actin, and cell adhesion [review]. *Immunol Rev* 2007;218:45-64.
7. Schmidt U, Boucheron N, Unger B, Ellmeier W. The role of Tec family kinases in myeloid cells. *Int Arch Allergy Immunol* 2004;134:65-78.
8. Fiedler K, Sindlaru A, Terszowski G, Kokai E, Feyerabend TB, Bullinger L, et al. Neutrophil development and function critically depend on Bruton tyrosine kinase in a mouse model of X-linked agammaglobulinemia. *Blood* 2011;117:1329-39.
9. Khare A, Viswanathan B, Gund R, Jain N, Ravindran B, George A, et al. Role of Bruton's tyrosine kinase in macrophage apoptosis. *Apoptosis* 2011;16:334-46.
10. Koprulu AD, Ellmeier W. The role of Tec family kinases in mononuclear phagocytes [review]. *Crit Rev Immunol* 2009;29:317-33.
11. Bao Y, Zheng J, Han C, Jin J, Han H, Liu Y, et al. Tyrosine kinase BTK is required for NK cell activation. *J Biol Chem* 2012;287:23769-78.
12. Rawlings DJ, Saffran DC, Tsukada S, Largaespada DA, Grimaldi JC, Cohen L, et al. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science* 1993;261:358-61.
13. Marron TU, Rohr K, Martinez-Gallo M, Yu J, Cunningham-Rundles C. TLR signaling and effector functions are intact in XLA neutrophils. *Clin Immunol* 2010;137:74-80.
14. Honda F, Kano H, Kanegane H, Nonoyama S, Kim ES, Lee SK, et al. The kinase BTK negatively regulates the production of reactive oxygen species and stimulation-induced apoptosis in human neutrophils. *Nat Immunol* 2012;13:369-78.
15. Sochorová K, Horváth R, Rozková D, Litzman J, Bartunková J, Sedivá A, et al. Impaired Toll-like receptor 8-mediated IL-6 and TNF- α production in antigen-presenting cells from patients with X-linked agammaglobulinemia. *Blood* 2007;109:2553-6.
16. Doyle SL, Jefferies CA, Feighery C, O'Neill LA. Signaling by Toll-like receptors 8 and 9 requires Bruton's tyrosine kinase. *J Biol Chem* 2007;282:36953-60.
17. González-Serrano ME, Estrada-García I, Mogica-Martínez D, González-Garay A, López-Herrera G, Berrón-Ruiz L, et al. Increased pro-inflammatory cytokine

- production after lipopolysaccharide stimulation in patients with X-linked agammaglobulinemia. *J Clin Immunol* 2012;32:967-74.
18. Marron TU, Martinez-Gallo M, Yu JE, Cunningham-Rundles C. Toll-like receptor 4-, 7-, and 8-activated myeloid cells from patients with X-linked agammaglobulinemia produce enhanced inflammatory cytokines. *J Allergy Clin Immunol* 2012; 129:184-90.
 19. Dalpke AH, Lehner MD, Hartung T, Heeg K. Differential effects of CpG-DNA in Toll-like receptor-2/-4/-9 tolerance and cross-tolerance. *Immunology* 2005;116: 203-12.
 20. Honigberg LA, Smith AM, Sirisawad M, Verner E, Loury D, Chang B, et al. 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* 2010;107:13075-80.
 21. De Rooij MF, Kuil A, Geest CR, Eldering E, Chang BY, Buggy JJ, et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood* 2012;119:2590-4.
 22. Winer ES, Ingham RR, Castillo JJ. PCI-32765: a novel Bruton's tyrosine kinase inhibitor for the treatment of lymphoid malignancies. *Expert Opin Investig Drugs* 2012;21:355-61.
 23. Advani RH, Buggy JJ, Sharman JP, Smith SM, Boyd TE, Grant B, et al. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies. *J Clin Oncol* 2013; 31:88-94.
 24. Brown JR. Ibrutinib in CLL and B cell malignancies. *Leuk Lymphoma* 2014;55: 263-9.

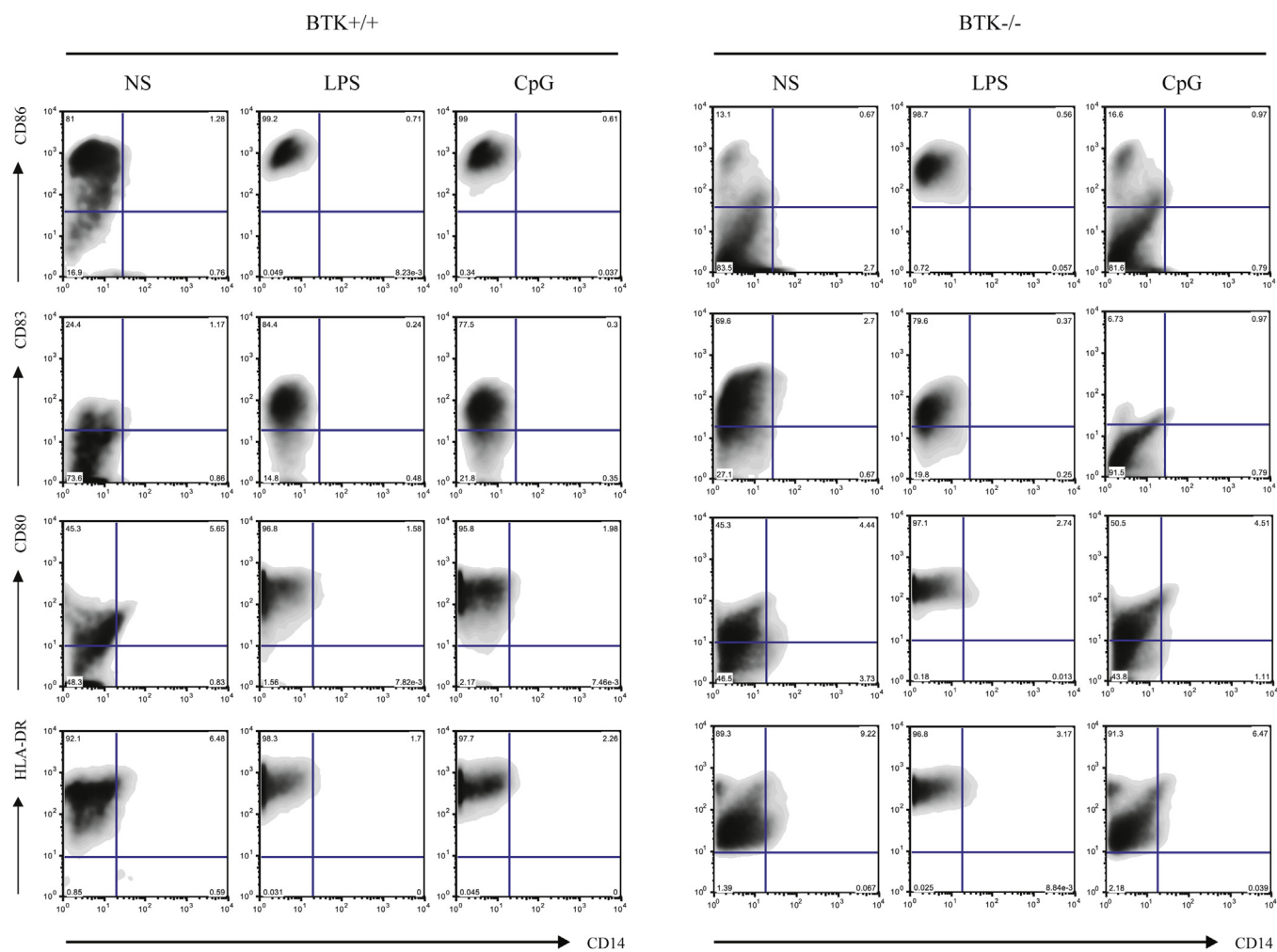


FIG E1. Human DCs activation from healthy control (BTK^{+/+}) (left panel) and XLA patient (BTK^{-/-}) (right panel) upon LPS or CpG stimulation in terms of CD86, CD83, CD80, and HLA-DR upregulation (representative experiment) (density plot).

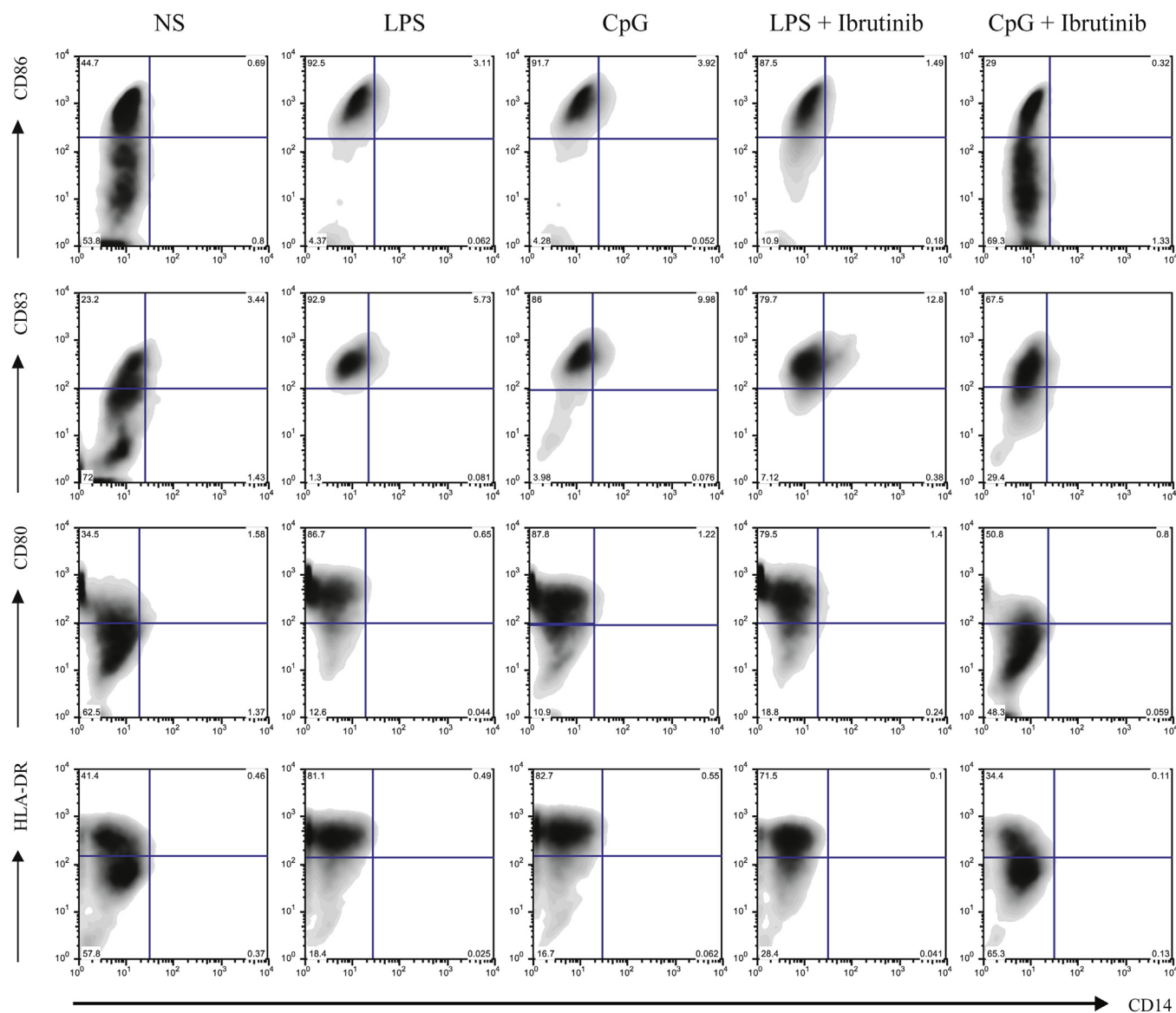


FIG E2. Human DCs activation from healthy donors without and with ibrutinib upon LPS or CpG stimulation in terms of CD86, CD83, CD80, and HLA-DR upregulation (representative experiment) (density plot).

TABLE E1. Data of patients with XLA: *BTK* mutations, residual B cells, and relevant clinical findings (arthritis, inflammatory bowel disease, autoinflammation)

Patient no.	<i>BTK</i> mutations	Residual B cells (%)	Clinical findings
XLA 1	R255X 858 C>T	0.5	NO
XLA 2	Q328X 1114 C>T	0.4	NO
XLA 3	G594E 1913 G>A	2	NO
XLA 4	Y461X 1516-1517delTG	0.5	NO
XLA 5	R228W 994 C>T	2	NO
XLA 6	G594E 1913 G>A	1	NO
XLA 7	L512P 1667 T>C	0.4	NO
XLA 8	K19X 188 A>T	1	Sarcoidosis
XLA 9	R562W 1816 C>T	1	NO
XLA 10	L175 fs X193 656/657 insT	2	NO
XLA 11	IVS8+2T>G splice donor def	0.4	NO
XLA 12	IVS15(-12-9)del ttg splice acc.def	0.5	NO
XLA 13	W147X 573 G>A	1	NO

NO, No clinical/autoinflammatory complications.

TABLE E2. Sequences of primers designed for quantitative real-time PCR analysis

Gene	Primers sequence	
	Forward	Reverse
<i>IL-6</i>	5'-gat gag tac aaa agt cct gat cca-3'	5'-ctg cag cca ctg gtt ctg t-3'
<i>IL-12</i>	5'-gaa tgc aaa gct tct gat gga-3'	5'-gtg gca cag tct cac tgt tga-3'
<i>TNF-α</i>	5'-cgc tcc cca aga aga cag-3'	5'-aga ggc tga gga aca agc ac-3'
<i>IL-10</i>	5'gat gcc ttc agc aga gtg aa-3'	5'-gca acc cag gta acc ctt aaa-3'
<i>STAT-1</i>	5'-tga gtt gat ttc tgt gtc tga agt t-3'	5'-aca cct cgt caa act cct cag-3'
<i>STAT-3</i>	5'-ctc tgc cgg aga aac agg-3'	5'-ctg tca ctg tag agc tga tgg ag-3'
<i>GAPDH</i>	5'-ccc ttc att gac ctg aac tac atg-3'	5'-tgg gat ttc cat tga tga caa gc-3'