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Nonresolving Inflammation in gp91<sup>phox</sup>−/− Mice, a Model of Human Chronic Granulomatous Disease, Has Lower Adenosine and Cyclic Adenosine 5'-Monophosphate

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In chronic granulomatous disease (CGD), there is failure to generate reactive oxygen metabolites, resulting in recurrent infections and persistent inflammatory events. Because responses to sterile stimuli in murine models of CGD also result in nonresolving inflammation, we investigated whether defects in endogenous counterregulatory mechanisms and/or proresolution pathways contribute to the etiology of CGD. To this end, we conducted a series of experiments finding, in the first instance that adenosine and cAMP, which dampen innate immune-mediated responses, show a biphasic profile in resolving peritonitis; peaking at onset, waning as inflammation progresses, and rising again at resolution. We also found elevations in adenosine and cAMP in resolving human peritonitis. In gp91<sup>phox</sup>−/− mice, an experimental model of CGD, levels of adenosine and cAMP were significantly lower at onset and again at resolution. Corroborating the finding of others, we show that adenosine, signaling through its A<sub>2A</sub> receptor and therefore elevating cAMP, is not only anti-inflammatory, but, importantly, it does not impair proresolution pathways, properties typical of nonsteroidal anti-inflammatory drugs. Conversely, antagonizing the A<sub>2A</sub> receptor worsens acute inflammation and prolongs resolution. Taking this further, activating the A<sub>2A</sub> receptor in gp91<sup>phox</sup>−/− mice was dramatically anti-inflammatory regardless of the phase the inflammatory response A<sub>2A</sub> agonists were administered, i.e., onset or resolution, demonstrating wide and robust pharmacological flexibility that is unlikely to subvert proresolution pathways. Therefore, we describe the biphasic profile of adenosine and cAMP throughout the time course of acute inflammation that is dysregulated in CGD. The Journal of Immunology, 2009, 182: 3262–3269.

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There are many clinical examples in which chronic inflammation could be hypothesized to be potentially derived from dysregulated resolution pathways. Thus, a human disease that fails to resolve and for which there is an experimental animal model amenable to scientific interrogation would aid enormously in elucidating the etiology of chronic inflammation. To this end, we turned to chronic granulomatous disease (CGD), which is an inherited immunodeficiency syndrome caused by a defect in the oxygen metabolic-burst machinery, resulting in the inability to neutralize infection, leading to persistent and recurrent inflammatory responses and granulomatous tissue formation (24). Activity of NADPH oxidase system (gp91$^{phox}$, p22$^{phox}$, p47$^{phox}$, and p67$^{phox}$) is either absent or dysregulated in these patients, with the most common being X-linked CGD (~65%) with defects in the gene encoding gp91phox. Fortunately, for the purpose of understanding the etiology of CGD, gp91$^{phox}$-deficient mice display all of the hallmarks of the human condition in response to infection (25). Interestingly, there are reports showing that inflammation in CGD mice is also prolonged and dysregulated in response to sterile stimuli (26, 27), suggesting potential irregularities in endogenous anti-inflammatory and/or proresolution pathways. We conducted a series of studies in gp91$^{phox}$−/− mice and found that of the factors known to control resolution, few appeared to be consistently dysregulated, with the exception of adenosine and cAMP, levels of which are significantly lower in experimental CGD compared with wild types.

Under physiological conditions, adenosine is continuously formed intracellularly and extracellularly. The intracellular production is mediated either by an intracellular 5′-nucleotidase, which dephosphorylates AMP, or by hydrolysis of 5′-adenosine-monophosphate (28). Adenosine generated within cells is transported into the extracellular space via bidirectional transporters through facilitated diffusion that efficiently equilibrates intracellular and extracellular levels of adenosine. Following trauma, there is a decrease of intracellular ATP, accompanied by an accumulation of 5′-AMP and subsequently adenosine by the above pathways, which may be sequentially metabolized to inosine, hypoxanthine, and xanthine. Expressed on cells of the hematopoietic system, adenosine receptors (A1, A2A, A2B, A3) belong to the family of G protein-coupled heptahelical transmembrane receptors, which either stimulate (Gs) or inhibit (Gi) adenylyl cyclase, the enzyme that catalyzes the formation of cAMP (28). Adenosine A1 and A3 receptors are high- and low-affinity receptors for adenosine, respectively, with both being inhibitors of adenylyl cyclase. High-affinity A2A and low-affinity A2B receptors, in contrast, activate adenylyl cyclase, thereby increasing intracellular levels of cAMP, resulting in potent immune suppression and regulation of inflammatory leukocyte trafficking. Besides controlling adenylyl cyclase, adenosine receptors are also coupled by distinct G proteins to several other effector systems, including calcium and potassium channels, phospholipase C, phospholipase D, phospholipase A2, cGMP, phosphodiesterases, and mitogen-activated protein kinases that modulate different cell functions. Thus, adenosine, released after tissue injury or low oxygen tension associated with inflammation, has been regarded by some to act as a first-line sensor of immune damage, where it could either amplify or inhibit (Gi) adenylyl cyclase, the enzyme that catalyzes the formation of cAMP (10, 29).

In this study, we report the biphasic synthesis of both adenosine and cAMP, first at the traditional early-onset phase of acute inflammation and again during resolution, with synthesis of these immunosuppressive agents being significantly lower in CGD (gp91$^{phox}$−/−) mice associated with a severe and prolonged innate immune response to a sterile stimulus. We also show that hyperinflammation in gp91$^{phox}$−/− mice can be rescued by A2A receptor activation, as defined by reduction in inflammatory leukocytes. Importantly, A2A receptor activation in gp91$^{phox}$−/− mice did not bring about resolution because this drug strategy was not associated with innate-type lymphocyte repopulation that is typical of events that occur during normal resolution in wild type leading to tissue homeostasis.

Materials and Methods

Animal maintenance, induction of inflammation, and human peritonitis sampling

The gp91$^{phox}$−/− mice (The Jackson Laboratory), along with wild-type mice, were bred under standard conditions, maintained in a 12-h/12-h light/dark cycle at 22 ± 1°C, and given food and tap water ad libitum in accordance with United Kingdom Home Office regulations. The murine 7-day air pouch was elicited by the injection of 5 ml of sterile air, followed 7 days later with the intrapouch injection of 0.5 ml of 1% carrageenin. Peritonitis was induced by the i.p. injection of 1 mg of type A zymosan (Sigma-Aldrich), and cells were enumerated by hemocytometer at time points stated in Results by sterile PBS washout. Ethical approval (P03/136A) for collection of human peritonitis samples was obtained from St. Bartholomew’s and the Royal London Hospitals from end-stage renal failure patients undergoing peritoneal dialysis.

Macrophage isolation, culture, and stimulation

Peripheral venous blood samples were collected from subjects into heparinized syringes (5 U/ml). Mononuclear cells were isolated by differential centrifugation (2000 rpm, 30 min, 20°C) over Lymphoprep (Axis-Shield) and washed twice with sterile PBS (Life Technologies) at 1200 rpm (5 min, 20°C). Cells were resuspended in 10 ml of RPMI 1640 medium (Invitrogen) supplemented with 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), and 20 mM HEPES buffer (Sigma-Aldrich) (RPMI 1640), and plated at a density of ~5 × 10⁶ cells/ml in 8-cm² Nunclon Surface tissue culture dishes (Nunc). After an initial culture period of 2 h at 37°C, 5% CO₂, the nonadherent cells were discarded, and 10 ml of fresh RPMI 1640 supplemented with 10% FBS (Sigma-Aldrich) (10% FBS/RPMI 1640) was added to each tissue culture dish. Cells were then cultured for 5 days at 37°C, 5% CO₂ with the addition of a further 10 ml of fresh 10% FBS/RPMI 1640 after 24 h. Adherent cells were scraped on day 5 and replated in 96-well culture plates at equal densities (10⁴/well) in X-Vivo-15 medium (Cambrex). These primary monocyte-derived macrophages were incubated overnight at 37°C, 5% CO₂ to adhere, and then stimulated for 24 h with 200 ng/ml LPS (Alexis). These studies were approved by the Joint University College London/University College London Hospital Committees on the Ethics of Human Research (02/0324). Written informed consent was obtained from all volunteers. No patient was studied more than once in each of the different sets of experiments.

Pharmacological rescue experiments

The gp91$^{phox}$−/− and wild-type mice were pretreated (30 min) with the A2A receptor agonist CGS 21680 (2 mg/kg) with/without ZM241385 (2 mg/kg, A2A receptor antagonist), followed by zymosan i.p. Rolipram was dosed at 30 mg/kg. The peritoneal cavity was lavaged at 4 h, and cell number was counted using a hemocytometer. For experiments in the resolution phase, inflammation was first induced with zymosan, followed by CGS 21680 or A2A receptor antagonist, followed by zymosan. Caffeine as well as its stable analog, 8-(3- chlorostyril)-caffeine (CSC), were dosed at 3 mg/kg.

Eicosanoid analysis

Samples stored at −20°C were thawed at room temperature and acidified to pH 3. Samples were extracted using C18 columns (Waters). For PGD₂ samples, methoxylamine, hydrochloride, and the resulting stable PGD₂-methoxylamine was measured by enzyme immunoassay (Cayman Chemical). PGE₂ was measured by enzyme immunoassay (Cayman Chemical), whereas lipoxin A₄ was quantified by ELISA (Neogen).

Purine and cAMP measurements

Proteins in exudates were removed by ultrafiltration (30,000-Da cutoff). Purine concentrations in samples were measured by high pressure liquid chromatography, as previously described (30).
In the first set of experiments, we characterized the profile of inflammation in wild-types vs gp91\(^{phox-/-}\) mice. Zymosan injection into the mouse peritoneal cavity resulted in an exaggerated influx of Ly6G-positive PMNs (Fig. 1A; Ly6G-positive cells) in knockouts along with F4/80-positive monocyte-derived macrophages (B) that persisted in these animals past the equivalent time point of resolution in wild types, with the persistence of this response not due to reduced apoptosis (C) in gp91\(^{phox-/-}\) mice. \(n = 8–10\) animals per group, with data expressed as mean ± SEM.

**Results**

**Inflammation is more severe and fails to resolve in gp91\(^{phox-/-}\) mice**

In the first set of experiments, we characterized the profile of inflammation in wild-types vs gp91\(^{phox-/-}\) mice. Zymosan injection into the mouse peritoneal cavity resulted in an exaggerated influx of PMNs (Fig. 1A; Ly6G-positive cells) in knockouts during the early-onset phase, with numbers declining up to 96 h. F4/80-positive macrophage numbers were similarly increased at onset in knockouts, with levels remaining elevated throughout the entire response (Fig. 1B), underlining the nonresolving nature of inflammation that is characteristic of CGD. Because there was little difference in leukocyte apoptotic rates as determined by annexin V/propidium iodide labeling between both animals (Fig. 1C), failure of resolution in CGD mice most likely resulted from continual influx and/or failed clearance of inflammatory leukocytes.

*Reduced synthesis of purines and cAMP in gp91\(^{phox-/-}\) mice*

The classic view as to why inflammation is more severe in CGD is explained by defects in phagocytic oxidase resulting in impaired bacterial killing and, consequently, delayed removal of the injurious agent. That notwithstanding, injection of sterile inflammatory stimuli also results in an exaggerated inflammatory event that fails to resolve (26, 27). Certainly, whereas this may arise from a defect in phagocytosis (31), we questioned whether it may also point to a possible defect in endogenous braking systems that counterregulate acute inflammation (23, 32, 33). To this end, we screened for alterations in levels of anti-inflammatory and proresolution mediators, finding no consistent trend in arachidonic acid metabolism between CGD and wild-type mice bearing a zymosan-triggered peritonitis. For instance, PGD\(_2\) (18, 22, 34), the lipoxins (35), and PGE\(_2\) (14) have all been shown to trigger inflammatory resolution, yet, with the exception of PGE\(_2\), there was little evidence for defects in arachidonic acid metabolism involved in etiology of CGD (Fig. 2, A–C). However, we noted a clear and robust reduction in levels of cAMP in CGD mice compared with wild-type animals (Fig. 2D). Specifically, intracellular cAMP was elevated during the early-onset phase of zymosan-induced peritonitis in wild-type controls (4–6 h), waning as inflammation progressed and became elevated again postresolution (Fig. 2D). In gp91\(^{phox-/-}\) mice bearing a zymosan-triggered peritonitis, cAMP was significantly lower than wild types at onset and failed to show the postresolution elevation seen in wild types (Fig. 2D). The functional relevance of raised cAMP postresolution is being answered in another body of work, which shows that cAMP controls the phenotype of resolution-phase macrophages imparting upon them an immunosuppressive state. We next examined why cAMP levels are lower in gp91\(^{phox-/-}\) mice. Certainly, PGs are well-known elevators of cAMP (via EP2, EP4, DP1) (36) and are also present and functional during the early as well as later stages of acute inflammation (22). Nonetheless, there was no substantial difference in cyclooxygenase activity between wild types and gp91\(^{phox-/-}\) mice. In fact, there was an elevation of PGE\(_2\) (Fig. 2A) in gp91\(^{phox}\) knockouts, indicating possible signaling of this PG through its EP1 (increased Ca\(^{2+}\)\(^+\)) and/or EP3 (inositol 1,4,5-triphosphate/1,2-diacylglycerol) receptors in CGD mice. However, there was a biphasic profile of adenosine synthesis mirroring that of cAMP: raised at onset and then again at resolution (Fig. 2E). Adenosine showed lower levels in gp91\(^{phox-/-}\) mice than wild types, and is a well-described immunomodulator of acute inflammation serving to dampen PMN function and prevent chemical-induced collateral liver injury (37). With its molecular actions exerted through four receptors, \(A_1\), \(A_2\) (\(A_{2A}\) and \(A_{2B}\)), as well as \(A_3\), signaling through \(A_{2A}\) has received most attention as a trigger for immunosuppressive cAMP. Thus, from these studies, we report the biphasic synthesis of adenosine and cAMP in resolving models of acute inflammation, with their levels diminished in inflammation associated with CGD. It could be argued that it is the absence of these endogenous anti-inflammatory agents in CGD that is responsible for its hyperinflammatory, nonresolving nature. Alternatively, persistence of the inflammatory stimulus would certainly prevent resolution and consequently deactivate or override endogenous pro-resolution pathways. The latter may indeed be the case because levels of cAMP released from monocyte-derived macrophages obtained from CGD patients released similar quantities of cAMP per...
FIGURE 2. Adenosine and cAMP synthesis is abrogated in gp91<sup>±/−</sup> mice. In an attempt to identify potential dysregulations in the synthesis of endogenous anti-inflammatory/proresolution factors in gp91<sup>±/−</sup> mice, arachidonic acid metabolism, as determined by PGD<sub>2</sub> (A), lipoxin A<sub>4</sub> (B), as well as PGE<sub>2</sub> (C), was determined and found not to show a consistent reduction in gp91<sup>±/−</sup> mice compared with wild types. However, cAMP was elevated at the early-onset phase of a murine peritonitis and then again at resolution in gp91<sup>±/−</sup> wild-type animals, but was significantly lower in gp91<sup>±/−</sup> mice at both phases (D). E. Adenosine, which signals through A2a receptors to elevate cAMP, was also measured in these peritonitis samples, peaking at onset and again at resolution, with levels being reduced in gp91<sup>±/−</sup> mice (■) compared with wild types (□). Although this would suggest dysregulated cAMP signaling in CGD, release of cAMP (F) from LPS-stimulated monocyte-derived macrophages obtained from healthy human volunteers was equivalent to that from CGD patients. This indicates that there is no direct interaction between NADPH oxidase and adenosine/cAMP, with the latter most likely being diminished or overridden by suppressive factors released during severe inflammation typical of CGD. n = 6–10 animals per group, with data expressed as mean ± SEM.

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Adenosine and cAMP profiles in human resolving peritonitis

As an alternative to hemodialysis, patients on end-stage renal failure may undergo chronic ambulatory peritoneal dialysis, in which a catheter is inserted to fill/drain the peritoneal cavity with a high glucose solution, with the peritoneal lining acting as a dialyzing membrane. Occasionally, patients experience bacterial infection (commonly *Staphylococcus aureus* or *Staphylococcus epidermidis*), resulting in acute resolving peritonitis from which both effluent and cells can be analyzed for markers of inflammation and resolution (38). We measured levels of cAMP (Fig. 3A) as well as adenosine (Fig. 3B) in these samples and found that when overlaid on the inflammatory leukocyte profile (Fig. 3C), which peaked 24 h after infection, cAMP as well as adenosine were elevated as inflammation resolved. Although these results corroborate that found in mice, we have no data on the very early-onset (~6-h) phase in humans to assess the very early release of adenosine and cAMP in human inflammation. Thus, as in rodents, levels of cAMP and adenosine were elevated as inflammation resolved.

Adenosine and cAMP synthesis is abrogated in gp91<sup>±/−</sup> mice. In an attempt to identify potential dysregulations in the synthesis of endogenous anti-inflammatory/proresolution factors in gp91<sup>±/−</sup> mice, arachidonic acid metabolism, as determined by PGD<sub>2</sub>, lipoxin A<sub>4</sub>, and PGE<sub>2</sub>, was determined and found not to show a consistent reduction in gp91<sup>±/−</sup> mice compared with wild types. However, cAMP was elevated at the early-onset phase of a murine peritonitis and then again at resolution in gp91<sup>±/−</sup> wild-type animals, but was significantly lower in gp91<sup>±/−</sup> mice at both phases. E. Adenosine, which signals through A2a receptors to elevate cAMP, was also measured in these peritonitis samples, peaking at onset and again at resolution, with levels being reduced in gp91<sup>±/−</sup> mice compared with wild types. Although this would suggest dysregulated cAMP signaling in CGD, release of cAMP from LPS-stimulated monocyte-derived macrophages obtained from healthy human volunteers was equivalent to that from CGD patients. This indicates that there is no direct interaction between NADPH oxidase and adenosine/cAMP, with the latter most likely being diminished or overridden by suppressive factors released during severe inflammation typical of CGD. n = 6–10 animals per group, with data expressed as mean ± SEM.

Adenosine via A<sub>2a</sub> is anti-inflammatory and not resolution toxic

As with the plethora of effects that NSAIDs exert on acute inflammation, it is well established that by signaling through its A2A receptor, adenosine exerts protective effects during acute inflammation at multiple levels. However, whereas NSAIDs dampen the inflammatory leukocyte profile, they also obstruct pro-resolution processes (22). Therefore, we next determined whether A2A signaling interferes with inflammatory resolution. In the first instance, rats bearing a carrageenin-induced pleurisy were administered rolipram, a PDE4 inhibitor, and therefore elevator of cAMP, as well as CGS 21680, a specific A2A receptor agonist 30 min before carrageenin injection. Both drugs dampened leukocyte trafficking to the inflamed cavity at onset, i.e., 4 h (Fig. 4A), being associated with an expected rise in cAMP (Fig. 4B). In a mouse zymosan-induced peritonitis at 4 h, CGS 21680 also dampened inflammation in an A2A receptor-dependent manner using ZM 241385 (A2A receptor antagonist) (Fig. 4C) associated with a significant increase in anti-inflammatory IL-10 (Fig. 4D). Other drugs, including theophylline, as well as beverages, are known to alter cAMP signaling, and therefore may unwittingly affect inflammation. A single cup of coffee, for instance, contains ~100 mg of caffeine, implying that an average person drinking one cup of coffee per day will ingest caffeine at 1.5 mg/kg. To investigate whether caffeine, a methylxanthine with antagonistic effects on the A2A receptor (39), affects acute inflammation, we administered caffeine as well as its stable analog, CSC at 3 mg/kg 30 min before i.p. zymosan injection, and found that both worsened inflammation possibly by decreasing protective IL-10 levels (Fig. 4E and F). Using a continual pharmacological dosing regimen, mice were administered not only 30 min before zymosan injection, but were also given caffeine and CSC again at 12 and 18 h after zymosan, and their effects were assessed at 24 h. The idea being that as inflammation resolves by 24 h, any resolution-toxic effects of these drugs would be detected, an important consideration with anti-inflammatories that must be highlighted. CGS 21680 maintained a dampening of acute inflammation without interfering with resolution (Fig. 4G). In contrast, caffeine at doses representative of reasonable caffeine daily intake as well as its analog, CSC, maintained their proinflammatory effects (Fig. 4G). Taking the doses of caffeine up to 10 and 30 mg/kg, equivalent to 6–20 cups of coffee, resulted in a loss of proinflammatory effects. Therefore, from these experiments, A2A agonists exert anti-inflammatory effects not only during the early-onset phase of acute inflammation, the phase traditionally tested experimentally for novel anti-inflammatories, but importantly, do not interfere with proresolution pathways. An interesting observation was the corollary to these experiments,
which revealed that caffeine has antagonistic effects on endogenous protective pathways and is not only proinflammatory, but potentially antiresolution.

**Adenosine via A2A is anti-inflammatory, but not proresolving in gp91<sub>phox</sub>/H<sub>11002</sub>/H<sub>11002</sub> mice**

Being anti-inflammatory is not the same as possessing proresolving properties (23). Therefore, in these final experiments, we determined whether drugs that signal through A2A and raise cAMP rescued the hyperinflammatory phenotype typical of gp91<sub>phox</sub>/H<sub>11002</sub>/H<sub>11002</sub> mice, and importantly, whether they bring about resolution of inflammation in these animals. Thus, CGS 21680 was dosed l.p. 30 min before zymosan to gp91<sub>phox</sub>/H<sub>11002</sub>/H<sub>11002</sub>, and wild types with inflammation were assessed 4 h later. Data revealed that leukocyte influx was greater in knockouts than wild types, and that A2A receptor activation in knockouts reversed inflammation back to levels seen in drug-treated wild types (Fig. 5A), with the principal effect being on PMN numbers (Fig. 5B). We then investigated whether CGS 21680, given therapeutically during the equivalent of resolution in wild types, could alter the progression of inflammation in gp91<sub>phox</sub>/H<sub>11002</sub>/H<sub>11002</sub> mice. In wild types, CGS 21680, given at 24 h and again at 36 h after established inflammation, had surprisingly no effect on leukocyte numbers at 48 h, i.e., CGS 21680 was neither anti-inflammatory nor resolution toxic in normal animals (Fig. 5C). Interestingly, an identical dosing regime in knockouts revealed that CGS 21680 was not only anti-inflammatory in these animals, but that it lowered inflammation below that of controls (Fig. 5C) concomitant with an elevation in cAMP (Fig. 5F). cAMPucci, caffeine (E) and its stable analog (F), CSC, worsened inflammation and depressed IL-10, respectively, when administered in a similar manner to receptor agonists. In the final set of experiments, we demonstrated that A2A receptor activation maintained its anti-inflammatory effects and did not interfere with resolution pathways because injection of CGS 21680 (G), given 30 min before zymosan and again 12 and 18 h later, continued to dampen inflammation, whereas caffeine and its analog worsened the response, as determined at 24 h. n = 8 animals per group; *, p ≤ 0.05; **, p ≤ 0.01, as determined by ANOVA, followed by Bonferroni t test, with data expressed as mean ± SEM.
peritoneal cavity (20). Lymphocyte repopulation is not required to bring about resolution, but is critical in restoring tissue homeostasis and conferring protection against superinfection. In C57BL/6J-treated gp91<sup>phox</sup><sup>−/−</sup> mice, whereas there was a classic anti-inflammatory effect, reducing predominantly PMN numbers, we argue that this is not a resolution effect because repopulation lymphocytes were not seen (data not included).

Discussion

In this study, we report an immediate increase in adenosine and cAMP at the early on-set phase of acute inflammation that wanes as the response progresses, only to increase again as inflammation resolves, with levels being significantly lower in experimental CGD (gp91<sup>phox</sup><sup>−/−</sup> mice). Whether this is a direct result of an interaction between NADPH oxidase systems and adenosine synthesis is unlikely, because levels of cAMP in isolated cells from CGD patients produce similar levels, on a cell-for-cell basis, to normal healthy volunteers. Therefore, does the persistent nature of CGD arise from an inherent defect in counterregulatory/proresolving pathways, or a much simpler explanation of CGD patients being incapable of clearing inflammatory stimuli, resulting in a persistent, almost frustrated innate immune response that consequently nullifies endogenous protective pathways? As emphasized previously, one of the most critical determinants for resolution of inflammation is clearance of the inflammatory stimulus (23, 32). CGD is an example of where defects in clearance may be one of the primary causes of exacerbated and prolonged responses. Certainly, PMNs from CGD patients have impaired phagocytosis of immune complexes, whereas CGD macrophages are equally defective in their clearance of apoptotic PMNs (31). Equally, the formation of granulomatous synovitis in response to intra-articular zymosan injection in NADPH oxidase-deficient mice was suggested to result from incomplete zymosan clearance from the joint due to impaired phagocytosis (26). This, therefore, suggests that dysregulation in cAMP and adenosine is secondary to that of an overwhelming inflammatory event, whose proinflammatory signals deactivate or override endogenous anti-inflammatory and/or proresolving pathways.

From the above argument, it should not be assumed that all endogenous protective pathways are depressed during CGD. Among some of the signals that counterbalance inflammatory onset and/or trigger resolution, neither PGD<sub>2</sub> nor native lipoxin A<sub>4</sub> levels were statistically different in gp91<sup>phox</sup><sup>−/−</sup> mice compared with wild types at onset, with the exception of PGD<sub>2</sub>, which showed a trend toward a reduction in knockouts as inflammation resolved. That notwithstanding, data presented in this study show a more consistent dysregulation in the synthesis of adenosine/cAMP in gp91<sup>phox</sup><sup>−/−</sup> mice in response to sterile zymosan. We went on to investigate and show that A<sub>2A</sub> receptor activation rescues the hyperinflammatory response in gp91<sup>phox</sup><sup>−/−</sup> mice without subverting resolution in wild-type animals. This latter point is important because existing anti-inflammatory agents, NSAIDS for instance, are being protec-tive by virtue of their ability to dampen the early-onset phase of acute inflammation, pirate resolution, and prolong inflammation (22). A<sub>2A</sub> receptor activation, in contrast, is anti-inflammatory without being resolution toxic, thereby displaying broader pharmacological flexibility and potentially fewer side effects in terms of prolonging inflammation. However, these data are counterintuitive based on current understanding of cAMP in inflammatory leukocyte longevity and clearance as derived from in vitro studies. For instance, elevating cAMP in PMNs delays their apoptosis (40), whereas raising cAMP in monocyte-derived macrophages impairs their phagocytic capacity (41), suggesting that activating A<sub>2A</sub> during inflammation and consequently elevating cAMP would lengthen the life span of PMN, impair their clearance, and prolong inflammation. Despite these data from isolated cell systems, in vivo derived data from Figs. 4 and 5 clearly show that A<sub>2A</sub> receptor activation is anti-inflammatory without being resolution toxic, and that activation of this receptor at any phase of CGD dampens inflammation. This implies that CGD is in a constant state of perpetual acute inflammation, and that A<sub>2A</sub> receptors inhibit PMN influx. The current treatment regime for CGD patients is antibacterial and antifungal prophylaxis (42), but for exacerbations of inflammatory events, perhaps concomitant A<sub>2A</sub> receptor activation would dampen associated inflammatory responses without subverting proresolving pathways. At the very least, such patients should avoid caffeine (and perhaps other dietary methylxan-thines such as theobromine) because it may nullify whatever protection residual adenosine may confer in CGD during inflammatory events.

As mentioned previously, being anti-inflammatory in pharmacological terms is distinct from being proresolving (23). We make this assertion based on ongoing work from our laboratory, where we have shown in the resolving peritoneum not only a disappearance of PMN via apoptosis and macrophages via lymphatic drainage, but the influx of innate-type lymphocytes as inflammation resolves (20). These repopulating lymphocytes do not switch off inflammation, but modulate postinflammatory responses to bacteria in the context of secondary infection. In fact, when the cellular composition of the naïve peritoneal cavity is examined, innate-type lymphocytes are a predominant cell type along with resident macrophages. These lymphocytes disappear in response to inflammatory stimulus not before secreting cytokines that modulate the severity of the inflammatory response such that in RAG1<sup>−/−</sup> mice, for instance, inflammation is more exaggerated in terms of PMN trafficking. Therefore, as inflammation resolves, we suspect that repopulating lymphocytes simply reflect the inflamed tissue reverting to its prior physiological state under the control of as yet
unidentified endogenous factors. Interestingly, we found no repopulating lymphocytes in the peritoneal cavity of gp91$^{phox-/-}$ mice at the equivalent time points of resolution in wild types. Moreover, whereas activation of A$_2A$ was certainly anti-inflammatory in gp91$^{phox-/-}$ mice by virtue of its inhibition of PMN numbers when administered therapeutically at the equivalent phase of resolution in wild types, it did not bring about resolution in gp91$^{phox-/-}$ as defined by its inability to trigger lymphocyte repopulation. Along these lines, activating A$_2A$ with CGS 21680 at an earlier time point, which exerted classic anti-inflammatory effects as defined by reduced PMN numbers (Figs. 4C and 5, A and B), was also without effect on lymphocyte numbers. This suggests that other factors, besides that which signal cAMP, are responsible for lymphocyte repopulation and reversion to homeostasis.

Our finding of adenosine being secreted and cAMP expressed during the early-onset phase of the zymosan-induced peritonitis was not surprising given the established role these factors play in counterregulating innate immune-mediated tissue damage (9, 10). The reappearance of cAMP and adenosine again at resolution, however, is a reflection of our growing understanding of resolution being an active, immunosuppressive event controlled by endogenous counterregulatory stop signals. The point, however, is that adenosine/cAMP appears after the bulk of the inflammatory cells, including PMNs and monocyte-derived macrophages, have disappeared either by apoptosis or lymphatic drainage. In another report, we have identified the presence of a population of resolution-phase macrophages that have not vacated the peritoneum and are derived from a common Ly6C-positive monocyte precursor and possess a unique phenotype (manuscript submitted). These resolution-phase macrophage cells express all the typical markers of alternatively activated M2 cells along with inducible NO synthase and cyclooxygenase 2. It transpires that this phenotype is controlled by cAMP, which, if inhibited or elevated transforms the phenotype of resolution-phase macrophage to that of M1 cells and vice versa, respectively. Thus, the postresolution expression of cAMP is not required to switch off inflammation per se, but is most likely the next step in postinflammation tissue restitution and attempts to restore homeostasis.

From data presented in this study, A$_2A$ is shown to be anti-inflammatory, although not affecting proresolutton pathways. However, it is well known that caffeine is a nonspecific A$_2A$ receptor antagonist because it can antagonize A$_1$ as well as A$_2A$, but possesses a lower affinity for the A$_3$ receptor (39). Resulting from its nonspecific inhibition of A$_2A$, caffeine may therefore worsen inflammation and negatively affect proresolutton pathways. Indeed, feeding animals with 2 mg/kg caffeine or its stable analog just before zymosan injection exaggerated the inflammatory response 4 h later and also impaired resolution. This is important because the amount of caffeine administered to mice is equivalent to a realistic 1–2 cups of coffee. Increasing doses of caffeine to unrealistic 10–30 mg/kg, as also done in this current study, caused a loss of caffeine’s proinflammatory impact because at these levels and higher (100 mg/kg) caffeine may become a PDE4 inhibitor, resulting in cAMP elevation, as shown in vivo recently (43). Given the wide consumption of caffeine in the form of coffee and tea at least, we need to be aware of the data presented in this study and that presented by others (43), which emphasizes that interfering with endogenous protective pathways, adenosine in this case, at realistic levels of socially consumed beverages will hamper innate immune responses, thus impairing the ability to combat infections concomitant with prolonging resolution. However, any attempts to increase caffeine intake in the hope of inhibiting PDE4 to dampen inflammation via cAMP elevation would require prohibitively high quantities of the drug. Thus, the most likely result of social caffeine consumption would be proinflammatory and resolution toxicity.

In conclusion, we show that of the endogenous anti-inflammatory pathways examined in CGD, both adenosine and its intracellular signaling molecule, cAMP, show dysregulation in their synthesis at onset and resolution, suggesting that CGD is in a constant state of proinflammation and PMN trafficking, with no apparent attempts at resolution due to the persistence of the inflammatory stimulus. Rescuing this hyperinflammatory state with A$_2A$ agonists shows powerful anti-inflammation that does not bring about resolution because it inhibits PMN trafficking, but does not initiate lymphocyte repopulation and reversal to homeostasis. Nonetheless, it does suggest a potential treatment regime to dampen the hyperinnate immune component of CGD-associated infections.

Disclosures
The authors have no financial conflict of interest.

References
phox and gp91
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