Neutrophil FcγRIIA promotes IgG-mediated glomerular neutrophil capture via Abl/Src kinases

Mark J. Miller  
Washington University School of Medicine in St. Louis

Samantha L. Hamilton  
Washington University School of Medicine in St. Louis

Lihua Yang  
Washington University School of Medicine in St. Louis

Spencer P. Pittman  
Washington University School of Medicine in St. Louis

et al

Follow this and additional works at: https://digitalcommons.wustl.edu/open_access_pubs

Recommended Citation  
https://digitalcommons.wustl.edu/open_access_pubs/6202
Neutrophil FcγRIIA promotes IgG-mediated glomerular neutrophil capture via Abl/Src kinases


Department of Pathology, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts, USA. Division of Infectious Diseases, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri, USA. Woodruff School of Mechanical Engineering and Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia, USA. Donald Danforth Plant Science Center, St. Louis, Missouri, USA. Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts, USA. Division of Rheumatology, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts, USA.

The kidney glomerular capillaries are frequent sites of immune complex deposition and subsequent neutrophil accumulation in post-infectious and rapidly progressive glomerulonephritis. However, the mechanisms of neutrophil recruitment remain enigmatic, and there is no targeted therapeutic to avert this proximal event in glomerular inflammation. The uniquely human activating Fc receptor FcγRIIA promotes glomerular neutrophil accumulation and damage in anti–glomerular basement membrane–induced (anti-GBM-induced) glomerulonephritis when expressed on murine neutrophils. Here, we found that neutrophils are directly captured by immobilized IgG antibodies under physiological flow conditions in vitro through FcγRIIA-dependent, Abl/Src tyrosine kinase–mediated F-actin polymerization. Biophysical measurements showed that the lifetime of FcγRIIA-IgG bonds increased under mechanical force in an F-actin–dependent manner, which could enable the capture of neutrophils under physiological flow. Kidney intravital microscopy revealed that circulating neutrophils, which were similar in diameter to glomerular capillaries, abruptly arrested following anti-GBM antibody deposition via neutrophil FcγRIIA and Abl/Src kinases. Accordingly, inhibition of Abl/Src with bosutinib reduced FcγRIIA-mediated glomerular neutrophil accumulation and renal injury in experimental, crescentic anti-GBM nephritis. These data identify a pathway of neutrophil recruitment within glomerular capillaries following IgG deposition that may be targeted by bosutinib to avert glomerular injury.

Introduction

Infiltration of myeloid-derived cells in the glomerulus of the kidney is a key pathogenic event in autoimmune-mediated glomerulonephritis (GN), a leading cause of end-stage renal disease (1–4). Glomerular neutrophil accumulation occurs in Goodpasture’s (or anti–glomerular basement membrane [anti-GBM]) disease, infection-related GNs, and proliferative lupus nephritis (1–3). Neutrophils are one of the earliest leukocyte subsets to be recruited to deposited autoantibodies and are known to promote glomerular injury (5). Yet, the molecular mechanisms driving immune complex–mediated (IC-mediated) neutrophil recruitment in the glomerulus, where capillaries are the major sites of leukocyte recruitment, remain poorly understood.

Low-affinity FcγRs, receptors for IgG that avidly bind ICs, are key determinants of leukocyte influx and injury following glomerular IgG deposition in models of proliferative GN. Mice deficient in the FcγR γ chain (γ−/−), essential for the expression and function of all murine activating FcγRs, exhibit a marked reduction in leukocyte accumulation and renal injury in acute and rapidly progressive anti-GBM antibody–induced GN, and in a lupus-prone NZB/W background (6, 7). The low-affinity activating FcγRs on neutrophils in mice and humans structurally differ. Mice express FcγRII and FcγRIV that rely on the FcγR γ chain, while humans express the uniquely human homologs FcγRIIA and FcγRIIB, respectively. FcγRIIA has an immunotyrosine activating motif (ITAM) in its cytoplasmic domain, and is a mediator of destructive antibody-based inflammation in autoimmunity, while FcγRIIB is a glycosylphosphatidylinositol-linked (GPI-linked) receptor whose physiological functions remain to be fully elucidated (8). Generation of mice expressing either of these human FcγRs selectively on neutrophils of γ−/− mice lacking their endogenous activating FcγRs allowed us to assess the role of the human FcγRs, and specifically FcγRII on neutrophils, in disease pathogenesis (9). The surface expression level of FcγRIIA on transgenic murine neutrophils and human neutrophils was comparable, while the surface expression level of FcγRIIB on transgenic neutrophils was similar to that of FcγRIIA but lower than that on human neutrophils (9). Neutrophil human FcγRIIA or FcγRIIB expression restored glomerular neutrophil influx in γ−/− mice following crescentic anti-GBM–induced nephritis. However, only FcγRIIA sustained neutrophil influx and promoted glomerular injury, suggesting that FcγRIIA on neutrophils is a key molecular link...
Here, we explored the mechanisms driving neutrophil capture by immobilized ICs in vitro and assessed the physiological relevance of our findings by intravital microscopy and in a model of crescentic, anti-GBM nephritis. Using a large small-molecule screen on primary neutrophils, we identified the Abl/Src inhibitor bosutinib as the lead inhibitor of several FcγRIIA functions and both Abl1 and Src kinases as key mediators of FcγRIIA-mediated neutrophil capture on immobilized ICs under flow. Abl1 silencing or Src inhibition prevented FcγR-mediated F-actin polymerization, which was required for IC-mediated neutrophil capture. Biophysical approaches showed that FcγR binding to IgG was strengthened under mechanical force and required F-actin polymerization, which may enable rapid FcγR-mediated capture under flow.

Figure 1. A small-molecule screen identifies bosutinib as the lead inhibitor of FcγRIIA functions in neutrophils. (A) FcγRIIA+/γ–/– mouse bone marrow neutrophils (BMNs), suspended in fluorescent ROS probes with library compounds (light blue, at 10 μM), were loaded on BSA/anti-BSA IC-coated plates, and fluorescence was read 1 hour later. The Src inhibitor PP2 (red) and the diluent DMSO (vehicle, deep blue) were positive and negative controls, respectively. A profile with representative “hits” (arrows) is shown. (B) Bosutinib (Bos) and PP2 at indicated doses were evaluated and plotted relative to vehicle (0 nM) (n = 3). (C) BMNs were preincubated with mouse anti–human FcγRIIA and then Bos, PP2, or vehicle (Veh). ROS generation was evaluated upon addition of F(ab′)-anti-mouse IgG in a luminol-based assay. A representative profile is shown (left). The average peak level of ROS was normalized to vehicle (right) (n = 3). (D) Bos-pretreated BMNs were stimulated with PMA, and ROS generation was assessed as in C (n = 3). (E) The number of adherent (left) and spread (right) cells on BSA or IC under static conditions of BMNs pretreated with vehicle (–) or Bos (n = 3). (F) Adhesion under shear flow (0.5 dyn/cm²) to BSA- or IC-coated coverslips, of vehicle- or Bos-pretreated BMNs. The data are presented as the average fold induction normalized to the average value of the vehicle (–)/BSA control (n = 3). Neutrophils were treated with 100 nM Bos or 1 μM PP2 unless indicated otherwise (n = 3). Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, 1-way ANOVA followed by Dunnett’s multiple comparison test for B and C to assess dose responsiveness of the drugs compared with vehicle, and for E and F.
mode of IC-mediated neutrophil capture was evident by intravital microscopy. Endogenous mouse neutrophils rapidly arrested in glomerular capillaries in response to anti-GBM deposition in the absence of prior rolling or crawling. Simultaneous adoptive transfer of FcγR-deficient and FcγRIIA-expressing neutrophils or vehicle-treated and bosutinib-treated neutrophils demonstrated the importance of FcγRIIA and Abl/Src in this process, respectively. Bosutinib treatment inhibited neutrophil accumulation and renal injury in mice subjected to crescentic anti-GBM nephritis, a model of Goodpasture’s syndrome (14). Thus, bosutinib, a drug currently used for the treatment of chronic myelogenous leukemia (CML) patients (15, 16), may be repurposed to inhibit IgG-mediated glomerular neutrophil accumulation, potentially one of the earliest steps in IgG-mediated renal inflammation.

Results

FcγRIIA-mediated neutrophil functions are inhibited by bosutinib, identified in a high-throughput screen. We established an automated, neutrophil reactive oxygen species–based (ROS-based) high-throughput drug screen in primary neutrophils for FcγRIIA inhibitors using plate-bound ICs (BSA and anti-BSA) and a small-molecule library in which FDA-approved drugs are highly represented (50%). Mature neutrophils isolated from γ−/− mice showed minimal H2O2 generation following their incubation with plate-bound ICs, while the same expressing human FcγRIIA (FcγRIIA+/γ−) gave a robust ROS response (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI94039DS1). Next, the ability of library compounds to inhibit FcγRIIA+/γ−–induced ROS was evaluated. Vehicle control (DMSO) and PP2, an inhibitor of Src family tyrosine kinases known to signal downstream of FcγRs (17, 18), served as negative and positive controls, respectively. The screen of 8,483 compounds yielded 84 hits (1% hit rate), defined as those that have an inhibitory potential of ≥95% of PP2 (Figure 1A). Of the 30 compounds cherry-picked for a secondary screen (Table 1), the most potent was bosutinib, with an IC50 of 50–100 nM, which approximated that of PP2 (Figure 1B). Bosutinib is a dual Abl/Src inhibitor currently used in CML patients to inhibit Bcr-Abl, a fusion protein that leads to constitutively active Abl1 (19). Adhesion-dependent ROS generation (20), as occurs in the primary screen with plate-bound ICs, also relies on the CD18 integrin Mac-1 (Supplemental Figure 1B). This is consistent with previous observations that Mac-1 activation by FcγRs sustains neutrophil adhesion (21, 22), which in our case may occur following binding of activated Mac-1 to BSA (23) in the anti-BSA/BSA ICs. To discern the effects of bosutinib on FcγRIIA alone, ROS was evaluated after FcγRIIA cross-linking of neutrophils in suspension; bosutinib reduced ROS generation with an IC50 of 10–50 nM (Figure 1C) while having no effect on ROS generated after PMA treatment (Figure 1D), which directly activates the NADPH oxidase (24). FcγR engagement leads to the release of enzymes from granules (8) and neutrophil extracellular traps (NETs) (3, 25–27), a network of DNA fibers extruded by neutrophils. Bosutinib reduced FcγRIIA-mediated release of enzymes (Supplemental Figure 1C), albeit reduced cell adhesion may also contribute to these results. Bosutinib also inhibited IC-induced NET release but not receptor-independent NETosis induced by the calcium ionophore ionomycin (Supplemental Figure 1D).

Next, the effect of bosutinib on FcγR-dependent neutrophil adhesion to plate-bound ICs under static and physiological flow conditions was evaluated. Bosutinib reduced the adhesion and spreading of FcγRIIA+/γ− neutrophils on ICs under static conditions (Figure 1E), which is both FcγR and Mac-1 dependent (21, 22). Importantly, bosutinib significantly reduced FcγRIIA+/γ− neutrophil tethering to immobilized ICs under physiological flow (Figure 1F), which is strictly FcγR dependent (10, 11) and may serve as a surrogate for neutrophil accumulation following intravascular IC deposition. Similarly, human neutrophils treated ex vivo with bosutinib exhibited a significant reduction in FcγRIIA-mediated (but not PMA-mediated) ROS generation (IC50 of 1–5 nM) (Figure 2, A and B), release of enzymes (Supplemental Figure 1E), adhesion and spreading on ICs under static conditions (Figure 2C), and neutrophil capture on plate-bound ICs under flow (Figure 2D). Likewise, the Abl inhibitors imatinib and nilotinib, which, unlike bosutinib, do not affect Src activity (28), reduced human neutrophil FcγRIIA-mediated ROS generation (Figure 2B) and adhesion to ICs under static (Figure 2C) and flow (Figure 2D) conditions. This suggests that Abl proteins play a key role in these FcγR functions. The Src kinase inhibitor PP2 also reduced ROS generation (Figure 2A) and neutrophil adhesion to ICs under static conditions (Figure 2C) as expected (18). The aforementioned studies evaluating the capture of human neutrophils on immobilized ICs were largely dependent on FcγRIIB (10, 11), which is GPI-linked but still signals via nonreceptor tyrosine kinase (29). To interrogate the effects of bosutinib specifically on FcγRIIA-mediated capture in human neutrophils, we evaluated neutrophil adhesion to TNF-activated human dermal microvascular endothelial cells (HDMECs) coated with ICs in situ, which results in an increase in neutrophil adhesion compared with TNF alone that is FcγRIIA and not FcγRIIB dependent (11). As reported (11), ICs increased neutrophil adhesion to TNF-activated endothelial cells, which was abrogated by functional blocking anti-FcγRIIA but not anti-FcγRIIB antibody (Figure 2E). Importantly, human neutrophil pre-treatment with bosutinib or imatinib markedly reduced neutrophil adhesion to TNF-activated/IC-coated HDMECs to levels observed for endothelial cells treated with TNF alone (Figure 2E).

Table 1. List of top 10 biologically active compounds that significantly suppressed mouse bone marrow–derived neutrophil ROS generation induced by immobilized ICs

<table>
<thead>
<tr>
<th>Compounds (1.1 μM)</th>
<th>Mode of action</th>
<th>ROS RLU ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bosutinib</td>
<td>Bcr-Abl and Src-Abl kinase inhibitor</td>
<td>0.033</td>
</tr>
<tr>
<td>A-443654</td>
<td>Akt inhibitor</td>
<td>0.266</td>
</tr>
<tr>
<td>PF43196</td>
<td>FAK and Pyk-2 inhibitor</td>
<td>0.301</td>
</tr>
<tr>
<td>GF109203X</td>
<td>PKC inhibitor</td>
<td>0.338</td>
</tr>
<tr>
<td>BX795</td>
<td>PKC and TBK1 inhibitor</td>
<td>0.396</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>Ryanodine Ca++ receptor activator</td>
<td>0.431</td>
</tr>
<tr>
<td>Lck inhibitor</td>
<td>Lck inhibitor</td>
<td>0.462</td>
</tr>
<tr>
<td>Parachlorophenol</td>
<td>Topical antibacterial</td>
<td>0.606</td>
</tr>
<tr>
<td>Cresol</td>
<td>Antiseptic and disinfectant</td>
<td>0.761</td>
</tr>
<tr>
<td>Ro 31-8220</td>
<td>PKC inhibitor</td>
<td>0.778</td>
</tr>
</tbody>
</table>
Figure 2. Bosutinib inhibits FcγRIIA functions in human neutrophils. (A) ROS after FcγRIIA cross-linking (XL) (left) or PMA (right) in bosutinib-pretreated (Bos) and PP2-pretreated neutrophils as in Figure 1, C and D (n = 3). (B) Representative profile of XL-induced ROS after treatment with vehicle or 100 nM Bos, imatinib (Ima), or nilotinib (Nil) (n = 3). (C) Neutrophils pretreated with 100 nM of the indicated compounds, 1 μM PP2, or vehicle (DMSO, −) on BSA- or IC-coated plates, stained with fluorescent phalloidin, and quantitated for adherent cells (left) and spread cell area (right) (n = 3). Representative images are shown. Original magnification, ×600. (D) The number of adherent cells pretreated as in C and drawn across BSA- or IC-coated plates at 1.0 dyn/cm² was calculated and is presented as the fold induction compared with vehicle (−)/BSA control (n = 3, except for Nil, n = 2). (E) HDMECs were treated without (−) or with (+) TNF and/or anti-CD105 plus a secondary antibody (2°ab). Neutrophils pretreated with anti-FcγRIIIB (3G8), FcγRIIA (IV.3), or isotype (Iso) controls (left panel), or with 100 nM Bos or Ima (right panel), were drawn across HDMECs at 1.5 dyn/cm². The number of rolling and adherent cells was assessed. Fold induction was relative to untreated HDMECs (n = 3). (F) XL- or PMA-induced ROS in blood leukocytes from 3 CML patients receiving Bos and matched normal volunteers (Ctrl). A representative ROS profile (left panel) and average fold induction relative to the XL (Ctrl) sample (right panel) are shown (n = 3). (G) CML and control leukocytes were analyzed as in D at 1.0 and 1.5 dyn/cm². Fold induction is relative to the control sample on BSA (Ctrl/BSA) (n = 3). All data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, 1-way ANOVA followed by Dunnett’s multiple comparisons test for A to assess dose responsiveness of the drugs compared with vehicle and also for C–E and G, and by 2-tailed unpaired t test in F.
Figure 3. Role for Abl kinases in FcγRIIA-mediated neutrophil functions. (A) Differentiated HL-60 cells pretreated with vehicle (–), bosutinib (Bos), imatinib (Ima), nilotinib (Nil), or PP2 adherent to ICs or BSA under flow. Fold induction is relative to vehicle (–) on BSA (n = 3). (B) A representative Western blot of cells expressing Abl1 (Ab1β #01 and #00) or control shRNA and analyzed for Abl or β-actin (loading control) (n = 3). ROS in cells in B subjected to FcγRIIA cross-linking (XL) or treated with PMA. The peak level was normalized to control shRNA cells (n = 3). (C) Cells on BSA- or IC-coated plates were stained for actin, and the number of adherent cells (left) and the area of a single spread cell (right) were calculated (n = 3, except in the right panel, n = 2 for treatment with shRNA #00). (E) Cell adhesion to BSA or IC at 0.35 dyn/cm². Fold induction is relative to control cells on BSA (n = 3). (F) Western blot analysis of indicated proteins with or without FcγRIIA XL for times in seconds. β-Actin is the loading control. One representative of 3 is shown. (G and H) F-actin polymerization analyzed by flow cytometry (mean fluorescence intensity, MFI) at indicated times after XL and staining with NBD-phallacidin. Omission of the primary antibody is the negative control. Shown is 1 representative of 3 experiments for Ab1- or control shRNA-expressing cells (G), and for cells treated with vehicle (–) or indicated inhibitors before XL (H). For A–H, cells were treated with 1 μM of Bcr-Abl inhibitors or PP2, or 3 μM cytochalasin D (cytD). All data are mean ± SEM. *P < 0.05, **P < 0.01, 1-way ANOVA followed by Dunnett’s multiple comparison test for A and C–E.

Thus, Abl/Src inhibition reduced FcγRIIA-mediated adhesion to ICs while sparing the classical adhesion cascade associated with TNF activation of the endothelium (30). To determine whether bosutinib reduces FcγR-mediated activity when administered in vivo, FcγR functions were evaluated in neutrophils from CML patients receiving bosutinib. Total leukocytes in peripheral blood of CML patients and matched normal volunteers were evaluated immediately after red blood cell (rbc) lysis, as bosutinib’s effect on neutrophils is reversible over time (data not shown). The percentage of neutrophils and neutrophil FcγRIIA levels on the surface were similar between samples (Supplemental Figure 2, A and B). An 80% reduction in FcγRIIA-mediated ROS generation was observed in patients compared with normal volunteers, while PMA-induced ROS was similar in both groups (Figure 2F). The interaction of neutrophils from bosutinib-treated patients with immobilized ICs under flow was significantly reduced compared with the interaction of those from the normal donors (Figure 2G). Neutrophils obtained from newly diagnosed, untreated CML patients exhibited a partial reduction in FcγRIIA-IgG interactions. This, together with the observed ability of FcγR to bind ICs under flow, prompted us to determine whether mechanical force increases FcγRIIA-IgG bond lifetimes, referred to as catch bonds (35), which we reasoned would facilitate capture of neutrophils under flow and may require F-actin polymerization. For this, Jurkat cells lacking CD18 integrins (ref. 11 and data not shown). Treatment with bosutinib or other Bcr-Abl inhibitors significantly inhibited HL-60 cell interaction with ICs under flow (Figure 3A) as demonstrated for human neutrophils (Figure 2D). HL-60 cells were transduced with Abl1 shRNAs to 2 independent sequences that stably reduced Abl1 protein compared with the shRNA control (Figure 3B). Both Abl1 shRNAs significantly attenuated FcγRIIA-mediated ROS generation compared with control shRNA (Figure 3C), while PMA-induced ROS was only partially affected (Figure 3C). Abl1 silencing reduced cell adhesion and spreading on ICs under static conditions (Figure 3D), and markedly inhibited adhesion to ICs under flow (Figure 3E). Abl1-silenced cells exhibited a decrease in FcγRIIA-induced phosphorylation of Crkl but not Pyk-2, Vav, ERK1/2, and Akt (Figure 3F), suggesting that Abl1 was not essential for proximal FcγR-mediated signaling events. Thus, the reductions in signaling observed with bosutinib in human neutrophils (Supplemental Figure 3A) likely reflect its known targeting of Src and the STE20 serine/threonine kinases (28, 32).

Among tyrosine kinases, Abl kinases (Abl and the Abelson-related gene, Arg) are unique in being able to directly bind and regulate the polymerization of F-actin (33). FcγRIIA cross-linking increased actin polymerization within seconds in control shRNA–transduced HL-60 cells as assessed by FACS analysis of NBD-phallacidin–stained cells (34). The maximal change in fluorescence intensity, which reflects actin polymerization (Figure 3G), was completely prevented in Abl1-silenced cells (Figure 3G) or following treatment with Bcr-Abl inhibitors (Figure 3H). The Src inhibitor PP2 similarly inhibited F-actin polymerization (Figure 3H). F-actin polymerization was required for FcγRIIA-mediated ROS generation and neutrophil capture by ICs under flow, with a greater requirement for F-actin in the latter. That is, treatment with cytochalasin D, an inhibitor of F-actin polymerization, partially reduced FcγRIIA-induced ROS generation (RLU at peak: vehicle, 1,879 ± 971; 20 μM cytochalasin D, 1,037 ± 483), while it markedly inhibited HL-60 tethering to plate-immobilized ICs (data not shown) as well as human neutrophil interaction with immobilized ICs (Figure 4A) or IC-coated HDMECs (Figure 4B) under flow. However, cytochalasin D had no effect on neutrophil adhesion to ICs under static conditions (Figure 4C). Thus, polymerized actin was not required when the on-rate of FcγR-IgG binding was not limiting, as is the case under static conditions, while it was essential under shear flow, which is governed by the force-dependent off-rate of the FcγR-IgG interactions. This, together with the observed ability of FcγR to bind ICs under flow, prompted us to determine whether mechanical force increases FcγRIIA-IgG bond lifetimes, referred to as catch bonds (35), which we reasoned would facilitate capture of neutrophils under flow and may require F-actin polymerization. For this, Jurkat cells lacking CD18 integrins (36) were engineered to express FcγRIIA to evaluate the role specifically of this FcγR, in the absence of FcγRIIB, in the aforementioned cell responses. FcγRIIA-expressing Jurkat cells bound ICs under flow, and cytochalasin D reduced this binding (Figure 4D) without affecting cell binding to ICs under static conditions (Figure 4E). Importantly, using a biomembrane force probe, we demonstrated that FcγRIIA formed catch bonds with IgG at forces ranging from 5 to 25 pN,
while at higher forces the catch bonds transitioned to slip bonds. Cytochalasin D treatment had no significant effect on bond life-times at 0–8 pN force but markedly inhibited bond stabilization at higher mechanical forces (Figure 4F). Thus, FcγRIIA forms catch bonds with IgG, and this relies on F-actin polymerization. Together, these data demonstrate that FcγRIIA induces F-actin polymerization via Abl1 and Src kinases and that F-actin polymerization supports force-induced FcγRIIA interactions with IgG, which may facilitate neutrophil capture by IgG under flow.

Rapid neutrophil capture within glomerular capillaries following anti-GBM antibody. We exploited 2-photon intravital microscopy to examine neutrophil behavior in the kidney under steady-state conditions and 1.5 hours after administration of rabbit nephrotic antisera, which leads to IgG deposition on the endothelium and GBM and will herein be referred to as anti-GBM antibody. We used LysM-GFP mice to evaluate endogenous neutrophils, focusing on early neutrophil–vessel wall interactions. Notably, neutrophils are readily distinguishable from monocytes and macrophages, as they are 5–10 times brighter than CD11b+, Ly6G– monocytes with the brightest GFP+ cells (>2 logs over background) being preferentially detected with our imaging settings. These cells are more than 95% neutrophils based on flow cytometric analysis (37, 38) and intravital microscopy analysis of LysM-GFP mice injected with a neutrophil-specific anti-Ly6G antibody (1A8) (Supplemental Figure 4, A–C). The right kidney was surgically exteriorized and imaged (39), and tissue perfusion was verified by bright-field observation of robust capillary flow and by injection of Q-dots into the retro-orbital sinus. In both control and anti-GBM–treated mice, neutrophils were visible passing through and arresting transiently (“fluttering”) in glomerular capillaries, which we show approximated the diameter of a neutrophil (Figure 5, A and B). This “fluttering” behavior was also observed with small beads injected i.v. (data not shown), suggesting that hemodynamic flow variations in the tortuous glomerular capillary bed account for this behavior. Time-lapse recordings (13 frames per second) were taken for up to 20 minutes, and the number of arrested neutrophils at \( t = 0 \) and \( t = 9 \) minutes was counted in both groups. In control animals, a few arrested neutrophils were present in glomeruli, but in anti-GBM–treated mice, these numbers increased significantly (Figure 5C). Neutrophil duration time was plotted for each group, and both distributions were clear-
increased proportion of adhesion events in this time period. The mean duration time increased in the anti-GBM group compared with control (Figure 5F), as did the median value (control, 36.09 ± 37.90 seconds; anti-GBM, 80.73 ± 17.40 seconds). The “stop and go” behavior of neutrophils in the glomerular capillaries (Supplemental Videos 1 and 2) was strikingly different from the slow neutrophil rolling and intraluminal crawling observed in larger vessels (20–30 μm) in other organs and inflammatory settings (37, 38). Indeed, neutrophil crawling was observed infrequently, with rates of 4.29 ± 1.1 and 7.2 ± 2.24 cells per glomer-

Despite the qualitative similarities, neutrophils in the anti-GBM group had significantly increased duration times compared with those in the control group (2-tailed Mann-Whitney U, P < 0.01) (Figure 5D). We also compared the shape of the duration time distributions using a 2-sample Kolmogorov-Smirnov (KS) test (Figure 5E). The KS test comparison percentile plot showed that the duration time distributions were significantly different (P < 0.01, D = 0.3813), with the plots diverging quickly at short durations (less than 60 seconds) and the anti-GBM group having an increased proportion of adhesion events in this time period. The mean duration time increased in the anti-GBM group compared with control (Figure 5F), as did the median value (control, 36.09 ± 37.90 seconds; anti-GBM, 80.73 ± 17.40 seconds). The “stop and go” behavior of neutrophils in the glomerular capillaries (Supplemental Videos 1 and 2) was strikingly different from the slow neutrophil rolling and intraluminal crawling observed in larger vessels (20–30 μm) in other organs and inflammatory settings (37, 38). Indeed, neutrophil crawling was observed infrequently, with rates of 4.29 ± 1.1 and 7.2 ± 2.24 cells per glomer-

![Figure 5](http://www.jci.org/). In vivo neutrophil trafficking in glomerular capillaries at steady state and after anti-GBM treatment in LysM-GFP mice. In vivo 2-photon time-lapse imaging of glomeruli in untreated LysM-GFP mice at steady state (untreated, control) and 1.5–2 hours after anti-GBM (αGBM) treatment (n = 3 mice, 5 glomeruli for control; n = 4 mice, 6 glomeruli for αGBM). Imaging parameters were set to preferentially visualize GFP+ neutrophils (see Supplemental Figure 4A). (A and B) Glomerular capillaries (GC, red, Q-dot 565) and neutrophils (Np, green, LysM-GFP) in a control (left) and αGBM-treated (right) mouse are shown (A). Capillary lumen and neutrophil diameters were calculated and found to be similar in size with no significant differences between control and αGBM groups (B) by a 2-tailed unpaired t test. (C) The mean ± SEM of neutrophils per glomerulus (glm) (arrows) in untreated and αGBM-treated LysM-GFP mice were counted at 0 and 9 minutes, and representative images are shown. SHG, second-harmonic generation. Scale bars: 25 μm (A), 50 μm (C). (D) Distributions of glomerular duration times for control and αGBM groups plotted in minutes. Duration times were clearly non-normal, and control and αGBM groups were significantly different using a 2-tailed Mann-Whitney U test, P < 0.01. (E) Distributions using a 2-sample Kolmogorov-Smirnov (KS) test comparison percentile plot. The group duration time distributions were significantly different using a KS test, P < 0.01, D = 0.3813. (F) A bar graph of mean ± SEM is shown to provide a sense of scale for the dwell times. *P < 0.01 by 2-tailed unpaired t test.
lar capillaries (Figure 6A, left panel, and Supplemental Video 4). In contrast, tracks in the anti-GBM–treated group were longer in duration and often had multiple periods of stable adhesion (Figure 6A, right panel, and Supplemental Video 5). We compared the distribution of video-rate duration times in control and anti-GBM groups in a single optical plane (Figure 6B). Similar to the 3D time-lapse data (Figure 5D), duration time distributions were heavily skewed toward shorter-duration interactions, but these events were better resolved. Control and anti-GBM groups were significantly different.

In order to better characterize the dynamics of the short-duration neutrophil–vessel wall interactions in glomeruli, we imaged in a single optical plane at high temporal resolution (40 ms per frame) (Figure 6, A–I). Individual cell tracks in glomeruli of control mice were brief and had few periods of stable adhesion with glomerular capillaries (Figure 6A, left panel, and Supplemental Video 4). In contrast, tracks in the anti-GBM–treated group were longer in duration and often had multiple periods of stable adhesion (Figure 6A, right panel, and Supplemental Video 5). We compared the distribution of video-rate duration times in control and anti-GBM groups in a single optical plane (Figure 6B). Similar to the 3D time-lapse data (Figure 5D), duration time distributions were heavily skewed toward shorter-duration interactions, but these events were better resolved. Control and anti-GBM groups were significantly dif-
Figure 7. Rapid neutrophil capture in glomerular capillaries is FcγRIIA and Abl/Src dependent. (A–F) FcγRIIA+/γ– recipient mice were given anti-GBM antibody (αGBM) (A–C) or left untreated (D–F), and 2-photon time-lapse imaging of the kidney was conducted 2 hours later following the simultaneous adoptive transfer of FcγRIIA+/γ– (IIAγ–) and γ– neutrophils differentially labeled with green or blue fluorescent dye and a red dextran vascular dye. Duration times were calculated as the time in seconds that neutrophils remained arrested in the capillary and the distribution of dwell times plotted as histograms. (A–C) In αGBM-treated recipients given IIAγ– and γ– neutrophils, the group and dwell time distributions were compared using a Mann-Whitney U test (A, P < 0.001), a 2-sample KS test comparison percentile plot (B, P = 0.001, D = 0.2485), and a 2-tailed unpaired t test (C, *P < 0.001) (n = 4 recipient mice with n = 2 for each neutrophil dye combination, 33 glomeruli). (D–F) Untreated recipients were analyzed following the adoptive transfer of labeled IIAγ– and γ– neutrophils, and statistics were conducted as in A–C (D, P = 0.138; E, P = 0.138, D = 0.1859; F, P = 0.138) (n = 3 recipient mice with 1 dye combination, 21 glomeruli). (G–I) FcγRIIA+/γ– recipient mice were given αGBM, and time-lapse imaging was conducted 2 hours after the adoptive transfer of differentially labeled FcγRIIA+/γ– neutrophils pretreated with 100 nM bosutinib (Bos) or vehicle (DMSO). Duration time (seconds) and group dwell time distributions were compared as in A–C. Statistics are as follows: G, P < 0.001; H, P < 0.001 and D = 0.2346; I, *P < 0.001 (n = 4 recipient mice with n = 2 for each neutrophil dye combination, 22 glomeruli). Bar graphs in C, F, and I are mean duration time ± SEM in seconds.
of our video-rate imaging experiments was designed to catch the difference in the midline versus the vessel wall. Notably, the short duration of narrow glomerular capillaries as it requires changes in shear stress is substantially slower and may not occur in larger vessels, which is why the short duration times (Figure 6F). Furthermore, adhesive interactions (S2 transitions) were more frequent in the anti-GBM group, occurring approximately once on average per track (Figure 6G), and S2 adhesive events were longer in duration in comparison with the control group (Figure 6H). S1 velocities were higher in the control group than in the anti-GBM group (Figure 6I), possibly as a result of more frequent upstream S2 events in the latter. Neither group displayed sustained classic selectin-mediated rolling behavior reported in larger vessels, which is substantially slower and may not occur in narrow glomerular capillaries as it requires changes in shear stress in the midline versus the vessel wall. Notably, the short duration of our video-rate imaging experiments was designed to catch the earliest steps of neutrophil recruitment. The early, subsecond adhesive events that we describe herein may precede the previously reported glomerular intraluminal crawling behavior (ref. 40 and Supplemental Figure 4).

Rapid neutrophil capture within glomerular capillaries is dependent on FcγRIIA and inhibited by bosutinib. Our single-cell analysis shows that when anti-GBM antibody is deposited, the frequency of S2 stable interactions increases as does the average duration of S2 adhesion events (from <0.25 in the steady state to ~1.6 seconds in the anti-GBM treatment group). Our video-rate analysis using KS percentile plots suggests that IgG-mediated capture events are likely to involve other cell adhesion mechanisms. Based on this duration window, we evaluated the role of FcγRIIA in neutrophil binding using a time resolution of 0.42 seconds, which should be sufficient to document the majority of Fc-mediated capture events. Moreover, we evaluated a potential cell-autonomous role for human FcγRIIA in the capture of neutrophils using adoptive transfer approaches: FcγRIIA–/– recipient mice were given an i.v. injection of rabbit anti-GBM antibody, and 1.5 hours later injected i.v. with differentially labeled, fluorescent FcγRIIA+/–/− neutrophils (Mann-Whitney U, P < 0.01) (Figure 7A). Neutrophil duration time was also analyzed using a KS test comparison...
percentile plot (Figure 7B), which showed group duration time distributions that were significantly different ($P < 0.001, D = 0.2485$) with $FcyRIIA^-/-/^- gamma$ neutrophils having increased mean duration times compared with $gamma^+$ (Figure 7C). The observed binding defect in injected $gamma^+$ neutrophils despite the presence of $FcyRIIA^-/-/^- gamma$ neutrophils demonstrates cell-autonomous roles for $FcyRIIA$ in neutrophil recruitment. Notably, there was no difference in the distribution of duration times between $FcyRIIA^-/-/^- gamma$ and $gamma^+$ neutrophils injected into untreated recipient mice (Figure 7, D–F).

To evaluate the role of Abl/Src in $FcyRIIA$-dependent recruitment, $FcyRIIA^-/-/^- gamma$ neutrophils were pretreated with either bosutinib or vehicle control ex vivo, differentially labeled, and adoptively transferred into $FcyRIIA^-/-/^- gamma$ recipient mice given anti-GBM antibody. Bosutinib treatment significantly altered neutrophil duration time distributions in glomerular capillaries compared with vehicle-treated counterparts (Mann-Whitney U, $P < 0.001$; KS test, $P < 0.001, D = 0.2346$) (Figure 7, G and H) and reduced the mean neutrophil duration time (Figure 7I and Supplemental Video 6). As observed in LysM-GFP mice, adoptively transferred neutrophils were rapidly captured in the absence of prior rolling or crawling (Supplemental Video 6).

**Discussion**

Our studies show that unlike the hierarchical cascade of neutrophil recruitment described in postcapillary venules that begins with rolling and subsequent adhesion of neutrophils to the activated endothelium, IgG-IC deposition within glomerular capillaries directly recruits neutrophils via their $FcyRIIA$. Moreover, the observation that glomerular capillaries are similar in size to neutrophils suggests that neutrophils may circumferentially interact with IgG deposits as they pass through. This unconventional IgG-mediated neutrophil capture occurs via $FcyRIIA$ and is an active process, as bosutinib, an inhibitor of Abl/Src kinases, markedly inhibited the number and duration of capture events both in vitro and in vivo. Mechanistically, these capture events may be facilitated by Abl1/Src kinase regulation of F-actin polymerization, which strengthens FcγRI-IgG interactions when the cells experience mechanical force, as may be the case when neutrophils transit through glomerular capillaries. Intravital microscopy analysis suggests that captured neutrophils may be released into circulation after transient interaction or sustain adhesion. The latter could trigger the intravascular generation of ROS, proteases, and NETs, which could promote glomerular damage (40, 42, 43). Bosutinib’s inhibition of IC-mediated cytotoxic functions in vitro suggests that it may both reduce neutrophil recruitment and dampen neutrophil cytotoxicity in vivo. Accordingly, in an experimental model of proliferative, crescentic anti-GBM nephritis, bosutinib significantly averted $FcyRIIA$-mediated neutrophil accumulation and glomerular injury.

Intravital microscopy revealed that neutrophils make brief, subsecond interactions with the endothelium in the steady state, presumably due to irregular hydrodynamic flow through the small-diameter and tortuous glomerular capillary bed. However, following anti-GBM deposition, the frequency of stable interactions increased, as did the average duration of adhesion events. $FcyRIIA$ on neutrophils played a significant, cell-autonomous role in the rapid capture of neutrophils following anti-GBM antibody deposition, suggesting that $FcyRIIA$ participates in one of the earliest steps of autoimmune-mediated neutrophil accumulation in the kidney. Binding of cells under shear requires the rapid formation of longer-lived adhesive bonds called catch bonds that resist shear-induced detachment forces of flowing blood; catch bonds have been shown to occur with selectin/ligand pairs, which are known to promote neutrophil capture and rolling (44, 45). We demonstrate that $FcyRIIA$-IgG forms catch bonds in vitro that may be reflected in vivo in the subsecond transition of flowing neutrophils to arrest in the presence of anti-GBM antibody. The observed frequent $FcyRIIA$-mediated neutrophil interactions with anti-GBM antibody within capillaries may lead to CD18 integrin activation and thus substitute for selectin-mediated rolling, which facilitates integrin-mediated firm adhesion (46). Indeed, activated Mac-1 sustains FcγR-mediated neutrophil adhesion under static and flow conditions (10, 21, 22) and following acute anti-GBM nephritis (21, 40). Interestingly, Mac-1 activation by ICs but not GPCR agonists (e.g., FMLP) relies on actin polymerization (47), which suggests the possibility that F-actin polymerization, essential for FcγR-mediated neutrophil capture and for catch bond formation in vitro, may concurrently promote Mac-1 activation.

Although the molecular mechanisms driving FcγR-mediated neutrophil cytotoxic functions such as phagocytosis and ROS generation are relatively well defined (48, 49), those that support FcγR-mediated neutrophil capture by ICs were unknown. Our studies revealed a role for Abl1/Src in rapid neutrophil capture by ICs under flow conditions. The significant effect of Abl1 silencing on $FcyRIIA$-mediated neutrophil capture and F-actin polymerization contrasts with the partial effects of silencing Abl family mem-
In summary, our studies have revealed a new pathway of neutrophil recruitment in the glomerulus that depends on FcyRIIA-mediated neutrophil capture by IgG deposited within glomerular capillaries. This may occur through FcyRIIA-mediated Abl1 and Src kinase–induced F-actin polymerization, which in turn may strengthen FcyRIIA-IgG interactions under flow conditions. We also provide evidence that bosutinib, currently used for the treatment of CML (65), averts this very early step in glomerular inflammation and may thus potentially be repurposed for Goodpasture’s syndrome and possibly other proliferative GN.

**Methods**

*Mice.* Human FcyRIIA-expressing, γ chain–deficient (FcyRIIA/γ−/−) mice and FcyRIIA-expressing, γ chain–deficient mice were as previously described (9, 66). These mice were maintained in a virus- and antibody-free facility at the New Research Building animal housing facility at Harvard Medical School. Mice used for each experiment were 8–10 weeks of age and were age- and sex-matched. LysM-GFP mice were provided by Klaus Ley (La Jolla Institute for Allergy and Immunology, La Jolla, California, USA), who backcrossed the originally generated mice from T. Graf (Albert Einstein College of Medicine, Bronx, New York, USA) to the B6 background. These mice were maintained and bred under specific pathogen–free conditions at Washington University School of Medicine. The Harvard Medical Association IACUC at Harvard Medical School approved all animal experiments.

*Neutrophil ROS-based high-throughput small-molecule screen.* In collaboration with the Institute of Chemistry and Cell Biology Screening facility at Harvard Medical School (ICCB-Longwood), 8,483 compounds were screened. Details are in Supplemental Methods. For a secondary screen, serial dilutions of the 30 top-ranked compounds that suppressed ROS generation were tested on a single 384-well plate using the same protocol as the primary screen. Reagents for subsequent studies were as follows: cytochalasin D and PP2 from Sigma-Aldrich and bosutinib (B1788), imatinib (I5508), and nilotinib (N8207) from LC Laboratories.

*Human neutrophils, mouse bone marrow neutrophils, and HL-60 cells.* Human polymorphonuclear neutrophils (>95% pure) were isolated from whole blood drawn from healthy volunteers as previously described (67) under Partners IRB approval (protocol 1999P001694). Mouse bone marrow neutrophils (BMNs) were isolated by Percoll gradient separation. HL-60 cells (ATCC) were cultured in RPMI 1640 supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. The cells were differentiated into neutrophils with 0.8% dimethylformamide (DMF; Sigma-Aldrich) for 4 days. Sequence-verified shRNA lentiviral plasmids against Abl1 (Mission library, Sigma-Aldrich, TRCN0000121100 and TRCN0000121101) were used to produce lentiviral particles in HEK293 T cells (clone 17, ATCC) by standard methods. Undifferentiated HL-60 cells were transduced with lentiviral shRNA particles, cultured for 5 days, selected for 10 days with 10 μg/mL puromycin, and differentiated for an additional 4 days with DMF.

*Human CML blood samples.* Anticoagulated blood samples (collected in citrate buffer) were obtained from 3 patients on 200, 400, or 500 mg/d bosutinib and age-, sex-, and ethnicity-matched normal volunteers. The blood was subjected to 2–3 sequential rbc lysis steps using ammonium-chloride-potassium (ACK) lysis buffer, resuspended in PBS at 1 × 107 cells/mL, and adjusted to 1 × 106 cells with RPMI plus 0.1%
BSA before experiments. Aliquots were evaluated by FACS analysis for FcγRIIA expression using anti-CD32 FITC (555448, BD Biosciences). Granulocytes were identified by forward scatter and side scatter.

Fcγ R cross-linking-induced generation of ROS, neutrophil degranulation, and Western blot analysis. Additional details and the complete unedited Western blots are provided in the supplemental materials.

Adhesion and spreading assay under static conditions. The adhesion assay and F-actin staining were as previously described (21). Briefly, neutrophils were plated on glass coverslips coated with BSA and anti-BSA (B7276, Sigma-Aldrich), and coverslips were fixed 20 minutes later. Adherent cell numbers were quantitated in 3 independent fields at magnification ×200, and the average cell number per field was determined. Cells were stained with Alexa Fluor 568 phalloidin, the area of a single spread cell was calculated with ImageJ (NIH), and the values of 20 cells were averaged.

Adhesion assay under shear stress. For binding assay on ICs, coverslips were coated with BSA or preformed soluble ICs of BSA-anti-BSA for 2 hours at room temperature, which were generated as previously described (10, 68). Coverslips were mounted on a flow chamber, and cells were perfused for 4.5 minutes at the indicated shear stresses. Live imaging of cell adhesion was recorded with a video camera on a Nikon TE2000 inverted microscope equipped with a 20× 0.75 NA phase contrast objective and VideoLab software (Mitov).

Human dermal microvascular endothelial cells (HDMECs) (HMVEC-D, Lonza) were grown to confluence on fibronectin-treated glass coverslips and activated with human TNF-α (10 ng/ml) for 4 hours where indicated. ECs were incubated with mouse anti-endothelin mAb (anti-CD105, clone 43A3, BioLegend) for 15 minutes at 37°C, gently washed, and subsequently incubated with rabbit anti-mouse IgG (1:250 dilution) for 15 minutes. HDMECs were assembled into a parallel plate flow chamber maintained at 37°C. Human peripheral blood neutrophils were purified from freshly drawn blood and incubated with anti-FcγRIIB (clone 3G8, BioLegend), anti-FcγRIIA (clone IV.3, Stemcell Technologies), appropriate isotype (Iso) controls or bosutinib (112-006-072, Jackson ImmunoResearch) was added to induce cross-linking, and the reaction was stopped with ice-cold 1.5% PFA solution at 0°C. For binding assay on ICs, coverslips were fixed 20 minutes later. HDMECs were assembled into a parallel plate flow chamber maintained at 37°C. Human peripheral blood neutrophils were purified from freshly drawn blood and incubated with anti-FcγRIIB (clone 3G8, BioLegend), anti-FcγRIIA (clone IV.3, Stem cell Technologies), appropriate isotype (Iso) controls or bosutinib (100 nM), imatinib (100 nM), or cytochalasin D (20 μg/ml) for 30 minutes at 37°C. The human PMNs were diluted 10-fold to 10⁶ cells/ml and drawn across the endothelial cell layer at 1.5 dyn/cm². For all flow experiments, the number of cells that accumulated in 4 fields in each coverslip was calculated and averaged. For each independent experiment, 2 coverslips were evaluated per condition and averaged.

Flow cytometric analysis of F-actin polymerization. 10 × 10⁶ HL-60 cells/ml in PBS were incubated with 10 μg/ml mouse anti-hFcγRIIA (clone IV.3, Stemcell Technologies) and thereafter stained with Zombie Aqua (423302, BioLegend) on ice for 20 minutes. After washing, cells were incubated with 1 ng/ml human GM-CSF (R&D Systems) in PBS with Ca²⁺/Mg²⁺ at 37°C for 15 minutes and pretreated with indicated reagents at 37°C for 30 minutes. Goat anti-mouse F(ab)², (36 μg/ml; 112-006-072, Jackson ImmunoResearch) was added to induce cross-linking, and the reaction was stopped with ice-cold 1.5% PFA solution at 4°C for 30 minutes. After permeabilization with 0.1% saponin (Sigma-Aldrich) for 2 minutes at room temperature, cells were stained with NBD-phallacidin (Life Technologies) at 4°C for 20 minutes, and the fluorescence signal intensity was analyzed with a DxFP12 Analyzer (Cytek).

rbc and hlgG1/streptavidin bead preparation for biomembrane force probe experiments. Human rbc were isolated from whole blood and biotinylated as previously described (69), and hlgG1/streptavidin-coated beads were prepared as detailed in Supplemental Methods.

Biomembrane force probe experiments. The biomembrane force probe technique has been previously described (69). In a chamber filled with L15 buffer with 1% BSA and 1 mM Ca²⁺/Mg²⁺, a hlgG1/streptavidin bead was attached to the apex of a micropipette-aspirated biotinylated rbc, which together acted as an ultrasensitive force transducer. The aspiration pressure was set to control the probe stiffness at 0.2 or 0.3 pN/μm, based on calculations (70). The axial displacement of the bead-rbc interface was monitored by high-speed camera, which reflected the force on the rbc by Hooke’s law. Jurkat cells lacking CD18 integrins (36), provided by Timothy A. Springer (Harvard Medical School, Boston, Massachusetts, USA), were engineered to express FcγRIIA. The Jurkat-FcγRIIA–expressing cell was then aspirated by an opposing micropipette and driven by a piezo actuator with subnanometer spatial precision. The Jurkat cell was programmed to approach and briefly interact with the probe bead to allow bond formation, which was signified by a tensile force on the rbc upon retraction of the cell. Following that, distance clamping was performed to measure the lifetime of this bond under a certain force. After dissociation of the bond, the cell was retracted to the original position to wait for the next cycle. To measure the bond lifetimes under zero force, after touching the bead, the Jurkat-FcγRIIA–expressing cell was retracted to the zero-force position and then held stationary. Bond association/dissociation was manifested as a sudden drop/increase in the thermal fluctuations of the bead position, which was quantified by the average standard deviation of a sliding interval of 70 time points (71). The site density of the hlgG1 was titrated to keep the adhesion frequency approximately 20%, to ensure that approximately 90% of adhesion events were mediated by single bonds. As a confirmation of the binding specificity, beads coated with streptavidin alone contacting the same batch of Jurkat cells only rendered approximately 3% adhesion frequency. For experiments with cytochalasin D, Jurkat-expressing FcγRIIA cells were incubated with 20 μM of cytochalasin D for 30 minutes under 37°C before being placed into the chamber.

Two-photon intravital microscopy of the glomerulus. FcγRIIA/γ⁻/⁻ isolated mouse BMNs (1 × 10⁶) were stained with Cell Tracker Green CMFDA or Cell Tracker Blue CMF2HC (5 μM; Life Technologies), washed with PBS, and incubated with 100 nM bosutinib or DMSO vehicle, respectively, at 37°C for 30 minutes. A recipient FcγRIIA⁻/⁻ mouse was given anti-GBM serum, and labeled neutrophils were transferred i.v. Alternatively, Lyn-M-GFP reporter mice were given anti-GBM serum to image endogenous neutrophil trafficking during glomerular injury and in some cases additionally given a retro-oral injection of 3.5 μg of PE-anti-Ly6G antibody (clone 1A8, eBioscience). The right kidney of the mouse was surgically exteriorized 1.5 hours later and prepared for intravital microscopy, which took 30 minutes. Details for intravital microscopy imaging are in Supplemental Methods. Glomerular lumen diameter was measured manually in z-sections using the Imaris 8.2 line measurement tool. The diameter of Lyn-M-GFP neutrophils in glomerular capillaries was measured along the smaller axis. For all intravital microscopy experiments, 2 or more glomeruli were analyzed per mouse.

Two-photon imaging data analysis and the HMM 2-state model. Velocities were plotted over time and fit to an HMM 2-state plot. The rationale for using an HMM 2-state model stems from the fact that neutrophils do not flow smoothly through glomerular capillaries in the steady state. This unusual behavior may in part be due to irregularities in hydrodynamic flow in small tortuous vessels, interactions with other cells in the capillaries, or adhesive interactions independent of FcγRs. The HMM 2-state model allows us to discriminate fluctuations in velocity and tran-
Adhesive interactions that are common in the steady-state neutrophil behavior (defined as S1 behaviors) from bona fide FcγR-dependent binding events in anti-GBM–treated mice. We used the HMM 2-state to assign cell decelerations from S1 as coming from the “hidden state” (i.e., S2) when track speed drops below ≤50 μm/s for 2 consecutive frames, which filters out steady-state fluctuations and respiration artifacts. S2 was defined as a genuine binding interaction and presumably occurred as a result of FcγR catch bond formation. Track durations were obtained and plotted to demonstrate the number of cells at time intervals of 0.56 seconds for each group. Average velocities per individual track from control and anti-GBM groups were calculated, along with the average duration spent in each state (S1, S2). In order to demonstrate the difference in neutrophil behavior between the control and anti-GBM, the average frequency of neutrophil adhesion in kidney glomeruli was plotted. The mean ± SEM or median ± median absolute deviation was calculated for distributions as descriptive statistics, and reported for non-normal distributions to provide a rough value for discussion. Group distributions were plotted as histograms, and non-normal data were compared using a nonparametric 2-tailed Mann-Whitney U test where a confidence interval of 95% was used to determine statistical significance. In addition, a 2-sample Kolmogorov–Smirnov (KS) test was performed to compare non-normal distributions. The KS test identifies distances between distributions, and the maximum distance (D) is shown to asymptotically converge to a statistical distribution of the exponential family and thus can assign a probability that the 2 distributions are equivalent. The maximum distance D is reported for each test, and the P value associated with the distance is also reported; a confidence interval of 95% was used to determine statistical significance.

Cutaneous reverse passive Arthus reaction. The reverse passive Arthus reaction was induced in the dorsal skin, and neutrophil accumulation and edema were evaluated as we have described (72). Details are provided in the Supplemental materials.

Nephritis model. Proliferative, crescentic anti-GBM glomerulonephritis was induced and neutrophil accumulation and renal injury were evaluated as we have described (73). Details are provided in Supplemental Methods.

Statistics. All in vitro data and in vivo disease models are presented as the mean ± SEM. One-way ANOVA followed by Dunnett’s test was used for multiple comparisons. Kidney histology scoring and infiltrated neutrophil counts were compared by the Mann-Whitney U test.

For other data, statistical differences were analyzed with the unpaired 2-tailed t test. P less than 0.05 was considered significant. For statistical analysis of data presented as fold induction, an average of data from control samples is assigned a value of 1, and all data are calculated relative to this value.

Study approval. The Harvard Medical School Animal Care and Use Committee approved all procedures in this study. Human peripheral blood was obtained from healthy volunteers under a protocol approved by the Brigham and Women’s Hospital IRB, and all subjects gave written informed consent. Blood samples from CML patients were obtained under a Dana-Farber Cancer Institute IRB-approved protocol.

Author contributions
HN, XC, KF, GC, FR, JL, and JMH conducted the experiments and acquired and analyzed the data. SLH, LY, SPP, and MJM conducted 2-imaging experiments, analyzed data, and edited the manuscript, and JCB performed calculations and provided guidance on the statistical analysis of the intravitral microscopy data. DJD provided blood samples from CML patients. TNM, HN, and XC wrote the manuscript. TNM, HN, XC, KF, GC, and CZ designed the research studies.

Acknowledgments
We thank the Harvard Medical School Center for Immune Imaging Core (director: Ulrich von Andrian) for advice and use of their 2-photon intravitral microscope. This work was supported by a Target Identification in Lupus Grant/Alliance for Lupus Research Foundation grant (to TNM) and a Distinguished Innovator Award/Lupus Research Initiative (to TNM); NIH HL065095 (to TNM), DK099507 (to TNM), AI42269 (to GCT), AI044902 (to CZ), DK097317 (to MJM), AI095776 (to MJM), AI077600 (to MJM), and T32 HL007627 (to FR); and a Japan Society for the Promotion of Science Postdoctoral Fellowship for Research Abroad (to HN).

Address correspondence to: Tanya N. Mayadas, Department of Pathology, Brigham and Women’s Hospital and Harvard Medical School, 77 Avenue Louis Pasteur, NRH Room 7520, Boston, Massachusetts 02115, USA. Phone: 617.525.4336; Email: tmayadas@rics.bwh.harvard.edu.
The Journal of Clinical Investigation


