in resting neutrophils from patients with XLA, we first used PTK inhibitors to investigate whether PTKs were involved in the PI(3)K activation. Inhibition of the activity of Src-family kinases (SFKs) by dasatinib (at a concentration of 10 nM)[36] led to normalized phosphorylation of PI(3)K-p85 in neutrophils derived from patients with XLA. Similarly, a Syk inhibitor (at a concentration of 15 nM)[37] but not a FAK inhibitor (at a concentration of 10 nM)[38] abrogated the hyperphosphorylation of PI(3)K (**Fig. 8a** and data not shown). The lower PI(3)K phosphorylation produced by dasatinib or the Syk inhibitor was accompanied by normalized production of ROS (**Fig. 8b**), which indicated that SFKs and Syk were involved in the augmented production of ROS in neutrophils from patients with XLA.

The findings noted above prompted us to determine whether the activated PTKs associated with Mal. SFKs are recruited to lipid rafts when activated for the assembly of signal components[39,40]. Coprecipitation assays showed that Lyn, c-Src and Syk interacted with Mal at the rafts of Btk-deficient neutrophils before stimulation (**Fig. 8c**). We also observed the colocalization of Mal and c-Src at the membrane by confocal fluorescence microscopy (**Fig. 8d**). We observed the interaction at the rafts of control neutrophils only after stimulation with PMA (**Fig. 8c** and **Supplementary Fig. 6**).

SFKs are cytoplasmic kinases and are anchored to the plasma membrane through myristoylation and palmitoylation[39,40]. Coprecipitation assays showed that Lyn, c-Src and Syk were associated with Mal in the cytosol of neutrophils from healthy controls but not in Btk-deficient neutrophils (**Fig. 8c**). We also confirmed by immunofluorescence staining the presence of c-Src associated with Mal in the cytoplasm but not in the membrane of normal resting neutrophils (**Fig. 8d**).

We next studied whether the membrane localization of Mal was regulated by SFKs or by Syk. The localization of Mal to the membrane in Btk-deficient neutrophils was diminished to normal amounts in cells treated with dasatinib but not those treated with the Syk inhibitor (**Fig. 8e**), which suggested that kinase activity of SFKs was required for membrane recruitment or maintenance of membrane-anchoring of Mal. Treatment of neutrophils from patients with XLA with dasatinib resulted in less baseline Syk phosphorylation, whereas incubation with the Syk inhibitor did not abrogate the hyperphosphorylation of c-Src (**Fig. 8f**), which indicated that Syk was downstream of SFKs in the steady-state signaling cascade of Btk-deficient neutrophils.

Collectively, the data reported above indicated that at least some PTKs associated with Mal together with Btk in the cytoplasm; in the absence of Btk, SFKs and Mal translocated to the membrane. The membrane-recruited PTKs formed a complex with and phosphorylated PI(3)K-p85 (**Supplementary Fig. 7**). It is still unclear which neutrophil SFK contributes to PI(3)K activation. Our findings may indicate that c-Src (or other SFKs) but not Lyn is (are) directly involved in the PI(3)K activation in Btk-deficient neutrophils; however, the possibility of an indirect contribution of Lyn to the phosphorylation of PI(3)K-p85 cannot be excluded solely by the inhibitor assay.

DISCUSSION

So far, most data have posited Btk as an essential molecule in innate immune responses[12–15,23,25]. Here we have shown that Btk is a negative regulator of signal transduction that leads to activation of NADPH oxidase and a molecule that prevents excessive neutrophil responses. Neutropenia in patients with XLA is usually induced by infection and is observed less often after immunoglobulin supplementation. This phenomenon can most probably be explained by ROS-mediated apoptosis of neutrophils triggered by the engagement of innate receptors and not by abnormal myeloid differentiation.

Our study suggested that Btk serves as a cytosolic component that interacts with Mal to prevent its translocation to the membrane and its interactions with PI(3)K until the appropriate stimulation is received. Both the PH and kinase domains of Btk were necessary for association with cytoplasmic Mal and were important for proper and coordinated initiation of the TLR and TNF receptor responses in human neutrophils. A similar mode of interaction has been demonstrated for the association of Btk with the cell-surface death receptor Fas (CD95) in B cells. Btk associates with Fas via its PH and kinase domains and prevents the interaction of Fas with the Fas-associated death domain and thus serves as a negative regulator of the Fas death-inducing signaling complex[41]. Notably, Btk serves as a negative regulator of apoptosis in both signaling systems.

SFKs were also involved in the baseline activation of PI(3)K in Btk-deficient neutrophils. We detected the association of c-Src, Lyn and Syk with Mal in the membrane raft in the absence of Btk. In addition, localization of Mal to the membrane in Btk-defective neutrophils was dependent on SFKs. These findings may indicate that SFKs serve as a substitute for the function of Btk in guiding the localization of Mal, albeit in an unregulated way. In neutrophils from control subjects, SFKs and Mal were associated in the cytoplasm and localized to the raft after stimulation. The mode of the SFK-Mal interaction remains unclear; however, we speculate that the kinase domain is involved, as SFKs lack a PH domain and the kinase domains of SFKs and Btk share 40–45% homology. Precise mapping of the Mal-binding site in the Btk kinase domain would help to clarify the SFK-Mal association site. Notably, neutrophils had more abundant expression of Mal than did monocytes (data not shown). Our data suggest that Mal is a critical coordinator of the priming signal and that its localization is tightly controlled by Btk.

Limited data indicate a role for PTKs in the production of ROS in neutrophils, particularly in humans. Lyn is reported to be a signaling component of the immunoglobulin receptors FcγRI and FcγRII or the receptor for the hematopoietic cytokine G-CSF, as well as an activator of PI(3)K[30,42], but is also noted for its ability to negatively regulate myeloid-cell signaling through phosphorylation of inhibitory receptors and recruitment of phosphatases[29]. Lyn-deficient neutrophils produce less ROS than Lyn-sufficient neutrophils do after stimulation with G-CSF[30] but show an enhanced respiratory burst after integrin-mediated signaling[29,31]. ROS responses triggered by *Aspergillus* species are totally dependent on Syk in mouse neutrophils[43]. The phosphorylation at different regulatory sites in Lyn versus c-Src in Btk-deficient neutrophils is notable. However, overall, PTKs in unstimulated neutrophils from patients with XLA seem to function as positive signal regulators. These data, along with our observations, suggest a potential contribution of SFKs and Syk to the early phase of NADPH oxidase activation in human neutrophils.

Activation of TFKs occurs downstream of SFKs in signaling pathways[40]. However, in neutrophils, Btk regulates baseline SFK activation. There are several possible mechanisms to explain how defective Btk is connected to SFK activation. We first speculated that Btk controls SFKs through the activation of negative SFK regulators. We investigated the Src kinase Csk and its regulatory molecule Cbp[44], but found no difference in the expression, localization or phosphorylation of Csk or Cbp (data not shown). As a second possible mechanism, SFKs but not TFKs may have been activated to compensate for Btk function in neutrophils. It is noteworthy that Btk regulates PtdIns(4,5)P_2 synthesis, acting as a shuttle to bring type I phosphatidylinositol-4-phosphate 5-kinases to the plasma membrane in B cells[45]. Although the role of Btk in PtdIns(4,5)P_2 production in human neutrophils has not been addressed, the generation of PtdIns(4,5)P_2 is a critical

step in the activation of NADPH oxidase. SFKs may have directly or indirectly served as a substitute for the function of Btk in neutrophils from patients with XLA. Finally, the cytoplasmic association of SFKs with Mal but without Btk may have resulted in SFK activation and Lyn inhibition. The phosphorylation of SFKs and subsequent modification of Mal by SFKs may have led to the translocation of Mal in the absence of Btk.

Neutrophils from patients with XLA show excessive production of ROS, but neutrophils from mice with X-linked immunodeficiency show poor ROS induction[15]. One possibility that could explain this discrepancy is the difference between mice and humans in the involvement of Btk in the NADPH oxidase pathway. Another possibility is the difference in the contributions of various members of the PI(3)K family to neutrophil activation. The primed production of ROS requires sequential activation of PI(3)Kγ and PI(3)Kδ in humans, whereas the production of ROS is largely dependent on PI(3)Kγ alone in mice[46]. A third possibility is differences in the methods of neutrophil collection from mice and in our study. Neutrophils collected from the peritoneum after treatment with thioglycolate broth may have been stimulated by that treatment[15]. The production of ROS was not augmented or compromised in neutrophils from patients with XLA in one study[26]. That may also have resulted from a relatively harsh isolation technique of hypotonic shock or from non–endotoxin-free conditions (for example, lipopolysaccharide in FBS) at any point of the experiment.

In this study, we have reported that Btk serves as a critical gatekeeper of neutrophil response. Our study suggests that the regulation of neutrophil activation and apoptosis in various human diseases could be achieved by manipulation of Btk. Future studies should explore the role of Btk in controlling the production of ROS and apoptosis of basophils, mast cells and eosinophils. Finally, ROS-mediated induction of apoptosis after suboptimal or optimal stimuli may be worth investigating in immature and precursor cells of the immune reponse to determine the role of Btk in their survival, proliferation and differentiation.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

F.H. did experiments; E.-S.K. and S.-K.L. contributed to protein-delivery experiments and provided some technical support; H. Kano and H. Kane made suggestions on data analysis and interpretation; S.N. and S.M. provided advice on project planning and data interpretation; M.T. provided advice on project plan and edited the manuscript; T.M. directed the project, designed research and wrote the manuscript; and all authors reviewed and approved the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Flannagan, R.S., Cosio, G. & Grinstein, S. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat. Rev. Microbiol.* **7**, 355–366 (2009).
2. Nauseef, W.M. How human neutrophils kill and degrade microbes: an integrated view. *Immunol. Rev.* **219**, 88–102 (2007).
3. Lambeth, J.D. NOX enzymes and the biology of reactive oxygen. *Nat. Rev. Immunol.* **4**, 181–189 (2004).
4. Babior, B.M. NADPH oxidase. *Curr. Opin. Immunol.* **16**, 42–47 (2004).
5. Sumimoto, H. Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species. *FEBS J.* **275**, 3249–3277 (2008).
6. Fang, F.C. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat. Rev. Microbiol.* **2**, 820–832 (2004).
7. Singh, A., Zarember, K.A., Kuhns, D.B. & Gallin, J.I. Impaired priming and activation of the neutrophil NADPH oxidase in patients with IRAK4 or NEMO deficiency. *J. Immunol.* **182**, 6410–6417 (2009).
8. Woollard, K.J. & Geissmann, F. Monocytes in atherosclerosis: subsets and functions. *Nat. Rev. Cardiol.* **7**, 77–86 (2009).
9. Finkel, T. Radical medicine: treating ageing to cure disease. *Nat. Rev. Mol. Cell Biol.* **6**, 971–976 (2005).
10. Conley, M.E. *et al.* Genetic analysis of patients with defects in early B-cell development. *Immunol. Rev.* **203**, 216–234 (2005).
11. Winkelstein, J.A. *et al.* X-linked agammaglobulinemia: report on a United States registry of 201 patients. *Medicine (Baltimore)* **85**, 193–202 (2006).
12. Mohamed, A.J. *et al.* Bruton's tyrosine kinase (Btk): function, regulation, and transformation with special emphasis on the PH domain. *Immunol. Rev.* **228**, 58–73 (2009).
13. Gray, P. *et al.* MyD88 adapter-like (Mal) is phosphorylated by Bruton's tyrosine kinase during TLR2 and TLR4 signal transduction. *J. Biol. Chem.* **281**, 10489–10495 (2006).
14. Doyle, S.L., Jefferies, C.A., Feighery, C. & O'Neill, L.A. Signaling by Toll-like receptors 8 and 9 requires Bruton's tyrosine kinase. *J. Biol. Chem.* **282**, 36953–36960 (2007).
15. Mangla, A. *et al.* Pleiotropic consequences of Bruton tyrosine kinase deficiency in myeloid lineages lead to poor inflammatory responses. *Blood* **104**, 1191–1197 (2004).
16. Fiedler, K. *et al.* Neutrophil development and function critically depend on Bruton tyrosine kinase in a mouse model of X-linked agammaglobulinemia. *Blood* **117**, 1329–1339 (2011).
17. Conley, M.E. *et al.* Primary B cell immunodeficiencies: comparisons and contrasts. *Annu. Rev. Immunol.* **27**, 199–227 (2009).
18. Kerner, J.D. *et al.* Impaired expansion of mouse B cell progenitors lacking Btk. *Immunity* **3**, 301–312 (1995).
19. Khan, W.N. *et al.* Defective B cell development and function in Btk-deficient mice. *Immunity* **3**, 283–299 (1995).
20. O'Neill, L.A.J. & Bowie, A.G. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat. Rev. Immunol.* **7**, 353–364 (2007).
21. Piao, W. *et al.* Tyrosine phosphorylation of MyD88 adapter-like (Mal) is critical for signal transduction and blocked in endotoxin tolerance. *J. Biol. Chem.* **283**, 3109–3119 (2008).
22. Jenkins, K.A. & Mansell, A. TIR-containing adaptors in Toll-like receptor signalling. *Cytokine* **49**, 237–244 (2010).
23. Taneichi, H. *et al.* Toll-like receptor signaling is impaired in dendritic cells from patients with X-linked agammaglobulinemia. *Clin. Immunol.* **126**, 148–154 (2008).
24. Pérez de Diego, R. *et al.* Bruton's tyrosine kinase is not essential for LPS-induced activation of human monocytes. *J. Allergy Clin. Immunol.* **117**, 1462–1469 (2006).
25. Horwood, N.J. *et al.* Bruton's tyrosine kinase is required for TLR2 and TLR4-induced TNF, but not IL-6, production. *J. Immunol.* **176**, 3635–3641 (2006).
26. Marron, T.U., Rohr, K., Martinez-Gallo, M., Yu, J. & Cunningham-Rundles, C. TLR signaling and effector functions are intact in XLA neutrophils. *Clin. Immunol.* **137**, 74–80 (2010).
27. Honda, F. *et al.* Transducible form of p47phox and p67phox compensate for defective NADPH oxidase activity in neutrophils of patients with chronic granulomatous disease. *Biochem. Biophys. Res. Commun.* **417**, 162–168 (2012).
28. Dang, P.M. *et al.* A specific p47phox -serine phosphorylated by convergent MAPKs mediates neutrophil NADPH oxidase priming at inflammatory sites. *J. Clin. Invest.* **116**, 2033–2043 (2006).
29. Scapini, P., Pereira, S., Zhang, H. & Lowell, C.A. Multiple roles of Lyn kinase in myeloid cell signaling and function. *Immunol. Rev.* **228**, 23–40 (2009).
30. Zhu, Q.S. *et al.* G-CSF induced reactive oxygen species involves Lyn-PI3-kinase-Akt and contributes to myeloid cell growth. *Blood* **107**, 1847–1856 (2006).
31. Pereira, S. & Lowell, C. The Lyn tyrosine kinase negatively regulates neutrophil integrin signaling. *J. Immunol.* **171**, 1319–1327 (2003).
32. Vlahos, C.J., Matter, W.F., Hui, K.Y. & Brown, R.F. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* **269**, 5241–5248 (1994).
33. Sadhu, C., Masinovsky, B., Dick, K., Sowell, C.G. & Staunton, D.E. Essential role of phosphoinositide 3-kinase δ in neutrophil directional movement. *J. Immunol.* **170**, 2647–2654 (2003).
34. Morris, A.C. *et al.* C5a-mediated neutrophil dysfunction is RhoA-dependent and predicts infection in critically ill patients. *Blood* **117**, 5178–5188 (2011).
35. Santos-Sierra, S. *et al.* Mal connects TLR2 to PI3Kinase activation and phagocyte polarization. *EMBO J.* **28**, 2018–2027 (2009).

36. Nam, S. *et al.* Action of the Src family kinase inhibitor, dasatinib (BMS-354825), on human prostate cancer cells. *Cancer Res.* **65**, 9185–9189 (2005).
37. Lai, J.Y. *et al.* Potent small molecule inhibitors of spleen tyrosine kinase (Syk). *Bioorg. Med. Chem. Lett.* **13**, 3111–3114 (2003).
38. Slack-Davis, J.K. *et al.* Cellular characterization of a novel focal adhesion kinase inhibitor. *J. Biol. Chem.* **282**, 14845–14852 (2007).
39. Korade-Mirnics, Z. & Corey, S.J. Src kinase-mediated signaling in leukocytes. *J. Leukoc. Biol.* **68**, 603–613 (2000).
40. Bradshaw, J.M. The Src, Syk, and Tec family kinases: distinct types of molecular switches. *Cell. Signal.* **22**, 1175–1184 (2010).
41. Vassilev, A., Ozer, Z., Navara, C., Mahajan, S. & Uckun, F.M. Bruton's tyrosine kinase as an inhibitor of the Fas/CD95 death-inducing signaling complex. *J. Biol. Chem.* **274**, 1646–1656 (1999).
42. Wang, A.V., Scholl, P.R. & Geha, R.S. Physical and functional association of the high affinity immunoglobulin G receptor (FcγRI) with the kinases Hck and Lyn. *J. Exp. Med.* **180**, 1165–1170 (1994).
43. Boyle, K.B. *et al.* Class IA phosphoinositide 3-kinase β and δ regulate neutrophil oxidase activation in response to *Aspergillus fumigatus* hyphae. *J. Immunol.* **186**, 2978–2989 (2011).
44. Kawabuchi, M. *et al.* Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. *Nature* **404**, 999–1003 (2000).
45. Saito, K. *et al.* BTK regulates PtdIns-4,5-P2 synthesis: importance for calcium signaling and PI3K activity. *Immunity* **19**, 669–678 (2003).
46. Condliffe, A.M. *et al.* Sequential activation of class IB and class IA PI3K is important for the primed respiratory burst of human but not murine neutrophils. *Blood* **106**, 1432–1440 (2005).
47. Uckun, F.M. *et al.* Anti-breast cancer activity of LFM-A13, a potent inhibitor of Polo-like kinase (PLK). *Bioorg. Med. Chem.* **15**, 800–814 (2007).
48. Mahajan, S. *et al.* Rational design and synthesis of a novel anti-leukemic agent targeting Bruton's tyrosine kinase (BTK), LFM-A13 [alpha-cyano-β-hydroxy-β-methyl-N-(2,5-dibromophenyl)propenamide]. *J. Biol. Chem.* **274**, 9587–9599 (1999).

ONLINE METHODS

Reagents and antibodies. The following reagents were used: lipopolysaccharide derived from *Escherichia coli* or *Pseudomonas aeruginosa*, fMLP, PMA, DHR123, luminol, N-acetyl cysteine, aprotinin, leupeptin, pepstatin and phenylmethyl sulfonyl fluoride (all from Sigma-Aldrich); recombinant human TNF (R&D Systems); Pam_3CSK_4, LFM-A13, LFM-A11, Syk inhibitor, FAK inhibitor and Ly294002 (all from Calbiochem); and dasatinib, IC87114 and AS-605240 (all from Biovision). Oligodeoxynucleotide CpG-A (5′-GGT GCATCGATGCAGGGGGG-3′) was from Operon Biotechnologies.

The antibodies used were as follows: goat polyclonal antibody to PI(3)K-p85α phosphorylated at Tyr508 (sc-12929), Hck phosphorylated at Tyr411 (sc-12928), rabbit polyclonal antibody to Hck (N-30), anti-PTEN (FL-403), anti-PTP-PEST (H130), anti-FAK (A-17), anti-Vav (C-14), anti-Syk (C-20), anti-SHP2 (C-18) and anti-SHP 1 (C-19), as well as mouse monoclonal antibody (mAb) to $p47^{phox}$ (D-10), $p40^{phox}$ (D-8) or $p22^{phox}$ (CS-9; all from Santa Cruz). Rabbit polyclonal antibody to p101-PI(3)K (07-281) and to $gp91^{phox}$ (07-024) and anti-Rac2 (07-604), biotin-labeled mouse mAb to phosphorylated tyrosine (4G10), as well as horseradish peroxidase–conjugated antibody to goat IgG (AP-180P) were from Upstate; fluorescein isothiocyanate–conjugated mouse mAb to gp91 (7D5) or goat antibody to mouse IgG (238) were from MBL; and mouse mAb to flotillin-1(18), $p67^{phox}$ (29) or PI(3)K-p85 (U15), and fluorescein isothiocyanate–conjugate mouse isotype-matched IgG antibody (MOPC-21) was from BD Pharmingen. Rabbit polyclonal antibody to PI(3)K-p85 (4292), to Lyn (2732), to Lyn phosphorylated at Tyr507 (2731), to Syk phosphorylated Tyr525-Tyr526 (2711), to Src phosphorylated Tyr416 (2101), to FAK phosphorylated Tyr576-Tyr577 (3281), to $p40^{phox}$ phosphorylated at Thr154 (4311) and to caspase-3 (9662), as well as mouse mAb to proliferating cell nuclear antigen (PC-19), were from Cell Signaling. Rabbit mAb to SOD1 (ep1727y), Mal (ep1231y) and catalase (ep1929), as well as rabbit polyclonal antibody to SOD2 (NB100-1992) and to Yes (NBP1-85369), were from Novus Biologicals. Rabbit polyclonal antibody to Bmx (ab73887), to Bmx phosphorylated at Tyr566 (ab59409), to Lyn phosphorylated at Tyr396 (EP503Y), to Vav phosphorylated at Tyr160 (ab4763) and to Prx1 (ab15571), and mouse mAb to Prx2 (12B1), as well as rabbit mAb to Btk (Y440), to CSK (CSK-04), to SHIP (EP378Y) and to Tec (Y398), were from Abcam. Rat mAb to Mal (TIRAP; sebi-1) was from ENZO Life Sciences. Goat polyclonal antibody to CBP (LS-C14699) was from LIFESPAN; anti-β-actin (Ab1) was from Calbiochem; and horseradish peroxidase–conjugated antibody to mouse IgG (NA931), to rabbit IgG (NA934) or to rat IgG (NA9350) was from GE Healthcare. Alexa Flour 546–anti–rabbit IgG (A11035), Alexa Flour 680–anti–rabbit IgG (A10043), Alexa Flour 594–anti–rat IgG (A21209) and Alexa Flour 488–anti–mouse IgG (A21202) were from Invitrogen. Mouse IgG (015-000-003) and rabbit IgG (011-00000-3) were from Jackson ImmunoResearch. Rat IgG2a (eBR2a) was from eBioscience. Horseradish peroxidase–conjugated streptavidin was from Cell Signaling.

The 482H mAb to Btk has been described[49]. Polyclonal antibody to human Btk was raised in rabbits with a Btk peptide of amino acids 169–187 (ENRNGSLKPGSSHRKTKKPC) conjugated to ovalubumin. The antibody collected was further affinity-purified with that same Btk peptide conjugated to thiol–Sepharose 4B (Pharmacia) and was used for immunoprecipitation in some experiments. The specificity of the antibody was confirmed by immunoblot analysis of lysates of Btk-deficient mononuclear cells. Antibody to phosphorylated Ser345 was generated in rabbits by injection of ovalbumin conjugated to a peptide of $p47^{phox}$ phosphorylated at Ser345 (QARPGPQSpPGSPLEEE, where 'Sp' indicates phosphorylated Ser345 (p-Ser345-pep)). The antibody raised was positively affinity-purified with activated thiol–Sepharose 4B adsorbed with p-Ser345-pep. The antibody was further purified by elimination of the fraction that bound to the same peptide of $p47^{phox}$ without phosphorylation at Ser345 (QARPGPQSPGSPLEEE (Ser345-pep)) by passage through thiol–Sepharose 4B conjugated to Ser345-pep; then, the antibody was used for immunoblot analysis. The specificity of the antibody was confirmed by direct enzyme-linked immunosorbent assay with plates coated with Ser345-pep or p-Ser345-pep and by immunoblot analysis experiments showing blockade of the p-$p47^{phox}$ signal by p-Ser345pep but not by Ser345-pep.

Subjects. Patients with XLA (n = 17) with stable health were studied (ages and Btk mutations, **Supplementary Fig. 3**). Healthy volunteers (n = 18) and patients with CVID (n = 5) were enrolled as healthy controls and disease control, respectively. Written informed consent was obtained from all subjects (or their parents). The study protocol was approved by the ethics committee of the Faculty of Medicine, Tokyo Medical and Dental University.

Isolation of neutrophils, monocytes and lymphocytes. Neutrophils were purified from heparinized peripheral blood by a standard technique. All samples were processed within 12 h of blood collection. Peripheral blood diluted in PBS was layered onto a MonoPoly mixture (Flow Laboratories) and centrifuged at 400*g* for 20 min. Layers with enrichment for neutrophils were collected and further purified to a purity of >97% by immunomagnetic negative selection (StemCell Technologies). Sterile and endotoxin-free conditions were used for all procedures. Monocytes were purified from the mononuclear cell–rich fraction with a human monocyte enrichment kit (StemCell Technologies), and lymphocytes were prepared as described[50].

Measurement of production of ROS. Purified neutrophils were loaded for 5 min at 37 °C with DHR123 (5 μg/ml). Cells were washed and then stimulated for 30 min at 37 °C with PMA (100 ng/ml), and the production of ROS was quantified via flow cytometry by measurement of intracellular rhodamine (FACSCalibur; Becton Dickinson). DHR123-loaded neutrophils were also stimulated for 60 min at 37 °C with a TLR ligand (lipopolysaccharide from *E. coli* or *P. aeruginosa*; 100 ng/ml), CpG-A (100 ng/ml) or TNF (1 μg/ml). After incubation, treated and untreated neutrophils were incubated for 5 min at 37 °C with or without fMLP (1 μM), followed by flow cytometry. Results are presented as MFI of treated cells – MFI of untreated cells.

Production of ROS was quantified by standard chemiluminescence. Neutrophils (1.0×10^6) were suspended in 0.5 ml PBS containing luminol (10 μM) preheated to 37 °C. After a baseline measurement was obtained, cells were stimulated with a TLR agonist and then with fMLP (1 μM) or with PMA (100 ng/ml); luminescence signals were monitored throughout the reaction.

Detection of apoptosis. Apoptotic cells were identified by staining with annexin V–fluorescein isothiocyanate and 7-AAD (7-amino-actinomycin D; BD Biosciences). Apoptosis was also identified by immunoblot analysis through the detection of cleaved caspase-3 or degraded proliferating cell nuclear antigen.

Flow cytometry. A FACSCalibur (Beckton Dickenson) was used for all flow cytometry analyzing surface expression of gp91, DHR123 staining, annexin V–7-AAD staining, and JC-1 mitochondrial membrane detection as described[50]. All analyses were undertaken after calibration of the fluorescence intensity with CaliBRITE Beads (BD Biosciences).

Subcellular fractionation of neutrophils. Isolated neutrophils were resuspended at a density of 5×10^7 cells per ml in ice-cold sonication buffer (HEPES (10 mM), pH 7.2, sucrose (0.15 M), EGTA (1 mM), EDTA (1 mM), NaF (25 mM), leupeptin (10 μg/ml), pepstatin (10 μg/ml), aprotinin (1 μg/ml) and PMSF (1 mM)). After sonication and pelleting on ice, 200 μl supernatant was layered on a discontinuous sucrose gradient consisting of 200 μl of 52% (wt/vol) sucrose, 200 μl of 40% (wt/vol) sucrose and 200 μl of 15% (wt/vol) sucrose. After centrifugation (100,000*g* for 60 min), 160 μl supernatant (cytosol source) and 120 μl interface of the 15%–40% sucrose layers (plasma-membrane source) were collected.

Immunoprecipitation and immunoblot analysis. Lysates were prepared from monocytes and lymphocytes as described[51]. For the preparation of lysates from neutrophils, cells were resuspended in lysis buffer (Tris-HCl (50 mM), pH 7.5, NaCl (150 mM), sucrose (0.25 M), EGTA (5 mM), EDTA (5 mM), leupeptin (15 μg/ml), pepstatin (10 μg/ml), aprotinin (10 μg/ml), PMSF (2.5 mM), 1.0% Nonidet-P40, 0.25% sodium deoxycholate, sodium pyrophosphate (10 mM), NaF (25 mM), Na_3VO_4 (5 mM), β-glycerophosphate (25 mM) and DNase I (1 μg/ml)), incubated for 30 min on ice and centrifuged at 15,000*g* for 30 min at 4 °C, then supernatants were collected. For extraction of the membrane-raft fraction, 1% n-dodecyl-β-D-maltoside was added to the lysis buffer. Immunoprecipitation and immunoblot analysis were done as described[52]. For immunoprecipitation of cytosolic proteins from neutrophils, cytosolic proteins

obtained as described above were diluted in four volumes of immunoprecipitation buffer (Tris-HCl (20 mM), pH 7.5, NaCl (150 mM), sucrose (0.25 M), EGTA (5 mM), EDTA (5 mM), leupeptin (15 μg/ml), pepstatin (10 μg/ml), aprotinin (10 μg/ml), PMSF (2.5 mM), 0.5% Triton-X, sodium pyrophosphate (10 mM), NaF (25 mM), Na_3VO_4 (5 mM), β-glycerophosphate (50 mM) and levamisole (1 mM)); supernatants were used for immunoprecipitation.

Measurement of phosphatidylinositol-(3,4,5)-trisphosphate. Phosphatidylinositol-(3,4,5)-trisphosphate in unstimulated neutrophils prepared from healthy controls and patients with XLA was measured with an enzyme-linked immunosorbent assay kit in accordance with the manufacturer's instructions (K-2500; Echelon).

Immunofluorescence staining. Cytospin preparations of neutrophils were air-dried and fixed for 10 min with paraformaldehyde in PBS, pH 7.4, then were made permeable for 20 min at −20 °C with acetone, washed, and incubated with the appropriate antibodies. After labeling and washing with 0.2% BSA in PBS, coverslips were mounted with Fluoromount G and the prepared specimens. Nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole). Slides were analyzed with a fluorescence microscope (FV10i; Olympus) equipped with Fluoview viewer and review station (Olympus). At least 100 cells were inspected for each slide.

Generation of Hph-1-Btk, Hph-1-Btk mutants, and transduction of recombinant protein into cells. Hph-1–tagged Btk constructs were generated by amplification of a full-length Btk cDNA fragment with the appropriate primers (**Supplementary Table 1a**). After the sequence of each PCR product was verified by DNA sequencing, the fragment was ligated into sites of a pET28b vector (Merck) cleaved by *Xma1* and *Sal1*; the vector has a six-histidine site for protein purification and two tandem Hph-1 sequences for protein transduction. Constructs with deletion of the Tec homology domain, SH3 domain or SH2 domain were generated by mutagenesis with the QuikChange SiteDirected Mutagenesis Kit (Stratagene) and the appropriate primers (**Supplementary Table 1b**). The Hph-1-Gal4 construct has been described[52]. Proteins were induced in BL21 Star competent cells (Novagen) as described[52]. Proteins were treated with Detoxi-Gel Endotoxin Removing Gel (Takara Bio) for elimination of endotoxins and were frozen at −80 °C until further use. Neutrophils (1×10^6 per ml) were incubated for 1 h with 1 μM Hph-1–tagged proteins (80 μg recombinant Hph-1–tagged full-length-Btk was used for 1×10^6 neutrophils for transduction at a concentration of 1 μM) and washed, then ROS production was assayed.

Btk-precipitation assay. Lysates of neutrophils from healthy controls were prepared on ice for 30 min with immunoprecipitation lysis buffer. Supernatants were then treated with protein G beads (GE Health Care) for removal of immunoglobulin G from the neutrophil lysate. For the Btk-precipitation assay, purified Btk recombinant proteins or control recombinant protein were eluted and proteins were measured by BCA protein assay (Pierce). Bacterial supernatants were bound to nickel–nitrilotriacetic acid Sepharose beads (Qiagen) and bound recombinant proteins were eluted, then equimolar amounts of recombinant proteins were rebound to the nickel beads; afterward, samples were washed and then incubated overnight at 4 °C with the cell lysates. Beads were washed four times with lysis buffer and assessed by immunoblot analysis with anti-Mal. Before incubation with cell lysates, the amount of the recombinant protein rebound to nickel beads was assessed by immunoblot analysis with anti-histidine, and the 'dose' was readjusted for further precipitation assays.

Statistical analysis. Student's *t*-test was used for statistical analysis. The software GraphPad Prism 4 was used for these analyses.

49. Futatani, T. *et al.* Deficient expression of Bruton's tyrosine kinase in monocytes from X-linked agammaglobulinemia as evaluated by a flow cytometric analysis and its clinical application to carrier detection. *Blood* **91**, 595–602 (1998).
50. Takahashi, N. *et al.* Impaired CD4 and CD8 effector function and decreased memory T cell populations in ICOS-deficient patients. *J. Immunol.* **182**, 5515–5527 (2009).
51. Morio, T. *et al.* Ku in the cytoplasm associates with CD40 in human B cells and translocates into the nucleus following incubation with IL-4 and anti-CD40 mAb. *Immunity* **11**, 339–348 (1999).
52. Choi, J.M. *et al.* Intranasal delivery of the cytoplasmic domain of CTLA-4 using a novel protein transduction domain prevents allergic inflammation. *Nat. Med.* **12**, 574–579 (2006).

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GATA-2 *anomaly and clinical phenotype of a sporadic case of lymphedema, dendritic cell, monocyte, B- and NK-cell (DCML) deficiency, and myelodysplasia*

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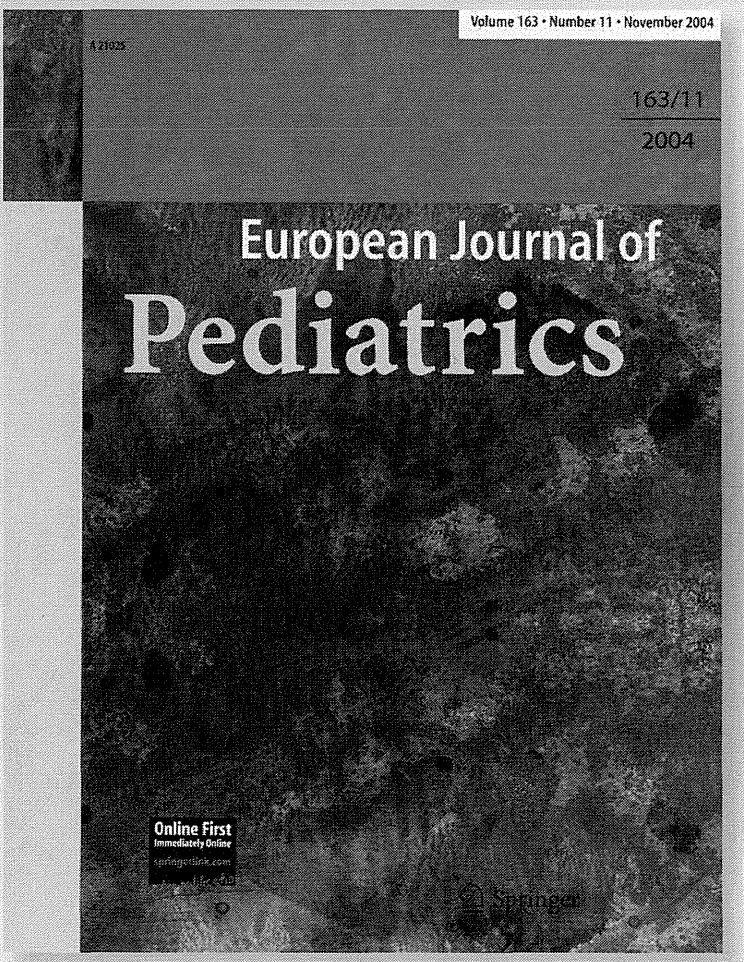

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CASE REPORT

GATA-2 anomaly and clinical phenotype of a sporadic case of lymphedema, dendritic cell, monocyte, B- and NK-cell (DCML) deficiency, and myelodysplasia

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Abstract A Japanese patient presented with lymphedema, severe *Varicella zoster*, and *Salmonella* infection, recurrent respiratory infections, panniculitis, monocytopenia, B- and NK-cell lymphopenia, and myelodysplasia. The phenotype was a mixture of the monocytopenia and mycobacterial infection (MonoMAC) and Emberger syndromes. Sequencing of the *GATA-2* cDNA revealed the heterozygous missense mutation 1187 G>A. This mutation resulted in the amino acid mutation Arg396Gln in the zinc fingers-2 domain, which is predicted to cause significant structural change and prevent a critical interaction with DNA. Functional analysis of the patient's *GATA-2* mutation is required to understand the relationship between these distinctive syndromes.

Keywords Emberger syndrome · MonoMAC · Monocytopenia · B- and NK-cell lymphopenia · Immunodeficiency · Myelodysplasia

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Recent studies have characterized a novel primary immunodeficiency known as monocytopenia and mycobacterial infection (MonoMAC), also known as dendritic cell, monocyte, B and NK lymphoid (DCML) deficiency. This form of immunodeficiency occurs either as an autosomal dominant form or sporadically. It is primarily characterized by persistent and profound peripheral monocytopenia, diagnostic B- and NK-cell lymphocytopenia, and variable T cell lymphocytopenia, along with increased susceptibility to mycobacterium or papilloma virus infections [1, 2, 13]. Moreover, most patients with MonoMAC eventually develop acute myelogenous leukemia (AML) following myelodysplastic syndrome (MDS). Another rare disorder called Emberger syndrome (MIM614038) is characterized by congenital deafness and primary lymphedema of the lateral lower limb; typically, onset occurs in childhood and is associated with a predisposition to MDS or AML in addition to other minor anomalies such as hypotelorism and long tapering fingers. It is also a sporadic or familial disorder [8]. Familial MDS/AML without other hematopoietic defects has also been reported [6]. Surprisingly, it was reported recently that these three distinctive syndromes are all caused by *GATA-2*

mutations, which suggests that these syndromes are different phenotypes caused by the same genetic alteration [5–7, 9]. Here, we report the case of a patient with a *GATA-2* mutation bearing the characteristic features of MonoMAC/Emberger syndrome.

Case report

The patient was the second child of non-consanguineous parents. Neither the parents nor the elder brother had a history of increased susceptibility to infection. The medical history of the patient included BCG vaccination 3 months after birth without any side effects and a severe *Varicella zoster* infection at 2 years of age. After that, she suffered repeated upper and lower respiratory tract infections that required antibiotics. At 4 years of age, the patient's peripheral blood showed mild neutropenia and profound monocytopenia ($0–20\times10^6$/L), and mild hypocellularity but no dysplasia was observed in the bone marrow. At 8 years of age, she experienced a prolonged *Salmonella* enterocolitis infection. Lymphedema in the left leg first appeared at 13 years of age. She subsequently developed recurrent panniculitis. Recently, the patient (now 19 years old) was admitted to hospital with fever (with no apparent cause) and panniculitis (Fig. 1a). She had mild hypotelorism and lymphedema, with warts on her left leg (Fig. 1b). Her mental ability was appropriate for her age. An immunodeficiency was first suspected after the severe *Varicella zoster* and *Salmonella* infections during early childhood. The most recent recurrent episode of fever supported this suspicion.

Peripheral blood analysis revealed a white blood cell count of 1.5×10^9/L with 45% neutrophils, 54 % lymphocytes, and 1 % monocytes, a hemoglobin level of 11.0 g/dl, and a platelet count of 146×10^9/L. Flow cytometric analysis of the peripheral blood also revealed a deficiency in dendritic cells (lineage$^-$/DR$^+$/CD123$^+$ or CD11c$^+$ cells, 0%), B cells (CD19$^+$ cells, 0.7%), and NK cells (CD3$^-$/CD56$^+$ cells, 0.5%), and profound monocytopenia (CD14$^+$ cells, 0.2%). Lymphocytes comprised 97% T cells (CD4/8 ratio, 0.54), 33% of which were TCR $\gamma\delta^+$ T cells. Immunological analyses revealed IgG, IgA, IgM, and IgE levels of 711, 65, 131,

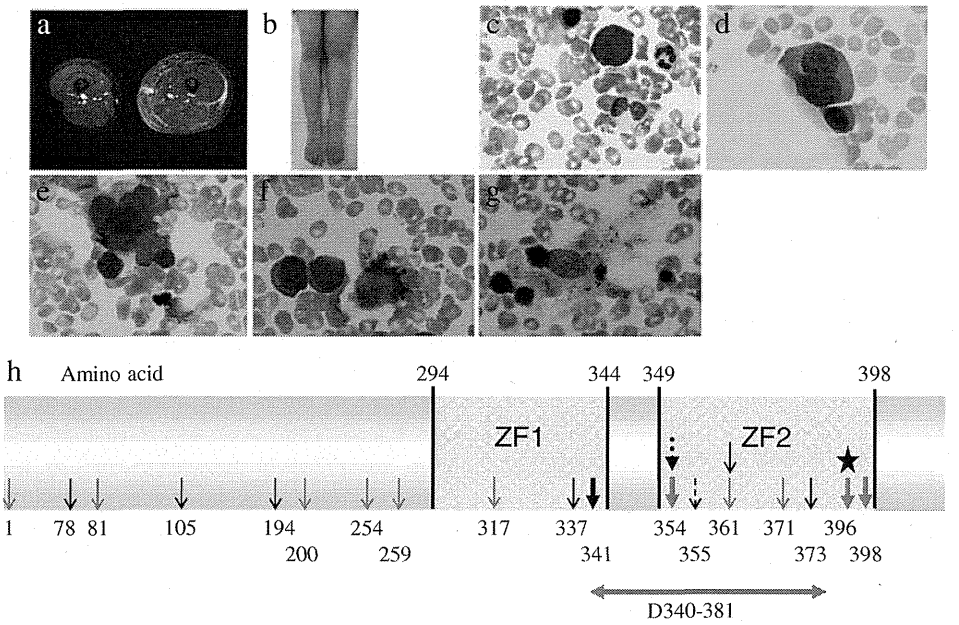

Fig. 1 Clinical and bone marrow features and *GATA-2* protein mutation sites. **a** A gadolinium-enhanced T2-weighted MRI image of the left thigh was performed when the patient developed panniculitis at 19 years of age. An increased signal was observed in the subcutaneous tissue and fascial layers. **b** After she was cured, the patient showed lymphedema in her left leg. **c–g** Bone marrow taken at the same time revealed decreased granule numbers within neutrophils and a pseudo-Pelger anomaly (**c**), binucleation (**d**), and megaloblastic changes in erythroblasts, dysplastic nuclei in megakaryocytes (**e**) and micromegakaryocytes (**f**), and hemophagocytosis (**g**). **h** Depiction of the *GATA-2* protein mutations previously identified in MonoMAC/DCML deficiency and Emberger syndrome. ZF1 and ZF2 are functional DNA-binding domains. The *star* indicates the Arg396Gln mutation identified in the present case. *Arrows* indicate previously reported mutations. These include missense, nonsense, and frameshift mutations (*short downward arrows*, respectively) and long deletions (*horizontal arrows*). *Black arrows* denote mutations associated with Emberger syndrome, *gray arrows* denote mutations associated with MonoMAC syndrome/DCML deficiency, *long horizontal arrows* indicate long deletions that have been observed in MonoMAC syndrome/DCML deficiency, *broken black arrows* denote mutations associated with familial MDS/AML, and *bold arrows* denote multiple pedigrees with the same mutation

and 5 mg/dl, respectively, and lymphocyte stimulation responses to phytohemagglutinin at the lower limits of the normal range. Antibody memory responses to infections contracted in early childhood (*Varicella* and *measles*) were maintained, and fibroblast sensitivity to radiation was normal. Flow FISH analysis of peripheral blood lymphocytes revealed normal telomere length; however, the peripheral blood contained 160 copies/μg WT1-mRNA (the upper limit of normal is 50 copies/μg RNA), and bone marrow aspirates showed hypocellularity, particularly of myeloid and lymphoid cells. Strikingly, despite monocytopenia in the peripheral blood, $CD64^+$ macrophages (accompanied by a few hemophagocytes) were observed in bone marrow specimens. Significant trilineage dysplasia was also present (Fig. 1c–g). Cytogenetic and chromosomal breakage analyses showed normal results. Meanwhile, profiles of familial peripheral blood showed a white blood cell count of 5.6×10^9/L with 50% neutrophils, 30% lymphocytes, and 8% monocytes, a hemoglobin level of 15.1 g/dl, and a platelet count of 199×10^9/L in the father; 5.1×10^9/L with 51 % neutrophils, 36% lymphocytes, and 9% monocytes, 10.7 g/dl, and 225×10^9/L in the mother; and 6.6×10^9/L with 41% neutrophils, 45% lymphocytes, and 10% monocytes, 15.4 g/dl, and 208×10^9/L in the brother. Flow cytometric analysis of peripheral blood samples taken from these family members showed a normal frequency of B cells ($CD19^+$ cells) and NK cells ($CD3^-/CD56^+$ cells) (the father 11 and 8%, the mother 10 and 12%, and the brother 9 and 15%, respectively). Taken together, these findings suggested that the patient might have sporadic MonoMAC/Emberger syndrome.

Sequencing of *GATA-2* cDNA revealed a 1187 G>A heterozygous missense mutation. This mutation resulted in an Arg396Gln substitution in the zinc finger-2 domain, which is predicted to cause significant structural changes that prevent critical interactions with DNA (Fig. 1h). Furthermore, sequencing of cDNA from her healthy familial members revealed no mutations, including 1187 G>A in *GATA-2* gene. Ultimately, the patient was diagnosed with MonoMAC/Emberger syndrome with a de novo *GATA-2* mutation.

Discussion

GATA-2 plays a critical role in both hematopoietic stem cell development and the maintenance of normal adult stem cell homeostasis [10]. It is likely that the significant protein structural alterations caused by mutations in *GATA-2* result in loss-of-function or have a dominant-negative effect on the DNA-binding ability of wild-type *GATA-2* [9] It seems reasonable to suggest that the loss of hematopoiesis-indispensable transcription factor activity results in impaired hematopoietic-cell differentiation and hematopoietic stem cell exhaustion; this in turn may promote the development of related diseases such as MDS and AML. Additional genetic alterations may also be required.

The patient's phenotype included hypotelorism, primary lymphedema (which had an onset during childhood before the recurrent episodes of panniculitis), peripheral monocytopenia, B- and NK-cell lymphocytopenia, neutropenia since early childhood, and myelodysplasia. The Arg396Glu mutation in *GATA-2* identified in this patient was not detected in 150 healthy individuals [7]. Taken together, these factors confirmed the diagnosis of MonoMAC/Emberger syndrome with a de novo *GATA-2* mutation; however, the *GATA-2* mutations alone cannot explain the phenotypic diversity between these three syndromes (MonoMAC, Emberger syndrome, and familial MDS/AML) and the presented patient. Interestingly, she developed neither BCG dissemination nor severe lymphadenitis after her BCG

Table 1 Summary of the clinical features of MonoMAC, Emberger syndrome, and the present case

	MonoMAC/DCML deficiency	Emberger syndrome	Present case
DCML[a] deficiency	+	+/–	+
MDS/AML	+	+	+
Lymphedema	ND	+	+
Deafness	ND	+/–	–
Hypotelorism	ND	+/–	+
Long slender fingers	ND	+/–	–
Mycobacterial infection	+	ND	–
Fungal infection	+/–	+/–	–
Papillomaviral infection/warts	+	+	+
Severe *varicella* and/or *Salmonella* infection	+/–	ND	+
Pulmonary alveolar proteinosis	+/–	ND	–
Panniculitis/erythema nodusa	+/–	+/–	+

ND not described, *MDS* myelodysplastic, *AML* acute myelogenous syndrome, + most cases, +/– some cases

[a]Dendritic cell, monocyte, B and NK lymphoid syndrome