Immunity

Supplemental Information

The Neutrophil Btk Signalosome Regulates Integrin Activation during Sterile Inflammation

Stephanie Volmering, Helena Block, Mark Boras, Clifford A. Lowell, and Alexander Zarbock

Figure S1

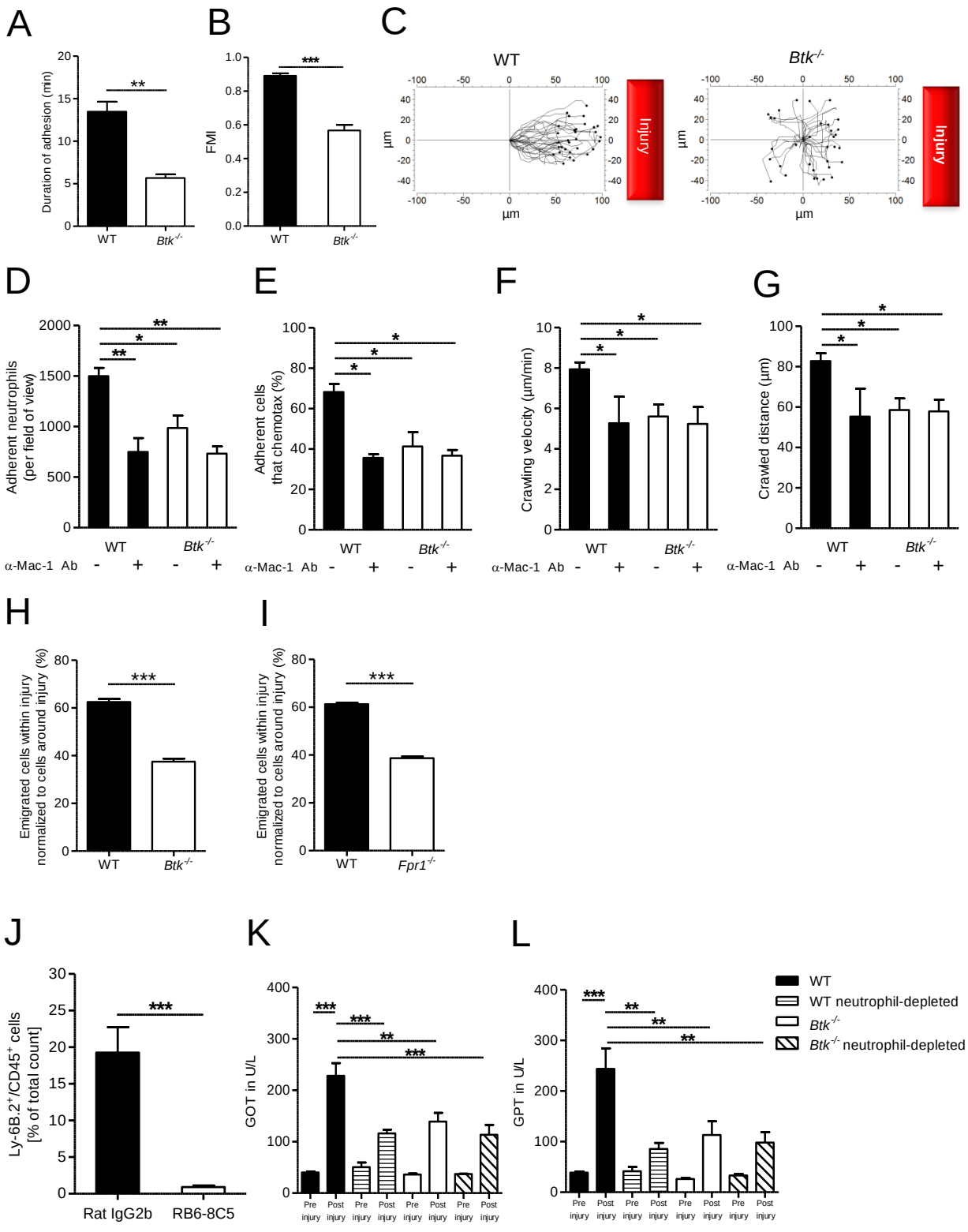

A
Duration of adhesion (min)
WT
Btk-/-
B
FMI
C
WT
Btk-/-
Injury
µm
D
Adherent neutrophils (per field of view)
α-Mac-1 Ab
E
Adherent cells that chemotax (%)
F
Crawling velocity (µm/min)
G
Crawled distance (µm)
H
Emigrated cells within injury normalized to cells around injury (%)
I
Fpr1-/-
J
Ly-6B.2+/CD45+ cells [% of total count]
Rat IgG2b
RB6-8C5
K
GOT in U/L
Pre injury
Post injury
L
GPT in U/L
WT
WT neutrophil-depleted
Btk-/-
Btk-/- neutrophil-depleted

Figure S1, related to Figure 1: Btk, Fpr1 and Mac-1 are required for neutrophil recruitment during sterile inflammation and protection of neutrophil-dependent liver damage. (A) Duration of adhesion of WT and *Btk*$^{-/-}$ neutrophils within the necrotaxis zone and (B) FMI of migrating neutrophils 2.5 h after sterile liver injury. (C) Representative trajectory plots (30 cells from 3 independent experiments) of WT and *Btk*$^{-/-}$ neutrophils migrating toward the site of injury. (D-G) Neutrophil recruitment during sterile liver injury w/ or w/o blocking Mac-1 antibody. (D) Number of adherent neutrophils per field of view in WT and *Btk*$^{-/-}$ mice in response to focal hepatic necrosis 4 h after sterile injury. (E) Percentage of neutrophils that chemotax toward the injury site, (D) neutrophil crawling velocity and (E) crawled distance 2.5 h post injury. (H-I) Isolated murine bone marrow from WT, *Btk*$^{-/-}$ or *Fpr1*$^{-/-}$ mice was labeled with celltrackers (green or orange) and co-injected prior to sterile liver injury in a ratio 1:1 into a WT recipient. Number of normalized emigrated cells within the necrotic area 4 h after injury for (H) WT/*Btk*$^{-/-}$ or (I) WT/*Fpr1*$^{-/-}$. (J-L) Depletion of endogenous neutrophils was achieved via an intraperitoneal injection of 7.5 µg mAb directed against murine neutrophils (RB6-8C5) 24 h prior the experiment. (J) Efficiency of neutrophil depletion in percentage as determined by flow cytometry. Quantity of the transaminases GOT (K) and GPT (L) in U/L were determined in serum pre and 4 h post liver injury in WT and *Btk*$^{-/-}$ mice and in neutrophil-depleted WT and *Btk*$^{-/-}$ mice. Depicted are mean + SEM; n≥3 individual mice/group; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. FMI, (Forward Migration Index).

Figure S2

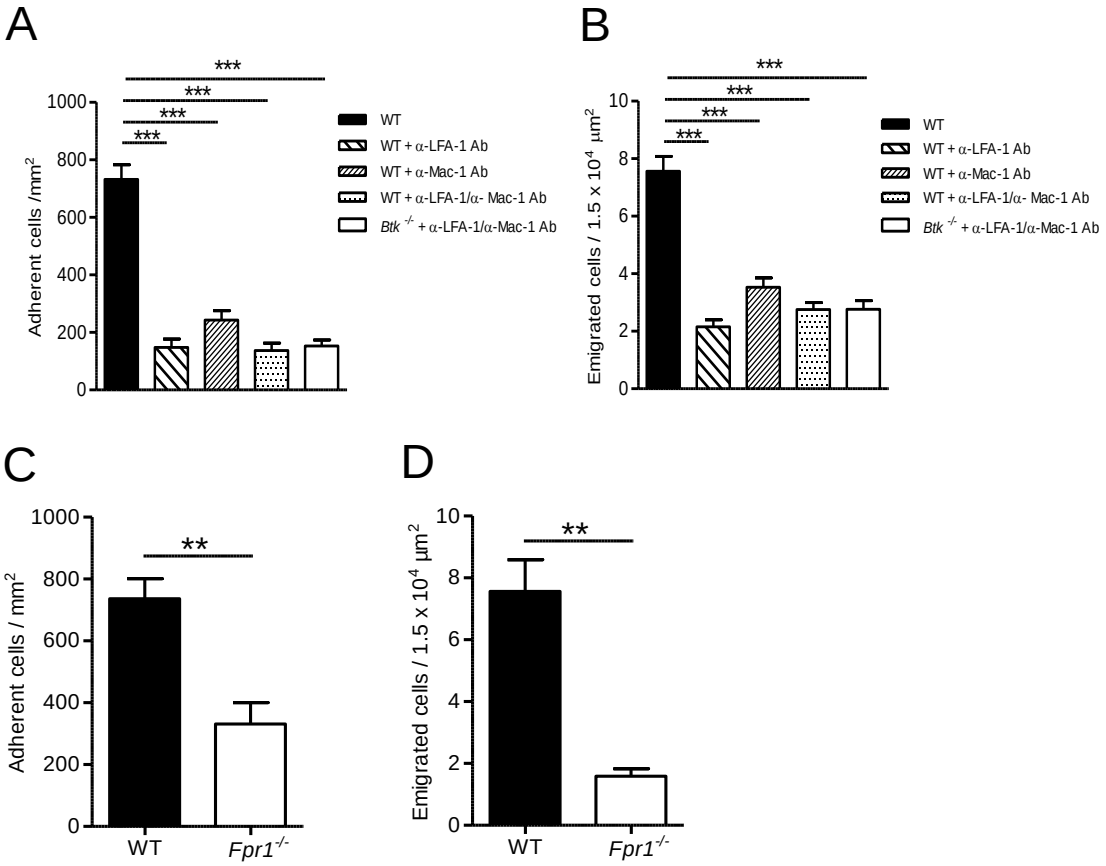


Figure S2, related to Figure 2: Fpr1 and the β_2-integrins LFA-1 and Mac-1 are required for fMLF-mediated neutrophil recruitment in the murine cremaster muscle. Intravital microscopy of postcapillary venules in the murine cremaster was performed in WT, *Btk*$^{-/-}$ and *Fpr1*$^{-/-}$ mice. (A+C) Number of adherent cells per mm^2 and number of (B+D) transmigrated cells per 1.5 x 10^4 µm^2 4 h after intrascrotal injection of fMLF or after injection of blocking antibodies (LFA-1, Mac-1 or both) prior to fMLF injection. Depicted are mean + SEM; n=4 individual mice/group; ** $p < 0.01$; *** $p < 0.001$.

Figure S3

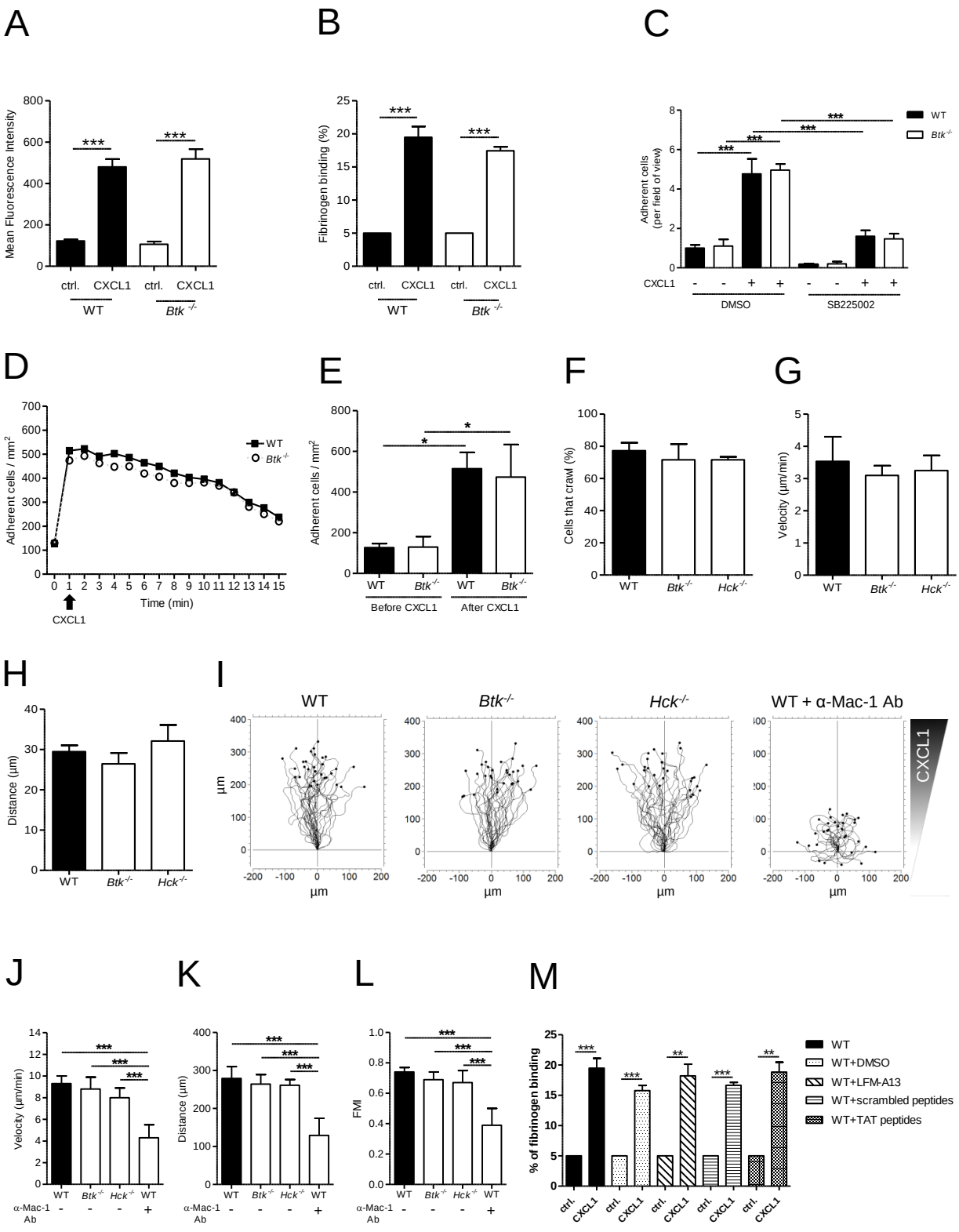
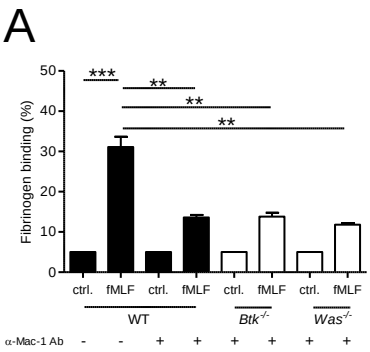
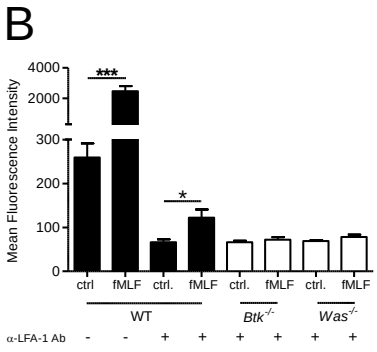
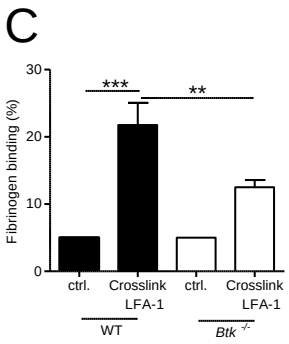

A
Mean Fluorescence Intensity
ctrl.
CXCL1
WT
Btk -/-
B
Fibrinogen binding (%)
C
Adherent cells (per field of view)
CXCL1
DMSO
SB225002
D
Adherent cells / mm2
Time (min)
E
Before CXCL1
After CXCL1
F
Cells that crawl (%)
G
Velocity (µm/min)
H
Distance (µm)
I
WT + α-Mac-1 Ab
CXCL1
J
K
L
FMI
M
% of fibrinogen binding
WT+DMSO
WT+LFM-A13
WT+scrambled peptides
WT+TAT peptides

Figure S3, related to Figure 2 + 3: Btk and Hck are not involved in chemokine-induced signaling. (A) Binding of ICAM-1 or (B) fibrinogen by unstimulated and CXCL1-stimulated WT and *Btk*$^{-/-}$ neutrophils was determined by flow cytometry. (C) Chemokine-induced adhesion of WT and *Btk*$^{-/-}$ neutrophils on fibrinogen under flow after injection of vehicle control or the selective CXCR2-inhibitor SB225002. (D) GPCR-induced arrest of neutrophils in postcapillary venules of WT and *Btk*$^{-/-}$ mice following CXCL1 injection (*i.v.*). (E) Respective statistics 1 min after CXCL1 injection. (F-H) Intravascular crawling of WT, *Btk*$^{-/-}$ and *Hck*$^{-/-}$ neutrophils during superfusion with MIP-2. (F) Percentage of adherent cells that crawled, (G) crawling velocity and (H) crawled distance. (I) Representative trajectory plots (30 cells from 3 independent experiments) of chemotaxing WT, *Btk*$^{-/-}$ and *Hck*$^{-/-}$ neutrophils towards indicated CXCL1 gradient *in vitro*. (J) Velocity, (K) migration distance and (L) FMI of WT and *Btk*$^{-/-}$ neutrophils. (M) Fibrinogen binding of WT neutrophils stimulated with CXCL1 after treatment with vehicle, scrambled or TAT peptides, LFM-A13 or their respective controls. (A-H, J-M) Depicted are mean + SEM; n=3 individual mice/group; * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. FMI, (Forward Migration Index).

Figure S4





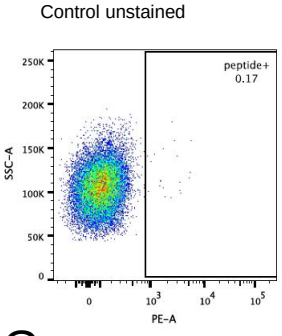


D

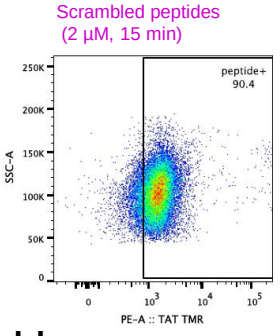
Titration of peptides

Scrambled peptides concentration	Time	Incorporated peptides in %	MFI
0.1 µM	15 min	1.3	171
1 µM	15 min	73.7	1378
2 µM	**15 min**	**89.2**	**1824**
5 µM	15 min	98.2	2412
TAT peptides concentration	**Time**	**Incorporated peptides in %**	**MFI**
0.1 µM	15 min	2.2	226
1 µM	15 min	77.2	1570
2 µM	**15 min**	**90.4**	**1913**
5 µM	15 min	99.3	3042
Scrambled peptides concentration	**Time**	**Incorporated peptides in %**	**MFI**
0.1 µM	30 min	1.1	164
1 µM	30 min	85.8	1719
2 µM	30 min	94.1	2001
5 µM	30 min	99.6	2760
TAT peptides concentration	**Time**	**Incorporated peptides in %**	**MFI**
0.1 µM	30 min	2.3	206
1 µM	30 min	74.3	1625
2 µM	30 min	96.2	2358
5 µM	30 min	99.8	3539

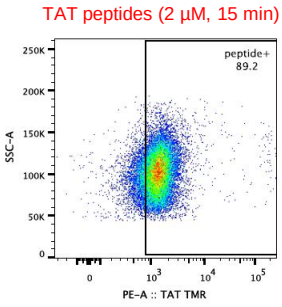
E



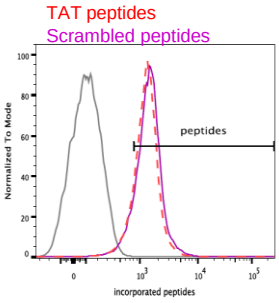
F



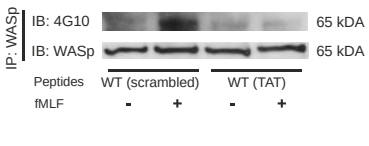
G

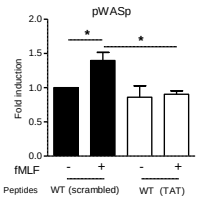


H

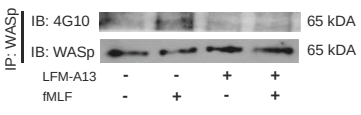


I

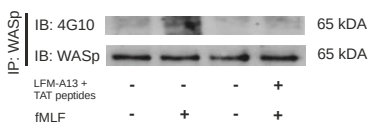




J



K



L

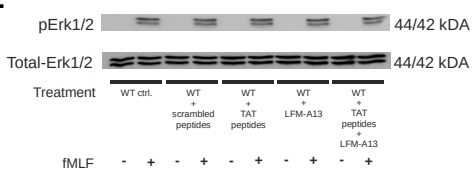


Figure S4, related to Figure 5: Eliminating or blocking Btk and WASp results in disturbed signaling events. (A) Fibrinogen binding of unstimulated or fMLF-stimulated WT, *Btk*$^{-/-}$ and *Was*$^{-/-}$ neutrophils w/ or w/o blocking Mac-1 antibody. (B) ICAM-1 binding of unstimulated or fMLF-stimulated WT, *Btk*$^{-/-}$ and *Was*$^{-/-}$ neutrophils in presence of a blocking LFA-1 antibody. (C) Fibrinogen binding of WT and *Btk*$^{-/-}$ neutrophils after crosslinking of LFA-1 w/o any other stimulus. (D-H) Murine bone marrow-derived neutrophils were isolated and incubated with either scrambled peptides or TAT peptides conjugated with TMR. Optimal working concentration was determined by flow cytometry. (I-L) After preincubation with peptides, LFM-A13 or a combination of both neutrophils were stimulated with fMLF or left unstimulated. Then lysates were prepared and either immunoprecipitated (IP) with an antibody against WASp followed by immunoblotting with a phosphotyrosine (4G10) antibody, or total-WASp antibody (I-K) or immunoblotted with a phospho-p44 and 42 MAPK (Erk1 and 2) antibody or total-p44 and 42 MAPK (Erk1 and 2) antibody (L). (A-H+I) Depicted are mean + SEM of $n \geq 3$ independent performed experiments; * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. (I-L) Depicted are representative Western Blots of $n \geq 3$ independent performed experiments.

Figure S5

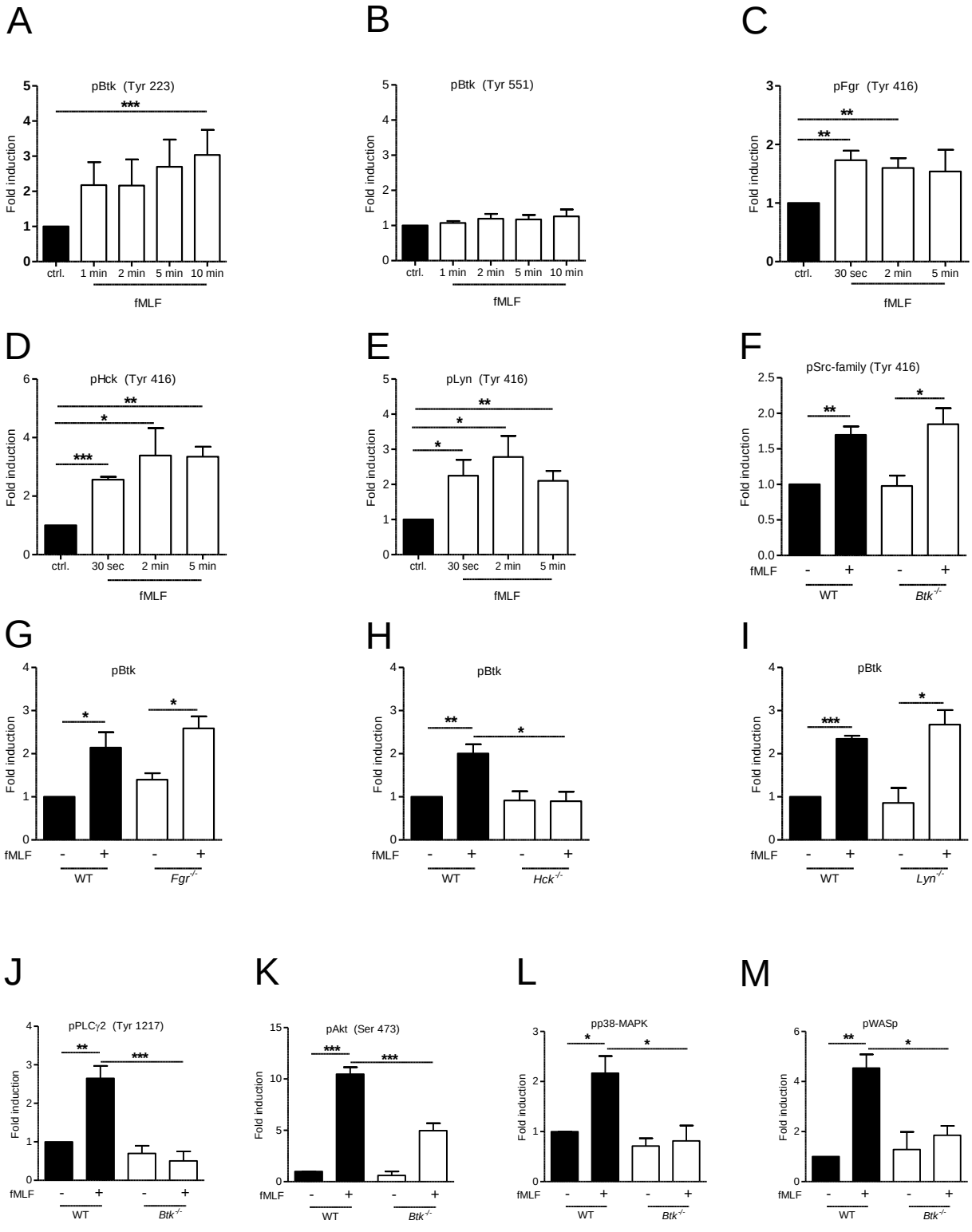

A
pBtk (Tyr 223)
Fold induction
ctrl.
1 min
2 min
5 min
10 min
fMLF
B
pBtk (Tyr 551)
C
pFgr (Tyr 416)
30 sec
D
pHck (Tyr 416)
E
pLyn (Tyr 416)
F
pSrc-family (Tyr 416)
WT
Btk-/-
G
pBtk
Fgr-/-
H
Hck-/-
I
Lyn-/-
J
pPLCγ2 (Tyr 1217)
K
pAkt (Ser 473)
L
pp38-MAPK
M
pWASp

Figure S5, related to Figure 6: Densitometric analysis of immunoprecipitations and Western blots. (A-M) Densitometric quantification of Western blots and immunoprecipitations demonstrating the phosphorylation of (A) Btk at tyrosine 223, (B) Btk at tyrosine 551, (C) Fgr at tyrosine 416, (D) Hck at tyrosine 416 and (E) Lyn at tyrosine 416 after fMLF stimulation in WT neutrophils for indicated time-points. Densitometric quantification of Western blots and immunoprecipitations comparing (F) the phosphorylation of Src family kinases at tyrosine 416 in WT and *Btk*$^{-/-}$ neutrophils, (G) the phosphorylation of Btk in WT and *Fgr*$^{-/-}$ neutrophils, (H) in WT and *Hck*$^{-/-}$ neutrophils and (I) in WT and *Lyn*$^{-/-}$ neutrophils following fMLF stimulation. Densitometric quantification of Western Blots and immunoprecipitations comparing the phosphorylation of (J) PLCγ2 at tyrosine 1217, (K) Akt at tyrosine 473, (L) p38-MAPK and (M) WASp following fMLF stimulation in WT and *Btk*$^{-/-}$ neutrophils. Depicted are mean + SEM of n≥3 independent performed experiments; * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$.

Figure S6

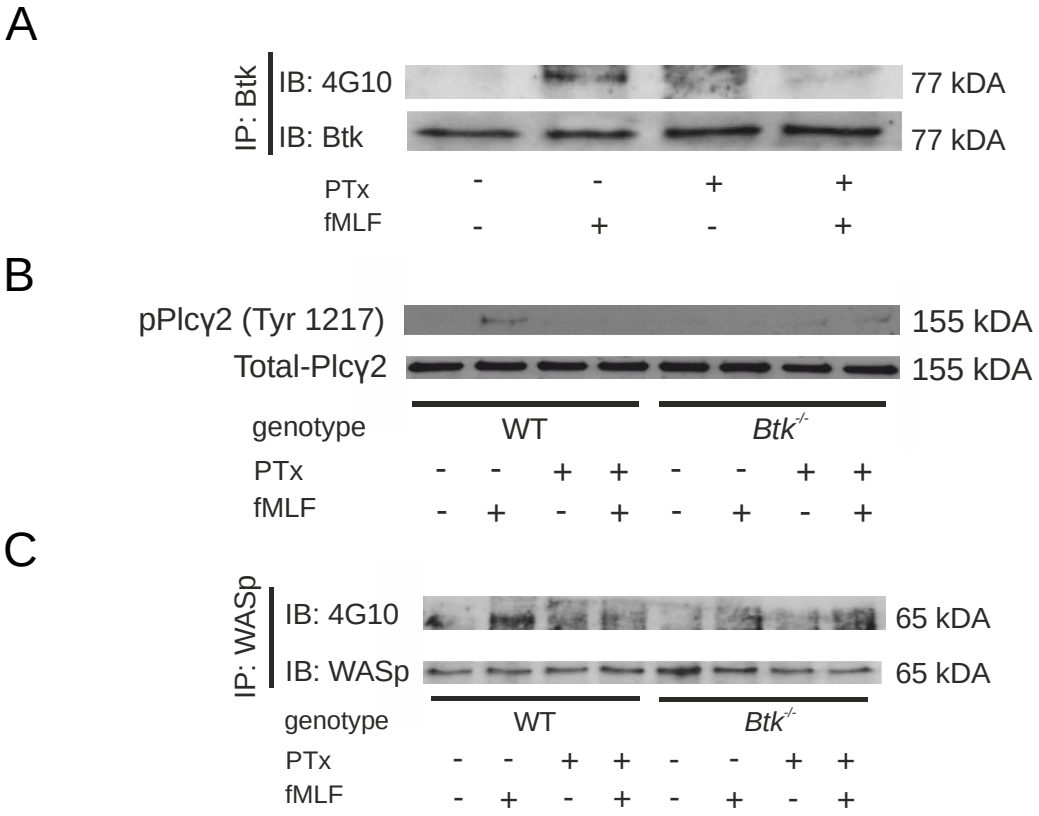


Figure S6, related to Figure 6: Phosphorylation of Btk, PLCγ2 and WASp is dependent on Gα_i-signaling. (A-C) Bone marrow-derived neutrophils were isolated, pretreated with PTx (400 ng/ml) and either left unstimulated or stimulated with fMLF. Lysates of WT and *Btk*$^{-/-}$ neutrophils were immunoprecipitated (IP) with an antibody against Btk followed by immunoblotting with a phosphotyrosine (4G10) antibody and total-Btk antibody (A), immunoblotted with antibodies to phospho-PLCγ2 (Tyr 1217) and total-PLCγ2 (B) or immunoprecipitated (IP) with an antibody against WASp followed by immunoblotting with a phosphotyrosine (4G10) antibody and total-WASp antibody (C). Depicted are representative Western Blots of n=3 independent performed experiments

Figure S7

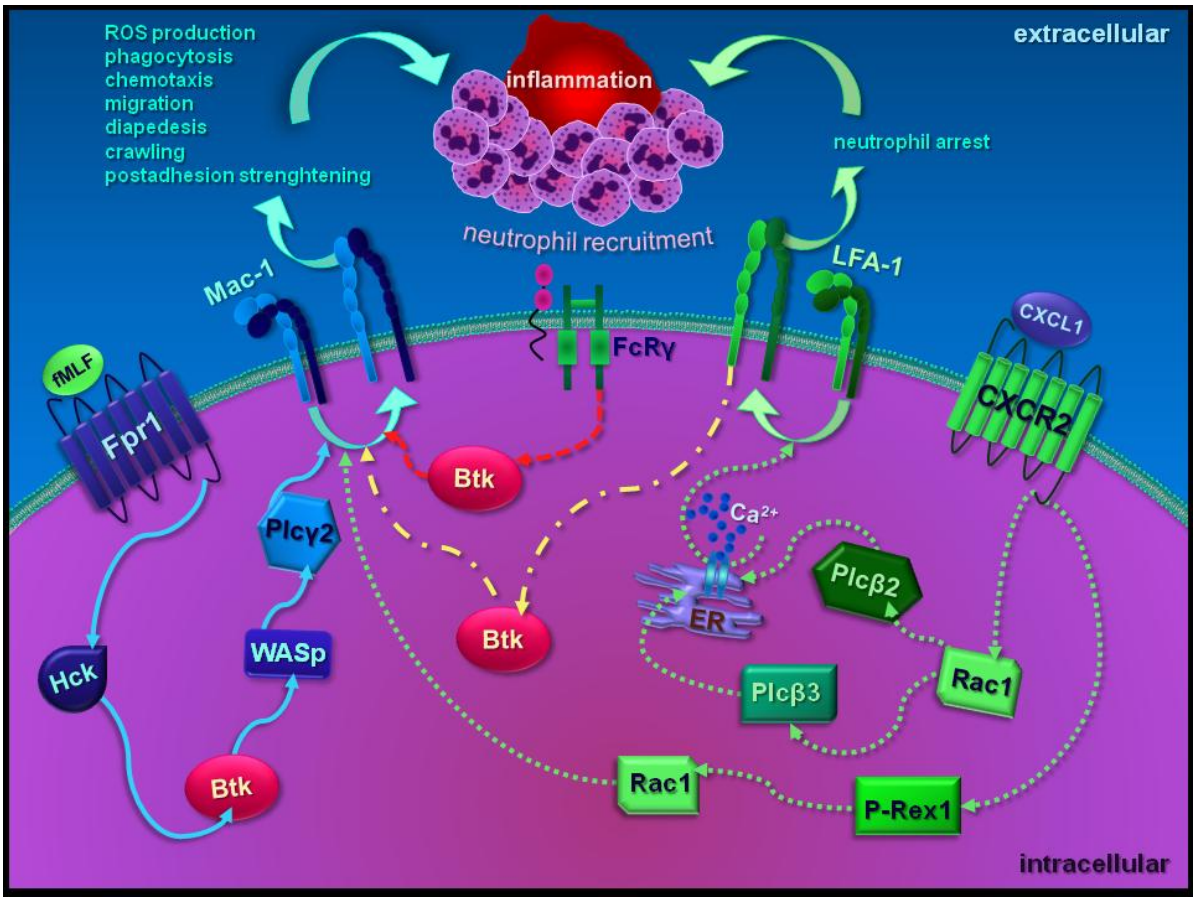


Figure S7, related to Figure 1-7: Schematic model of Btk signaling during sterile inflammation. After fMLF binding to its respective receptor Fpr1, Src family kinase Hck gets activated and triggers the phosphorylation of Btk. Btk initiates the activation of Mac-1 via WASp and Plcγ2, consequently of inside-out and outside-in signaling events resulting in neutrophil recruitment. Additionally, Btk is involved in FcRγ signaling, thereby mediating Mac-1 activation and outside-in signaling. Btk is also required for LFA-1 activation-dependent Mac-1 activation. After CXCL1-CXCR2 engagement, the signaling pathway divides into two different branches.

On the one hand, Rac1 activates either Plcβ2 or Plcβ3 leading to Ca^{2+} release, subsequently to LFA-1 activation and neutrophil arrest (Block et al., 2015). On the other hand, P-Rex1 activates Rac1 resulting in Mac-1 activation (Herter et al., 2013). Btk is not involved in CXCR2-mediated signaling. Blueish arrows= Fpr1-signaling; greenish arrows= CXCR2-signaling; reddish arrows= FcRγ-signaling; yellowish arrows= LFA-1-dependent signaling. ER=endoplasmic reticulum.

Supplemental Experimental Procedures

Animals

The 8- to 15-weeks-old C57Bl/6 (Janvier, Le Genest Saint Isle, France), LysM-GFP^{+} (Faust et al., 2000), *Btk*$^{-/-}$ (The Jackson Laboratory), *Fgr*$^{-/-}$ (Zarbock et al., 2008), *Hck*$^{-/-}$ (Zarbock et al., 2008), *Lyn*$^{-/-}$ (Zarbock et al., 2008), *Plcg2*$^{-/-}$ (Wang et al., 2000), *Was*$^{-/-}$ (Snapper et al., 1998) and Lyz2-eGFP^{+}-*Btk*$^{-/-}$ mice were housed in the specified pathogen-free (SPF) facility. The Animal Care and Use Committees of the University of Muenster (Muenster, Germany) approved all animal experiments. For some experiments, chimeric mice were generated by performing bone marrow transplantation as described previously (Mueller et al., 2010). In brief, bone marrow was isolated from gene-deficient mice (*Fgr*$^{-/-}$, *Hck*$^{-/-}$, *Lyn*$^{-/-}$, *Plcg2*$^{-/-}$ or *Fpr1*$^{-/-}$ mice). These cells were injected *i.v.* (2×10^6/recipient) into lethally irradiated WT mice (9.5 Gy). Experiments were performed 6–8 weeks after bone marrow transplantation. *Fpr1*$^{-/-}$ mice were a gift from Oliver Söhnlein (Munich).

Sterile inflammation induced by focal hepatic necrosis after bone marrow reconstitution, neutrophil depletion or injection of blocking Mac-1 antibody

In order to perform reconstitution experiments for liver IVM, bone marrow from WT and *Btk*$^{-/-}$ mice or WT and *Fpr1*$^{-/-}$ mice was isolated and then labeled with 2 mM green (CMFDA) or orange (CMRA) cell trackers (Life Technologies), according to the manufacturer's instructions. Cells were counted and co-injected *i.v.* in a ratio 1:1 WT/*Btk*$^{-/-}$ or WT/*Fpr1*$^{-/-}$ in WT mice. In this way, recruitment of both WT and knockout cells were

assessed in the same recipient. The number of emigrated cells was determined within the necrotic area 4 h after injury and related to the number of cells around injury.

For blocking experiments, WT and Btk-deficient mice received an intravenous injection of 30 µg anti-Mac-1 (clone M1/70, eBioscience) 30 min prior to liver injury. Neutrophil depletion experiments were conducted as previously described (Maus et al., 2002). In brief, mice received an intraperitoneal injection of 7.5 µg anti-Gr-1 mAb (RB6-8C5) or respective isotype control (Rat IgG2b). After 24 h, the levels of the transaminases GOT and GPT pre and 4 h post focal hepatic necrosis in serum in WT and *Btk*$^{-/-}$ mice was determined with a high-volume hematology analyzer (ADVIA, Siemens). Successful neutrophil depletion (more than 90%) was verified by flow cytometry. Peripheral blood was collected, red blood cells were removed by hypotonic lysis and samples were stained with FITC-conjugated anti-Ly-6B.2 (AbD Serotec) and PE-conjugated anti-CD45 (BD Biosciences). The percentage of total cell counts of Ly-6B.2^{+}/CD45^{+} cells was determined.

Intravascular *in vivo* crawling assay

By intravital microscopy, intravascular crawling of neutrophils was examined as described previously (Phillipson et al., 2006). Briefly, the anti-Gr-1 antibody (clone RB6-8C5), labeled with Alexa Fluor 488 (Molecular Probes), was injected via the cannulated carotid artery prior to the experiment. Following preparation and exteriorization, the *M. cremaster* was superfused with fMLF (10 µM, Sigma-Aldrich) or rmMIP-2 (5 nM, R&D Systems) and time-lapse microscopy was performed for 2 h.

The number of adherent cells was determined, and the neutrophil crawling velocity and crawled distance were analyzed using SlideBook software (Intelligent Imaging Innovations).

Btk TAT-fusion peptides

In brief, isolated bone marrow-derived neutrophils were incubated with TAT-fusion peptides (GenScript, 2 µM, 37°C, 15 min) and subsequently used in the *in vitro* crawling assay. Cells were incubated either with a Btk TAT–fusion peptide, directed against the tyrosine 223 of Btk within the SH3 domain plus a TAT sequence (YGRKKRRQRRRGKVVALYDYMPM) or a scrambled control peptide (YGRKKRRQRRRGMYKAYDVPVLM). Both peptides were TMR-conjugated (N-terminal modification) for experiments ensuring the intake of peptides.

***In vitro* crawling assay**

In vitro crawling was performed as described previously (Phillipson et al., 2006; Herter et al., 2013). Briefly, using a discontinuous Percoll gradient (72%, 64%, 54%; 4°C) neutrophils were purified and applied to polystyrene cell culture dishes pre-coated with murine blood serum or ICAM-1 (2.5 µg/ml). Cells were incubated for 15 min in a 5% CO_2 atmosphere at 37°C. Subsequently they were stimulated with fMLF for 5 min. In some experiments, blocking (Btk-TAT-fusion peptide) or scrambled control peptides were added to the neutrophils during the 15 min of incubation. The cell culture dishes were then

placed onto a parallel plate flow chamber (GlycoTech) and time-lapse microscopy was performed. To investigate *in vitro* crawling, neutrophils were subjected to time-lapse microscopy without flow (5 min), during flow (shear stress 2 dyn/cm^2, 5 min) and after flow (5 min). Neutrophil crawling was analyzed separately for each timeframe using Ibidi's Chemotaxis and Migration Tool (Ibidi).

MAC-1 activation in isolated human neutrophils

Human neutrophils were isolated from healthy donor blood. Neutrophils were resuspended in PBS (1 mM $CaCl_2$/$MgCl_2$) and stimulated with 10 µM fMLF for 1, 5, 15 or 20 min at 37°C in the presence of the PE-conjugated reporter antibody recognizing the activated conformation of human MAC-1 (clone CBRM1/5, BioLegend) or a isotype control antibody (mouse IgG1κ). After stimulation, neutrophils were fixed with 1% PFA in cold PBS and analyzed using flow cytometry (BD FacsCanto, Becton Dickinson). For blocking experiments, neutrophils were preincubated with a Btk inhibitor LFM-A13 (25 µM, Merck Millipore) or a Phospholipase C inhibitor U-73122 (10 µM, Sigma-Aldrich) for 30 min at 37°C.

Phagocytosis

Fluorescent polystyrene beads (1 µm, Polysciences) were washed twice with PBS, resuspended in 1 ml PBS containing 100 µg/ml mouse IgG (Sigma), and incubated for 1 h at room temperature with gentle agitation. Beads were washed twice with PBS and

subsequently incubated with bone marrow-derived neutrophils at a 10:1 ratio in PBS containing $CaCl_2$ (1 mM), $MgCl_2$ (1 mM) and HEPES (10 mM, pH 7.4) with or without 1 µM fMLF. After 10 min incubation on ice, cells were incubated at 37°C for indicated time-points. Cells were then washed three times with ice-cold PBS and fixed with 4% PFA. Phagocytosis was analyzed by confocal laser scanning microscopy:

Fibrinogen-coated bead binding

Fluorescent polystyrene beads were washed twice in 0.1 M borate buffer (pH 8.5) and absorbed with 150 µg/ml fibrinogen (Sigma) in borate buffer over night at room temperature with gentle agitation. Beads were washed and resuspended in borate buffer containing 10 µg/ml BSA for 30 min at room temperature. After washing twice, beads were resuspended in PBS and incubated with cells at a 20:1 ratio in PBS containing $CaCl_2$ (1 mM), $MgCl_2$ (1 mM) and HEPES (10 mM, pH 7.4). Cells were incubated for 10 min at 37°C without any stimulus, with 1 µM fMLF, 10 µg/ml IgG immune complexes or combination of both. Immune complexes were generated by incubating 120 µg/ml rabbit anti-chicken ovalbumin (Sigma) with 30 µg/ml ovalbumin (Sigma) in PBS for 1 h at 37°C and over night at 4°C, forming a visible insoluble immune complex precipitate after centrifugation. Quantitative binding of fibrinogen-coated bead was determined by flow cytometry.

Superoxide production

Superoxide release of bone marrow-derived neutrophils was measured by a superoxide dismutase-inhibitable cytochrome c reduction assay. Cells were applied to uncoated or 20 µg/ml polyRGD (Sigma) precoated Immunolon-4 HBX 96-well plates with $CaCl_2$ (1 mM), $MgCl_2$ (1 mM) and cytochrome c (0.1 mM) in the presence or absence of fMLF (3 µM). 45 units Superoxide Dismutase (SOD, Sigma) was added to each control sample to confirm that reduction of cytochrome c is dependent on superoxide anion radicals. Plates were immediately inserted into a plate reader preheated to 37°C and absorbance at 550 and 490 nm was recorded every 10 min for 90 min. For calculation each measurement was corrected by its SOD control measurement.

***In vitro* chemotaxis**

Purified bone marrow-derived neutrophils were seeded on chemotaxis µ-slides (Ibidi) precoated with 50 µg/ml fibronectin (Sigma) in RPMI medium. A gradient of rmCXCL1 (1.15 µM) or fMLF (1 µM) colored with Patentblue (100 ng/ml) was applied and chemotaxis was recorded with a Nikon Eclipse Ti equipped with an incubation module (37°C, 5% CO_2) by using time-lapse microscopy at 3 frames per minute for 30 min. Blocking experiments were performed in presence of monoclonal antibodies against Mac-1 (10 µg/ml, eBioscience). Cells were manually tracked with Manual Tracking (ImageJ) and analyzed with Ibidi's Chemotaxis and Migration Tool (Ibidi).

for average velocity and accumulated distance. Forward migration index (FMI) represents the directness toward the gradient.

Autoperfused adhesion flow chamber

To investigate chemokine-induced adhesion, rectangular glass capillaries (20x200 µm) were coated with rmP-selectin (50 µg/ml) and mfibrinogen (50 µg/ml) or rmP-selectin and mfibrinogen in combination with rmCXCL1 (25 µg/ml) for 2 h and then blocked for 1 h using 1% casein. One end of the capillary was connected to a PE 10 tubing (Becton Dickinson) and surgically inserted into a mouse carotid artery. To control wall shear stress, the other end of the capillary was connected to a PE50 tubing (Becton Dickinson). For blocking experiments, animals were pretreated with the selective CXCR2 antagonist SB225002 (30 µg/mouse), administered *i.v.* 30 min before performing experiments. Representative fields of view were recorded after an initial perfusion period of 6 min and the number of adherent cells per field of view was determined.

Biochemical Assays

Bone marrow-derived WT, *Btk*$^{-/-}$, *Fgr*$^{-/-}$, *Hck*$^{-/-}$ or *Lyn*$^{-/-}$ neutrophils were isolated and resuspended in PBS (containing 1 mM each $CaCl_2$ and $MgCl_2$). Neutrophils were stimulated with fMLF (10 µM for indicated time-points) or were left unstimulated. For some experiments neutrophils were treated prior to stimulation with scrambled or TAT peptides, with the Btk inhibitor LFM-A13 (25 µM, Merck Millipore), pertussis toxin (400 ng/ml, Calbiochem) or respective controls at 37°C. Subsequently neutrophils were lysed in

RIPA buffer, and lysates were boiled with sample buffer (10 min, 95°C) or incubated with Sepharose A/G beads (Santa Cruz Biotechnology, Inc.) and anti-Btk, anti-Fgr, anti-Hck, anti-Lyn and anti-WASp (Santa Cruz Biotechnology, Inc.) antibody for 4 h at 4°C. Beads were washed four times, and bound proteins were eluted by adding boiling sample buffer. Cell lysates and immunoprecipitates were run on 10% SDS-PAGE and immunoblotted using antibodies against phosphotyrosine (4G10; EMD Millipore), PLCγ2, phospho-PLCγ2 (Tyr1217), Akt, phospho-Akt (Ser473), p38-MAPK, phospho-p38-MAPK, phospho-Btk (y223 and y551), phospho-Src (Tyr416), Erk1/2 or phospho-Erk1/2 (all from Cell Signaling Technology) and Btk, Fgr, Hck, Lyn, and WASp (Santa Cruz Biotechnology, Inc.). Immunoblots were developed using an ECL system (GE Healthcare). Densitometric quantifications were conducted using ImageJ software.

Rap1 activation assay

Rap1 activation was investigated as previously described (Grommes et al. 2014). Briefly, bone marrow-derived WT and *Btk*$^{-/-}$ neutrophils were isolated and resuspended in PBS (containing 1 mM each $CaCl_2$ and $MgCl_2$). Neutrophils were stimulated with fMLF (10 µM) for 30 sec or were left unstimulated and immediately lysed with EDTA-free ice-cold lysis buffer. Recombinant effector protein GST-RalGDS-RBD was produced in *E.coli* (BL21) and immobilized to Glutathion Sepharose 4B (GE Healthcare). GTP-bound Rap1 (Rap1-GTP) was precipitated from whole cell lysates using 25 µg GST effector fusion protein and subsequently immunoblotted against Rap1 (Santa Cruz).

Statistics

All data are presented as mean ± standard error of the mean (SEM) and were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Differences between the groups were evaluated by one-way ANOVA with post-hoc Bonferroni correction or Student's *t* test where appropriate. The number of experiments is indicated by the n values and a P value of <0.05 was considered statistically significant, where *=$p<0.05$, **= $p<0.01$, and ***= $p<0.001$. Each experiment was independently performed at least three times.

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