おわりに

DKCは，X連鎖劣性遺伝型の古典的なDKCが発見され，その原因がテロメアの機能不全であることが明らかになり，その後テロメアの機能不全という観点より，不全型のDKCの存在が明らかになってきた。しかし，依然として確立した治療法はなく，さらなる病態の解析による新たな治療法の確立が期待される。最近になり，再プログラム化されたDKC由来のinduced pluripotent stem (iPS) 細胞において，OCT4やNANOGといった分化増殖万能性の維持に必須の転写因子が*TERC*や*DKC1*の発現を亢進させ，DKC由来のテロメラーゼ複合体の機能障害を克服し，テロメアの再伸長が認められた[54]。このことは，DKC由来のiPS細胞はテロメア関連遺伝子変異によるテロメア伸長の機能障害があってもテロメア伸長が回復することを示しており，将来の治療法の開発に発展するものと期待する。

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Matched sibling donor stem cell transplantation for Fanconi anemia patients with T-cell somatic mosaicism

Yabe M, Shimizu T, Morimoto T, Koike T, Takakura H, Tsukamoto H, Muroi K, Oshima K, Asami K, Takata M, Yamashita T, Kato S, Yabe H. Matched sibling donor stem cell transplantation for Fanconi anemia patients with T-cell somatic mosaicism.

Abstract: SCT from HLA-identical sibling donors is generally associated with an excellent survival in FA patients if performed prior to the development of MDS or leukemia. However, the optimal conditioning regimen has not been defined. We report here our experience with 15 Japanese FA patients who underwent HLA-matched sibling donor SCT. The aim of this study is to compare radiation-based conditioning to Flu-based conditioning for FA patients in a Japanese population where the T-cell somatic mosaicism is higher than in the Caucasian population. Eight patients (a-group) received a radiation-based conditioning (500–600 cGy of thoracoabdominal/TBI) with CY dose modification (20–120 mg/kg), and ATG; two patients exhibited rejection. Seven patients (b-group) received CY (40 mg/kg), 150–180 mg/m^2 of Flu, and ATG. Durable engraftment was demonstrated in all patients. In FA patients, Flu-based conditioning may allow stable engraftment in matched sibling donor transplantation without radiation, even in patients with T-cell somatic mosaicism.

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Key words: Fanconi anemia – stem cell transplantation – HLA-matched sibling donors – fludarabine – T-cell somatic mosaicism

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Somatic mosaicism, the presence of non-FA cells among FA hematopoietic cells, has been considered a risk factor for engraftment in SCT from alternative donors, because DEB-resistant T-cells may increase the risk of graft rejection (1). Wagner et al. (2) reported that engraftment was poorer in unrelated donor recipients with T-cell somatic mosaicism not treated with a Flu-containing regimen. We reported that there is a high frequency of T-cell somatic mosaicism in Japanese FA patients (3). The current study presents the results of matched sibling donor SCT in 15 FA patients undergoing two types of conditioning regimens: radiation-based conditioning with CY dose modification and Flu-based regimen without radiation.

Abbreviations: ALG, antilymphocyte globulin; ATG, antithymocyte globulin; BMT, bone marrow transplantation; CsA, cyclosporine A; CY, cyclophosphamide; DEB, diepoxybutane; FA, Fanconi anemia; Flu, fludarabine; GVHD, graft-vs.-host disease; HLA, human leukocyte antigen; INFA, International Fanconi Anemia Registry; MDS, myelodysplastic syndrome; MMC, mitomycin-C; MTX, methotrexate; RA, refractory anemia; RRT, regimen-related toxicity; SCT, stem cell transplantation; TAI, thoracoabdominal irradiation; TBI, total body irradiation.

Patients and methods

Patients and donor selection

Between 1987 and 2008, 15 patients with FA received 17 SCTs from their HLA-matched sibling donors. The diagnosis of FA was confirmed by chromosomal breakages induced by MMC or 0.1 μg/mL DEB. The chromosomal fragility test for CY metabolites was performed using the serum obtained from other CY-treated, non-FA SCT patients with severe aplastic anemia as previously described (4). We chose the concentration of 0.4 μg/mL for CY metabolites in chromosomal fragility testing of FA patients because it induces multiple chromosomal breaks in FA patients while having little clastogenic effect on normal cells (4). Peripheral lymphocytes from 78 FA patients underwent both CY metabolite and DEB tests in our laboratory. Patients with 50% or more cells insensitive to treatment with 0.1 μg/mL DEB and/or 0.4 μg/mL CY metabolite were classified as high-mosaic in this study for comparison with patients in the IFAR (5). Patient characteristics are shown in Table 1. Age at transplant ranged from 5 to 24 yr. Twelve patients had severe aplastic anemia, and three had RA. Determination of complementation groups was informative in 11 patients, of whom six were placed in group A and five in group C. Of our 15 patients, seven had received androgen therapy, and all 15 were transfusion-dependent at the time of SCT. All 15 donors (14 bone marrow, one cord blood for patient 8) had negative results of DEB/MMC test prior to transplant and were matched (at HLA loci A, B, and DR) to HLA-identical siblings. Harvested marrow was not manipulated.

Conditioning regimen and GVHD prophylaxis

The conditioning regimen of eight patients of a-group (Nos. 1–8) consisted of the following: modified CY (20–120 mg/kg), either TAI (500–600 cGy) or TBI, and ATG or ALG. We provisionally reduced the dose of CY according to the increase of chromosomal breaks case by case, although we set a minimum dose of 20 mg/kg.

Since 2000, Flu – an antimetabolite and immunosuppressive agent – has been used as a part of conditioning. The regimen of seven patients of b-group (Nos. 9–15) included CY (40 mg/kg), Flu (150–180 mg/m^2), and ATG, without radiation. GVHD prophylaxis was carried out using CsA; short-term MTX (15 mg/m^2 on day 1; 10 mg/m^2 on days 3, 5, and 11) administration was utilized in patients older than 10 yr.

Analysis of chimerism

Engraftment on the bone marrow was assayed using short tandem repeats analysis, XY chromosomal analysis, or fluorescence *in situ* hybridization with XY chromosome-specific probes.

The Tokai University Hospital institutional review board approved the collection and reporting of these data.

Results

Chromosomal fragility test

Table 2 shows a summary of the results of cytogenetic testing of 78 FA patients; there was a linear correlation between the percentage of aberrant metaphases in lymphocytes treated with CY metabolites and the percentage in lymphocytes treated with DEB (r = 0.868) (Fig. 1). Among the 12 patients tested for DEB, four were shown to be high-mosaic: two in a-group and two in b-group (Table 1). In the CY metabolite test, eight of 15 patients (four in each group) were shown to have high-mosaic.

Table 1. Patient characteristics; (a) radiation-based conditioning with CY dose modification group; (b) fludarabine-based conditioning without radiation group

No.	Age at SCT (yr)	Sex	Status at SCT (% blasts)	Clonal abnormality	Complementation group	CY test	CY mosaic (%)	DEB mosaic (%)	Prior therapy	No. of prior blood transfusions
(a)										
1	8	F	SAA (0)	No	A	0.16	91.0	56.0	PSL, Androgen	>20
2	6	M	SAA (0)	No	C	0.43	73.2	NT	None	1–20
3	14	M	SAA (0)	No	C	0.44	79.4	NT	Androgen	1–20
4	11	M	SAA (0)	No	Unknown	0.23	77.0	NT	PSL	1–20
5	5	M	SAA (0)	No	C	1.12	47.0	34.0	GCSF, Epo, CsA	1–20
6	10	M	SAA (0)	No	C	2.85	20.0	13.3	PSL, Androgen	1–20
7	24	F	RA (<5)	del(7)(p12)	Unknown	2.66	1.0	0	PSL, CsA, Androgen	1–20
8	5	F	RA (<5)	No	C	0.96	49.5	56.0	PSL, Androgen	1–20
(b)										
9	8	F	SAA (0)	No	A	0.52	58.0	54.0	None	1–20
10	15	F	SAA (0)	No	Unknown	0.81	50.0	8.0	None	1–20
11	6	M	SAA (0)	No	Unknown	2.91	13.0	4.9	Androgen	1–20
12	6	M	SAA (0)	No	A	1.05	27.0	17.0	Androgen	1–20
13	1	M	RA (<5)	Add(2)(q33)	A	0.20	82.0	63.0	None	>20
14	9	F	SAA (0)	No	A	0.61	66.0	16.0	None	1–20
15	4	F	SAA (0)	No	A	2.60	15.0	3.0	None	>20

F, female; M, male; SAA, severe aplastic anemia; CY test, cyclophosphamide metabolites-induced (0.4 μg/mL) test, with results listed as mean number of chromosome breaks per cell; CY mosaic, percentage of cells with 0.4 μg/mL cyclophosphamide metabolites-insensitivity; DEB mosaic, percentage of cells with 0.1 μg/mL diepoxybutane insensitivity; NT, not tested; PSL, prednisolone; GCSF, granulocyte colony-stimulating factor; Epo, erythropoietin.

Table 2. Chromosome fragility test performed with DEB and CY metabolites in 78 FA patients

Agent	Breaks/cell, *n*	Aberrant cells, %	Aberrations/aberrant cell, *n*
Spontaneous	0.0 6 ± 0.09 (0–0.49)	7.93 ± 10.63 (0–66)	1.04 ± 0.16 (1–2)
DEB (0.1 μg/mL)	3.10 ± 2.67 (0.03–12.0)	68.63 ± 27.64 (2–100)	3.85 ± 2.37 (1–12)
CY metabolites (0.4 μg/mL)	1.38 ± 1.16 (0–5.57)	54.91 ± 24.61 (1–100)	2.20 ± 1.02 (1–5.93)

DEB, diepoxybutane; CY, cyclophosphamide, FA, Fanconi anemia.

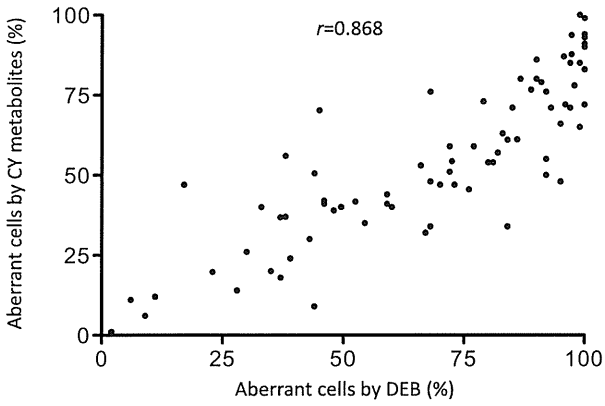

Fig. 1. Linear correlation between the percentage of aberrant metaphases in lymphocytes treated with 0.4 μg/mL CY metabolites and the percentage in lymphocytes treated with 0.1 μg/mL-DEB.

Engraftment and chimerism

The transplantation characteristics and the follow-up characteristics after transplantation are summarized in Table 3 and 4, respectively. Seven of eight patients in a-group engrafted. Patient 1, who was high-mosaic with 56% DEB-insensitive and 91% CY metabolites-insensitive lymphocytes, did not engraft; this patient received an infusion of marrow cells from another HLA-matched sibling after a conditioning regimen of TBI (8 Gy) + CY (150 mg/kg) + ALG, and engrafted. Patient 7 achieved successful engraftment after receiving 20 ng/kg of CY without any RRT. Although she was not high-mosaic, she developed late graft rejection on day 205 after pure red cell aplasia. Chromosomal analysis of peripheral blood cultured with phytohemagglutinin and that of bone marrow showed mixed chimerism. She received a second BMT from the same brother on day 240 after TBI (7.5 Gy) + CY (60 mg/kg) + ALG, and successfully engrafted.

Successful engraftment was achieved in all seven patients in b-group, independent of the proportion of DEB- or CY-insensitive cells

Table 3. Transplantation characteristics; (a) radiation-based conditioning with CY dose modification group; (b) fludarabine-based conditioning without radiation group

No.	Conditioning	TNC/kg ($\times 10^8$)	Engraftment		Organ toxicity (Bearman)	GVHD	
			Day ANC >0.5 × 10^9/L	Day platelet >50 × 10^9/L	Grade I/II	Acute	Chronic
(a)							
1-1	CY(40)* + TAI(6) + ALG	4.7	Rejection	Rejection	No	NE	NE
1-2	CY(150) + TBI(8) + ALG	4.5	21	74	Stomatitis, mucositis	0	Lim
2	CY(40) + TAI(6) + ALG	4.5	27	22	Stomatitis	0	–
3	CY(45) + TAI(6) + ALG	2.5	12	27	Stomatitis	0	Lim
4	CY(120) + TAI(6) + ALG	1.7	13	26	No	0	–
5	CY(20) + TAI(6)	6.6	16	21	No	0	–
6	CY(20) + TAI(6) + ATG	3.1	17	33	No	0	–
7-1	CY(20) + TAI(5) + ALG	≥2.0	14/No	16/No	No	0	–
7-2	CY(60) + TBI(7.5) + ALG	≥2.0	15	Unknown/Yes	Stomatitis, liver	II	Ext
8	CY(40) + TBI(6) + ATG	0.21† CB	15	38	Stomatitis	0	–
(b)							
9	CY(40) + Flu(180) + ATG	6.2	10	12	No	0	–
10	CY(40) + Flu(150) + ATG	5.2	14	23	Stomatitis, liver	0	–
11	CY(40) + Flu(150) + ATG	3.5	14	31	Stomatitis	0	–
12	CY(40) + Flu(150) + ATG	3.3	11	24	No	0	–
13	CY(40) + Flu(150) + ALG	7.8	9	17	Stomatitis	0	–
14	CY(40) + Flu(150) + ATG	4.1	11	17	Stomatitis	0	–
15	CY(40) + Flu(150) + ATG	3.9	12	24	No	0	–

TNC, total nucleated cells; NE, not evaluable.
*The number in parentheses indicates the dose, and units are Gy for TAI and TBI, mg/m^2 for Flu, and mg/kg for CY.
†Post-thawing cell dose of cord blood.

Table 4. Follow-up characteristics after transplantation; (a) radiation-based conditioning with CY dose modification group; (b) fludarabine-based conditioning without radiation group

No.	Chimerism status (% donor cells) At the first time (source/days after SCT)	At the last time (source/months after SCT)	Complication (months after SCT)	Outcome (months after SCT)
(a)				
1-1	NE	NE	Hepatitis B and C	Rejection. 2nd SCT
1-2	100 (BM/42)	NT	Ovarian dysfunction Hepatic carcinoma (145)	Dead/hepatic carcinoma (147)
2	100 (BM/41)	100 (PB/280)	Prediabetic state	Alive (280)
3	NT	NT	Tongue carcinoma (102)	Dead/tongue carcinoma (114)
4	NT	NT	No	Alive (250)
5	NT	NT	No	Alive (220)
6	100 (BM/14)	100 (BM/2)	No	Dead/accident (154)
7-1	100 (BM/15)	50 (PB/3)	Ovarian dysfunction	PRCA/late rejection, 2nd SCT (eight months)
7-2	100 (BM/15)	100 (PB/60)	Esophagus carcinoma (138) Tongue carcinoma (177)	Alive with cancer disease (234)
8	100 (PB/15)	100 (PB/178)	Pheochromocytoma (147) Prediabetic state	Alive (178)
(b)				
9	100 (BM/12)	100 (PB/105)	No	Alive (129)
10	100 (BM/32)	100 (PB/81)	No	Alive (117)
11	100 (BM/14)	100 (PB/60)	No	Alive (86)
12	98.8 (BM/14)	100 (PB/36)	No	Alive (48)
13	100 (BM/28)	100 (PB/17)	No	Alive (46)*
14	100 (BM/14)	100 (PB/46)	No	Alive (46)
15	100 (BM/14)	95 (PB/41)	No	Alive (41)

NE, not evaluable; BM, bone marrow; NT, not tested; PB, peripheral blood; PRCA, pure red cell aplasia.
*This case was reported by Oshima et al. (21).

(ranges, 3–63% and 13–82%, respectively). The time to an absolute neutrophil count (ANC) $>0.5 \times 10^9$/L was 9–14 days, and to a platelet count of 50×10^9/L was 12–31 days. In patients of b-group, chimerism of their BM mononuclear cells in the early stages after SCT was 98.8–100% of donor type. Although two of these seven patients showed transient mixed chimerism (patient 14 and 15) in the peripheral blood mononuclear cells during the first year after SCT, all patients achieved >95% donor chimerism.

Toxicity

The grade of toxicity was low both in two groups. None of the patients showed grade III/VI RRT (Bearman's criteria) at any evaluation point.

GVHD

Only one patient (patient 7) developed grade II acute GVHD after the second transplant, and chronic GVHD was observed in three patients who developed malignancies as late effects (patient 1, 3 and 7). No patients of b-group had acute and/or chronic GVHD.

Outcome

In patients of a-group, hepatocarcinoma and tongue/esophagus carcinoma were observed in patients 1 and 7, at 12 and 10/14 yr after BMT, respectively. Tongue carcinoma and pheochromocytoma were also observed in patients three and eight, at nine and 12 yr after BMT, respectively. Causes of death in a-group were solid cancer in two of eight, and one died of accident. Two patients suffered from ovarian dysfunction (patient 1 and 7), and two patients are in prediabetic state (patient 2 and 8). None of the b-group required a second SCT (median follow-up, 48 months; range, 41–129 months); all patients are alive with a Lansky/Karnofsky score of 100%, and there are no late side effects such as ovarian failure or other endocrinopathy.

Discussion

Graft rejection, RRT, and severe acute GVHD have been the major causes of SCT failure in FA patients. However, in FA patients, SCT from an HLA-identical sibling donor is generally associated with an excellent outcome when performed before leukemic transformation. The approach used by Gluckman et al. (6), including low-dose

CY (20–40 mg/kg) + TAI/TBI (400–450 cGy), has been the standard SCT conditioning regimen. This conditioning regimen provided good results, with >80% survival at 3–10 yr (7, 8). Non-radiation regimens have been increasingly used for FA patients to reduce the late effects associated with radiation, such as endocrinopathies, infertility, and cataracts.

Bonfim et al. (9) reported using only CY (60 mg/kg) in 43 patients from matched-related SCT donors; Ayas et al. (10) also reported using a CY (60 mg/kg) + ATG regimen without radiation in 34 patients with matched-related donor SCT. Overall survival rate in these studies were 93% (median follow-up, 3.7 yr) and 96.9% (median follow-up, 33.7 months), respectively. However, MacMillan et al. (1) observed a high rate of graft failure in FA patients receiving unrelated donor transplants with T-cell somatic mosaicism, suggesting that the presence of DEB-insensitive T-cells increased the risk of graft rejection.

We showed that there was a linear correlation between the percentage of aberrant metaphases in lymphocytes treated with CY metabolites and those treated with DEB (Fig. 1). On the basis of these data, we suggested that DEB-insensitive cells are also CY-insensitive cells. Therefore, incomplete ablation of DEB-resistant host lymphocytes might increase the risk of graft failure. If patients with 50% or more DEB-insensitive cells are classified as high-mosaic, only 10% of FA patients have been reported to exhibit >50% insensitive cells in IFAR patients (5); however, in our study, among the 78 patients, 24 were high-mosaic (30.8%). A conditioning regimen that exhibits strong cytotoxic activity against lymphocytes and minimized exposure to DNA cross-linking agents may be necessary for Japanese FA patients because the T-cell somatic mosaicism in Japanese population is higher than the Caucasian population. The patients in a-group received high-dose therapy, particularly those who received a second transplant. This might explain the high incidence of cancer. Furthermore, the two patients who developed tongue carcinoma in a-group had oral chronic GVHD. There was a significant association of the oral squamous cell carcinoma with chronic GVHD (11, 12).

In recent years, Flu-containing conditioning regimens for FA have become more popular and have been successfully employed, especially in SCT from alternative donors (2, 13, 14). Flu is an antimetabolite and immunosuppressive agent that is not a DNA cross-linking agent. The first FA patient with leukemic transformation successfully treated by matched sibling BMT following a Flu-based conditioning regimen was reported in 1997 (15). Flu is an attractive and tolerable agent for FA because it is not an alkylating agent and has an antileukemic effect. Furthermore, omission of irradiation from a conditioning regimen has been considered to reduce the late effects. Tan et al. (16) reported that 11 patients with 0–20% DEB-insensitive cells had received a conditioning regimen of CY (20 mg/kg) + Flu (175 mg/m^2) + ATG without irradiation, followed by an infusion of HLA-genotypically identical T-cell-depleted bone marrow or cord blood. Neutrophil engraftment was observed in all patients, but secondary graft failure was observed in one patient. No patients experienced severe RRT or either acute or chronic GVHD, and nine are alive and well at a median follow-up of 2.9 yr. Ertem et al. (17), who used a similar regimen (CY 20 mg/kg + Flu 150 mg/m^2 + ATG), reported successful engraftment in 6 FA patients. Stepensky et al. (18) also reported that a combination of Flu with ATG and low-dose CY without radiation was safe and demonstrated low rejection rates when compared with alternative regimens in patients with FA. After 2000, we selected Flu, a consistent, reduced dose of CY 40 mg/kg, and ATG without radiation as conditioning for HLA-matched sibling donor SCT, and successful engraftment was achieved in all seven patients with stable chimerism, independent of the T-cell somatic mosaicism. They are all disease free and in good clinical condition without any late side effects.

Age at SCT (>10 yr) is also a risk factor of acute GVHD (19). To prevent moderate-to-severe acute GVHD, we have used the combination of CsA plus short-term MTX in patients older than 10 yr in matched sibling donor SCT and used tacrolimus plus short-term MTX after alternative donor transplant (13). No patients had severe MTX toxicity, and none of them died of acute GVHD in either a- and b-group. Bonfim et al. (9) have also used CsA plus short-term MTX in HLA-matched related donor SCT in 43 FA patients. They found a very low incidence of acute GVHD and suggested that less regimen-related tissue damage enabled the delivery of all four scheduled MTX doses in the majority of patients; MTX dosing was previously shown to be important in controlling the incidence of GVHD (20). These combinations of GVHD prophylaxis including MTX considerably decreased the severe acute GVHD for FA patients, which could have varied in accordance with ethnic differences.

Our study indicates that a Flu-based regimen without radiation enabled successful engraftment

in HLA-matched sibling donor SCT even in FA patients with evidence of T-cell somatic mosaicism. It is very difficult to compare the outcome of two different regimens as there are two second transplant in a-group and the major differences between the follow-up times of two groups. Long-term follow-up and larger studies are warranted to confirm the high engraftment rates and reduction of post-transplant malignancies.

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Disclosure

The authors declare no competing financial interests.

Author contributions

M. Yabe, H. Yabe: concept/design; M. Yabe, T. Shimizu, T. Morimoto, T. Koike, H. Takakura, H. Tsukamoto, K. Muroi, K. Asami, K. Oshima, M. Takata, T. Yamashita: data analysis/interpretation; S. Kato, H. Yabe: approval of the article.

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nature immunology

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

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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].

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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

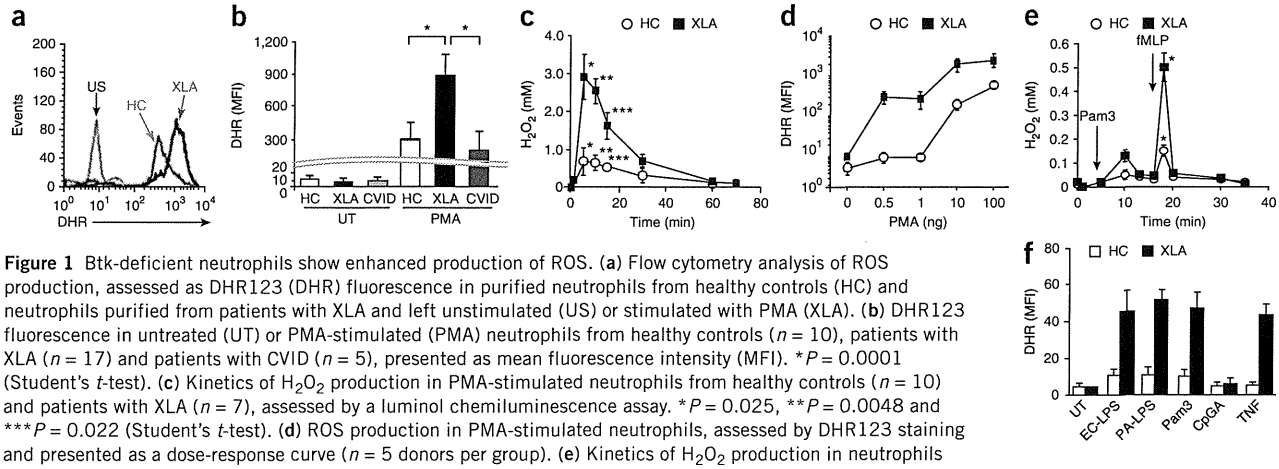

Figure 1 Btk-deficient neutrophils show enhanced production of ROS. (**a**) Flow cytometry analysis of ROS production, assessed as DHR123 (DHR) fluorescence in purified neutrophils from healthy controls (HC) and neutrophils purified from patients with XLA and left unstimulated (US) or stimulated with 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**).

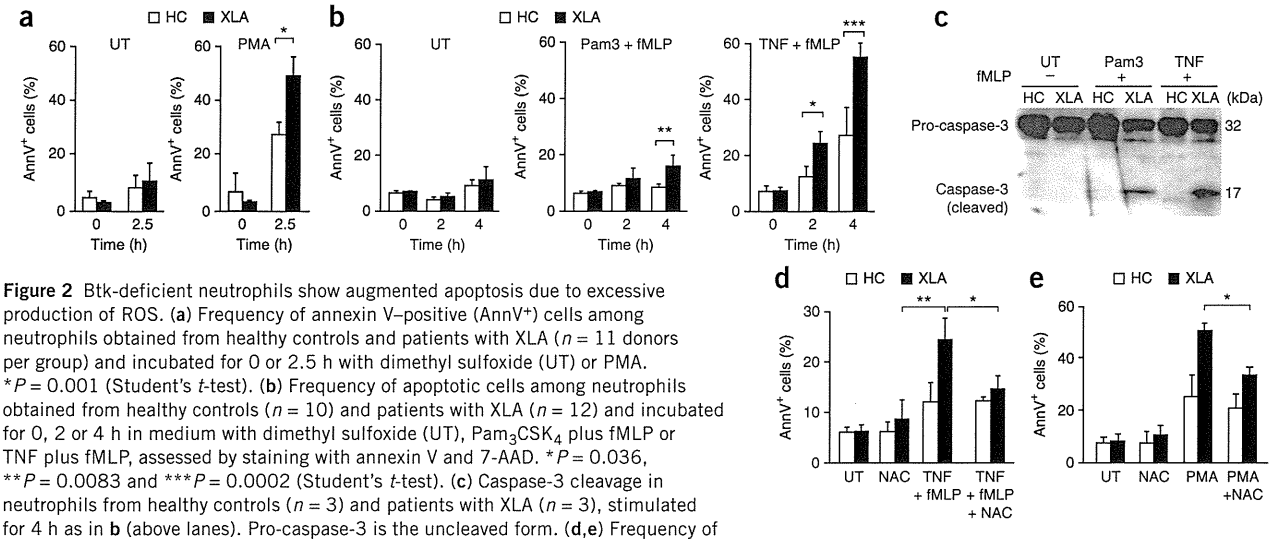

Figure 2 Btk-deficient neutrophils show augmented apoptosis due to excessive production of ROS. (**a**) Frequency of annexin V–positive ($AnnV^+$) cells among neutrophils obtained from healthy controls and patients with XLA ($n = 11$ donors per group) and incubated for 0 or 2.5 h with dimethyl sulfoxide (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

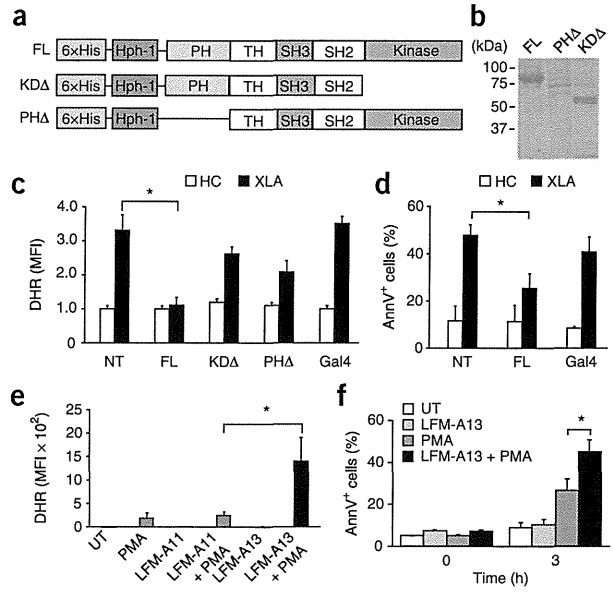

Figure 3 Excessive production of ROS and apoptosis in neutrophils from patients with XLA are abrogated by transduction of Hph-1–tagged full-length recombinant Btk but not by Hph-1–tagged Btk with deletion of the kinase or PH domain. (**a**) Hph-1–tagged Btk constructs: full-length Btk (FL); Btk with 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.

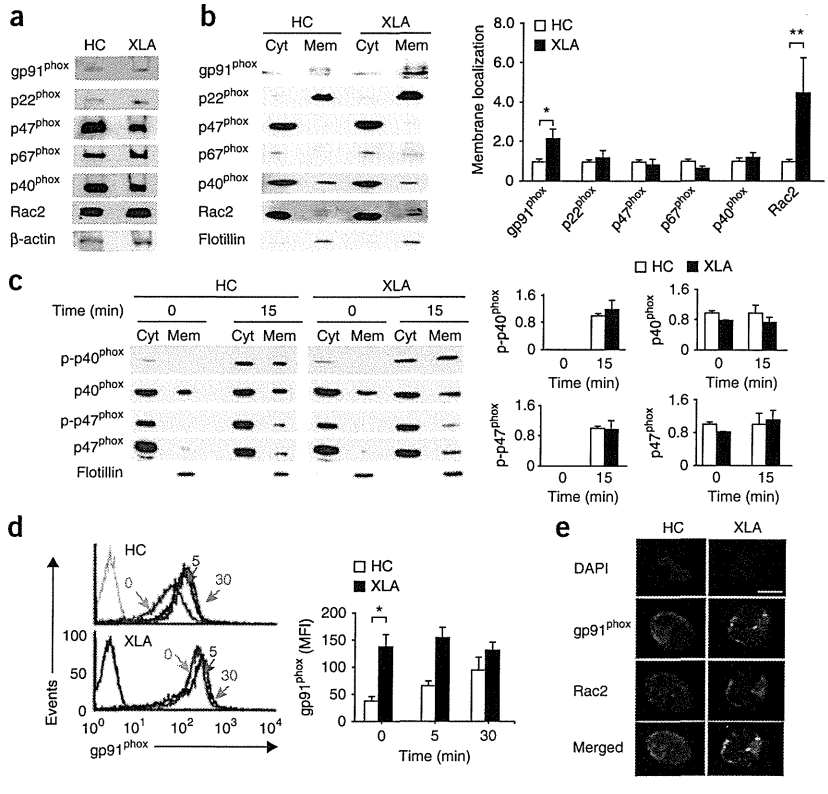

Figure 4 Btk-deficient neutrophils show targeting of Rac2 to the plasma membrane, colocalization of Rac2 with gp91^{phox} and higher membrane expression of gp91^{phox}. (**a**) Immunoblot analysis of the components of the NADPH oxidase complex in neutrophils from a healthy control and a patient with 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

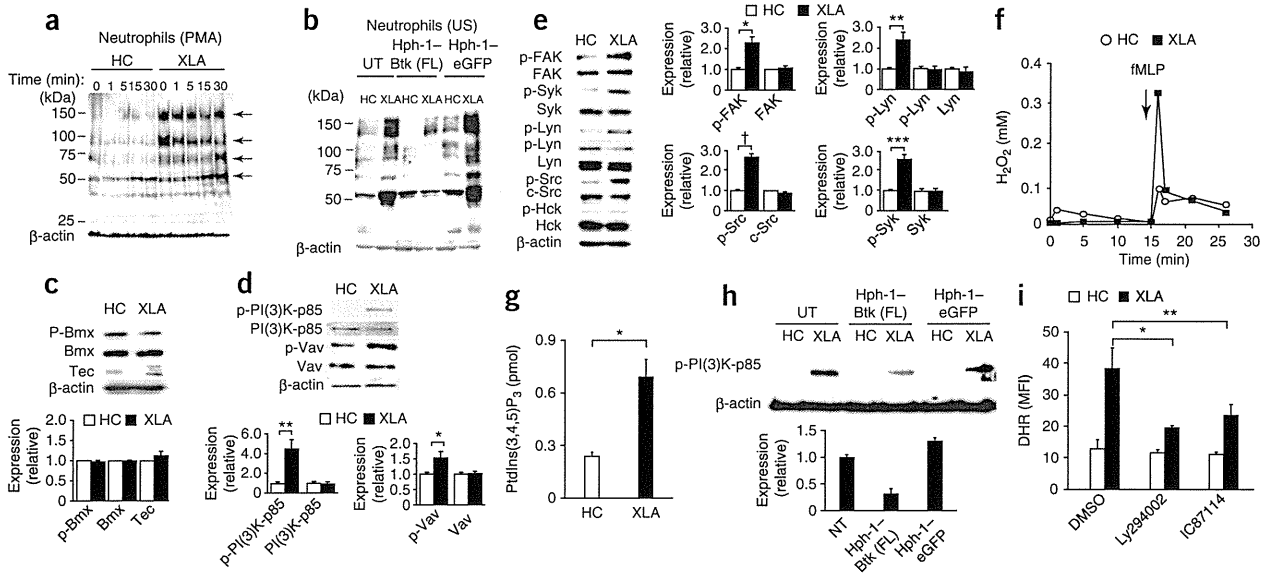

Figure 5 Btk-deficient neutrophils have higher baseline activity of PTKs and PI(3)K, which is reversed by transduction of recombinant Btk protein. (**a**) Immunoblot analysis of phosphorylated tyrosine in lysates of PMA-stimulated neutrophils from healthy controls ($n = 5$) and patients with XLA ($n = 7$). 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**).

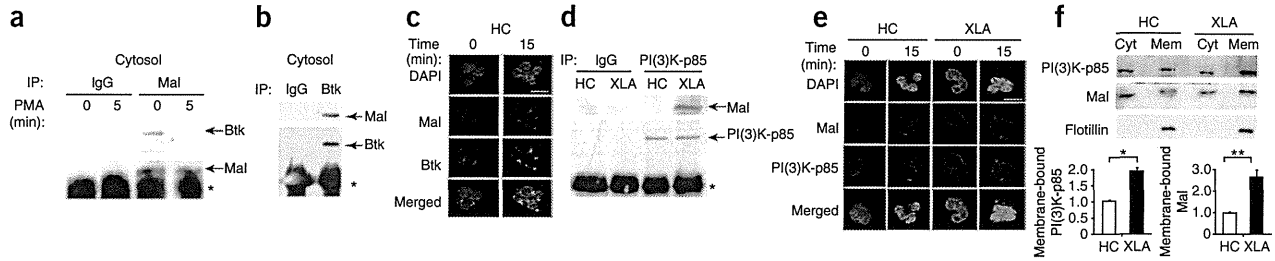

Figure 6 Mal in neutrophils from healthy controls associates with Btk in the resting state and translocates to the plasma membrane after stimulation, whereas Mal associates with PI(3)K at the plasma membrane in Btk-deficient neutrophils. (**a**,**b**) Coimmunoprecipitation analysis of Btk and Mal in 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**).

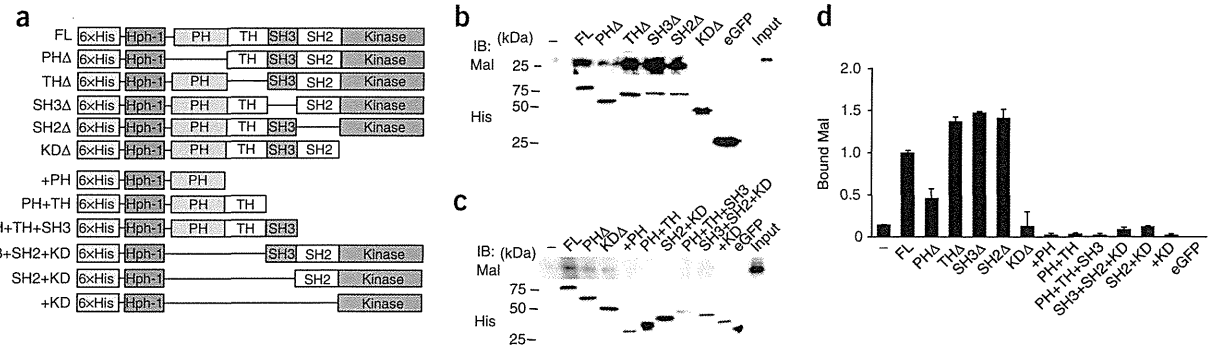

Figure 7 Btk associates with Mal at the PH and kinase domains. (**a**) Hph-1–tagged Btk constructs: full-length Btk (FL); Btk mutants with deletion of the PH domain (PHΔ), Tec homology (THΔ), SH3 domain (SH3Δ), SH2 domain (SH2Δ) or kinase domain (KDΔ); and Btk mutants with truncation retaining (+) only 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

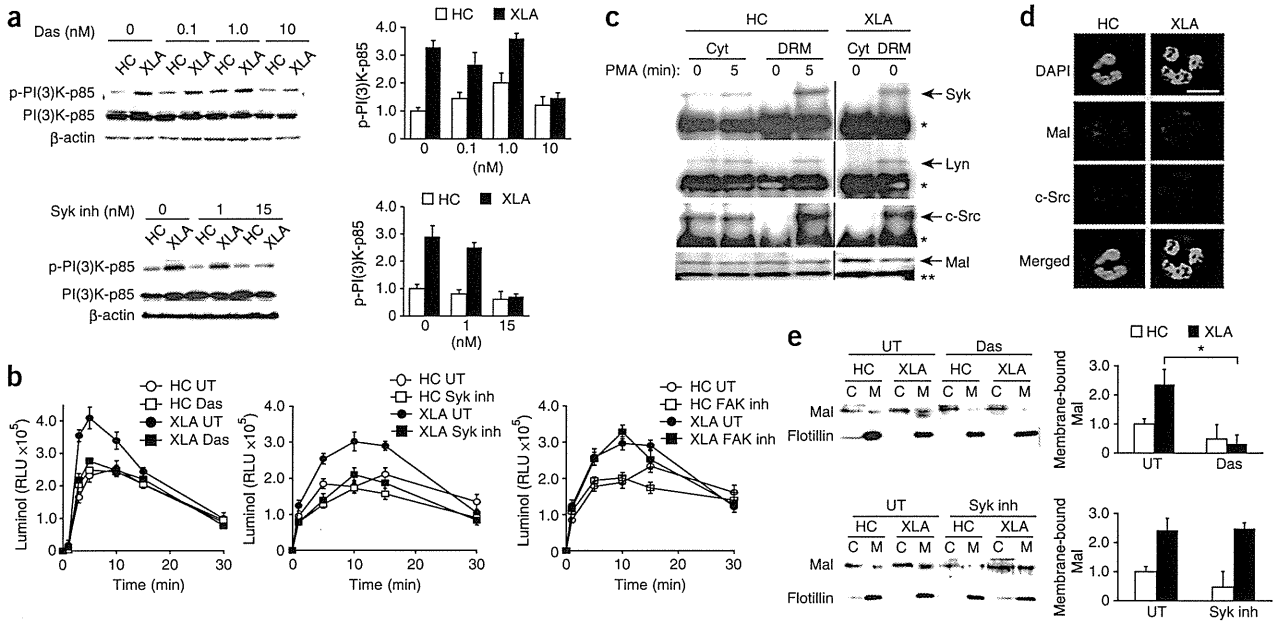

Figure 8 SFKs and Syk are involved in PI(3)K activation and the augmented production of ROS, whereas SFKs are involved in the membrane localization of Mal, in Btk-deficient neutrophils. (**a**) Immunoblot analysis (left of each pair) of total and phosphorylated PI(3)K-p85 in neutrophils from healthy 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**).

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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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