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Nonresolving Inflammation in gp91phox$^{-/-}$ Mice, a Model of Human Chronic Granulomatous Disease, Has Lower Adenosine and Cyclic Adenosine 5′-Monophoshate

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Abstract

In chronic granulomatous disease (CGD) there is failure to generate reactive oxygen metabolites resulting in recurrent infections and persistent inflammatory events. As responses to sterile stimuli in murine models of CGD also result in non-resolving inflammation, we investigated whether defects in endogenous counter-regulatory mechanisms and/or pro-resolution pathways contribute to the aetiology of CGD. To this end we carried out 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 gp91phox$^{-/-}$ 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, signalling through its A_{2A} receptor and therefore elevating cAMP is not only anti-inflammatory but, importantly, it does not impair pro-resolution pathways, properties typical of nonsteroidal anti-inflammatory drugs. Conversely, antagonising the A_{2A} receptor worsens acute inflammation and prolongs resolution. Taking this further, activating the A_{2A} receptor in gp91phox$^{-/-}$ mice was dramatically anti-inflammatory regardless of the phase of the inflammatory response A_{2A} agonists were administered i.e. onset or resolution demonstrating wide and robust pharmacological flexibility that is unlikely to subvert pro-resolution pathways. Therefore, we describe the biphasic profile of adenosine and cAMP throughout the time course of acute inflammation that is dysregulated in CGD.

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Keywords

Lipid mediators; Cytokines; Human peritonitis

INTRODUCTION

In response to injury/infection there is the sequential release of pro-inflammatory mediators including complement(1), histamine(2,3), bradykinin(4) and 5-hydroxytryptamine(5) along with cytokines and chemokines(6) that cause cell and edema accumulation. Concomitantly, there are braking signals that temper the severity of this early onset phase including adrenaline (7), adenosine(8-10), steroid hormones(11), cAMP(12) and counter-regulatory cytokines including IL-10(13). In attempts to enhance our understanding of acute inflammation, we are beginning to realise that, in addition to endogenous checkpoints that temper PMN trafficking there are also signals that control the transition of PMNs to phagocytosing macrophages (prostaglandin [PG]E_2(14), IL6/IL6ra complex(15)), scavenge cytokine/chemokine from the inflammatory environment (D6 scavenger receptor(16), lipoxins(17)) as well as clear macrophages to the draining lymph nodes (PGD_2(18) and resolvins(19)) leading to inflammatory resolution. Advancing this paradigm even more, our group recently identified the post-resolution influx of innate-type lymphocytes to sites of inflammation with a role in controlling host responses to secondary inflammation and superinfection(20). Indeed, we propose, for the first time, that one of the cardinal signs of resolution is the recruitment of innate-type lymphocytes to sites of tissue injury concomitant with inflammation switching off. It is argued that while inflammation has apparently resolved, classically defined by the clearance of inflammatory leukocytes, there are events occurring locally involving innate-type lymphocytes that have an impact on organ and host health long after inflammation has abated. Thus, innate immune responses are designed to combat infection and repair injured tissues in a timely manner. However, there are instances when the endogenous factors that control immune severity become dysregulated, resulting in progression to chronic inflammation and tissue injury. To combat this, anti-inflammatory agents were designed based on their inhibition of signals that propagate inflammation(21) with little consideration for any adverse impact on pro-resolution pathways, as has been demonstrated with non-steroidal anti-inflammatory drugs (NSAIDs)(22). Consequently, when treating inflammation we must also aim to trigger its resolution or, at least, not impair endogenous pathways that lead to tissue homeostasis(23), with one of the hallmarks for this, in the peritoneum at least, being innate-type lymphocyte repopulation(20).

There are many clinical examples where chronic inflammation could be hypothesised 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 aetiology 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 neutralise infection leading to persistent and recurrent inflammatory responses and granulomatous tissue formation(24). Activity of NADPH oxidase system (gp91phox, p22phox, p47phox and p67phox) 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 aetiology of CGD, gp91phox deficient mice display all 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 pro-resolution pathways. We carried out a series of studies in gp91phox$^{-/-}$ 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 to wild types.

Under physiological conditions, adenosine is continuously formed intracelluarly and extracellularly. The intracellular production is mediated either by an intracellular 5′-nucleotidase, which dephosphorylates AMP or by hydrolysis of *S*-adenosyl-homocysteine (28). Adenosine generated within cells is transported into the extracellular space *via* bi-directional 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 (A_1, A_{2A}, A_{2B}, A_3) 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 A_1 and A_3 receptors are high and low affinity receptors for adenosine, respectively, with both being inhibitors of adenylyl cyclase. High-affinity A_{2A} and low-affinity A_{2B} receptors, on the other hand, 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, D, 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 prevents further damage by inhibiting activated immune cells with its immune-suppression mediated by A_{2A} receptor elevation 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 (gp91phox$^{-/-}$) mice associated with a severe and prolonged innate immune response to a sterile stimuli. We also show that hyper-inflammation in gp91phox$^{-/-}$ mice can be rescued by A_{2A} receptor activation as defined by reduction in inflammatory leukocytes. Importantly, A_{2A} receptor activation in gp91phox$^{-/-}$ mice did not bring about resolution as 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

gp91phox$^{-/-}$ mice (Jackson Laboratories, Maine, USA),along with wild type mice, were bred under standard conditions and maintained in a 12h/12h 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 3ml of sterile air followed 7 days later with the intra-pouch injection of 0.5ml 1% carrageenin. Peritonitis was induced by the intraperitoneal injection of 1mg type A zymosan (Sigma) and cells enumerated by haemocytometer at time points stated in results section by sterile PBS washout. Ethical approval (P/03/136A) for collection of human peritonitis samples was obtained from St. Bartholomew's & 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 heparinised syringes (5U/ml). Mononuclear cells were isolated by differential centrifugation (2000rpm, 30 mins, 20°C) over Lymphoprep® (Axis-Shield, Oslo, Norway) and washed twice with sterile phosphate-buffered saline (PBS) (GIBCO, Paisley, UK) at 1200rpm (5 mins, 20°C). Cells were re-suspended in 10mls RPMI-1640 medium (Invitrogen, Paisley, UK) supplemented with 100U/ml of penicillin (GIBCO) and 100μg/ml streptomycin (GIBCO) and 20mM Hepes buffer (Sigma) (RPMI), and plated at a density of approximately 5×10^6 cells/ml in $8cm^2$ Nunclon™ Surface tissue culture dishes (Nunc, Roskilde, Denmark). After an initial culture period of 2 hours at 37°C, 5% CO_2, the non-adherent cells were discarded and 10mls of fresh RPMI supplemented with 10% foetal bovine serum (Sigma) (10% FBS/RPMI) added to each tissue culture dish. Cells were then cultured for 5 days at 37°C, 5% CO_2, with the addition of a further 10mls fresh 10% FBS/RPMI after 24 hours. Adherent cells were scraped on day 5 and re-plated in 96-well culture plates at equal densities (10^5/well) in X-Vivo-15 medium (Cambrex, Walkersville, MD, USA). These primary monocyte-derived macrophages were incubated overnight at 37°C, 5% CO_2 to adhere, and then stimulated for 24 hours with 200ng/ml lipopolysaccharide (LPS) (Alexis, San Diego, CA, USA). These studies were approved by the Joint UCL/UCLH 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

gp91phox$^{-/-/-}$ and wild type mice were pre-treated (30mins) with the A_{2A} receptor agonist CGS 21680 (2mg/kg) with/without ZM241385 (2mg/kg, A_{2A} receptor antagonist) followed by zymosan intraperitoneally. Rolipram was dosed at 30mg/kg. The peritoneal cavity was lavaged at 4 hours and cell number counted using a haemocytometer. For experiments in the resolution phase, inflammation was firstly induced with zymosan followed by CGS 21680 or vehicle (DMSO) at 24 and 36 hours. The peritoneal cavity was lavaged at 48 hours following zymosan. Caffeine as well as its stable analogue, 8-(3-chlorostyryl)-caffeine (CSC) were dosed at 3mg/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 PGD_2, samples were then treated with methoxylamine hydrochloride (MOX HCL) and the resulting stable PGD_2-MOX measured by EIA (Cayman Chemicals, USA). PGE2 was measured by EIA (Cayman Chemicals, USA) while lipoxin A4 was quantified by ELISA (Neogen, USA).

Purine and cAMP measurements

Proteins in exudates were removed by ultrafiltration (30,000 Dalton cut-off). Purine concentrations in samples were measured by high pressure liquid chromatography as previously described(30).

FACS analysis and cytokine/chemokine analysis

Cytokines were measured by ELISA according to manufacturer's instructions (eBiosciences). FACS was carried out on Becton Dickinson Facscalibur with data analysed by Cellquest. Leukocytes were incubated with antibodies for 30mins to either CD3/CD19 (Serotec, UK), B cells (Ly220, Serotec), GR1 (BD Pharmingen, UK) or F4/80 (Caltag laboratories, CA, USA) using respective isotype antibodies as controls (Serotec, UK) and compensated as appropriate for dual labelling. For apoptosis, cells were incubated with annexin V/propidium iodide

(Becton Dickinson) and analysed on Becton Dickinson Facscalibur with data analysed by Cellquest.

RESULTS

Inflammation is more severe and fails to resolve in gp91phox$^{-/-}$ mice

In the first set of experiments we characterised the profile of inflammation in wild types versus gp91phox$^{-/-}$ mice. Zymosan injection into the mouse peritoneal cavity resulted in an exaggerated influx of PMNs (Figure 1A, Ly6G positive cells) in knockouts during the early onset phase with numbers declining up to 96h. F4/80 positive macrophage numbers were similarly increased at onset in knockouts with levels remaining elevated throughout the entire response, Figure 1B, underlining the non-resolving nature of inflammation that is characteristic of CGD. As there was little difference in leukocyte apoptotic rates as determined by annexin V/propidium iodide labelling between both animals, Figure 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 gp91phox$^{-/-}$ 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, while 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 counter-regulate acute inflammation(23, 32,33). To this end we screened for alterations in levels of anti-inflammatory and pro-resolution 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 being involved in aetiology of CGD, Figures 2A-C. However, we noted a clear and robust reduction in levels of cAMP in CGD mice compared to wild type animals, Figure 2D. Specifically, intracellular cAMP was elevated during the early onset phase of zymosan-induced peritonitis in wild type controls (4-6h), waning as inflammation progressed and became elevated again post-resolution, Figure 2D. In gp91phox$^{-/-}$ mice bearing a zymosan-triggered peritonitis, cAMP was significantly lower than wild types at onset and failed to show the post-resolution elevation seen in wild types, Figure 2D. The functional relevance of raised cAMP post resolution is being answered in another body of work (paper enclosed), 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 gp91phox$^{-/-}$ 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 gp91phox$^{-/-}$ mice. In fact, there was an elevation of PGE_2 (Figure 2A) in gp91phox knockouts indicating possible signalling of this PG through its EP1 (increased Ca2+) and/or EP3 (IP_3/DAG) 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, Figure 2E. Adenosine showed lower levels in gp91phox$^{-/-}$ mice than wild types and is a well described immuno-modulator 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, signalling through A_{2A} has received most attention as a trigger for immuno-suppressive 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-inflammatories in CGD that is responsible for its hyper-inflammatory, non-resolving 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 as levels of cAMP released from monocyte-derived macrophages obtained from CGD patients released similar quantities of cAMP per equivalent cell numbers to those from healthy volunteers when stimulated with LPS, Figure 2F.

Adenosine and cAMP profiles in human resolving peritonitis

As an alternative to dialysis, patients on end-stage renal failure may undergo chronic ambulatory peritoneal dialysis (CAPD) where a catheter is inserted to fill/drain the peritoneal cavity with a high glucose solution with the peritoneal lining acting as a dialysing membrane. Occasionally, patients experience bacterial infection (commonly *S. aureus* or *S. epidermidis*) resulting in acute resolving peritonitis from which both effluent and cells can be analysed for markers of inflammation and resolution(38). We measured levels of cAMP (Figure 3A) as well as adenosine, (Figure 3B) in these samples and found that when overlaid on the inflammatory leukocyte profile (Figure 3C), which peaked 24h after infection, cAMP as well as adenosine was elevated as inflammation resolved. While these results corroborates that found in mice, we have no data on the very early onset (~ 6h) 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, *via* A_{2A} is anti-inflammatory and not resolution-toxic

As with the plethora of effects NSAIDs exert on acute inflammation, it is well established that by signalling through its A_{2A} receptor, adenosine exerts protective effects during acute inflammation at multiple levels. However, while NSAIDs dampen the early onset phase of acute inflammation, they also obstruct pro-resolution processes(22). Therefore, we next determined whether A_{2A} signalling 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 A_{2A} receptor agonist 30mins prior to carrageenin injection. Both drugs dampened leukocyte trafficking to the inflamed cavity at onset i.e. 4h (Figure 4A), being associated with an expected rise in cAMP, Figure 4B. In a mouse zymosan-induced peritonitis at 4h, CGS 21680 also dampened inflammation in an A_{2A} receptor dependent manner using ZM 241385 (A_{2A} receptor antagonist) (Figure 4C) associated with a significant increase in anti-inflammatory IL-10, Figure 4D. Other drugs, including theophylline as well as beverages are known to alter cAMP signalling and therefore may unwittingly affect inflammation. A single cup of coffee, for instance, contains about 100mg of caffeine implying that an average person drinking one cup of coffee per day will ingest caffeine at 1.5mg/kg. To investigate whether caffeine, a methylxanthine with antagonistic effects on the A_{2A} receptor(39) affects acute inflammation, we administered caffeine as well as its stable analogue, 8-(3-chlorostyryl)-caffeine (CSC) at 3mg/kg 30mins before i.p. zymosan injection and found that both worsened inflammation possibly by decreasing protective IL-10 levels, Figures 4E and F. Using a continual pharmacological dosing regimen, mice were administered not only 30mins before zymosan injection but were also given caffeine and CSC again at 12h and 18h after zymosan and their effects assessed at 24h. The idea being that as inflammation resolves by 24h, 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, Figure 4G. On the other hand, caffeine at doses representative of reasonable caffeine daily intake as well as its analogue, CSC, maintained their pro-inflammatory effects, Figures 4G. Taking the doses of caffeine up to 10 and 30mg/kg, equivalent to 6-20 cups of coffee, resulted in a loss of pro-inflammatory effects.

Therefore, from these experiments, A_{2A} 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 pro-resolution pathways. An interesting observation was the corollorary to these experiments, which revealed that caffeine has antagonistic effects on endogenous protective pathways and is not only pro-inflammatory but potentially anti-resolution.

Adenosine *via* A_{2A} is anti-inflammatory but not pro-resolving in gp91phox$^{-/-}$ mice

Being anti-inflammatory is not the same as possessing pro-resolution properties(23). Therefore, in these final experiments, we determined whether drugs that signal through A_{2A} and raise cAMP rescued the hyper-inflammatory phenotype typical of gp91phox$^{-/-}$ mice and importantly, whether they bring about resolution of inflammation in these animals. Thus, CGS 21680 was dosed orally 30mins before zymosan to gp91phox$^{-/-}$ and wild types with inflammation assessed 4h later. Data revealed that leukocyte influx was greater in knockouts than wild types and that A_{2A} receptor activation in knockouts reversed inflammation back to levels seen in drug-treated wild types (Figure 5A), with the principle effect being on PMN numbers, Figure 5B. We then investigated whether CGS 21680, given therapeutically during the equivalent of resolution in wild types, could alter the progression of inflammation in gp91phox$^{-/-}$ mice. In wild types, CGS 21680, given at 24h and again at 36h after established inflammation, had surprisingly no effect on leukocyte numbers at 48h i.e. CGS 21680 was neither anti-inflammatory nor resolution-toxic in normal animals, Figure 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 (Figure 5C) concomitant with an elevation of cAMP, Figure 5D. To determine whether this was an anti-inflammatory or a pro-resolution effect, we next quantified the number of lymphocytes in CGS 21680-treated gp91phox$^{-/-}$ mice. The rationale for using these criteria for resolution stemmed from our recent findings, which revealed that as inflammation resolves, lymphocyte repopulate the 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 CGS 21680-treated gp91phox$^{-/-}$ mice, while there was a classic anti-inflammatory effect, reducing predominantly PMN numbers, we argue that this is not a resolution effect as repopulation lymphocytes were not seen, data not included.

DISCUSSION

Here we report an immediate increase in adenosine and cAMP at the early onset 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 (gp91phox$^{-/-}$ mice). Whether this is a direct result of an interaction between NADPH oxidase systems and adenosine synthesis is unlikely as levels of cAMP in isolated cells from CDG 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 counter-regulatory/pro-resolution 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 emphasised 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 while 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 pro-inflammatory signals deactivates or overrides endogenous anti-inflammatory and/or pro-resolution 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 counter-balance inflammatory onset and/or trigger resolution, neither PGD_2 nor native lipoxin A_4 levels were statistically different in gp91phox$^{-/-}$ mice compared to wild types at onset with the exception of PGD_2, which showed a trend towards a reduction in knockouts as inflammation resolved. That notwithstanding, data presented here shows a more consistent dysregulation in the synthesis of adenosine/cAMP in gp91phox$^{-/-}$ mice in response to sterile zymosan. We went on to investigate and show that A_{2A} receptor activation rescues the hyper-inflammatory response in gp91phox$^{-/-}$ mice without subverting resolution in wild type animals. This latter point is important as existing anti-inflammatories, NSAIDS for instance, while being protective by virtue of their ability to dampen the early onset phase of acute inflammation, pirate resolution and prolong inflammation(22). A_{2A} receptor activation, on the other hand, 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 counter-intuitive 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) while raising cAMP in monocyte-derived macrophages impairs their phagocytic capacity(41) suggesting that activating A_{2A} 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 Figures 4 and 5 clearly show that A_{2A} 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_{2A} 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_{2A} receptor activation would dampen associated inflammatory responses without subverting pro-resolution pathways. At the very least, such patients should avoid caffeine (and perhaps other dietary methylxanthines such as theobromine) as 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 pro-resolution(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 post-inflammatory 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$^{-/-}$ 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 un-indentified endogenous factors. Interestingly, we found no repopulating lymphocytes in the peritoneal cavity of gp91phox$^{-/-}$ mice at the equivalent time points of resolution in wild types. Moreover, while activation of A_{2A} was certainly anti-inflammatory in gp91phox$^{-/-}$ mice by virtue of it inhibition of PMN numbers when administered therapeutically at the equivalent phase of resolution in wild types, it did not bring about resolution in gp91phox$^{-/-}$ as defined by its inability to trigger lymphocyte repopulation. Along these lines, activating A_{2A} with CGS 21680 at earlier time point, which

exerted classic anti-inflammatory effects as defined by reduced PMN numbers (Figure 4C and 5A-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-induce peritonitis was not surprising given the established role these factors play in counter-regulating innate-immune mediated tissue damage(9,10). The re-appearance of cAMP and adenosine again at resolution, however, is a reflection of our growing understanding of resolution being an active, immuno-suppressive event controlled by endogenous counter-regulatory 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 (paper enclosed) we have identified the presence of a population of resolution-phase macrophages (rM) that have not vacated the peritoneum and are derived from a common Ly6C-positive monocyte precursor and which possess a unique phenotype. These rM cells express all the typical markers of alternatively-activated M2 cells along with iNOS and COX 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 post-resolution expression of cAMP is not required to switch off inflammation *per se* but is most likely the next step in post-inflammation tissue restitution and attempts to restore homeostasis.

From data presented in this study, A_{2A} is shown to be anti-inflammatory whilst not affecting pro-resolution pathways. However, it is well known that caffeine is a non-specific A_{2A} receptor antagonist as it can antagonise A_1 as well as A_{2A} but possesses a lower affinity for the A_3 receptor(39). Resulting from its non-specific inhibition of A_{2A}, caffeine may therefore worsen inflammation and negatively affect pro-resolution pathways. Indeed, dosing animals with 2mg/kg caffeine or its stable analogue just before zymosan injection exaggerated the inflammatory response 4h later and also impaired resolution. This is important as 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 pro-inflammatory impact as at these levels and higher (100mg/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 paper and that presented by others(43), which emphasises 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 in order 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 pro-inflammatory and resolution toxicity.

In conclusion, we show that of the endogenous anti-inflammatory pathways examined in CGD, both adenosine and its intracellular signalling molecule, cAMP, show dysregulation in their synthesis at onset and resolution suggesting that CGD is in a constant state of pro-inflammation and PMN trafficking with no apparent attempts at resolution due to the persistence of the inflammatory stimulus. Rescuing this hyper-inflammatory state with A_{2A} agonists shows powerful anti-inflammation that does not bring about resolution as 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 hyper-innate immune component of CGD-associated infections.

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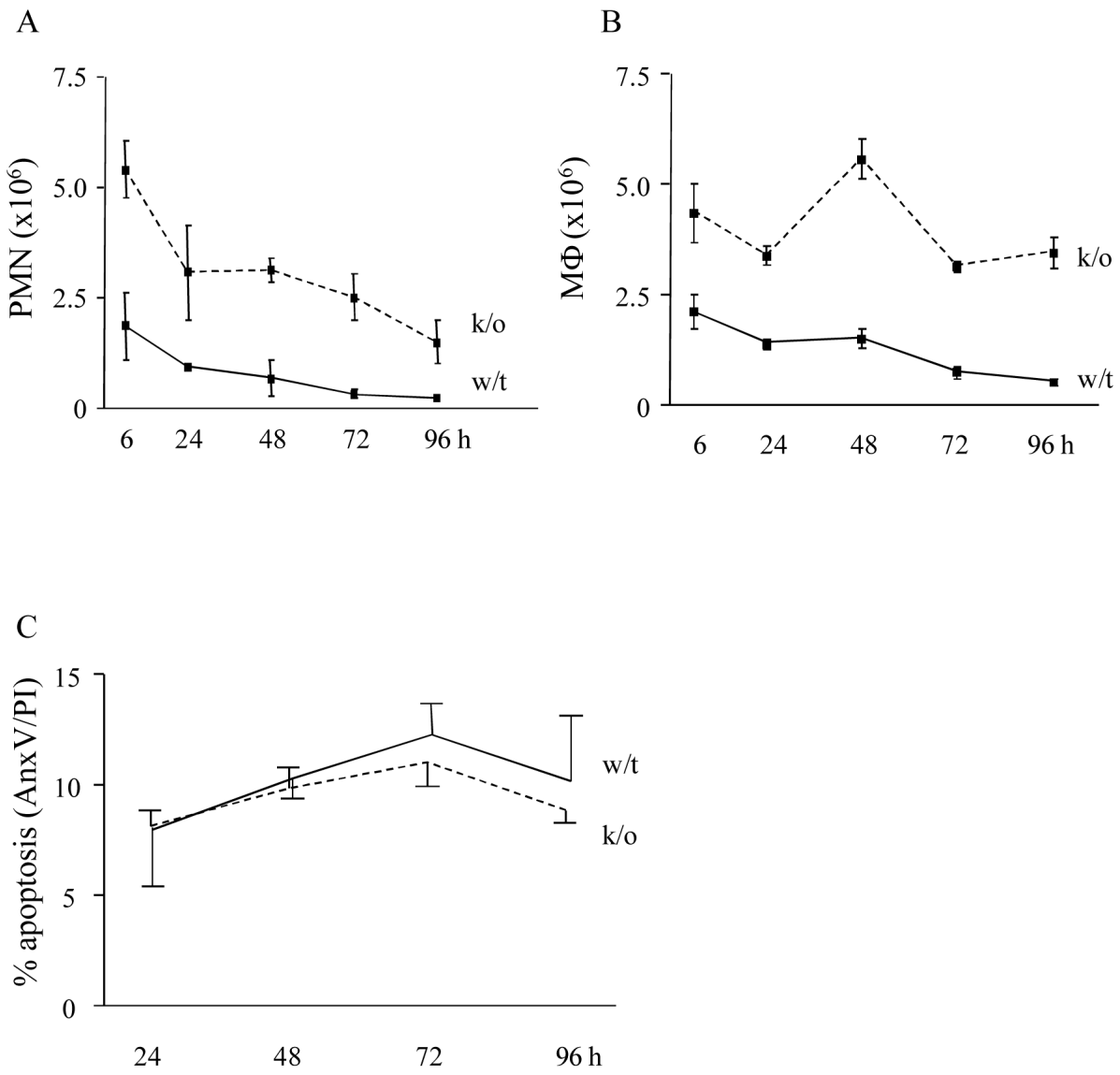

Figure 1.

Inflammation is more severe and fails to resolve in gp91phox$^{-/-}$ mice. Intraperitoneal zymosan injection resulted in a more exaggerated influx of (A) Ly6G-positive PMNs in gp91phox$^{-/-}$ mice along with (B) F4/80-positive monocyte-derived macrophages that persisted in these animals past the equivalent time point of resolution in wild types with the persistence of this response not due to (C) reduced apoptosis in gp91phox$^{-/-}$ mice. *n* = 8-10 animals per group with data expressed as mean ± SEM.

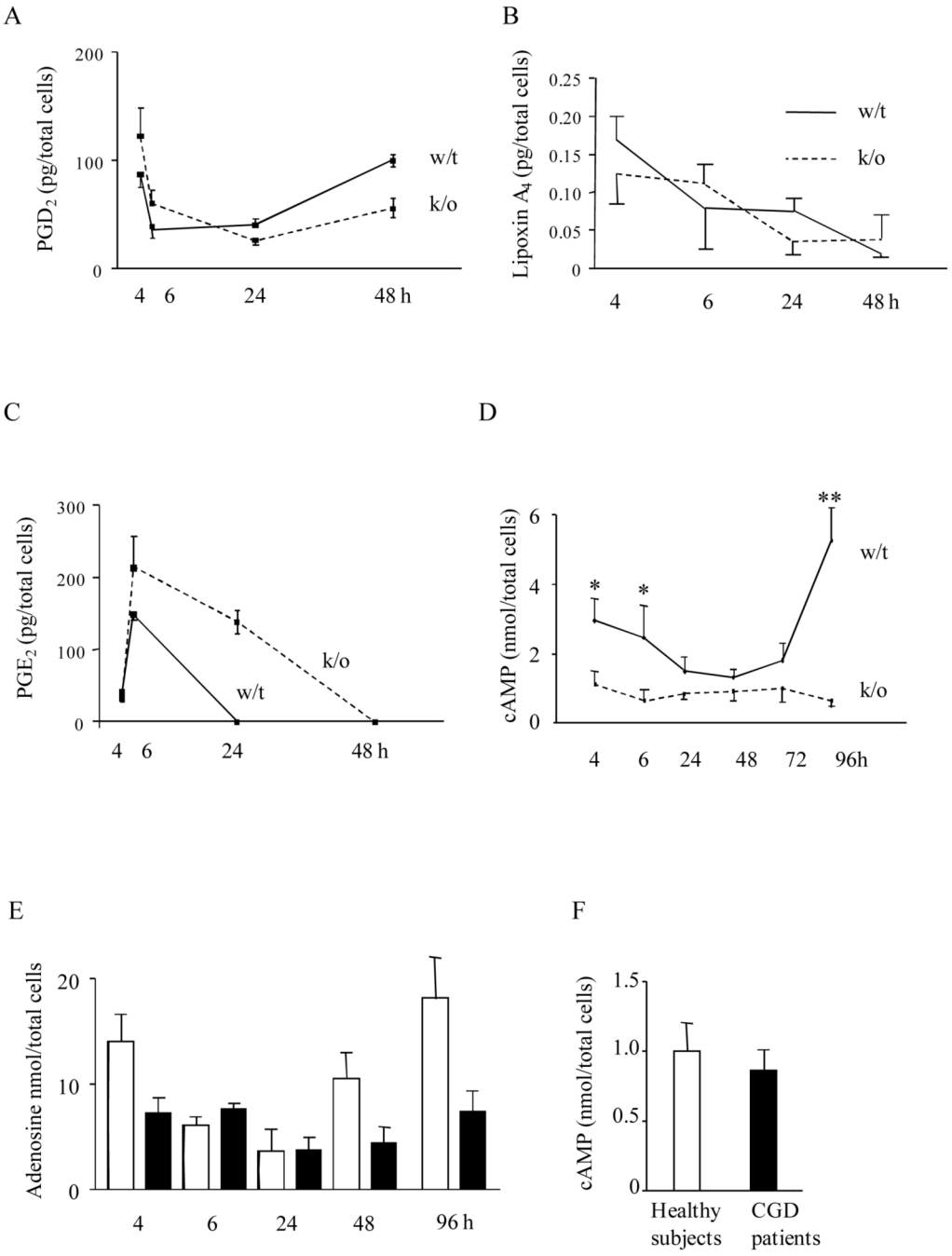

Figure 2.

Adenosine and cAMP synthesis is abrogated in gp91phox$^{-/-}$ mice. In an attempt to identify potential dysregulations in the synthesis of endogenous anti-inflammatory/pro-resolution factors in gp91phox$^{-/-}$ mice, arachidonic acid metabolism, as determined by (A) PGD_2, (B) lipoxin A_4 as well as (C) PGE_2 were determined and found not to show a consistent reduction in gp91phox$^{-/-}$ mice compared to wild types. However, (D) cAMP was elevated at the early onset phase of a murine peritonitis and then again at resolution in wild type animals, but was significantly lower in gp91phox$^{-/-}$ 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 gp91phox$^{-/-}$ mice (filled columns)

compared to wild types (empty columns). While this would suggest dysregulated cAMP signalling in CGD, (F) 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 over ridden by suppressive factors released during severe inflammation typical of CGD. *n* = 6-10 animals per group with data expressed as mean ± SEM.

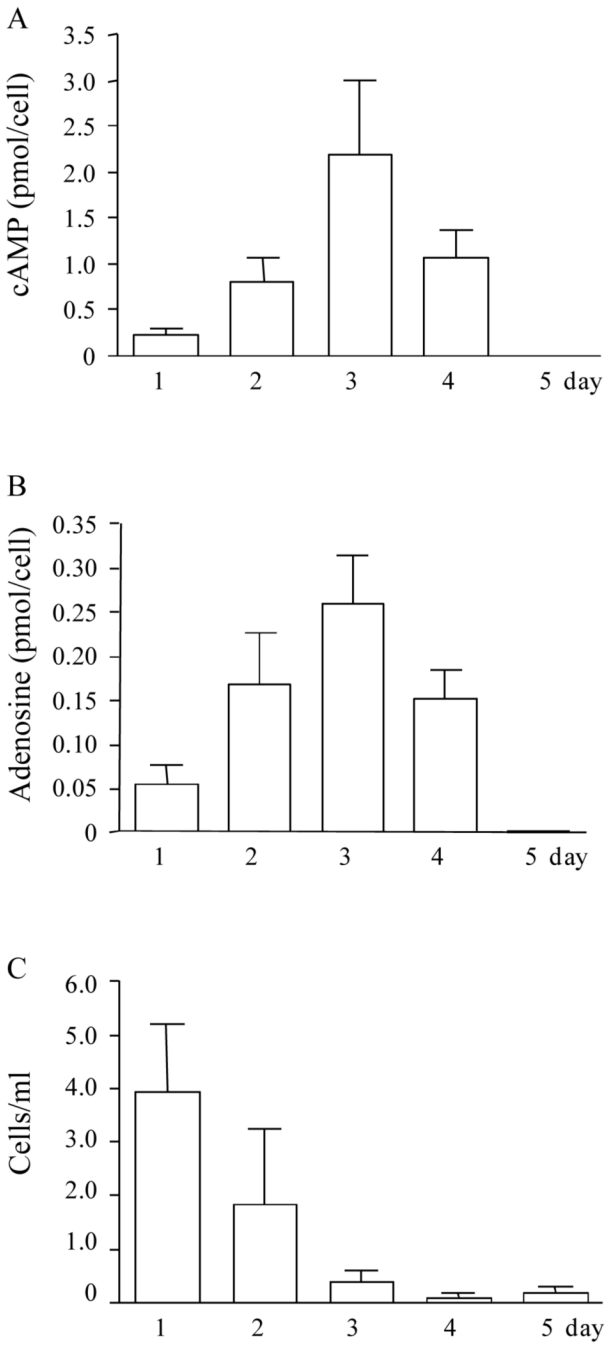

Figure 3.

Expression of adenosine and cAMP concomitant with resolution of human peritonitis. Peritonitis samples were obtained from patients undergoing chronic ambulatory peritoneal dialysis and who experience transient infection that usually resolves with a few days. Analysis of cell-free exudates revealed a peak in (A) cAMP and (B) adenosine as (C) inflammation resolved. n = 6-8 patients with data expressed as mean ± SEM.

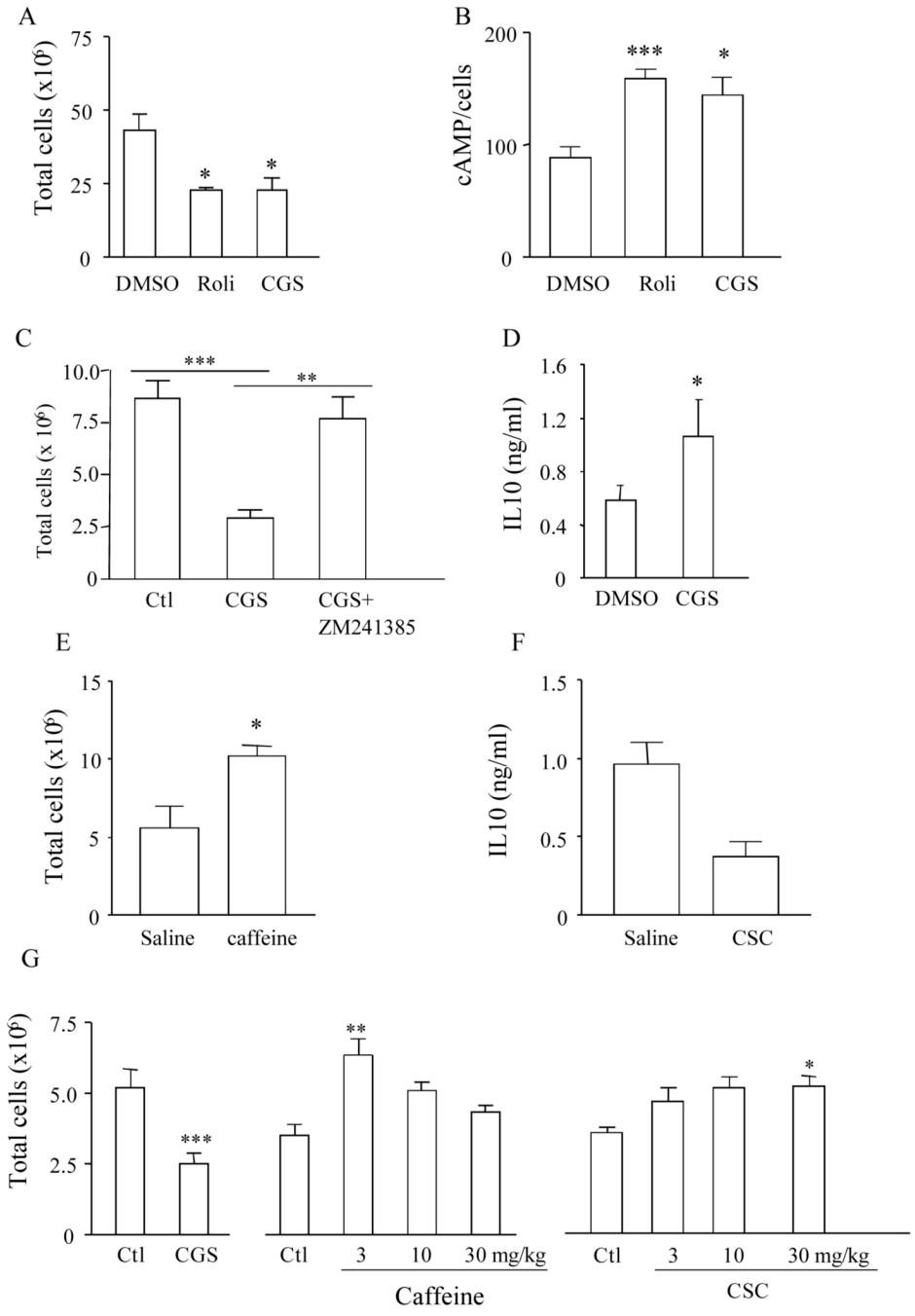

Figure 4.

Adenosine, *via* A_{2A} is anti-inflammatory and not resolution-toxic. The selective A_{2A} agonist CGS 21680 as well as the PDE4 inhibitor rolipram, which increases cAMP were dosed at 10 and 30 mg/kg respectively, to rats bearing a carrageenin-induced pleurisy. In (A) rolipram and CGS 21680 were dosed 30 mins prior to pleurisy induction and their effects assessed 4h later with A_{2A} receptor activation exerting an anti-inflammatory effect concomitant with an increase in (B) cAMP in this model. A_{2A} receptor activation was also examined in a zymosan-induced peritonitis dosed 30mins prior to zymosan injection and the effects of CGS 21680 with or without ZM241385 (A_{2A} receptor antagonist) determined 4h later revealing, again, that (C) CGS 21680 is anti-inflammatory in this model concomitant with an elevation in (D) anti-

inflammatory IL-10. Conversely, the A_{2A} receptor antagonist (E) caffeine and its (F) stable analogue, 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 A_{2A} receptor activation maintained its anti-inflammatory effects and did not interfere with resolution pathways as injection of (G) CGS 21680, given 30mins before zymosan and again 12h and 18h later continued to dampen inflammation while caffeine and its analogue worsened the response as determined at 24h. $n = 8$ animals per group; *, $P \leq 0.05$; **, $P \leq 0.01$, as determined by ANOVA, followed by Bonferroni *t* test, with data expressed as mean ± SEM.

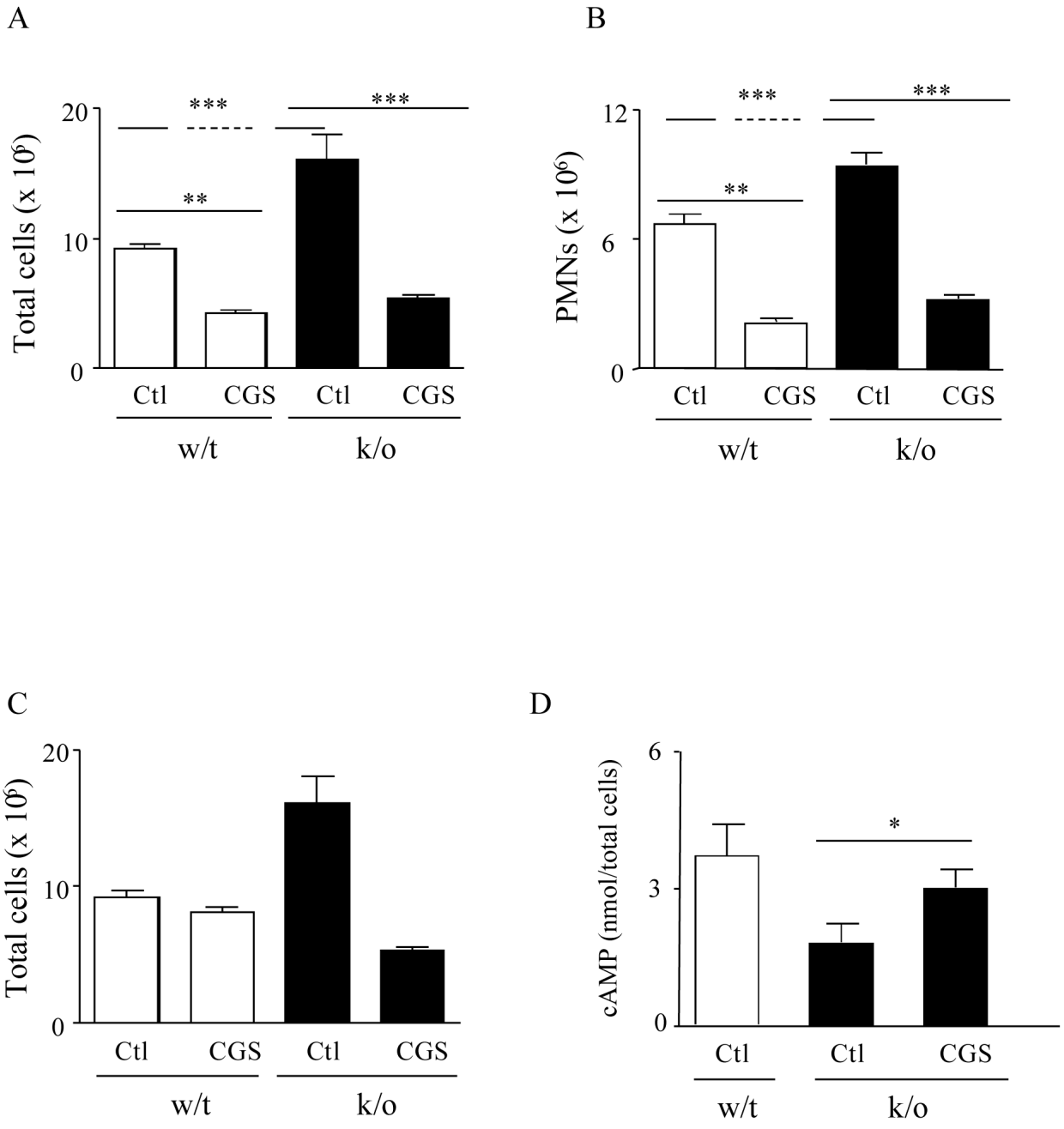

Figure 5.

Adenosine, *via* A_{2A} is anti-inflammatory but not pro-resolution in gp91phox$^{-/-}$ mice. Wild type and gp91phox$^{-/-}$ mice were injected with zymosan i.p. and the A_{2A} agonist CGS 21680 given either (A-B) 30mins before stimulus injection to both wild types and knockouts and its effects determined 4h later or (C) administered to both animal types therapeutically i.e. 24h and 36h after zymosan and its effects on resolution and (D) cAMP determined at 48h. $n = 6$ animals per group; *, $P \leq 0.05$; **, $P \leq 0.01$, as determined by ANOVA, followed by Bonferroni *t* test, with data expressed as mean ± SEM.