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Serum and Glucocorticoid–Regulated Kinase 1 Regulates Neutrophil Clearance during Inflammation Resolution


The inflammatory response is integral to maintaining health by functioning to resist microbial infection and repair tissue damage. Large numbers of neutrophils are recruited to inflammatory sites to neutralize invading bacteria through phagocytosis and the release of proteases and reactive oxygen species into the extracellular environment. Removal of the original inflammatory stimulus must be accompanied by resolution of the inflammatory response, including neutrophil clearance, to prevent inadvertent tissue damage. Neutrophil apoptosis and its temporary inhibition by survival signals provides a target for anti-inflammatory therapeutics, making it essential to better understand this process. GM-CSF, a neutrophil survival factor, causes a significant increase in mRNA levels for the known anti-apoptotic protein serum and glucocorticoid–regulated kinase 1 (SGK1). We have characterized the expression patterns and regulation of SGK family members in human neutrophils and shown that inhibition of SGK activity completely abrogates the antiapoptotic effect of GM-CSF. Using a transgenic zebrafish model, we have disrupted sgk1 gene function and shown this specifically delays inflammation resolution, without altering neutrophil recruitment to inflammatory sites in vivo. These data suggest SGK1 plays a key role in regulating neutrophil survival signaling and thus may prove a valuable therapeutic target for the treatment of inflammatory disease.

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M.K.B.W., S.A.R., and J.R.W. conceived the study and designed the experiments with J.B.; S.N.F. and W.J.Z. provided advice on pharmacological inhibition of SGK1 including supply of GSK1558634A and GSK398689A; P.I. and C.O.S. provided advice on pharmacological assessment of SGK1 inhibition; P.R. and A.F.H. provided the sgk1 morpholino and the SGK1 gene comparison and background advice; J.B., M.K.B.W., and S.A.R. wrote the manuscript; and J.B., A.L.R., F.S., E.H.-G., L.R.P., and E.E.H. performed the experiments. X.W. and P.W.I. generated the PH-AKT-line and provided advice on its use after background advice.

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Abbreviations used in this article: AUR, aurora kinase; DMOG, dimethyl oxaloylglycine; dpf, days postfertilization; EGFP, enhanced GFP; hpi, h postinjury; NDRG, N-myc downstream regulated; McI-1, myeloid cell leukemia-1; PDK, phosphoinositide-dependent kinase; SGK, serum and glucocorticoid–regulated kinase.

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and are an essential component of the cellular inflammatory response. Their main role is the removal of microbes through phagocytosis and intracellular killing. In addition, neutrophils may release intracellular contents either as neutrophil extracellular traps (3) or by degranulation of their potentially harmful granule contents into the inflammatory environment (4). Thus, tight regulation of neutrophil function is essential to the maintenance of health. In a perfectly balanced inflammatory response, there is removal of the initiating stimulus together with the avoidance of tissue damage by successful inflammation resolution. A number of prerequisites must be met for the resolution of inflammation; of which, the safe removal of leukocytes from the inflammatory site is of critical importance (5). An important mechanism of clearance is the apoptosis of neutrophils and their removal by macrophage phagocytosis in situ (6). At sites of inflammation, many signals are produced that suppress neutrophil apoptosis leading to an extended lifespan. Host-derived signals such as ATP (7) or GM-CSF (8) and pathogen-associated molecular patterns (9) combine with physical properties of the inflammatory environment, such as hypoxia (10), to suppress neutrophil apoptosis, leading to delay or failure of inflammation resolution. This in turn results in tissue damage, which is a further stimulus for neutrophil recruitment and survival via damage-associated molecular patterns and underlies many chronic inflammatory diseases (11, 12). Understanding how these survival signals act at a molecular level within the neutrophil should identify targets for novel drug therapies for inflammatory disease.

GM-CSF is an important host-derived signal influencing neutrophil lifespan and, upon binding to its receptor, initiates well-characterized intracellular signaling events. The heterodimeric GM-CSF receptor complex activates at least two distinct signaling pathways: activation of class I PI3Ks (13) and JAK/STAT signaling (14). PI3K activation leads to generation of the secondary...
messenger phosphatidylinositol 3,4,5-triphosphate and downstream phosphorylation of AKT (15, 16), which in turn phosphorylates a wide range of cellular substrates with diverse cellular effects (17). AKT is seen as a central player in the transduction of cell surface signals to the core apoptotic machinery, and AKT inhibitors have been shown to delay neutrophil apoptosis (18). However, IMLP receptors also signal via PI3K activation and activate AKT but without affecting neutrophil apoptosis (19). Therefore, there is a dissociation of apoptosis signaling and AKT activation, suggesting roles for other signaling molecules in addition to PI3K activation in apoptosis regulation.

Activation of the JAK/STAT pathway downstream of GM-CSF stimulation is thought to act predominantly via transcriptional effects. Traditionally, neutrophils were thought not to be subject to transcriptional regulation, but there is evidence of considerable regulation of neutrophil mRNA repertoire by inflammatory stimuli (including GM-CSF) (20, 21), with potential further regulation by microRNAs (22). In one study, mRNA changes in neutrophils were detected by microarray following GM-CSF stimulation (21). One of the most upregulated mRNAs was that of the known antiapoptotic protein serum and glucocorticoid–regulated kinase 1 (SGK1), which, with GM-CSF stimulation, was upregulated 13.2-fold. This protein had previously been shown to be regulated by GM-CSF and also upon treatment with other neutrophil survival agents such as LPS and TNF-α (23). SGK1 shares 54% amino acid homology with AKT (24) and is also regulated by phosphorylation downstream of PI3K (25). We hypothesized that SGK1 might be an important link between extracellular antiapoptotic stimuli and the downstream apoptosis machinery in neutrophils.

Materials and Methods

Reagents

Reagents were used qVD-OPh (R&D Systems, Abingdon, U.K.), GM-CSF (PeproTech, London, U.K.), dimethylxoylglycine (DMOG), LPS, ATP, and the DMSO control (all from Sigma-Aldrich, Poole, U.K.). Three SGK inhibitors were used: GSK650394 (Toctris Bioscience, Bristol, U.K.), GSK1558634A, and GSK398689A (GlaxoSmithKline, Hertfordshire, U.K.). The sgk1 morpholino and standard control morpholino were from GeneTools (Philomath, OR).

Purification of peripheral blood neutrophils

Peripheral venous blood was taken from healthy volunteers in accordance with the specific approval of the South Sheffield Research Ethics Committee (reference number STH13927), and neutrophils were prepared as described previously (26). Negatively selected neutrophil preparations were >98% pure. Apoptotic neutrophils were counted by an observer blind to the experimental condition, from cytospins stained with Quick-Diff (Gentaur, Bucks, U.K.) using a Trans-Blot semidry blotter (Bio-Rad). Abs used were anti-SGK (number 3272; Cell Signaling Technology, Hertfordshire, U.K.) at 1:1000, anti–phospho-N-myc downstream fractures, (reference number STH13927), and neutrophils were prepared as described previously (26). RNA was extracted from sorted cells using the mirVana kit as per manufacturer’s instructions (Ambion, Huntingdon, U.K.). Microarray analysis was performed using a Zebravish (V3) Gene Expression Microarray containing 43663 probes (Agilent Technologies, Amsterdam, Netherlands) as described previously (32).

Results

Multiple SGK isoforms are expressed in neutrophils and regulated by GM-CSF

Kobayashi et al. (21) showed GM-CSF stimulation increased levels of SGK1 mRNA and prevented time-dependent decreases in SGK1 protein levels. There are, however, three SGK genes present in humans: SGK1, SGK2, and SGK3, each sharing 80% homology at their catalytic domain (34). In addition, there are four alternatively spliced isoforms of SGK1 and two for both SGK2 and 3 (Supplemental Fig. 1A). We determined the expression profile of these different genes and isoforms in ultrapure human neutrophils (35). Using specific primer pairs in RT-PCRs, we identified full-length isoforms of SGK1, SGK2, and SGK3 mRNA in human neutrophils (Fig. 1A). SGK1 mRNA was detected at higher levels according to the manufacturer’s instructions. RT-PCR used Superscript III RT Enzyme (Invitrogen), and PCR used Finnzymes Phusion (New England Biolabs, Hertfordshire, U.K.) and was carried out in a C1000 Thermal Cycler. Primers used are shown in Supplemental Table I.

Agilent microarray expression analysis

Statistical analyses

Data were analyzed (Prism 5.0; GraphPad Software) using either unpaired, two-tailed Student t tests for comparisons between two groups or one-way ANOVA with Bonferroni posttest adjustment for other data, except where indicated in the figure legend.

Mass spectrometric analysis of drug penetration of larvae

Three dfp zebrafish samples (20 fish per time point) were sonicated (Soniprep 150; MCE, Sussex, U.K.) as described previously (33). Homogenized zebrafish samples were extracted by protein precipitation with the addition of 750 μl acetonitrile containing a structural analog internal standard. Samples were mixed and centrifuged at 2200 × g for 20 min. Liquid chromatography-tandem mass spectrometry analysis was performed using an API 4000 triple quadruple mass spectrometer (AB Sciex) with an electrospray ionization source operating in positive ion mode. The UHPLC system incorporated a Jasco XLC dual pump system with a gradient elution method using acetonitrile +0.1% formic acid (v/v) and water +0.1% formic acid (v/v) mobile phase and a Phenomenex Kinex analytical column (C18, 2.6 μm, 50 × 2.1 mm). Samples were analyzed against calibration standards prepared in a control zebrafish homogenate matrix over a concentration range of 1–5000 ng/ml, with an assay LLOQ of 1 ng/ml. We estimated the volume of a zebrafish larva to be 0.0375 μl.

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following GM-CSF stimulation at early time points, whereas SGK2 expression was unaltered with GM-CSF stimulation. SGK3 mRNA levels, in contrast to previous reports (34), appeared to be increased initially but declined over time. Because GM-CSF regulation was largely confined to SGK1, we examined expression of the different isoforms of this gene. We aligned the sequences of the four SGK1 isoforms and identified the important domains and regulatory regions of the gene (Supplemental Fig. 1B). We found that all isoforms varied only at their amino termini, where amino acids encoded by the first exons varied between isoforms both in length and sequence, with isoform 2 having additional exons. Functional domains within the protein were maintained between isoforms excepting the first 60 aa; these have been identified as playing a role in the ubiquitination and degradation of SGK1 (36). PCR analysis shows mRNAs for all SGK1 isoforms are regulated by GM-CSF (Fig. 1B) at early time points, although not at 20 h.

To confirm changes in mRNA expression were paralleled by changes at the protein level, we analyzed the change in SGK1 protein levels over time and following GM-CSF stimulation. We found, in agreement with Kobayashi et al. that GM-CSF prevented the reduction in SGK1 levels over time (Fig. 1C, 1D) in parallel with increases in neutrophil survival (Fig. 1E). Although the Ab used detects all members of the SGK protein family (SGK1, SGK2, and SGK3), no bands corresponding to full-length SGK2 or SGK3 were identified. A band was routinely identified corresponding to full-length SGK1 (isoform 1), which has a predicted size of 49 kDa. To investigate whether the different levels of SGK1 detected were due to its decrease over time or merely due to the different numbers of viable neutrophils at the time points analyzed, we used the caspase inhibitor, Q-VD-OPh, to prevent all neutrophil death (26). Following Q-VD-OPh treatment, SGK1 levels with or without GM-CSF stimulation were unchanged from DMSO-treated control levels, despite the almost complete absence of apoptosis. This suggests that levels of SGK1 do not fall as a consequence of engagement of neutrophil apoptotic pathways (Supplemental Fig. 2).

SGK1 inhibition blocks GM-CSF–induced human neutrophil survival

SGK1 is upregulated at both the mRNA and protein level with GM-CSF treatment. We hypothesized that this upregulation was functionally important and that the activity of SGK1 contributed to GM-CSF–induced neutrophil survival. To test this hypothesis, we studied the effects on neutrophil apoptosis of the only commercially available inhibitor of SGK enzymes, GSK650394. GM-CSF–induced neutrophil survival was clearly seen at 8 h of culture, but with addition of 10 μM GSK650394, this effect was totally abrogated (Fig. 2A). GSK650394 also increased constitutive neutrophil apoptosis, suggesting that the compound was directly influencing neutrophil lifespan. To confirm the caspase dependence of the morphological changes of apoptosis observed in these experiments, the pan-caspase inhibitor qVD-OPh was shown to prevent all cell death (Fig. 2B).

To investigate whether the inhibition of neutrophil survival signaling by GSK650394 was specific to GM-CSF survival, we investigated its effect on a number of other neutrophil survival stimuli including activation of hypoxia-inducible factor signaling (mimicking hypoxia, induced in this study by the chemical DMOG) (31), LPS (37), and ATPγs (7). All survival factors reduced the amount of apoptosis compared with the control, although to a lesser degree than GM-CSF (Fig. 2C). GSK650394 treatment abrogated the survival induced by GM-CSF and hypoxia but did not affect that induced by LPS and ATPγs (Fig. 2C), indicating that SGK1 is important to some, but not all, survival pathways.

To further probe the mechanism by which SGK1 inhibition might lead to neutrophil apoptosis, we tested whether SGK1 inhibi-
Acceleration of apoptosis in vitro may not always correspond to acceleration of inflammation resolution in vivo, particularly if antiapoptotic factors predominate at the inflammatory site. Therefore, we wished to study the effect of SGK1 inhibition on recruitment of neutrophils to the site of injury occurs within the first 6 hposinjury (hpi), followed by spontaneous resolution within 24 h. In this model, GSK650394 added during peak inflammation caused specific reduction in neutrophil numbers (Fig. 3C, 3D), without altering numbers of neutrophils elsewhere in the animal (Fig. 3E).

To see whether the effects on inflammation resolution were mediated by induction of neutrophil apoptosis in vivo, we dual stained for endogenous neutrophil peroxidase activity and for dsDNA breaks as a marker for apoptosis using TUNEL. These experiments were performed by adding the compound at peak neutrophil number (6hpi) and measuring apoptosis at 12hpi during peak inflammation resolution. Neutrophil numbers were reduced significantly in these experiments compared with control (Fig. 3F). Rates of apoptosis were comparable to previous studies (31) and were significantly higher for the GSK650394 group (Fig. 3G). Interestingly, these effects were specific to inflammatory neutrophils, because rates of neutrophil apoptosis were not increased with GSK650394 treatment of uninjured larvae (data not shown).

\[ GSK650394 \text{inhibits the recruitment of neutrophils to the site of injury through disruption of the direction and speed of neutrophil chemotaxis} \]

To test whether the SGK1 inhibitor had any effect on other aspects of neutrophil function in vivo, we assessed the effects of GSK650394 on recruitment assays in the injured transgenic zebrafish model. Using Velocity software, we tracked neutrophils as they moved toward the site of tailfin injury (Fig. 4A) and saw a marked reduction in the movement of neutrophils treated with GSK650394. Further analysis revealed that this was due to a reduction in neutrophil speed (Fig. 4B), whereas the meandering index (displacement divided by path length) of neutrophils was unaffected (Fig. 4C). We noted that many neutrophils remained in one area, and importantly for these experiments, the zebrafish *sgk1* gene is highly similar to the human gene in both amino acid sequence and conservation of key functional domains (Supplemental Fig. 1C). By microarray analysis of FACS-sorted zebrafish neutrophils, *sgk1* is present in zebrafish neutrophils at a level 7.84 times higher than in nonneutrophil cells, underlining its importance in this cell type.

To investigate whether the chemical inhibitor of human SGK1, GSK650394, could penetrate zebrafish at this larval stage, we incubated 3dpf larvae with GSK650394 at 10 µM for an hour. We then subjected the larvae to mass spectrometric analysis as described in Materials and Methods. We measured 0.089 nmol compound per larva, or 237 µM, confirming considerable concentration of compound within each larva. To further test whether the compound could successfully inhibit zebrafish *Sgk1*, we studied its phosphorylation activity in vivo. NDRG1 is known to be phosphorylated solely by SGK1 (39), and thus, its phosphorylation can be used as a read out for SGK1 activity. In larvae treated with GSK650394, there was a dose-dependent reduction in phosphorylated NDRG1 without any effect on the total amount of NDRG1 present (Fig. 3A). This indicates that GSK650394 successfully inhibits SGK1 activity in our assays.

Inflammation is induced in 3dpf zebrafish larvae by transection of the caudal fin (Fig. 3B), and the cellular component of the inflammatory response is quantified by counting the number of neutrophils present at the site of injury. In untreated larvae, recruitment of neutrophils to the site of injury occurs within the first 6hpi, followed by spontaneous resolution within 24h. In this model, GSK650394 added during peak inflammation caused specific reduction in neutrophil numbers (Fig. 3C, 3D), without altering numbers of neutrophils elsewhere in the animal (Fig. 3E).

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control neutrophils preferentially migrate toward the wound but GSK650394-treated larvae do not (Fig. 4E).

**Genetic inhibition of sgk1 has a different anti-inflammatory phenotype to pharmacological inhibition of Sgk1**

Pharmacological modulators of neutrophil function are prone to confounding results by off-target effects, and genetic confirmation of the phenotype is essential to confirm potential drug targets. Efficient knockdown of gene function in zebrafish can be achieved by injection of morpholino-modified antisense constructs into the fertilized egg (40). This can lead to altered expression of the target gene for many days. We therefore designed a “morpholino” to alter the pre-mRNA splicing of the *sgk1* gene by targeting the splice site of intron 5 and causing intron inclusion, introducing a premature stop codon and leading to protein truncation. Effective gene knockdown was confirmed by RT-PCR analysis on RNA extracted from morphant larvae, with maintenance of the intron seen as a 385-nt shift in the *sgk1* band detected (Fig. 5A).

To isolate and identify the specific stage of the inflammatory response at which Sgk1 inhibition was having its effect, we analyzed neutrophil number at the site of injury over the course of an entire inflammatory response by performing neutrophil counts at 2, 4, 6, 8, 12, and 24 hpi. In GSK650394-treated larvae, there was a clear inhibition of neutrophil recruitment (Fig. 5B).

To investigate whether reduction in neutrophil recruitment was due to specific inhibition of Sgk1 we repeated this time course using *sgk1* morpholino injected larvae. We found that genetic knockdown of *sgk1* caused an increase in the resolution of the inflammatory response (Fig. 5C), without affecting recruitment or total neutrophil number (Fig. 5D). Additional confirmation of the importance of

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**FIGURE 3.** Inhibition of Sgk1 enhances inflammation resolution in a model of tissue injury. (A) Protein extracts (30 μg) from 30 pooled 5-dpf zebrafish embryos exposed for 4 h to GSK650394 at the indicated concentrations were immunoblotted for p-NDRG1 and total NDRG1 showing inhibition of Sgk1 activity by GSK650394. (B–D) Three-dpf *mpx*:GFP zebrafish larvae were subjected to tailfin transection at the site shown in (B) and treated as indicated at 4 hpi; neutrophil number was counted at 8 hpi. (C) Representative micrographs of the larvae used for (D). (D) GSK650394 significantly reduced the number of neutrophils at the site of injury in a dose-dependent manner (*p < 0.05, **p < 0.01, n = 18 performed as three independent experiments). (E) Total neutrophil counts show no difference between GSK650394 treatment and the control. (F and G) Three-dpf *mpx*:GFP larvae were injured as above, treated at 6 hpi, and at 12 hpi, neutrophil number was counted or larvae were fixed and subsequently stained with neutrophil markers (TSA) and apoptosis markers (TUNEL) to allow calculation of percentage apoptosis. (F) GSK650394 causes a highly significant reduction in neutrophil number at the site of injury (**p < 0.0001, n = 18 performed as three independent experiments). (G) GSK650394 treatment causes a significant increase in neutrophil apoptosis (*p < 0.05, n = 135 performed as three independent experiments).
Sgk1 levels on resolution of inflammation was obtained by separating larvae into groups showing the highest and lowest rates of inflammation resolution. The group having fewer neutrophils had better knockdown of sgk1 (Fig. 5E), confirming its importance in regulating inflammation resolution.

**FIGURE 4.** GSK650394 inhibits neutrophil recruitment through disrupted chemotaxis. Three-dpf mpx:GFP larvae were pretreated for 2 h and injured, and a time-lapse image sequence was taken for 1 h. Neutrophil chemotaxis was analyzed using Volocity software. (A) Representative images of treated larvae with neutrophil tracks superimposed. (B) Neutrophil speed is significantly reduced with GSK650394 treatment (**p < 0.005, n = 14 performed as three independent experiments). (C) Neutrophil meandering index is unaffected. (D) GSK650394 treated neutrophils show a lack of movement toward the site of injury, as represented by positive values (**p < 0.005, n = 14 performed as three independent experiments). (E) Analysis of the bearing at which neutrophils move demonstrates that directional movement of neutrophils toward the wound is removed with GSK650394 treatment (**p < 0.005, ***p < 0.001, n = 14 performed as three independent experiments).

We have shown that sgk1 genetic inhibition removes neutrophils from a site of inflammation; however, for this to be useful in a clinical situation, SGK1 inhibition would have to be achieved using a chemical inhibitor. We have seen that GSK650394 has off-target effects and would not fulfill this role; we therefore used two potent SGK1 inhibitors from a chemical template distinct from that of GSK650394: GSK1558634A and GSK398689A. These compounds have a pIC50 of 8.2 and 8.8, respectively, with limited off-target effects. GSK1558634A inhibits only two other enzymes (data not shown). To further investigate this hypothesis, the effect of PI3K inhibition on neutrophil recruitment was studied in the zebrafish model. PI3K inhibition reduces the number of neutrophils recruited to a site of injury in a similar manner to GSK650394 (Fig. 6A). A more detailed investigation of neutrophil chemotaxis shows that this reduction in recruitment is due to an inhibition of neutrophil speed (Fig. 6B), similar to that seen with SGKgk inhibition. However, in contrast to findings with GSK650394-treated larvae, the bearing at which neutrophils moved was unaffected (Fig. 6C). Furthermore, using a PHAkt-EGFP line (29) to indicate the subcellular localization of phosphoinositides produced by PI3K activity, we investigated whether there were changes in PI3K activity in response to GSK650394 or Sgk1 knockdown. To quantify PI3K activity in individual cells in vivo, we used an assay we had previously developed to quantify PI3K activity in neutrophils in response to pharmacological treatments (29). This assay gives a numerical value of the cell polarization as indicated by PHAkt-EGFP distribution within neutrophils. Using this system, we were able to show that both PI3K inhibitors and GSK650394 reduced the polarity index (Fig. 6D, 6E) whereas genetic manipulation of Sgk1 did not (Fig. 6F).

This phenotype demonstrates the complexity of understanding off-target pharmacological inhibition, with only part of the effect of pharmacological Sgk inhibition likely to act via the PI3K pathway while underscoring the importance of genetic confirmation for all inhibitor studies.

**Highly specific SGK1 inhibitors block GM-CSF induced neutrophil survival and also lower neutrophil number at an in vivo site of injury**

We have shown that sgk1 genetic inhibition removes neutrophils from a site of inflammation; however, for this to be useful in a clinical situation, SGK1 inhibition would have to be achieved using a chemical inhibitor. We have seen that GSK650394 has off-target effects and would not fulfill this role; we therefore used two potent SGK1 inhibitors from a chemical template distinct from that of GSK650394: GSK1558634A and GSK398689A. These compounds have a pIC50 of 8.2 and 8.8, respectively, with limited off-target effects. GSK1558634A inhibits only two other enzymes with a pIC50 > 6; these are aurora kinase (AUR)A, AURB; GSK398689A additionally inhibits YAK3 (GSK internal data).

Treatment of human neutrophils with either of these new inhibitors removes the survival response to GM-CSF in a dose-dependent manner, with total abrogation by 100 µM (Fig. 7A–D). Importantly, these compounds had no effect upon constitutive apoptosis of human neutrophils.

Furthermore, zebrafish larvae treated with either GSK1558634A or GSK398689A after transection of the caudal fin show a signif-
significant and dose dependent reduction in the number of neutrophils at the site of injury (Fig. 7E, 7F). The ability of two additional, more specific, inhibitors of SGK1 to replicate both the inhibition of GM-CSF–induced neutrophil survival and the rapid resolution of the inflammatory response in vivo suggests that this occurs through inhibition of the SGK1 protein, not through off-target actions, adding support for a key role for SGK1 in the regulation of neutrophil lifespan during inflammation resolution.

Discussion

Previous work using human neutrophils has shown increased amounts of SGK1 mRNA and protein following GM-CSF stimulation (21); we have now produced genetic and pharmacological data showing the importance of SGK1 in controlling neutrophil lifespan in vitro and in vivo. We have not distinguished between new transcription and regulation of protein and mRNA stability, but we have shown by highly effective caspase inhibition that the changes in SGK1 protein level are independent of levels of apoptosis. Use of the only commercially available SGK inhibitor, GSK650394, together with newer more selective compounds and a morpholino against sgk1, has shown it to play an important role in maintaining neutrophils at a site of injury. Sgk inhibition leads to rapid resolution of the inflammatory response without affecting neutrophil recruitment. SGK1 is therefore an important component of the pathways that link certain survival stimuli with the neutrophil apoptotic machinery. In other cell types, SGK1 is known to act downstream of PI3K and is activated by phosphoinositide-dependent kinase (PDK)1 through phosphorylation of Thr256 and by PDK2 via Ser422 (25, 41). We do not yet know completely how, once activated, SGK1 plays its antiapoptotic role; in other cell types, SGK1 may also mediate control of cytokine production and cell migration.
types, it regulates this process through inactivation of the FOXO3a transcription factor (42) and upregulation of NF-κB signaling (43), both of which are important in neutrophils (44, 45). Interestingly, SGK1 phosphorylates and inactivates glycogen synthase kinase-3 (25), which is known to phosphorylate the key neutrophil antiapoptotic protein Mcl-1 leading to its destabilization and degradation (46), a key upstream event in neutrophil apoptosis (26). We now show that inhibition of Mcl-1 turnover in human neutrophils is a likely final mechanism for the antiapoptotic action of SGK1.

Chemical inhibition of Sgk enzymes with GSK650394 accelerates inflammation resolution and reduces neutrophil recruitment. Genetic ablation of Sgk1 alone has no effect on neutrophil recruitment. Studies of PI3K inhibition in vivo suggest that GSK650394, but not the genetic strategy, may act upstream of PI3K to modulate neutrophil recruitment, suggesting that inhibition of neutrophil recruitment is an off-target effect. It remains possible that the lack of effect on neutrophil recruitment with sgk1 morpholino could be due to incomplete knockdown of sgk1, as shown by the maintenance of a wild-type sgk1 band in our PCRs (Fig. 5A); the small amounts of wild-type sgk1 present may be sufficient for neutrophil recruitment, but not their maintenance, at a site of injury. More probable, given the different effects of treatment with the more specific inhibitors, is that the recruitment phenotype is an off-target effect of GSK650394. Over 30 enzymes are known to be inhibited by GSK650394 with a pIC50 ≥ 6, including activin receptor-like kinase 5, AURB, calcium/calmodulin-dependent protein kinase kinase 1 and 2, cyclin dependent kinase-2, IL-2–inducible T cell kinase, JNK1, JNK3, and the class III PI3K, and vacuolar protein sorting 34, and it is likely that additional kinases that have not been included in these kinase profiling studies are also inhibited by GSK650394 (S. Farrow and W. Zuercher, unpublished data). Thus, there is considerable scope for significant off-target effects at the dose used. To overcome this limitation of the pharmacological approach, we used new inhibitors, GSK1558634A and GSK398689A. These compounds have a pIC50 of 8.2 and 8.8, respectively, inhibiting only three and four other enzymes with a pIC50 ≥ 6. The fact that both compounds show the same pro-resolution phenotype as GSK650394 strongly supports the role of SGK1 in the regulation of neutrophil responses to survival signals. The effects on chemotaxis may relate to inefficient AKT2 recruitment following changes in PI3Kγ activation, because knockout of either gene causes frustrated chemotaxis (47, 48). Interestingly, the

FIGURE 6. PI3K inhibition attenuates neutrophil recruitment through a reduction in speed. Three-dpf mpox:GFP larvae were pretreated for 2 h and injured, and a time-lapse image sequence was taken for 1 h. Neutrophil chemotaxis was analyzed using Velocity. (A) Both GSK650394 and LY294002 reduce the number of neutrophils recruited to a site of injury (**p < 0.0001, n = 12 performed as two independent experiments). (B) Neutrophil speed is significantly reduced with LY294002 treatment (**p < 0.0001, n = 15 performed as two independent experiments). (C) The bearing at which neutrophils migrate is not affected by PI3K inhibition (**p < 0.0001, **p < 0.05, n = 15 performed as two independent experiments). (D–F) Three-dpf PHAkt-EGFP larvae were pretreated for 2 h as indicated and subsequently injured. Neutrophils were imaged, and their polarity index calculated. (D) Representative images of treated neutrophils and fluorescence intensity per pixel graphs. (E) GSK650394 and LY294002 significantly reduce the polarity index of neutrophils (**p < 0.005, n = 22 performed on three separate occasions). (F) Injection of the sgk1 morpholino has no effect upon the polarity index of neutrophils (n = 26 performed on three separate occasions).
morbidity of PI3Kγ knockout neutrophils is very similar to that seen in our time-lapse videos of GSK650394-treated neutrophils in the extending and retracting of lamellipodia.

The use of the zebrafish model of inflammation has allowed us to dissect the cellular consequences of SGK inhibition in ways that would not be possible in mammalian systems. The ability to dissociate effects of SGK inhibition on recruitment and neutrophil removal allows the pathway to be understood in detail. Although murine models might allow the identification of reduced neutrophil numbers, dissociating therapeutic differences in recruitment and clearance is more challenging but critically important. There is an important therapeutic difference between removing an unwanted neutrophil and preventing recruitment of all neutrophils to sites of potential infection.

SGK1, like AKT, is activated downstream of PI3K by PDK1/2 at similar residues (15, 25, 41), and most protein targets of SGK1 are also phosphorylated by other protein kinases such as AKT (49). However, it has previously been noted that some stress stimuli, which activated SGK1, did not result in AKT phosphorylation, showing separate rather than redundant roles for these two protein kinases (50). Although AKT activation is thought to delay neutrophil apoptosis (18), AKT can also be activated without influencing apoptosis, for example, by fMLP (19). Thus, SGK1 might in some circumstances act to transduce apoptotic signals downstream of PI3K, in parallel to AKT.

The upregulation of SGK1 by survival signals (21, 23) links to a clinically important increase in SGK1 expression seen in a number of inflammatory or fibrotic diseases during which the inflammatory response is involved, including Crohn’s disease (50) and glomerulonephritis (51). Prolonged neutrophil lifespan has been implicated in the extensive host tissue damage seen in these conditions, further underlining the therapeutic potential of SGK1 inhibition. Many current anti-inflammatory treatments block neutrophil recruitment to the site of injury but fail to remove neutrophils already there. We propose that SGK1 represents a possible target for anti-inflammatory therapeutics specifically targeting the resolution phase of inflammation while still allowing neutrophils to mount a response against infection, without the extended survival and degranulation associated with disease.

Disclosures
C.S., E.H.-G., W.Z., and S.F. are employees of GlaxoSmithKline, which has financial interest in inhibitors of SGK1 and other targets involved in inflammation resolution.

FIGURE 7. Highly specific SGK1 inhibitors removes GM-CSF induced neutrophil survival. (A–D) Human neutrophils were treated as incubated for 8 h, and percentage apoptosis was scored by cytopsin analysis. (A and C) GSK1558634A causes a dose-dependent inhibition of GM-CSF–induced neutrophil survival (**p < 0.01, n = 4). (B and D) GSK398689A causes an inhibition of GM-CSF–induced neutrophil survival (*p < 0.05, n = 5).
(E and F) Three-dpf mpx:GFP zebrafish larvae were subjected to tailfin transection and treated as indicated at 6 hpi; neutrophil number was counted at 12 hpi. Both GSK1558634A (E) and GSK398689A (F) significantly reduce the number of neutrophils present at the site of injury in a dose-dependent manner (**p < 0.05, ***p < 0.01, ****p < 0.005, n = 4).