雑誌(和文)

発表者名	論文タイトル名	発表雑誌	巻号	ページ	出版年
平田 修, 小林 正夫	【血液症候群(第2版)-その他の血液疾患を含めて-】リンパ球の異常 リンパ球機能異常と類縁疾患 原発性免疫不全症候群 単独IgGサブクラス欠損症	日本臨床別冊 血液症候群第2版II		250-253	2013
早川 誠一, 小林 正夫	【血液症候群(第2版)-その他の血液疾患を含めて-】リンパ球の異常 リンパ球機能異常と類縁疾患 原発性免疫不全症候群 IgM単独(選択的)欠損症	日本臨床別冊 血液症候群第2版II		246-249	2013
平田 修, 小林 正夫	【血液症候群(第2版)-その他の血液疾患を含めて-】リンパ球の異常 リンパ球機能異常と類縁疾患 原発性免疫不全症候群 選択的IgA欠損症	日本臨床別冊 血液症候群第2版II		242-245	2013
平田 修, 中村 和洋, 小林 正夫	【血液症候群(第2版)-その他の血液疾患を含めて-】白血球(顆粒球)の異常(悪性腫瘍を除く) 好中球の異常 好中球減少症 周期性好中球減少症	日本臨床別冊 血液症候群第2版II		57-60	2013
唐川 修平, 中村 和洋, 小林 正夫	【血液症候群(第2版)-その他の血液疾患を含めて-】白血球(顆粒球)の異常(悪性腫瘍を除く) 好中球の異常 好中球減少症 自己免疫性好中球減少症	日本臨床別冊 血液症候群第2版II		54-56	2013
溝口 洋子, 中村 和洋, 小林 正夫	【血液症候群(第2版)-その他の血液疾患を含めて-】白血球(顆粒球)の異常(悪性腫瘍を除く) 好中球の異常 好中球減少症 同種免疫性好中球減少症.	日本臨床別冊 血液症候群第2版II		50-53	2013
唐川 修平, 小林 正夫	【知っておきたい最新の免疫不全症分類-診断から治療まで】治療 原発性免疫不全症に対する造血幹細胞移植	小児科診療	76	476-480	2013
宮地 隆史, 丸山 博文, 小林 正夫, 松本 昌泰	【クローズアップ 呼吸管理】〈在宅呼吸ケア〉 在宅人工呼吸器装着者の災害時対策	小児内科	45	116-120	2013
原 寿郎	2.幹細胞異常と内科系疾患、現状と展望 1)造血幹細胞の異常:先天性免疫不全症	日本内科学会雑誌	102(9)	2255-61	2013
原 寿郎	小児感染・免疫疾患の発症におけるヒトー環境相互作用	小児感染免疫	25(1)	41-53	2013
原 寿郎	シリーズ小児医療第6回 原発性免疫不全症研究:最新の進歩	あいみっく	.34(3)	50-5	2013
原 寿郎	こどもの発熱の原因とその対処法	ふたば	77	18-24	2013
戸田 尚子、原 寿郎	2.疾患と栄養 先天性免疫不全症と低栄養	臨床栄養		印刷中	

書籍

著者氏名	論文タイトル	書籍全体の編集者名	書籍名	出版社名	出版地	ページ	出版年
原　寿郎	第1章:血液系疾患の医療ニーズ　第3節　原発性免疫不全症候群		希少疾患／難病の診断・治療と製品開発	(株)技術情報協会	東京	593-610	2013
原　寿郎	免疫疾患	原　寿郎, 高橋 孝雄, 細井 創	標準小児科学 第8版	医学書院	東京	258-279	2014
原　寿郎	原発性免疫不全症候群 Primary immunodeficiency syndrome	福井 次矢, 高木　誠, 小室 一成	今日の治療指針 2014年版－私はこう治療している	医学書院	東京	1270-1271	2014

Ⅲ　研究成果の刊行物・別刷

nature immunology

The kinase Btk negatively regulates the production of reactive oxygen species and stimulation-induced apoptosis in human neutrophils

Fumiko Honda[1], Hirotsugu Kano[2], Hirokazu Kanegane[3], Shigeaki Nonoyama[4], Eun-Sung Kim[5], Sang-Kyou Lee[5], Masatoshi Takagi[1], Shuki Mizutani[1] & Tomohiro Morio[1]

The function of the kinase Btk in neutrophil activation is largely unexplored. Here we found that Btk-deficient neutrophils had more production of reactive oxygen species (ROS) after engagement of Toll-like receptors (TLRs) or receptors for tumor-necrosis factor (TNF), which was associated with more apoptosis and was reversed by transduction of recombinant Btk. Btk-deficient neutrophils in the resting state showed hyperphosphorylation and activation of phosphatidylinositol-3-OH kinase (PI(3)K) and protein tyrosine kinases (PTKs) and were in a 'primed' state with plasma membrane–associated GTPase Rac2. In the absence of Btk, the adaptor Mal was associated with PI(3)K and PTKs at the plasma membrane, whereas in control resting neutrophils, Btk interacted with and confined Mal in the cytoplasm. Our data identify Btk as a critical gatekeeper of neutrophil responses.

Among 'professional' phagocytes with a sophisticated arsenal of microbicidal features, neutrophils are the dominant cells that mediate the earliest innate immune responses to microbes[1–3]. Neutrophils migrate to the site of infection, sense and engulf microorganisms, produce reactive oxygen species (ROS) and kill the invading microbes via ROS by acting together with antimicrobial proteins and peptides[1,2]. The enzyme responsible for the respiratory burst is NADPH oxidase, which catalyzes the production of superoxide from oxygen and NADPH. This enzyme is a multicomponent complex that consists of membrane-bound flavocytochrome b_{558} (gp91^{phox} and p22^{phox}), cytosolic components (p47^{phox}, p67^{phox} and p40^{phox}) and a GTPase (Rac1 or Rac2)[3–6]. Activation of NADPH oxidase is strictly regulated both temporally and spatially to ensure that the reaction takes place rapidly at the appropriate cellular localization. Activation of this system requires three signaling triggers, including protein kinases, lipid-metabolizing enzymes and nucleotide-exchange factors that activate the Rac GTPase[3–6].

Inadequate production of ROS is associated with various human pathological conditions. Deficiency of any component of the NADPH oxidase complex results in chronic granulomatous disease, in which bacterial and fungal infections are recurrent and life-threatening[4]. Abnormalities in the molecules involved in the signal-transduction pathway initiated by the recognition of pathogen-associated molecular patterns are accompanied by less production of ROS after exposure to specific stimuli and by susceptibility to bacterial infection. These abnormalities include deficiency in the kinase IRAK4, the adaptor MyD88 deficiency or the kinase NEMO (IKKγ)[7]. In contrast, many other human disorders are believed to be associated with or induced by excessive production of ROS that causes DNA damage, tissue damage, cellular apoptosis and neutropenia[8,9].

Here we focus on determining the role of the kinase Btk in production of ROS and cellular apoptosis in human neutrophils, as 11–30% of patients with X-linked agammaglobulinemia (XLA), a human disease of Btk deficiency, have neutropenia[10,11], and Btk is a critical signaling component of phagocytic cells[12–14]. The neutropenia of XLA is distinct from that of common variable immunodeficiency (CVID) in that the neutropenia is induced by infection, is usually ameliorated after supplementation with immunoglobulin and is not mediated by the autoimmune response[10,11,14]. Although a few reports have suggested that myeloid differentiation is impaired in mice with X-linked immunodeficiency[15,16], the reason for the infection-triggered neutropenia is unknown. The role of Btk in human neutrophils remains largely unexplored.

Btk is a member of the Tec-family kinases (TFKs) that are expressed in hematopoietic cells such as B cells, monocytes, macrophages and neutrophils[12]. It has a crucial role in cell survival, proliferation, differentiation and apoptosis, especially in cells of the B lineage. In humans with XLA, B cells fail to reach maturity and are presumably doomed to premature death by the *BTK* mutation that leads to the XLA phenotype[17]. Both mice with X-linked immunodeficiency that have natural mutations in *Btk* and mice in which *Btk* is targeted have B cell defects, but these are associated with much milder effects than those seen in XLA, which suggests species differences in the role of Btk[18,19].

[1]Department of Pediatrics and Developmental Biology, Tokyo Medical and Dental University Graduate School of Medical and Dental Sciences, Tokyo, Japan. [2]Department of Pediatrics, Teikyo University School of Medicine Hospital, Mizonokuchi, Kawasaki, Japan. [3]Department of Pediatrics, Toyama University School of Medicine, Toyama, Japan. [4]Department of Pediatrics, National Defense Medical College, Tokorozawa, Japan. [5]Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seoul, Republic of Korea. Correspondence should be addressed to T.M. (tmorio.ped@tmd.ac.jp).

Received 28 November 2011; accepted 12 January 2012; published online 26 February 2012; doi:10.1038/ni.2234

Btk is also an important signaling component of the innate immune system in phagocytic cells. Btk is involved in signaling via Toll-like receptors (TLRs) such as TLR2, TLR4, TLR7, TLR8 and TLR9, and is associated with the TLR adaptors MyD88, Mal (TIRAP) and IRAK1 (refs. 12–14,20–22). Defective innate immune responses have been observed in monocytes, dendritic cells, neutrophils and mast cells from Btk-deficient mice[12,14]. Neutrophils from mice with X-linked immunodeficiency have poor production of ROS and nitric oxide[15].

The contribution of Btk to the human innate immune system is less obvious. Stimulation via TLR2, TLR4, TLR7-TLR8 or TLR3 results in impaired production of tumor-necrosis factor (TNF) by dendritic cells from patients with XLA, whereas the TLR4-induced production of TNF and interleukin 6 (IL-6) by monocytes from patients with XLA remains intact[23–25]. Neutrophils from control subjects and patients with XLA show no substantial differences in their phosphorylation of the mitogen-activated protein kinases p38, Jnk and Erk induced by engagement of TLR4 or TLR7-TLR8 or production of ROS induced by the same stimuli[26].

Here we evaluate the role of Btk in the production of ROS and cellular apoptosis in human neutrophils through the use of Btk-deficient neutrophils, a protein-delivery system based on a cell-permeable peptide, and specific kinase inhibitors. Unexpectedly, and in contrast to published observations of mice with X-linked immunodeficiency[15], the production of ROS was substantially augmented in the absence of Btk in neutrophils stimulated via TLRs, the TNF receptor or phorbol 12-myristate 13-acetate (PMA) but not in monocytes or in lymphoblastoid B cell lines transformed by Epstein-Barr virus. Excessive production of ROS was associated with neutrophil apoptosis, which was reversed by the transduction of wild-type Btk protein. Btk-deficient neutrophils showed activation of key signaling molecules involved in the activation of NADPH oxidase, and this was accompanied by targeting of Rac2 to the plasma membrane. Mal was confined to the cytoplasm in association with Btk but was translocated to plasma membrane and interacted with protein tyrosine kinases (PTKs) and phosphatidylinositol-3-OH kinase (PI(3)K) in the absence of Btk. Here we present our findings on the mechanism by which Btk regulates the priming of neutrophils and the amplitude of the neutrophil response.

RESULTS

Excessive production of ROS in Btk-deficient neutrophils

To investigate the production of ROS in the absence of Btk, we monitored ROS in neutrophils, monocytes and Epstein-Barr virus–transformed lymphoblastoid B cell lines obtained from patients with XLA, healthy controls and patients with CVID (disease control) by staining with dihydrorhodamine 123 (DHR123) and a luminol chemiluminescence assay. PMA-driven production of ROS in Btk-deficient neutrophils was three to four times greater than that in neutrophils from healthy controls or patients with CVID, and we observed augmented production of ROS with a suboptimal dose of PMA, whereas the baseline production of ROS was similar (**Fig. 1a–d**). Similarly, and in contrast to published reports[26], engagement of TLR2 (with its ligand tripalmitoyl cysteinyl seryl tetralysine lipopeptide (Pam_3CSK_4)), TLR4 (with its ligand lipopolysaccharide) or the TNF receptor (with TNF) followed by stimulation with formyl-Met-Leu-Phe (fMLP), an agonist of G protein–coupled receptors, elicited augmented ROS responses in neutrophils from patients with XLA (**Fig. 1e,f**). The production of ROS was minimal after stimulation with the TLR9 agonist CpG-A in neutrophils from patients with XLA and was not significantly different from that of neutrophils from healthy controls. The observed phenomena were reproduced in Btk-deficient eosinophils but not in monocytes or Epstein-Barr virus–transformed lymphoblastoid B cell lines obtained from patients with XLA (**Supplementary Fig. 1**). These data indicated Btk-deficient neutrophils had excessive NADPH oxidase activity after various stimuli.

Augmented apoptosis in Btk-deficient neutrophils

Because high ROS concentrations are potentially harmful to cells, we investigated cell death induced by various stimuli in neutrophils from patients with XLA by staining with annexin V and the membrane-impermeable DNA-intercalating dye 7-AAD. Stimulation with PMA, TLR agonist plus fMLP, or TNF plus fMLP induced a significantly higher frequency of cells positive for annexin V among neutrophils from patients with XLA than among control neutrophils, whereas spontaneous cell death in the absence of stimulation was not significantly altered at 4 h in neutrophils from healthy controls versus those from patients with XLA (**Fig. 2a,b**). We observed cleavage of caspase-3, lower mitochondrial membrane potentials and degradation of proliferating

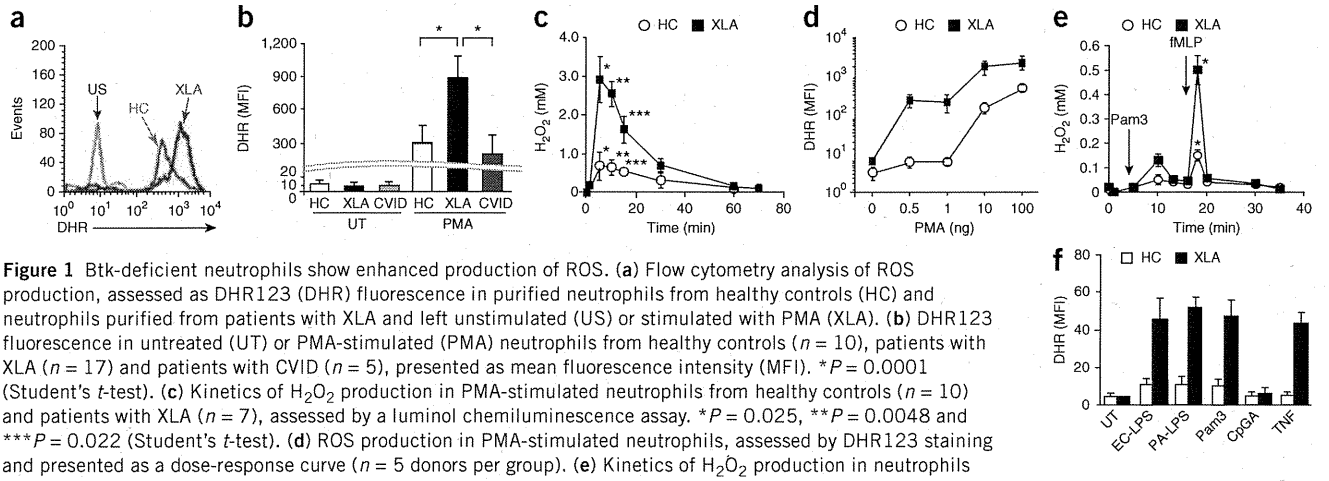

Figure 1 Btk-deficient neutrophils show enhanced production of ROS. (**a**) Flow cytometry analysis of ROS production, assessed as DHR123 (DHR) fluorescence in purified neutrophils from healthy controls (HC) and neutrophils purified from patients with XLA and left unstimulated (US) or stimulated with PMA (XLA). (**b**) DHR123 fluorescence in untreated (UT) or PMA-stimulated (PMA) neutrophils from healthy controls ($n = 10$), patients with XLA ($n = 17$) and patients with CVID ($n = 5$), presented as mean fluorescence intensity (MFI). $^*P = 0.0001$ (Student's t-test). (**c**) Kinetics of H_2O_2 production in PMA-stimulated neutrophils from healthy controls ($n = 10$) and patients with XLA ($n = 7$), assessed by a luminol chemiluminescence assay. $^*P = 0.025$, $^{**}P = 0.0048$ and $^{***}P = 0.022$ (Student's t-test). (**d**) ROS production in PMA-stimulated neutrophils, assessed by DHR123 staining and presented as a dose-response curve ($n = 5$ donors per group). (**e**) Kinetics of H_2O_2 production in neutrophils stimulated with Pam_3CSK_4 (Pam3) and fMLP, assessed by a luminol chemiluminescence assay ($n = 7$ donors per group). $^*P = 0.005$ (Student's t-test). (**f**) DHR123 fluorescence in neutrophils incubated with lipopolysaccharide from *Escherichia coli* (EC-LPS) or *Pseudomonas aeruginosa* (PA-LPS), Pam_3CSK_4, CpG-A or TNF, followed by stimulation with fMLP ($n = 7$ donors per group). Data are representative of seventeen experiments (**a**) or are pooled from at least five (**b,c,e,f**) or four (**d**) independent experiments (mean and s.d. in **b–f**).

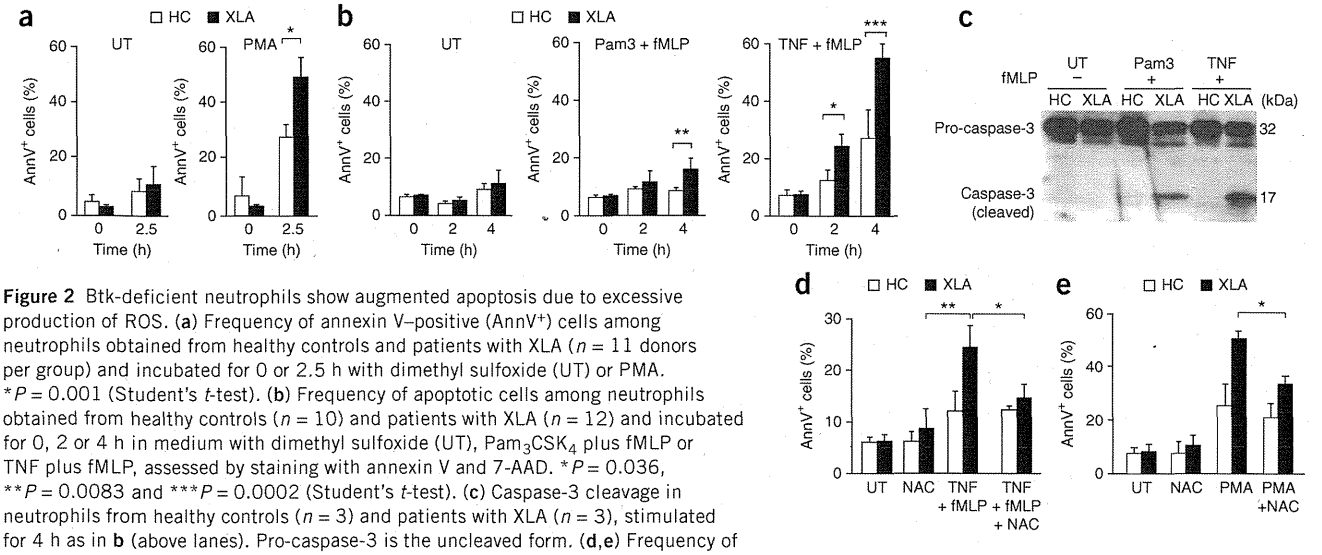

Figure 2 Btk-deficient neutrophils show augmented apoptosis due to excessive production of ROS. (**a**) Frequency of annexin V–positive ($AnnV^+$) cells among neutrophils obtained from healthy controls and patients with XLA ($n = 11$ donors per group) and incubated for 0 or 2.5 h with dimethyl sulfoxide (UT) or PMA. $^*P = 0.001$ (Student's *t*-test). (**b**) Frequency of apoptotic cells among neutrophils obtained from healthy controls ($n = 10$) and patients with XLA ($n = 12$) and incubated for 0, 2 or 4 h in medium with dimethyl sulfoxide (UT), Pam_3CSK_4 plus fMLP or TNF plus fMLP, assessed by staining with annexin V and 7-AAD. $^*P = 0.036$, $^{**}P = 0.0083$ and $^{***}P = 0.0002$ (Student's *t*-test). (**c**) Caspase-3 cleavage in neutrophils from healthy controls ($n = 3$) and patients with XLA ($n = 3$), stimulated for 4 h as in **b** (above lanes). Pro-caspase-3 is the uncleaved form. (**d**,**e**) Frequency of apoptotic Btk-deficient neutrophils ($n = 5$ donors) stimulated for 2 h (**d**) or 2.5 h (**e**) with the antioxidant N-acetyl-cysteine (NAC). $^*P = 0.043$ and $^{**}P = 0.036$ (**d**) or $^*P = 0.026$ (**e**; Student's *t*-test). Data are representative of three experiments (**b**) or at least five independent experiments (**a**,**c**–**e**; mean and s.d. in **a**,**b**,**d**,**e**).

cell nuclear antigen; hence, cell death was caused by apoptosis (**Fig. 2c** and **Supplementary Fig. 2**). Apoptosis assessed by these methods was augmented considerably for neutrophils from patients with XLA. The observed apoptosis was most probably triggered by ROS, as coincubation of neutrophils with N-acetyl cysteine, an antioxidant, rescued the cells from apoptosis induced by TNF plus fMLP or by PMA (**Fig. 2d**,**e**). We detected much more ROS release and stimulation-induced apoptosis of neutrophils from all patients with XLA regardless of the site or mode of their mutation (**Supplementary Fig. 3**). In addition, we found no correlation between genotype and the extent of neutrophil production of ROS. These data suggested that neutrophils from patients with XLA are susceptible to apoptosis triggered by pathogens.

Normalization of the ROS response by transduction of Btk

We next determined whether the enhanced apoptosis noted above was due to a defect in Btk itself or abnormal myeloid differentiation in the absence of Btk. For this, we prepared three recombinant Btk proteins (full-length Btk; Btk with deletion of the pleckstrin homology (PH) domain; and Btk with deletion of the kinase domain) fused to the cell-permeable peptide Hph-1 (**Fig. 3a**,**b**). We purified the products and transduced the proteins into neutrophils lacking Btk. The efficacy of transduction was more than 95%; and Hph-1–Btk expression was stable for at least 12–24 h (ref. 27). We adjusted the expression of Btk to that in neutrophils from healthy controls by incubating 1×10^6 cells for 1 h with 1 μM recombinant fusion protein. Transduction of full-length Btk into neutrophils from patients with XLA restored the production of ROS and the frequency of apoptotic cells after PMA stimulation to that observed for neutrophils from healthy controls (**Fig. 3c**,**d**). Transduction of the recombinant fusion of Btk with deletion of the PH domain only modestly reversed neutrophil overactivation (**Fig. 3c**), which indicated that appropriate cellular localization and interactions with other molecules were required for Btk function. Transduction of the recombinant fusion of Btk with deletion of the kinase domain minimally corrected excessive production of ROS (**Fig. 3c**), which suggested that the kinase activity of Btk or molecules that interacted via the kinase domain were critical for the regulation of ROS. We also confirmed the importance of the kinase domain by an experiment that showed excessive production of ROS in normal neutrophils treated with 50 μM LFM-A13, an inhibitor of the kinase activity of Btk, but not in those treated with LFM-A11, a control compound (**Fig. 3e**). We also documented augmented apoptosis in control neutrophils treated with LFM-A13 (**Fig. 3f**). These data demonstrated that the enhanced production of ROS and apoptosis was directly related to a defect in Btk.

NADPH oxidase components in Btk-deficient neutrophils

The NADPH oxidase complex consists of the transmembrane component ($gp91^{phox}$ and $p22^{phox}$), a cytosolic component ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$) and Rac2 (refs. 3–6). The activity of NADPH oxidase is controlled by targeting of the cytosolic components to the plasma membrane or phosphorylation of the cytosolic components or both. To assess the mechanism of the excessive production of ROS in Btk-deficient neutrophils, we investigated the abundance, phosphorylation and subcellular localization of each component by immunoblot analysis.

The expression of each component of the NADPH oxidase complex was similar in neutrophils from patients with XLA and those from healthy controls (**Fig. 4a**). The amount of $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ in the cytoplasm and the membrane was not substantially different in neutrophils from patients with XLA and those from healthy controls (**Fig. 4b**). Similarly, the amount in the membrane-targeted fraction after stimulation with PMA was not very different in neutrophils from patients with XLA and those from healthy controls (**Fig. 4c**). Phosphorylation of Ser345 in $p47^{phox}$ and of Thr154 in $p40^{phox}$ are important for translocation of the cytosolic components to the membrane[4,5,28]. Those modifications were not altered in Btk-deficient neutrophils (**Fig. 4c**). In contrast, we detected Rac2 in the plasma membrane of Btk-deficient neutrophils before stimulation with PMA. We observed four- to fivefold higher membrane expression of Rac2 in neutrophils from patients with XLA than in those from healthy controls in the resting state (**Fig. 4b**).

Typically, 10–15% of $gp91^{phox}$ is located in the plasma membrane of unstimulated neutrophils, whereas the majority of the molecule resides in specific granules. Membrane expression increases after

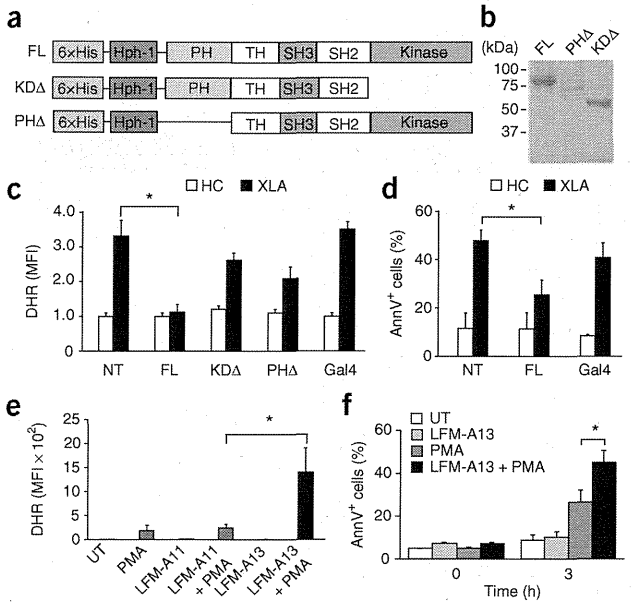

Figure 3 Excessive production of ROS and apoptosis in neutrophils from patients with XLA are abrogated by transduction of Hph-1–tagged full-length recombinant Btk but not by Hph-1–tagged Btk with deletion of the kinase or PH domain. (**a**) Hph-1–tagged Btk constructs: full-length Btk (FL); Btk with deletion of the kinase domain (KDΔ); Btk with deletion of the PH domain (PHΔ). 6×His, six-histidine tag; TH, Tec homology; SH3, Src homology 3; SH2, Src homology 2. (**b**) Size of purified Hph-1–tagged Btk proteins, confirmed by Coomassie brilliant blue staining. (**c**) ROS production in neutrophils from healthy controls ($n = 5$) and patients with XLA ($n = 5$), left untransduced (NT) or transduced with the constructs in **a** or Hph-1–tagged yeast transcriptional activator Gal4 (far right; control), presented as the MFI of DHR123 relative to that of untreated neutrophils from healthy controls, set as 1. (**d**) Frequency of apoptotic cells among neutrophils from healthy controls and patients with XLA, left untransduced or transduced with Hph-1–tagged full-length Btk or Gal4 (control). (**e**) DHR123 fluorescence in neutrophils from healthy controls ($n = 7$) left untreated or treated with PMA alone, or pretreated with LFM-A13 (Btk inhibitor) or LFM-A11 (control) alone or followed by stimulation with PMA (+ PMA). (**f**) Frequency of annexin V–positive cells among neutrophils from healthy controls ($n = 7$) left untreated or treated with PMA alone, or pretreated with LFM-A13 (50 μM, a concentration that does not inhibit other PTKs[47,48]) alone or followed by stimulation with PMA. $^*P = 0.0021$ (**c**), 0.019 (**d**), 0.021 (**e**) or 0.025 (**f**; Student's *t*-test). Data are representative of five experiments (**b**) or are pooled from six (**c**), three (**d**) or four (**e**,**f**) independent experiments (mean and s.d. in **c**–**f**).

signaling via TLRs or G protein–coupled receptors because of translocation to the plasma membrane[2]. Immunoblot analysis with antibody to gp91 (anti-gp91; **Fig. 4b**) and flow cytometry analysis of surface flavocytochrome b_{558} (**Fig. 4d**) showed higher gp91 expression in neutrophils from patients with XLA. Immunohistochemical analysis by confocal fluorescence microscopy showed localization of gp91 and Rac2 together in the membranes of resting Btk-deficient neutrophils but not in neutrophils from healthy controls (**Fig. 4e**). These results suggested that NADPH oxidase complex was partially assembled and ready to be activated in steady-state Btk-deficient neutrophils.

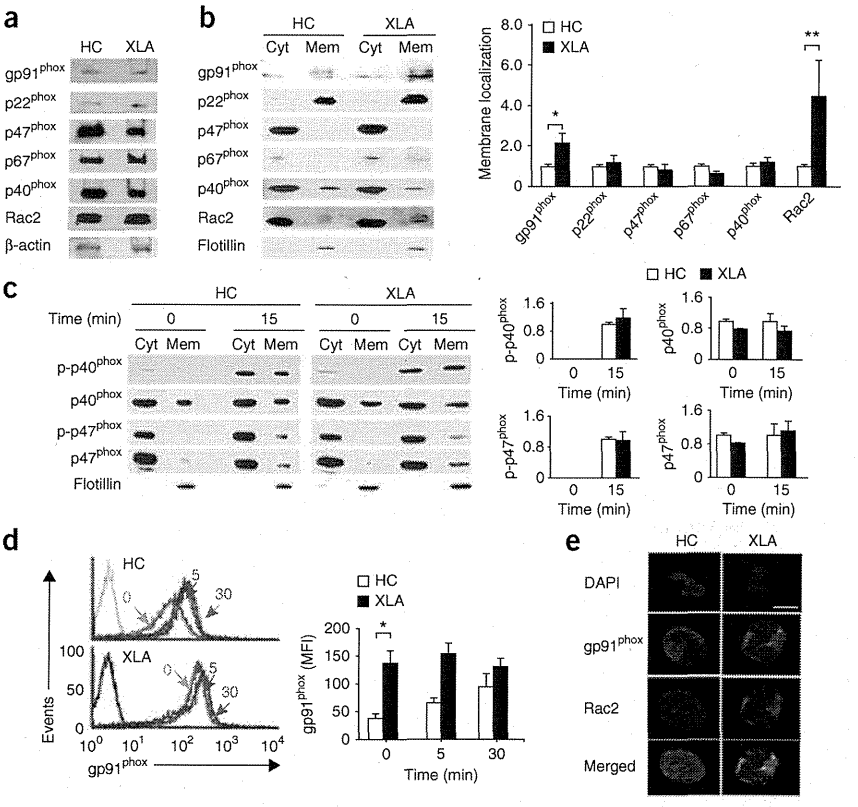

Figure 4 Btk-deficient neutrophils show targeting of Rac2 to the plasma membrane, colocalization of Rac2 with gp91^{phox} and higher membrane expression of gp91^{phox}. (**a**) Immunoblot analysis of the components of the NADPH oxidase complex in neutrophils from a healthy control and a patient with XLA. β-actin serves as a loading control throughout. (**b**) Immunoblot analysis (left) of the components of the NADPH oxidase complex in the cytoplasm (Cyt) and plasma membrane (Mem) of neutrophils from healthy controls and patients with XLA ($n = 9$ per group). Right, quantification of the membrane expression at left, presented as band intensity relative to that of flotillin (loading marker for the membrane-raft fraction) in membranes of neutrophils from healthy controls, set as 1. $^*P = 0.045$ and $^{**}P = 0.027$ (Student's *t*-test). (**c**) Immunoblot analysis of total and phosphorylated (p-) p40^{phox} and p47^{phox} in the cytoplasm and membrane of PMA-stimulated neutrophils from healthy controls and patients with XLA. Right, quantification as in **b**. (**d**) Flow cytometry analysis of gp91^{phox} on neutrophils from healthy controls and patients with XLA, left unstimulated (0) or stimulated for 5 or 30 min (above lines) with PMA, detected by staining with mAb 7D5 to gp91. Gray lines indicate staining with MsIgG (control). Right, quantification of the gp91 MFI in cells treated as at left. $^*P = 0.0039$ (Student's *t*-test). (**e**) Confocal microscopy of gp91^{phox} (green) and Rac2 (red) in healthy controls and neutrophils from patients with XLA; nuclei are counterstained with the DNA-intercalating dye DAPI (blue). Original magnification, ×600; scale bar, 10 μm. Data are from one representative of nine independent experiments with seven healthy controls and nine patients with XLA (**a**), are representative of nine experiments (**b**), are from nine independent experiments (**c**), are pooled from seven independent experiments (**d**) or are representative of four independent experiments (**e**; mean and s.d. in **b**–**d**).

Activated PTKs and PI(3)K in resting XLA neutrophils

Assembly and activation of the cytosolic components and Rac requires the involvement of kinases such as PTKs, PI(3)K and protein kinase C. We thus explored a potential signaling pathway that would lead to the partial assembly of NADPH oxidase. First, we examined the extent of tyrosine phosphorylation of cellular substrates in Btk-deficient and Btk-sufficient neutrophils before and after stimulation with PMA. Btk-deficient neutrophils showed hyperphosphorylation of protein species in the range of 50–53 kilodaltons (kDa), 72 kDa, 85 kDa and 150 kDa at baseline relative to phosphorylation in neutrophils from healthy controls (**Fig. 5a**). TLR4-mediated stimulation led to more phosphorylation of protein species 38 kDa, 50–53 kDa, 60 kDa, 72 kDa and 85 kDa in size in Btk-deficient neutrophils (**Supplementary Fig. 4a**).

In contrast, the baseline PTK activity in monocytes from patients with XLA was unaltered or slightly diminished relative to that of monocytes from healthy controls. TLR2-stimulated activation of PTKs was largely similar or slightly less in the absence of Btk (**Supplementary Fig. 4b**). We were able to directly ascribe the enhanced PTK activity to the absence of Btk, as transduction of recombinant Btk into neutrophils from patients with XLA restored baseline phosphorylation to that seen in neutrophils from healthy controls (**Fig. 5b**).

We next searched for tyrosine-phosphorylated proteins in Btk-deficient neutrophils through the use of phosphorylation-specific antibodies. The expression and activation of Tec and Bmx, TFKs present in neutrophils, was not upregulated in neutrophils from patients with XLA (**Fig. 5c**), which indicated that they did not compensate for Btk function. However, we found that the tyrosine-phosphorylated proteins 50–53 kDa, 72 kDa, 85 kDa and 150 kDa in size were the kinases Lyn and c-Src, Syk, the p85 subunit of PI(3)K (class IA) and FAK, respectively (**Fig. 5d,e**). We found that c-Src, Syk, PI(3)K-p85 and FAK were phosphorylated at their tyrosine residues that have a positive regulatory function. Notably, Lyn, a kinase known to have positive as well as negative roles in the modulation of myeloid function, was phosphorylated at Tyr507, a negative regulatory site[29–31].

We first focused on PI(3)K, as PI(3)K activation targets Rac2 to flavocytochrome b_{558}; this process is important for converting

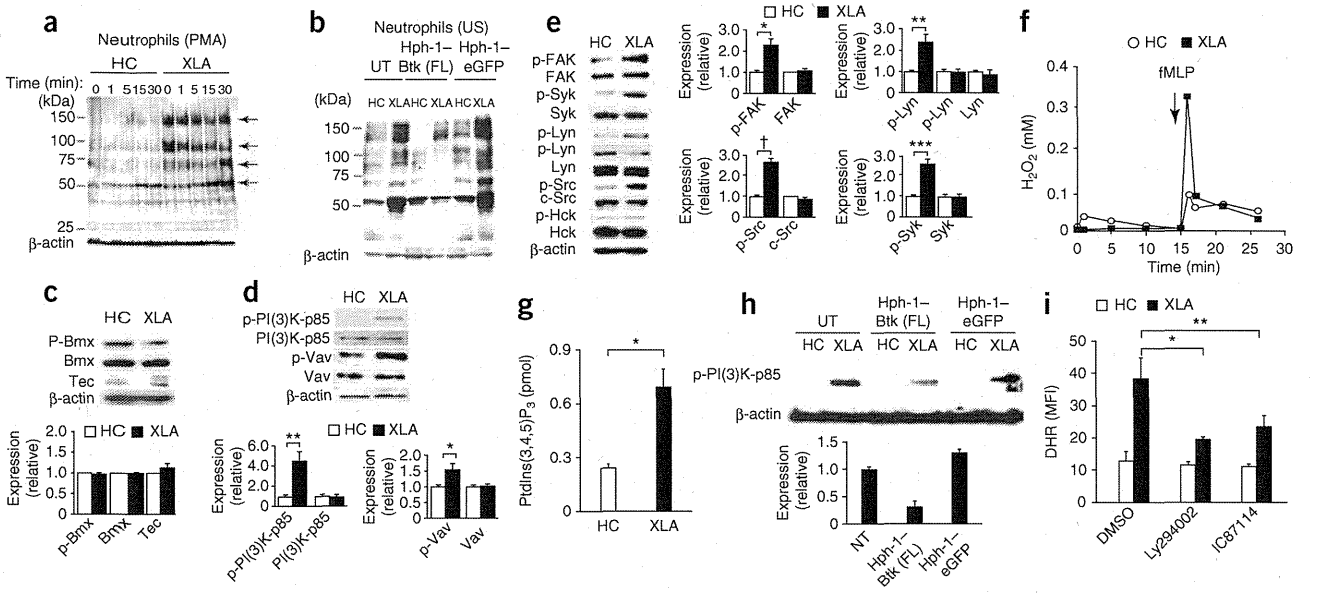

Figure 5 Btk-deficient neutrophils have higher baseline activity of PTKs and PI(3)K, which is reversed by transduction of recombinant Btk protein. (**a**) Immunoblot analysis of phosphorylated tyrosine in lysates of PMA-stimulated neutrophils from healthy controls ($n = 5$) and patients with XLA ($n = 7$). Arrows indicate hyperphosphorylated proteins in neutrophils from patients with XLA at 0 min. (**b**) Immunoblot analysis of phosphorylated tyrosine (as in **a**) in lysates from unstimulated (US) neutrophils from healthy controls ($n = 4$) and patients with XLA ($n = 5$), left untransduced or transduced with Hph-1-tagged full-length Btk or eGFP. (**c,d**) Immunoblot analysis (top) of whole-cell lysates of neutrophils from healthy controls ($n = 5$) and patients with XLA ($n = 7$), probed for total and phosphorylated Bmx and total Tec (**c**) or total and phosphorylated PI(3)K-p85 and Vav (phosphorylated at Tyr508 (PI(3)K-p85) or Tyr174 (Vav); **d**). Phosphorylated Tec was not detected by immunoblot analysis of phosphorylated tyrosine in samples immunoprecipitated with anti-Tec (data not shown). Bottom, quantification of the expression at top, presented relative to expression of β-actin in neutrophils from healthy controls, set as 1. $*P = 0.038$ and $**P = 0.0001$ (Student's *t*-test). (**e**) Immunoblot analysis (left) of neutrophils from healthy controls ($n = 5$) and patients with XLA ($n = 7$), probed for total PTKs and PTKs phosphorylated at Tyr576 and Tyr577 (FAK); Tyr524 and Tyr525 (Syk); Tyr507 (Lyn; top) or Tyr397 (Lyn; bottom); (Tyr416 (c-Src); and Tyr411 (the kinase Hck). Phosphorylated PTKs Fgr and Yes were undetectable (data not shown). Right, quantification as in **c,d**. $*P = 0.033$, $**P = 0.004$, $***P = 0.0007$ and $\dagger P = 0.0002$ (Student's *t*-test). (**f**) H_2O_2 production by fMLP-stimulated neutrophils from healthy controls and patients with XLA ($n = 5$ per group). (**g**) Enzyme-linked immunosorbent assay of phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) in unstimulated neutrophils from patients with XLA ($n = 5$). $*P = 0.0005$ (Student's *t*-test). (**h**) Immunoblot analysis (top) of phosphorylated PI(3)K-p85 in neutrophils from healthy controls and patients with XLA ($n = 5$ per group), left untransduced or transduced with Hph-1–tagged full-length Btk or eGFP. Detection of phosphorylated PI(3)K-p85 in neutrophils from healthy controls required longer exposure. Below, quantification of results above, presented relative to the expression of phosphorylated PI(3)K-p85 relative to that of β-actin in neutrophils from patients with XLA, set as 1. (**i**) Production of ROS in neutrophils from patients with XLA, treated with dimethyl sulfoxide (DMSO) or preincubated with Ly294002 (universal PI(3)K inhibitor; 50 μM)[32] or IC87114 (PI(3)Kδ inhibitor; 1 μM (a concentration that does not inhibit PI(3)Kα, PI(3)Kβ or PI(3)Kγ)[33]) and stimulated with fMLP. $*P = 0.006$ and $**P = 0.003$ (Student's *t*-test). Data are representative of or pooled from six (**a,f**), seven (**b–e**), four (**g**), eight (**h**) or five (**i**) independent experiments (mean and s.d. in **c–e,g–i**).

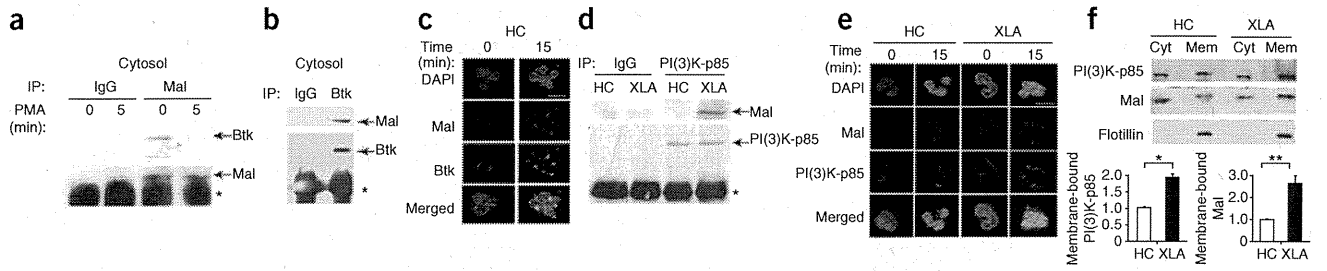

Figure 6 Mal in neutrophils from healthy controls associates with Btk in the resting state and translocates to the plasma membrane after stimulation, whereas Mal associates with PI(3)K at the plasma membrane in Btk-deficient neutrophils. (**a**,**b**) Coimmunoprecipitation analysis of Btk and Mal in the cytoplasmic fraction of neutrophils from healthy controls, left unstimulated (0 (**a**), **b**) or stimulated for 5 min with PMA (5 (**a**)). IP, immunoprecipitation; IgG, control antibody. *, immunoglobulin light chain (**a**) or heavy chain (**b**). (**c**) Confocal microscopy of neutrophils from healthy controls, left unstimulated (0) or stimulated for 15 min with PMA (15), then stained with anti-Mal (red) and anti-Btk (green) and counterstained with DAPI. Original magnification, ×600; scale bar, 10 μm. (**d**) Coprecipitation analysis of PI(3)K-p85 and Mal in membrane fraction of neutrophils from healthy controls and patients with XLA. *, immunoglobulin heavy chain. (**e**) Confocal microscopy of neutrophils from healthy controls and patients with XLA, left unstimulated or stimulated for 15 min with PMA, then stained with anti-Mal (red) and anti-PI(3)K-p85 (green) and counterstained with DAPI. Scale bar, 10 μm. (**f**) Immunoblot analysis (above) of PI(3)K-p85 and Mal in the cytoplasm and plasma membrane of unstimulated neutrophils from healthy controls and patients with XLA. Below, quantification of results above, presented relative to the expression of flotillin in neutrophils from healthy controls, set as 1. $*P = 0.0035$ and $**P = 0.0021$ (Student's *t*-test). Data are representative of three (**a**,**b**), four (**c**,**e**), six (**d**) or seven (**f**) independent experiments (mean and s.d. in **f**).

neutrophils into a 'primed' state in which they are ready for complete activation of NADPH oxidase triggered by stimuli such as fMLP. Indeed, Btk-deficient neutrophils were in a primed state, as fMLP alone elicited excessive production of ROS (**Fig. 5f**). Greater phosphorylation of PI(3)K-p85 was accompanied by more enzymatic activity, as shown by more baseline production of phosphatidylinositol-(3,4,5)-trisphosphate and by phosphorylation of the adaptor Vav (**Fig. 5d**,**g**). Furthermore, augmented PI(3)K activation was normalized, although only partially, by transduction of full-length Btk linked to Hph-1 (**Fig. 5h**).

The importance of PI(3)K in inducing the primed state was supported by data showing inhibition of fMLP-driven production of ROS by preincubation of Btk-deficient neutrophils with the universal PI(3)K inhibitor LY294002 at a concentration of 50 μM (refs. 32,33). We observed this inhibition in cells incubated with the PI(3)Kδ–specific inhibitor IC87114 at a concentration of 1 μM (ref. 33) but not in those incubated with the PI(3)Kγ–specific inhibitor AS605240 at a concentration of 8 nM (ref. 34; **Fig. 5i** and **Supplementary Fig. 5a**). These findings suggested PI(3)Kδ activation was involved in the excessive ROS response.

Interaction of membrane-targeted Mal with PI(3)K

We next sought the reason for the PI(3)K activation in the absence of Btk. For this, we first focused on a molecule that interacts with both Btk and PI(3)K. Evidence obtained with monocytes indicates that Mal is a critical component of TLR2-TLR4 signaling and is a target of Btk[13,14,20,21]. The TLR signal triggers activation of Btk, which in turn phosphorylates Mal. Phosphorylated Mal translocates to the plasma membrane via phosphatidylinositol-(4,5)-bisphosphate ($PtdIns(4,5)P_2$) and then interacts with and activates PI(3)K[35].

Unexpectedly, coimmunoprecipitation assays of neutrophils from human controls demonstrated that Mal was associated with Btk in the resting state (**Fig. 6a**,**b**). We observed colocalization of Mal and Btk in the cytoplasm and, after activation of cells with PMA, we detected the Mal-Btk complex at the membrane by immunofluorescence staining (**Fig. 6c**).

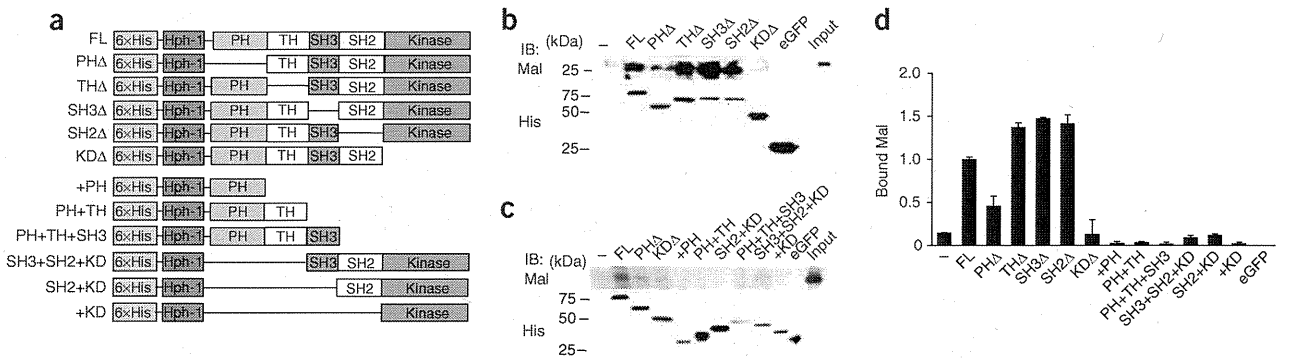

Figure 7 Btk associates with Mal at the PH and kinase domains. (**a**) Hph-1–tagged Btk constructs: full-length Btk (FL); Btk mutants with deletion of the PH domain (PHΔ), Tec homology (THΔ), SH3 domain (SH3Δ), SH2 domain (SH2Δ) or kinase domain (KDΔ); and Btk mutants with truncation retaining (+) only some domains (bottom six). (**b**,**c**) Immunoblot analysis (IB) of Mal (top) in extracts of cytoplasm of neutrophils from healthy controls, incubated with nickel beads bound to Hph-1–tagged recombinant full-length Btk or the deletion mutants (**b**) or truncation mutants (**c**) in **a**, or to Hph-1–tagged eGFP (negative control). Below, immunoblot analysis after rebinding to nickel beads, probed with anti-histidine (His). To make these as equimolar as possible, more beads were added for the +PH, PH+TH+SH3, SH3+SH2+KD and +KD constructs. Input, cytoplasmic extracts without precipitation. (**d**) Quantification of Mal bound to the recombinant Btk proteins based on the results in **b**,**c** ($n = 4$ donors), presented to results for full-length Btk, set as 1. Data are representative of four experiments (**b**,**c**) or are a summary of four independent experiments (**d**; mean and s.d.).

We did not detect the association of Mal with PI(3)K-p85 in unstimulated neutrophils from healthy controls; however, we did observe this association in Btk-deficient neutrophils before stimulation with PMA (**Fig. 6d**). Moreover, confocal fluorescence microscopy showed targeting of the PI(3)K-p85–Mal complex to the membrane in the absence of Btk, whereas we observed the complex at the membrane after stimulation with PMA in the presence of Btk (**Fig. 6e**). In addition, most of the PI(3)K-p85 and Mal was present in the membrane fraction in neutrophils from patients with XLA (**Fig. 6f**). These data suggested that Btk in resting neutrophils was involved in confining Mal to the cytoplasm.

The mode of the Btk-Mal association

Btk phosphorylates Mal at Tyr86, Tyr106 and Tyr187, and the Btk-Mal interaction requires Pro125, Tyr86, Tyr106 and Tyr159 in Mal, whereas the critical site in Btk for this association remains unknown[21,22]. To clarify the region of Btk required for the cytoplasmic Btk-Mal association, we generated various Btk deletion mutants fused to histidine-tagged Hph-1 (**Fig. 7a**) and assessed their binding to Mal (**Fig. 7**). We incubated nickel bead–bound recombinant proteins with the cytoplasmic fraction of control neutrophils and evaluated the associations by immunoblot analysis with anti-Mal. Full-length Btk effectively bound to cytoplasmic Mal prepared from control neutrophils, but a control fusion of histidine-tagged Hph-1 and enhanced green fluorescent protein (eGFP) did not. Btk with deletion of the kinase domain almost completely lost the ability to interact with Mal, and Btk with deletion of the PH domain showed less binding to Mal. In contrast, recombinant proteins lacking the Tec homology domain, the Src homology 3 domain or the Src homology 2 domain had slightly greater capacity to associate with Mal (**Fig. 7b,d**). Other truncated Btk recombinant proteins without either the PH domain or kinase domain failed to bind to Mal (**Fig. 7c,d**), which suggested that both the PH domain and kinase domain are critical for the Btk-Mal interaction.

PTKs associate with Mal and regulate PI(3)K activation

The precise mechanism of PI(3)K activation triggered by membrane-associated Mal is largely unknown. As several PTKs were phosphorylated

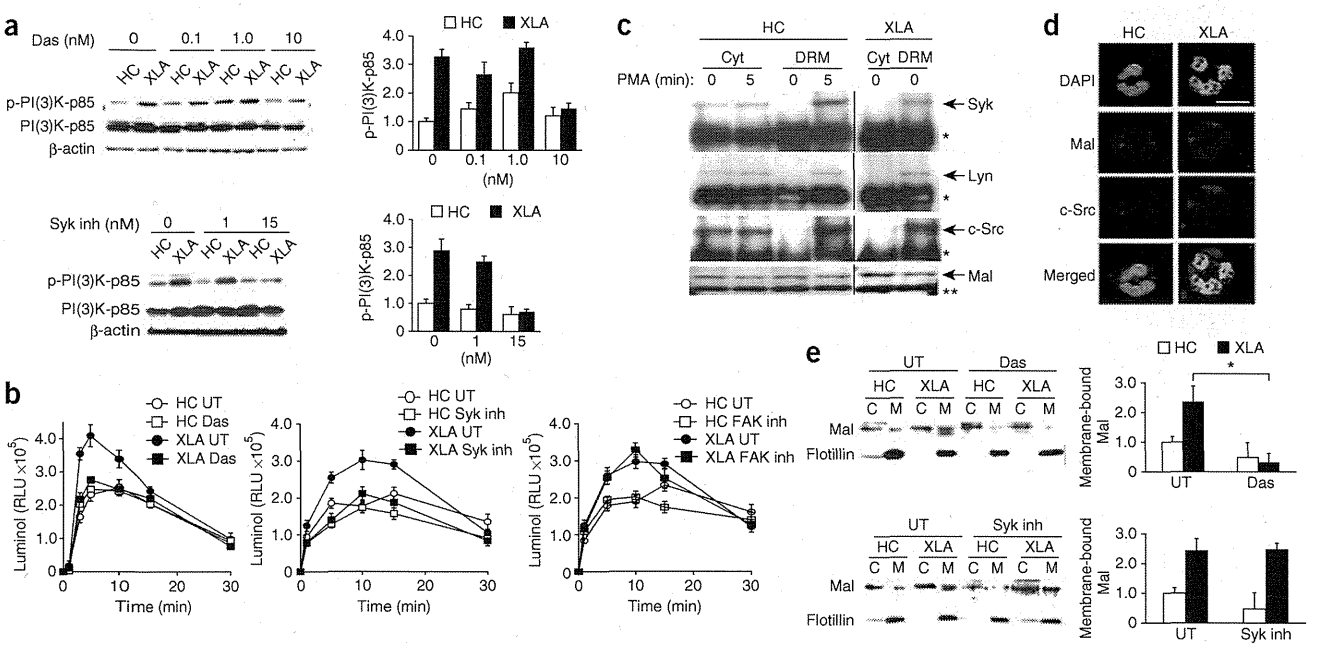

Figure 8 SFKs and Syk are involved in PI(3)K activation and the augmented production of ROS, whereas SFKs are involved in the membrane localization of Mal, in Btk-deficient neutrophils. (**a**) Immunoblot analysis (left of each pair) of total and phosphorylated PI(3)K-p85 in neutrophils from healthy controls and patients with XLA ($n = 4$ per group), treated with dasatinib (Das; at 10 nM, to inhibit the activity of c-Src, Lyn and Bcr-Abl but not FAK or Syk directly[36]) or a Syk inhibitor (Syk inh). Right (of each pair), densitometry of PI(3)K-p85 phosphorylated at Tyr508, presented as band intensity relative to that in untreated neutrophils from healthy controls, set as 1. (**b**) H_2O_2 production in neutrophils ($n = 4$ donors per group) left untreated (UT) or pretreated with dasatinib (10 nM), Syk inhibitor (15 nM) or FAK inhibitor (4 nM), and then stimulated with PMA, assessed by luminol assay and presented in relative light units (RLU). (**c**) Immunoassay of cytosolic fractions (Cyt) and detergent-resistant membrane fractions (DRM) of neutrophils from healthy controls and patients with XLA, left untreated (0) or treated for 5 min with PMA (5), followed by immunoprecipitation with anti-Mal and immunoblot analysis with anti-Syk, anti-Lyn, anti-c-Src or anti-Mal. *, immunoglobulin heavy chain; **, immunoglobulin light chain. (**d**) Confocal microscopy of neutrophils from healthy controls and patients with XLA ($n = 3$ per group), stained with anti-Mal (red) and anti-c-Src (blue) and counterstained with DAPI. Original magnification, ×600; scale bar, 10 μm. (**e**) Immunoblot analysis (left) of Mal in the cytoplasm (C) and membrane (M) of neutrophils from healthy controls and patients with XLA ($n = 5$ per group), left untreated or treated as in **a**. Right, quantification of results for Mal (left), presented relative to that of flotillin in the membrane fraction of neutrophils from healthy controls, set as 1. *$P = 0.0024$ (Student's *t*-test). (**f**) Immunoblot analysis of total Syk and Syk phosphorylated at Tyr524 and Tyr525 (top left) and of total c-Src and c-Src phosphorylated at Tyr416 (bottom left) in neutrophils from healthy controls and patients with XLA, left untreated or treated with dasatinib (top left) or Syk inhibitor (bottom left). Right, quantification of band intensity relative to that of β-actin in untreated neutrophils from healthy controls, set as 1. *$P = 0.013$ (Student's *t*-test). Data are from four (**a**) or five (**f**) independent experiments, are from one representative of four independent experiments (**c**) or are representative of four experiments (**b,e**) or three experiments (**c**; mean and s.d. in **a,b,e,f**).