Overexpression of CD39 in mouse airways promotes bacteria induced inflammation.[1]

Running Title: CD39 and lung inflammatory response

Emilie Théâtre,[2*] Kim Frederix,[2*] William Guilmain,[*] Christelle Lecut,[*] Lucien Bettendorff,[†] Vincent Bours,[*] Cécile Oury[*]

[*]GIGA-Inflammation, Infection, Immunity, Unit of Human Genetics, Laboratory of Thrombosis and Haemostasis, [†]GIGA-Neurosciences, Unit of Bionergetics and Cerebral Excitability, University of Liège, Liège, Belgium.

Corresponding author:

Dr. Cécile Oury, phone: +32(0)4 3662487, Fax: +32(0)4 3664534, E-mail address: cecile.oury@ulg.ac.be.

Keywords: Bacterial infection, inflammation, lung, transgenic/knockout mice.

Abstract

In airways, the ecto-nucleoside triphosphate diphosphohydrolase CD39 plays a central role in the regulation of physiological mucosal nucleotide concentrations and likely contributes to the control of inflammation since accelerated ATP metabolism occurs in chronic inflammatory lung diseases. We sought to determine whether constant elevated CD39 activity in lung epithelia is sufficient to cause inflammation and whether this affects the response to acute lipopolysaccharide or *Pseudomonas aeruginosa* exposure. We generated transgenic mice overexpressing human CD39 under the control of the airway-specific Clara cell 10-kDa protein gene promoter. Transgenic mice did not develop any spontaneous lung inflammation. However, intratracheal instillation of lipopolysaccharide resulted in accelerated recruitment of neutrophils to the airways of transgenic mice. Macrophage clearance was delayed, and the amounts of CD8+ T and B cells were augmented. Increased levels of KC, interleukin-6 and RANTES were produced in transgenic lungs. Similarly, higher numbers of neutrophils and macrophages were found in the lungs of transgenic mice infected with *Pseudomonas aeruginosa*, which correlated with improved bacteria clearance. The transgenic phenotype was partially and differentially restored by co-instillation of $P2X_1$ or $P2X_7$ receptor antagonists or of caffeine with lipopolysaccharide. Thus, a chronic increase of epithelial CD39 expression and activity promotes airway inflammation in response to bacterial challenge by enhancing P1 and P2 receptor activation.

Introduction

Airway epithelia constitute an essential protective barrier against lung infection, coordinating luminal and interstitial responses to inhaled pathogens through signals provided by epithelial, inflammatory, and immune cells. Extracellular nucleotides provide an elaborated cell communication system in mammalian tissues including the airways (1). The major source of extracellular nucleotides in normal airways is the epithelium, secreting ATP under resting conditions and in response to various mechanical stimulations, including membrane stretch, shear stress, hypotonicity-induced swelling, and after physical interaction with air contaminants or microbes (2). Extracellular ATP and adenosine contribute to mucociliary clearance (3), a process critical for maintaining the airways clear of inhaled particles or pathogens. In inflammatory conditions, ATP can also be abundantly released upon cell damage as well as by activated leukocytes and platelets (4).

Upon release, extracellular ATP acts in an autocrine or paracrine manner on specific cell surface P2 receptors belonging to two subclasses, the G protein-coupled P2Y receptors and the ATP-gated P2X non-selective cation channels (5). At present, eight human P2Y receptors, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11\text{-}14}$, and seven P2X subtypes, $P2X_{1\text{-}7}$, have been identified. P2 receptor-mediated signals are quickly terminated due to receptor desensitization and/or scavenging of ATP by cell surface ecto-nucleotidases. In airways, ATP is converted into adenosine through a two-step enzymatic process mediated by the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) CD39 (conversion of ATP and ADP to AMP) and the ecto-5'-nucleotidase CD73 (conversion of AMP to adenosine) that play major roles in the regulation of physiological mucosal nucleotide concentrations (6). Adenosine activates P1 receptors identified as A_1, A_{2A}, A_{2B} and A_3 receptors (5). Substantial preclinical evidence suggests that targeting of adenosine receptors may provide novel approaches for the treatment of asthma and chronic obstructive pulmonary disease (COPD) (7).

Recently, it has been reported that ATP accumulates in the airways in various animal models of airway inflammation or patients with asthma, cystic fibrosis, or COPD (8-10). Moreover, both *in vitro* and *in vivo* evidence indicates that ATP plays an active role in the pathogenesis of asthma and COPD (10) and antagonizing P2 receptors reduces inflammation in mouse models of these diseases (8, 11, 12).

Conversely, increased CD39 expression has been reported in coronary vasculature, lungs, intestine and pancreas during inflammation and tissue damage (13-16). Particularly, chronic lung disease is characterized by higher rates of nucleotide elimination, E-NTPDase expression and activity (6). Transgenic mice that lack CD39 develop increased pulmonary edema and inflammation compared with control littermates in a model of acute lung injury (17).

In order to study the consequences of a chronic increase of surface CD39 activity in lung inflammation, we generated transgenic mice overexpressing human CD39 (hCD39) in airway epithelia and characterized their response to a bacterial challenge. Some of the results of these studies have been previously reported in the form of an abstract (18).

Materials and Methods.

Generation of transgenic mice. A construct containing the mCC10 promoter upstream of the human CD39 cDNA was prepared in the pCDNA3 vector (Invitrogen, Carlsbad, CA). Human CD39 cDNA: total RNA extracted from HUVEC cells was reverse transcribed with the Transcription First Strand kit (Roche Biochemicals, Mannheim, Germany). The following primers were used for PCR: 5'-GAAAACAAAAGCTGCTACT-3' and 5'-CAGTAAAAGCCAAGGAAGC-3' and TOPO TA cloning was performed. Mouse CC10 promoter: we took advantage of the high density of Clara cells in the mouse respiratory epithelium and used the Clara cell 10-kDa protein (CC10) promoter to target the expression of CD39 to the airways. The approach used has previously been described by Zhu Z., *et al*. (19). The mouse CC10 (mCC10) promoter was amplified by PCR using the following primers: 5'-GGTAAGGCCTGGGAATGGCTAAC-3' and 5'-GGGTATGTGTGGGTGTGTGGC-3'. These primers were designed to incorporate *Hin*dIII and *Bam*HI restriction sites. The DNA fragment encompassing the transgene and the pCDNA3 polyadenylation signal was amplified by PCR, purified and resuspended in the microinjection buffer (0.5 mM Tris-HCl, 25 mM EDTA; pH 7.4). Transgenic mice were generated by zygote pronuclear microinjection according to classical procedures (C57BL/6J background) (20). Transgenic offspring was identified by PCR screening using genomic DNA extracted from tail samples. The following primer pair was used: mCC10 promoter, 5'-GTCTCCGGCCTCTGGTTCTC-3' and hCD39 cDNA, 5'-CGCCTGTGTCATTCTCCTTT-3'.

Mice were kept under "Specific Pathogen Free" conditions and all experiments were approved by the Animal care and use committee of the University of Liège.

Tissue RNA extraction and RT-PCR. Reverse-transcribed lung RNA was used in real-time PCR carried out on a ABI 7000 Sequence Detection System, with SYBR Green PCR Master

Mix (Applied Biosystems, Foster City, CA). The primers used for the amplification of CD39 cDNA were specific to human CD39 and did not amplify mouse CD39 cDNA.

Histological evaluation. Lungs were infused with 4 % paraformaldehyde, fixed overnight, embedded in paraffin, sectioned at 4 µm and stained with hematoxylin and eosin.

Immunohistochemistry. Frozen lung sections of 4 µm were incubated with the monoclonal anti-human CD39 antibody (Ancell, Bayport, MN) followed by Envision+® System Labelled Polymer-HRP Anti-Mouse Antibody (Dako, Glostrup, Denmark). Anti-CD45 staining (rat anti-mouse CD45, BD Pharmingen, San Jose, CA) was performed on PFA fixed paraffin-embedded sections according to classical procedures.

Lipopolysaccharide and P. aeruginosa instillation. C57BL/6J wild-type or transgenic mice (8-12 week-old) were anesthetized with isoflurane (Forene®, Abbott, Ottignies, Belgium). Ultra Pure *E. coli* lipopolysaccharides (InvivoGen, San Diego, CA) were administered by intratracheal instillation (5 µg/mice in 50 µl of sterile saline). Saline was instilled as control. In some experiments, lipopolysaccharide (LPS) and α,βMeATP (200 µM, Sigma-Aldrich, Bornem, Belgium), NF449 (0.1µmol/kg), A438079 (100 µM, Tocris Biosciences, Bristol, UK) or caffeine (20 mg/kg, Sigma-Aldrich) were instilled simultaneously. Acute infection with *P. aeruginosa* (PAK laboratory strain) was performed by intratracheal instillation of $1x10^7$ bacteria (CFU) in a volume of 50 µl. PBS was instilled as control. Bacteria load was determined by counting the number of viable bacteria in lung homogenates at different times post-infection. Serial dilutions of homogenates were plated on LB agar for 24 hours at 37°C.

Bronchoalveolar lavage, cell count and cytokine level quantification, alveolar permeability. Bonchoalveolar lavage (BAL) were performed according to classical procedures. Differential cell counts were determined on cytospins (Cytofuge®, StatSpin, Westwood, MA) using morphological criteria after Giemsa-Wright staining (Diff-Quick stain set, Medion Diagnostics, Düdingen, Switzerland). Levels of cytokines in BAL fluid (BALF) supernatants were quantified using Bio-Plex Cytokine Bead Array analyses according to manufacturer's instructions (Bio-Rad, Nazareth Eke, Belgium/ BD biosciences, Erembodegem, Belgium).

Flow cytometry. Cells isolated from BALF were stained with fluorescein isothiocyanate (FITC)-conjugated CD45R/B220 (RA3-6B2), FITC-conjugated CD8a (Ly-2), allophycocyanin (APC)-conjugated CD3e (145-2C11) and phycoerythrin (PE)-conjugated CD4 (GK1.5). These antibodies were purchased from BD Biosciences Pharmingen (San Jose, CA). Analyses were performed on a FACS Canto flow cytometer (BD Biosciences).

Luciferase assay and HPLC analysis. Supernatants of BALF were used to determine extracellular ATP levels in the airways of the mice with the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). For the analyses of adenyl purines, proteins from BALF supernatants were precipitated with 12 % trichloroacetic acid. Derivatization of purines was performed with 1 M chloroacetaldehyde, and the resulting fluorescent etheno-species were analysed by reversed-phase HPLC as previously described (2, 21).

Statistical analyses – One way anova tests with Bonferronni adjustment were used for multiple comparisons. Statistical significance was set at $p < 0.05$.

Results

Generation of transgenic mice overexpressing the human CD39 in the airway epithelium.

On three independent zygote injections of the mCC10-hCD39 transgene, 77 offspring were obtained, and 24 animals were found to be transgenic by PCR screening. Three founder lines were used throughout our study. The transgenic mice had no apparent physiologic abnormalities. Real-time RT-PCR analyses using primer pairs that selectively amplify human CD39 cDNA showed the presence of hCD39 transcripts in the lungs of the three independent lines of transgenic mice (Fig. 1A). The levels of the endogenous pulmonary mouse CD39, CD73 and adenosine deaminase transcripts remained similar to that of wild-type mice (supplemental Fig. S1).[5] No transgene expression was found in any of the other organs tested (data not shown). The expression of hCD39 protein was demonstrated by immunoblotting of mouse lung extracts (data not shown). Immunohistochemistry of lung sections revealed selective hCD39 staining in the transgenic epithelia (Fig. 1B). The ATP levels were reduced in bronchoalveolar lavage fluids (BALF) of the three transgenic lines as compared to wild-type animals (Fig. 1C), indicative of increased CD39 enzymatic activity. Accordingly, HPLC analyses revealed elevated AMP (data not shown) and adenosine levels in transgenic BALF (Fig. 1D). All three lines showed similar phenotypes.

Increased leukocyte infiltration in the lungs of hCD39 transgenic mice after acute LPS exposure.

Under basal conditions, inflammatory cell counts in BALF of wild type and transgenic mice were identical and only macrophages were present (data not shown). Lung histology was found to be normal. MUC5AC mRNA expression levels remained low (supplemental Fig. S2A) and mucus could not be detected following periodic acid-Schiff and alcian blue colorations (supplemental Fig. S2B).

We then performed intratracheal instillation of a unique moderate dose of bacterial LPS. Under these conditions, ATP levels in transgenic BALF remained lower than in WT BALF for up to 5 days post-instillation while adenosine levels were constantly increased (Fig. 1D). Histological examination of lung sections and anti-CD45 staining showed leukocyte infiltration (Fig. 2A), alveolar oedema and congestion, which all were more pronounced in transgenic lungs. Accordingly, the amounts of neutrophils in the BALF of transgenic mice were significantly increased after 2, 6 and 12 hours following LPS as compared to wild type mice (Fig. 2B). Although macrophage numbers were not different between wild type and transgenic animals until 3 days, macrophage amounts remained significantly higher in transgenic BALF 5 days after LPS treatment. At this time, about 25 % lymphocytes were present in BALF, among which the amounts of CD8+ T and B220+ B cells were significantly increased in transgenic *versus* wild-type BALF (Fig. 2B). CD4+ T cell amounts were normal. Inflammation was resolved by day 14 both for hCD39 and WT mice (Fig. 2B).

Elevated LPS-induced alveolar permeability and inflammatory cytokine production in the lungs of transgenic mice.

In agreement with a pro-inflammatory phenotype for LPS-treated hCD39 mice, alveolar permeability was significantly increased in hCD39 as compared to WT mice (Fig. 3A). To further analyse this phenotype, we then measured cytokine levels in BALF. LPS administration induced transient production of the cytokines tested, KC, IL-6, TNF-α, IL-1β, INF-γ, CCL-2, RANTES and G-CSF (data not shown). Among these cytokines, levels of KC, RANTES, and IL-6 were significantly augmented in transgenic *versus* wild-type BALF (Fig. 3B). KC and IL-6 levels reached higher values 2 hours after LPS treatment; IL-6 and RANTES levels were more elevated at 14 hours. As previously described (17), mouse CD39

mRNA transcripts were increased upon LPS treatment, both in wild-type and transgenic lungs (supplemental Fig. S3).

Increased P2 receptor activity in hCD39 transgenic lungs.

The pro-inflammatory response of transgenic mice may result from increased ATP degradation, adenosine accumulation, or both. Moreover, increased CD39 activity may also improve P2 receptor signaling by limiting receptor desensitization by released ATP (22, 23). To address this question, we studied the effects of P2 receptor antagonists on LPS-induced inflammation in wild type and transgenic mice. In transgenic mice, the broad spectrum P2 receptor antagonist suramin (data not shown), the selective $P2X_1$ and $P2X_7$ receptor antagonists, NF449 (24) and A438079 (25), reversed the early increase of neutrophil recruitment to levels found in wild type BALF (Fig. 4A). Neutrophil counts measured 48 hours after LPS instillation were also significantly reduced by NF449 and A438079. These antagonists did not affect the response of wild type mice. In contrast, neither NF449 (data not shown) nor A438079 (Fig. 4A) affected the macrophage recruitment measured after 48 hours. The delay of macrophage clearance observed in transgenic mice at 5 days following LPS was restored by A438079. At 5 days after LPS instillation, lymphocyte recruitment to transgenic lungs was significantly inhibited by treatment with A438079. The stable ATP analog, α,βMeATP, acts preferentially as an agonist for $P2X_1$ and $P2X_3$ receptor subtypes, but it can also target other P2X subtypes expressed on airway epithelia (26). *In vivo*, this compound has demonstrated $P2X_1$ receptor desensitizing properties (27). In order to further investigate the role of P2X receptors in the phenotype of CD39 transgenic mice, α,βMeATP was co-instilled with LPS. Similarly as treatment with NF449, this agonist prevented the recruitment of neutrophils specifically to transgenic lungs 2 hours after LPS (Fig. 4B); at later time points, it almost fully abolished neutrophil, macrophage and lymphocyte recruitment in both wild type

and transgenic lungs. Caffeine was then used to assess the implication of adenosine receptors in the pro-inflammatory phenotype of transgenic mice. Co-instillation of caffeine with LPS decreased neutrophil recruitment to transgenic lungs (Fig. 5). This treatment promoted macrophage clearance from transgenic and wild type lungs. Lymphocyte influx occurred earlier in the presence of caffeine. This effect was smaller (at 48 hours) or absent (at 120 hours) for transgenic mice compared to wild type, suggesting that the increased lymphocyte recruitment into transgenic lungs observed at 120 hours does not rely on caffeine-sensitive adenosine receptors.

We then determined whether co-instillation of P2 receptor antagonists with LPS would affect cytokine production in mouse lungs. A438079 could only lower KC levels measured in transgenic BALF 2 hours after LPS treatment (Fig. 6). Despite their ability to decrease leukocyte infiltration, neither NF449 nor α,βMeATP displayed significant inhibitory effect on KC (Fig. 6), IL-6 or RANTES production.

Increased leukocyte infiltration in the lungs of hCD39 transgenic mice upon acute infection with Pseudomonas aeruginosa.

In order to determine whether the pro-inflammatory phenotype of the hCD39 transgenic mice could be reproduced during infection with live bacteria, we instilled the mice with *Pseudomonas aeruginosa* and counted inflammatory cells in their BALF after 24 hours. In agreement with our LPS data, CD39 overexpression augmented inflammatory cell recruitment into the lungs (Fig. 7A). In transgenic lungs, both neutrophil and macrophage counts were increased in conditions where only neutrophil recruitment occurred in wild-type lungs. Similarly as with LPS, bacteria-induced KC and RANTES secretion was increased in hCD39 BALF as compared to WT BALF (Fig. 7B) while TNF-α, IL-1β, and CCL-2 levels did not differ between WT and hCD39 mice (data not shown). Kinetics of KC and RANTES

production displayed striking differences. Though KC levels were significantly higher in hCD39 BALF as compared to WT BALF at 2 hours post-infection, production of this chemokine declined more rapidly for transgenic mice. Also, RANTES production occurred earlier for transgenic mice and resolved when reaching peak values in WT. Such resolution differences were not observed in response to LPS challenge. Other differences with LPS concerned bacteria-induced IL-6 production that was identical in hCD39 and WT mice and IFN-γ levels that were significantly higher in hCD39 BALF. These results prompted us to analyse the ability of hCD39 mice to clear bacteria. Interestingly, transgenic mice could better eliminate bacteria than WT mice, as determined by counting live bacteria in lungs (Fig. 7C).

Discussion

By generating transgenic mice overexpressing human CD39 in airway epithelia, we created a model of chronic ATP removal and elevation of adenosine levels in the airways. These mice did not develop spontaneous lung inflammation, indicating that increased CD39 activity is not sufficient to cause inflammation. Nevertheless, the transgenic mice developed an enhanced inflammatory response upon intratracheal instillation of a moderate dose of LPS or lung infection with *P. aeruginosa*. Thus, a chronic increase of CD39-mediated nucleotide metabolism on the airway surface, as observed in chronic pulmonary inflammatory diseases (6), promotes the inflammatory response to bacterial challenge. Inflammation was initiated earlier than in wild type mice, resulting in improvement of bacteria clearance. Transgenic mice displayed accelerated neutrophil recruitment into the lungs at early time points following LPS instillation. At later time points, delayed clearance of macrophages was observed, as well as increased lymphocyte recruitment. Accordingly, production of the chemokines KC and RANTES was increased (LPS) or occurred earlier (*P. aeruginosa*) in transgenic lungs as compared to WT lungs. In response to live bacteria, KC and RANTES production ended sooner for transgenic mice, which coincided with strongly reduced bacteria load while neutrophil and macrophage counts were still higher. At this time, IFN-γ levels were increased in transgenic lungs, which may be in agreement with its important immunomodulatory role during infection (28). In light of the recently proposed pro-inflammatory role for ATP in the airways (8, 10, 29) and because CD39 deficiency in mice leads to increased LPS-induced lung inflammation (17), the observed transgenic mouse phenotype was unexpected. The use of $P2X_1$ and $P2X_7$ receptor antagonists (NF449 and A438079) enabled us to reconcile these apparent discrepancies. Indeed, these antagonists decreased the LPS-induced immune cell recruitment in transgenic mice while they were mainly inactive in wild-type animals, indicating that P2 receptor function is facilitated by CD39 overexpression. In animals with

smoke-induced lung injury, Cicko et al. (12) have observed a specific up-regulation of the $P2Y_2$ receptor on blood and lung neutrophils and macrophages. Lucattelli et al. (11) have shown that cigarette smoke-induced inflammation was associated with an up-regulation of the $P2X_7$ receptor on blood and airway neutrophils, alveolar macrophages and in whole lung tissue. Up-regulation of $P2X_7$ receptors on BAL macrophages and blood eosinophils has also been observed in patients with chronic asthma (30). In our study, $P2X_1$ and $P2X_7$ mRNA levels were found to be unchanged in transgenic lungs and were not up-regulated following LPS treatment (supplemental Fig. S4). Up-regulation of $P2X_1$ and $P2X_7$ receptor expression can therefore not explain the observed increased activity of these receptors in transgenic airways. In contrast, levels of $P2Y_2R$ transcripts were increased by about 5 fold in transgenic lungs. Thus, even if we were not able to show specific contribution of $P2Y_2$ receptors in our transgenic mouse phenotype due to lack of selective antagonists for this receptor, these receptors may be involved. Our data with the selective $P2X_7$ receptor antagonist A438079 depict an important role of this receptor in neutrophil and lymphocyte recruitment into the lungs as well as in macrophage survival, which may contribute to anti-bacterial immunity under conditions of CD39 overexpression. In agreement with its effect on neutrophil infiltration, A438079 also potently inhibited early KC production in transgenic lungs. These data are in line with recent studies using antagonists and $P2X_7$-deficient mice in models of lung fibrosis induced by airway-administered bleomycin (31), OVA- alum and/or host dust mite model of asthmatic airway inflammation (30), and cigarette smoke-induced lung inflammation and emphysema (12). $P2X_7$ receptors are involved in caspase-1-mediated IL-1β and IL-18 maturation and secretion by LPS-primed monocytes/macrophages (32); however, we could not detect higher IL-1β levels in the lungs of transgenic mice compared to wild-type. $P2X_7$ receptors also contribute to IL-6 secretion, to dendritic cell (33), and lymphocyte activation and migration (34-36). In addition, our findings with the selective $P2X_1$ antagonist,

NF449, reveal a novel role for $P2X_1$ receptors in LPS-induced neutrophil and lymphocyte recruitment into the lungs. This observation is compatible with the recent findings on a role for $P2X_1$ receptors in neutrophil chemotaxis (37), acting in cooperation with $P2Y_2$ and adenosine A3 receptors (38, 39). $P2X_1$ receptors have recently been found to be expressed on T cells and to participate in their activation *in vitro* (40). Our *in vivo* data suggest for the first time that $P2X_1$ receptors may also contribute to airway adaptive immunity by controlling lymphocyte influx into the lungs. In our study, we observed an increase of CD8+ T lymphocytes in the lungs of LPS-treated transgenic mice while CD4+ T lymphocyte counts were normal. Interestingly, CD8+ T cells predominate over CD4+ T cells in the airways and lung parenchyma of patients with COPD (41).

Upon LPS challenge, the stable ATP analog α,βMeATP, a P2X-desensitizing agonist, demonstrated potent anti-inflammatory properties both in wild type and transgenic mice that were characterized by an almost full inhibition of inflammatory cell recruitment. This agonist is more potent than the two other P2X antagonists used in our study, suggesting that it affects additional or multiple P2 receptor subtypes. Further investigations are required to identify the precise target(s) of this compound.

A contribution of adenosine in the transgenic airway phenotype was assessed by use of caffeine, a broad spectrum inhibitor of adenosine receptors. Adenosine has been involved in leukocyte migration and inflammatory cell death in several *in vitro* studies (42). More importantly, chronic adenosine elevations in the airways were found to serve a pro-inflammatory role (43-46). Notably, adenosine deaminase-deficient mice, showing lung adenosine accumulation, spontaneously develop lung inflammation and damage (46). In our study, co-instillation of caffeine with LPS suggested that elevated adenosine levels participated in increasing neutrophil influx into the lungs of transgenic mice, possibly through A3 receptors. Adenosine receptors were also involved in macrophage survival during

resolution of inflammation but this function did not differ between wild type and transgenic mice. Finally, caffeine increased lymphocyte influx in mice of both genotypes, which would support immunosuppressive function of adenosine. The increase of lymphocyte influx in transgenic lungs can therefore not be explained by elevated adenosine levels and adenosine receptor activation and would rather depend on P2 receptors. Since adenosine promotes the production of IL-6 by many cell types through engagement of the $A_{2B}R$, contributing to inflammation and fibrosis (47), it is possible that the increase of IL-6 levels in BALF of LPS-treated transgenic mice would be mediated at least partially by adenosine. Detailed assessment of differential contribution of adenosine receptor subtypes to our transgenic phenotype would require the availability of selective antagonists or their genetic ablation in hCD39 overexpressing background.

Thus, we propose a model in which CD39 overexpression in airways would promote immune responses triggered by interactions of epithelial cell TLR with bacteria (Fig. 8). The increased CD39 activity would result in increased $P2X_1$ and $P2X_7$ receptor mediated immune cell recruitment by preventing their desensitization through removal of excess released ATP and possible concomitant increase of $P2Y_2$ receptor expression levels. Breakdown of ATP into adenosine would also contribute to promote neutrophil-dependent inflammatory responses. Together, these events contribute to facilitate bacteria clearance.

Our model suggests that CD39 up-regulation in chronic lung diseases would be implicated in host adaptation to recurrent bacteria infection. Under these conditions, the use of P2 receptor antagonists, by preventing immune cell recruitment, could be deleterious to the host. It remains to be determined if CD39 is up-regulated in the lungs of mouse models of asthma (8), COPD (11, 12, 30) or idiopathic pulmonary fibrosis (31), in which P2 receptor antagonism or acute treatment with the ATP scavenger apyrase have proven anti-inflammatory efficacy, and, most importantly, in the airways of patients.

Our study provide *in vivo* evidence that ATP, referred as an important damage-associated molecular pattern (DAMP), contributes to the immune response by activation and recruitment of various inflammatory cells. Further research into this area should focus on the role of ATP in maintenance of chronic lung inflammation and induction of airway remodeling. In this sense, the mCC10-hCD39 transgenic mice, developing enhanced airway inflammatory response upon bacterial challenge, constitute a new tool to further investigate the role of ATP and adenosine in chronic lung infectious or allergic diseases, as well as to evaluate the therapeutic potential of aerosolized P1 or P2 receptor agonists and antagonists.

Acknowledgements

The authors are grateful to Prof. Simon C Robson for useful discussion and advices, and to Dr Laurence de Leval for assistance and anatomy pathology expertise in histological analyses. They wish to thank Sandra Ormenese, PhD, GIGA Imaging platform, Véronique Dhennin, GIGA Genomics platform, Fabien Ectors, MD,PhD, GIGA Transgenesis platform, University of Liège, Belgium.

Disclosures.

The authors have no financial conflicts of interest.

References

1. Volonte, C., and N. D'Ambrosi. 2009. Membrane compartments and purinergic signalling: the purinome, a complex interplay among ligands, degrading enzymes, receptors and transporters. *Febs J* 276:318-329.
2. Lazarowski, E. R., R. Tarran, B. R. Grubb, C. A. van Heusden, S. Okada, and R. C. Boucher. 2004. Nucleotide release provides a mechanism for airway surface liquid homeostasis. *J Biol Chem* 279:36855-36864.
3. Lazarowski, E. R., and R. C. Boucher. 2009. Purinergic receptors in airway epithelia. *Curr Opin Pharmacol* 9:262-267.
4. Lazarowski, E. R., R. C. Boucher, and T. K. Harden. 2003. Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol Pharmacol* 64:785-795.
5. Burnstock, G. 2007. Purine and pyrimidine receptors. *Cell Mol Life Sci* 64:1471-1483.
6. Burch, L. H., and M. Picher. 2006. E-NTPDases in human airways: Regulation and relevance for chronic lung diseases. *Purinergic Signal* 2:399-408.
7. Polosa, R., and M. R. Blackburn. 2009. Adenosine receptors as targets for therapeutic intervention in asthma and chronic obstructive pulmonary disease. *Trends Pharmacol Sci* 30:528-535.
8. Idzko, M., H. Hammad, M. van Nimwegen, M. Kool, M. A. Willart, F. Muskens, H. C. Hoogsteden, W. Luttmann, D. Ferrari, F. Di Virgilio, J. C. Virchow, Jr., and B. N. Lambrecht. 2007. Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat Med* 13:913-919.
9. Esther, C. R., Jr., N. E. Alexis, M. L. Clas, E. R. Lazarowski, S. H. Donaldson, C. M. Ribeiro, C. G. Moore, S. D. Davis, and R. C. Boucher. 2008. Extracellular purines are biomarkers of neutrophilic airway inflammation. *Eur Respir J* 31:949-956.

10. Mortaz, E., G. Folkerts, F. P. Nijkamp, and P. A. Henricks. 2010. ATP and the pathogenesis of COPD. *Eur J Pharmacol* 638:1-4.

11. Lucattelli, M., S. Cicko, T. Muller, M. Lommatzsch, G. De Cunto, S. Cardini, W. Sundas, M. Grimm, R. Zeiser, T. Durk, G. Zissel, S. Sorichter, D. Ferrari, F. Di Virgilio, J. C. Virchow, G. Lungarella, and M. Idzko. 2011. P2X7 receptor signaling in the pathogenesis of smoke-induced lung inflammation and emphysema. *Am J Respir Cell Mol Biol* 44:423-429.

12. Cicko, S., M. Lucattelli, T. Muller, M. Lommatzsch, G. De Cunto, S. Cardini, W. Sundas, M. Grimm, R. Zeiser, T. Durk, G. Zissel, J. M. Boeynaems, S. Sorichter, D. Ferrari, F. Di Virgilio, J. C. Virchow, G. Lungarella, and M. Idzko. 2010. Purinergic receptor inhibition prevents the development of smoke-induced lung injury and emphysema. *J Immunol* 185:688-697.

13. Eckle, T., L. Fullbier, M. Wehrmann, J. Khoury, M. Mittelbronn, J. Ibla, P. Rosenberger, and H. K. Eltzschig. 2007. Identification of ectonucleotidases CD39 and CD73 in innate protection during acute lung injury. *J Immunol* 178:8127-8137.

14. Eltzschig, H. K., J. C. Ibla, G. T. Furuta, M. O. Leonard, K. A. Jacobson, K. Enjyoji, S. C. Robson, and S. P. Colgan. 2003. Coordinated adenine nucleotide phosphohydrolysis and nucleoside signaling in posthypoxic endothelium: role of ectonucleotidases and adenosine A2B receptors. *J Exp Med* 198:783-796.

15. Kunzli, B. M., P. Nuhn, K. Enjyoji, Y. Banz, R. N. Smith, E. Csizmadia, D. Schuppan, P. O. Berberat, H. Friess, and S. C. Robson. 2008. Disordered pancreatic inflammatory responses and inhibition of fibrosis in CD39-null mice. *Gastroenterology* 134:292-305.

16. Neshat, S., M. deVries, A. R. Barajas-Espinosa, L. Skeith, S. P. Chisholm, and A. E. Lomax. 2009. Loss of purinergic vascular regulation in the colon during colitis is

associated with upregulation of CD39. *Am J Physiol Gastrointest Liver Physiol* 296:G399-405.

17. Reutershan, J., I. Vollmer, S. Stark, R. Wagner, K. C. Ngamsri, and H. K. Eltzschig. 2009. Adenosine and inflammation: CD39 and CD73 are critical mediators in LPS-induced PMN trafficking into the lungs. *Faseb J* 23:473-482.

18. Theatre, E., de Leval, L., Bettendorf, L., Bours, V., Oury, C. 2008. Overexpression of airway CD39 in transgenic mice enhances lipopolysaccharide induced inflammation In *Purinergic Signal*. S113.

19. Zhu, Z., R. J. Homer, Z. Wang, Q. Chen, G. P. Geba, J. Wang, Y. Zhang, and J. A. Elias. 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 103:779-788.

20. Holvoet, P., S. Danloy, and D. Collen. 1997. Role of the carboxy-terminal domain of human apolipoprotein AI in high-density-lipoprotein metabolism--a study based on deletion and substitution variants in transgenic mice. *Eur J Biochem* 245:642-647.

21. Gangolf, M., P. Wins, M. Thiry, B. El Moualij, and L. Bettendorff. 2010. Thiamine triphosphate synthesis in rat brain occurs in mitochondria and is coupled to the respiratory chain. *J Biol Chem* 285:583-594.

22. Enjyoji, K., J. Sevigny, Y. Lin, P. S. Frenette, P. D. Christie, J. S. Esch, 2nd, M. Imai, J. M. Edelberg, H. Rayburn, M. Lech, D. L. Beeler, E. Csizmadia, D. D. Wagner, S. C. Robson, and R. D. Rosenberg. 1999. Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. *Nat Med* 5:1010-1017.

23. Dwyer, K. M., S. C. Robson, H. H. Nandurkar, D. J. Campbell, H. Gock, L. J. Murray-Segal, N. Fisicaro, T. B. Mysore, E. Kaczmarek, P. J. Cowan, and A. J.

d'Apice. 2004. Thromboregulatory manifestations in human CD39 transgenic mice and the implications for thrombotic disease and transplantation. *J Clin Invest* 113:1440-1446.

24. Hulsmann, M., P. Nickel, M. Kassack, G. Schmalzing, G. Lambrecht, and F. Markwardt. 2003. NF449, a novel picomolar potency antagonist at human P2X1 receptors. *Eur J Pharmacol* 470:1-7.

25. King, B. F. 2007. Novel P2X7 receptor antagonists ease the pain. *Br J Pharmacol* 151:565-567.

26. Theatre, E., V. Bours, and C. Oury. 2009. A P2X ion channel-triggered NF-kappaB pathway enhances TNF-alpha-induced IL-8 expression in airway epithelial cells. *Am J Respir Cell Mol Biol* 41:705-713.

27. Oury, C., K. Daenens, H. Hu, E. Toth-Zsamboki, M. Bryckaert, and M. F. Hoylaerts. 2006. ERK2 activation in arteriolar and venular murine thrombosis: platelet receptor GPIb vs. P2X. *J Thromb Haemost* 4:443-452.

28. Desvignes, L., and J. D. Ernst. 2009. Interferon-gamma-responsive nonhematopoietic cells regulate the immune response to Mycobacterium tuberculosis. *Immunity* 31:974-985.

29. Koeppen, M., F. Di Virgilio, E. T. Clambey, and H. K. Eltzschig. 2011. Purinergic regulation of airway inflammation. *Sub-cellular biochemistry* 55:159-193.

30. Muller, T., R. P. Vieira, M. Grimm, T. Durk, S. Cicko, R. Zeiser, T. Jakob, S. F. Martin, B. Blumenthal, S. Sorichter, D. Ferrari, F. Di Virgillio, and M. Idzko. 2011. A potential role for P2X7R in allergic airway inflammation in mice and humans. *Am J Respir Cell Mol Biol* 44:456-464.

31. Riteau, N., P. Gasse, L. Fauconnier, A. Gombault, M. Couegnat, L. Fick, J. Kanellopoulos, V. F. Quesniaux, S. Marchand-Adam, B. Crestani, B. Ryffel, and I.

Couillin. 2010. Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis. *Am J Respir Crit Care Med* 182:774-783.

32. Ferrari, D., C. Pizzirani, E. Adinolfi, R. M. Lemoli, A. Curti, M. Idzko, E. Panther, and F. Di Virgilio. 2006. The P2X7 receptor: a key player in IL-1 processing and release. *J Immunol* 176:3877-3883.

33. Weber, F. C., P. R. Esser, T. Muller, J. Ganesan, P. Pellegatti, M. M. Simon, R. Zeiser, M. Idzko, T. Jakob, and S. F. Martin. 2010. Lack of the purinergic receptor P2X(7) results in resistance to contact hypersensitivity. *J Exp Med* 207:2609-2619.

34. Heiss, K., N. Janner, B. Mahnss, V. Schumacher, F. Koch-Nolte, F. Haag, and H. W. Mittrucker. 2008. High sensitivity of intestinal CD8+ T cells to nucleotides indicates P2X7 as a regulator for intestinal T cell responses. *J Immunol* 181:3861-3869.

35. Labasi, J. M., N. Petrushova, C. Donovan, S. McCurdy, P. Lira, M. M. Payette, W. Brissette, J. R. Wicks, L. Audoly, and C. A. Gabel. 2002. Absence of the P2X7 receptor alters leukocyte function and attenuates an inflammatory response. *J Immunol* 168:6436-6445.

36. Taylor, S. R., M. Gonzalez-Begne, S. Dewhurst, G. Chimini, C. F. Higgins, J. E. Melvin, and J. I. Elliott. 2008. Sequential shrinkage and swelling underlie P2X7-stimulated lymphocyte phosphatidylserine exposure and death. *J Immunol* 180:300-308.

37. Lecut, C., K. Frederix, D. M. Johnson, C. Deroanne, M. Thiry, C. Faccinetto, R. Maree, R. J. Evans, P. G. Volders, V. Bours, and C. Oury. 2009. P2X1 Ion channels promote neutrophil chemotaxis through Rho kinase activation. *J Immunol* 183:2801-2809.

38. Chen, Y., R. Corriden, Y. Inoue, L. Yip, N. Hashiguchi, A. Zinkernagel, V. Nizet, P. A. Insel, and W. G. Junger. 2006. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science* 314:1792-1795.

39. Junger, W. G. 2011. Immune cell regulation by autocrine purinergic signalling. *Nat Rev Immunol* 11:201-212.

40. Woehrle, T., L. Yip, A. Elkhal, Y. Sumi, Y. Chen, Y. Yao, P. A. Insel, and W. G. Junger. 2010. Pannexin-1 hemichannel-mediated ATP release together with P2X1 and P2X4 receptors regulate T-cell activation at the immune synapse. *Blood* 116:3475-3484.

41. Saetta, M., A. Di Stefano, G. Turato, F. M. Facchini, L. Corbino, C. E. Mapp, P. Maestrelli, A. Ciaccia, and L. M. Fabbri. 1998. CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 157:822-826.

42. Bours, M. J., E. L. Swennen, F. Di Virgilio, B. N. Cronstein, and P. C. Dagnelie. 2006. Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol Ther* 112:358-404.

43. Spicuzza, L., G. Di Maria, and R. Polosa. 2006. Adenosine in the airways: implications and applications. *Eur J Pharmacol* 533:77-88.

44. Schepp, C. P., and J. Reutershan. 2008. Bench-to-bedside review: adenosine receptors - promising targets in acute lung injury? *Crit Care* 12:226.

45. Blackburn, M. R., C. G. Lee, H. W. Young, Z. Zhu, J. L. Chunn, M. J. Kang, S. K. Banerjee, and J. A. Elias. 2003. Adenosine mediates IL-13-induced inflammation and remodeling in the lung and interacts in an IL-13-adenosine amplification pathway. *J Clin Invest* 112:332-344.

46. Blackburn, M. R. 2003. Too much of a good thing: adenosine overload in adenosine-deaminase-deficient mice. *Trends Pharmacol Sci* 24:66-70.

47. Pedroza, M., D. J. Schneider, H. Karmouty-Quintana, J. Coote, S. Shaw, R. Corrigan, J. G. Molina, J. L. Alcorn, D. Galas, R. Gelinas, and M. R. Blackburn. 2011. Interleukin-6 contributes to inflammation and remodeling in a model of adenosine mediated lung injury. *PLoS One* 6:e22667.

Footnotes

[1]At the time of the study E.T. was Research Fellow, L.B., is Research Director, and C.O., Research Associate at the Belgian National Fund for Scientific Research. K.F. was supported by the Belgian Science Policy (IAP 6/18). This work was supported by the Belgian National Fund for Scientific Research (Projects 3.4509.07 and 3.4584.09), Belgian Science Policy (IAP 6/18) and by the "Fonds spéciaux pour la recherche" (Project n° I-05/02) of the University of Liège, Belgium.

[2]E.T. and K.F. contributed equally to this work.

[3]Address correspondence and reprint requests to Dr Cécile Oury, PhD, University of Liège GIGA-Inflammation, Infection, Immunity, Unit of Human Genetics, Laboratory of Thrombosis and Haemostasis, Tour GIGA B34, Avenue de l'Hôpital 1, B-4000 Liège, Belgium. E-mail address: cecile.oury@ulg.ac.be.

[4]Abbreviations used in this paper: αβMeATP, alpha,beta-methylene ATP; ADOR, adenosine receptor; BALF, bronchoalveolar lavage fluid ; COPD, chronic obstructive pulmonary disease ; E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; hCD39, human CD39; RT, room temperature; WT, wild-type.

[5]The online version of this article contains supplemental material.

Figure legends

Figure 1. Characterization of transgenic hCD39 mice. A. hCD39 mRNA levels in the lungs for WT and 3 different founder lines of hCD39 transgenic mice generated through pronuclear injection. Data represent mean ± SEM from 3 independent experiments on 4 mice per founder line. *p≤0.004 *vs* WT. B. Immunohistochemistry of the lungs. Left: haematoxylin eosin staining (HE); middle: negative control by omitting primary antibody; right: staining with anti-human CD39 antibody. All stainings shown for WT (above) and transgenic hCD39 mice (below). 400x magnification. C. Luciferase assay to measure ATP levels in the broncho-alveolar lavage (BAL) fluid of WT and 3 transgenic lines. Data represent mean ±SEM from 2 independent experiments using 4 mice per founder line. *p<0.05 *vs* WT. D. ATP and adenosine levels in BAL fluid measured by luciferase assay and HPLC, respectively 5 days after a unique intratracheal instillation of 5 µg LPS or vehicle (control). Data represent mean ±SEM from 2 independent experiments with 3 mice per group, *p<0.05, **p<0.01 *vs* WT.

Figure 2. Increased inflammatory cell recruitment in hCD39 TG lungs in response to LPS challenge (5 µg per mouse via intratracheal instillation). A. Lung immunohistochemistry. Anti-CD45 staining on lung sections 2, 6, and 14 hours after LPS instillation shows increased leukocyte infiltration for hCD39 mice compared to WT. 400x magnification. B. Differential cell counts in BAL fluid after LPS challenge show increased recruitment and delayed clearance of inflammatory cells in hCD39 mice. Time courses after LPS for neutrophils (top), macrophages (middle) are shown. Data represent mean ±SEM for n≥6 mice per group. C (control) represents averaged data for vehicle instillation. *p<0.05, **p<0.01, *** p<0.001 *vs* WT. The bottom panel shows flow cytometric analyses of lymphocyte $CD4^+$, $CD8^+$ and $CD3^-$ $B220^+$ subpopulations in the BAL fluid of WT and hCD39 mice 120 hours following LPS instillation. **p<0.01 *vs* WT.

Figure 3. Elevated LPS-induced alveolar permeability and inflammatory cytokine production in the lungs of hCD39 TG mice. A. BAL protein concentration for hCD39 and WT mice measured 14 hours after LPS or vehicle (C) instillation. B. Cytometric bead array measuring pro-inflammatory cytokines KC, RANTES, and IL-6 in the supernatants of the BALF at different times following LPS challenge. Data represent mean ± SEM , n≥7 mice coming from at least 2 experiments. **p<0.01, *** p<0.001 *vs* WT, $^{\#}$p<0.05 *vs* C.

Figure 4. Inhibition of P2X receptors decreases LPS-induced inflammatory cell recruitment. A. Effects of $P2X_1$ (NF449) and $P2X_7$ (A438079) blockade on the pro-inflammatory phenotype of hCD39 mice. Differential BAL fluid cell counts 2, 48, and 120 hours after simultaneous instillation of LPS and P2 receptor antagonists, as indicated. B. Effects of instillation of αß-methylene-ATP as a desensitizing agonist. Bars represent mean ± SEM, n≥5 mice per group. *p<0.05, **p<0.01, *** p<0.001 *vs* WT, $p<0.001, #p<0.01, £p<0.05 *vs* C.

Figure 5. Inhibition of adenosine receptors with caffeine differentially affects LPS-induced inflammatory cell recruitment. Differential BAL fluid cell counts 2, 48, and 120 hours after simultaneous instillation of LPS and caffeine, as indicated. Data represent mean ± SEM, n≥6 mice per group; *** p<0.001 *vs* WT, £p<0.05 *vs* C.

Figure 6. Inhibition of $P2X_7$ receptors dampens LPS-induced KC production in hCD39 lungs. Mice treated with LPS with or without simultaneous instillation of $P2X_7$, A438079, or $P2X_1$, NF449, antagonists, as indicated. Cytometric bead array analyses of BAL fluid supernatants. Data are mean ±SEM for n≥4 mice per timepoint per group** p<0.01 *vs* WT, £p<0.05 *vs* C.

Figure 7. Increased inflammatory cell recruitment and improved bacteria clearance for hCD39 TG mice upon infection with *Pseudomonas aeruginosa*. A. Instillation of 10^7 CFU in the lungs of WT and hCD39 mice for 24 hours. Differential count of BAL fluid is represented, mean ±SEM, n≥6 per group. B. Cytometric bead array measuring pro-inflammatory cytokines KC, RANTES, IFN-γ, and IL-6 in the supernatants of the BALF at different times following bacteria challenge. Data represent mean ± SEM , n≥7 mice coming from 2 experiments. C. Bacteria load in the lungs of hCD39 and WT at different times after instillation. Data represent mean ± SEM , n≥7 mice coming from 2 experiments. *p<0.05, **p<0.01, *** p<0.001 *vs* WT, $p<0.001, £p<0.05 *vs* PBS.

Figure 8. Chronic CD39 overexpression in lung epithelia improves host immunity by locally removing desensitizing excess extracellular ATP and promoting $P2X_1$ and $P2X_7$ receptor mediated immune cell recruitment. See text for details. Grey arrows: not demonstrated in our study.

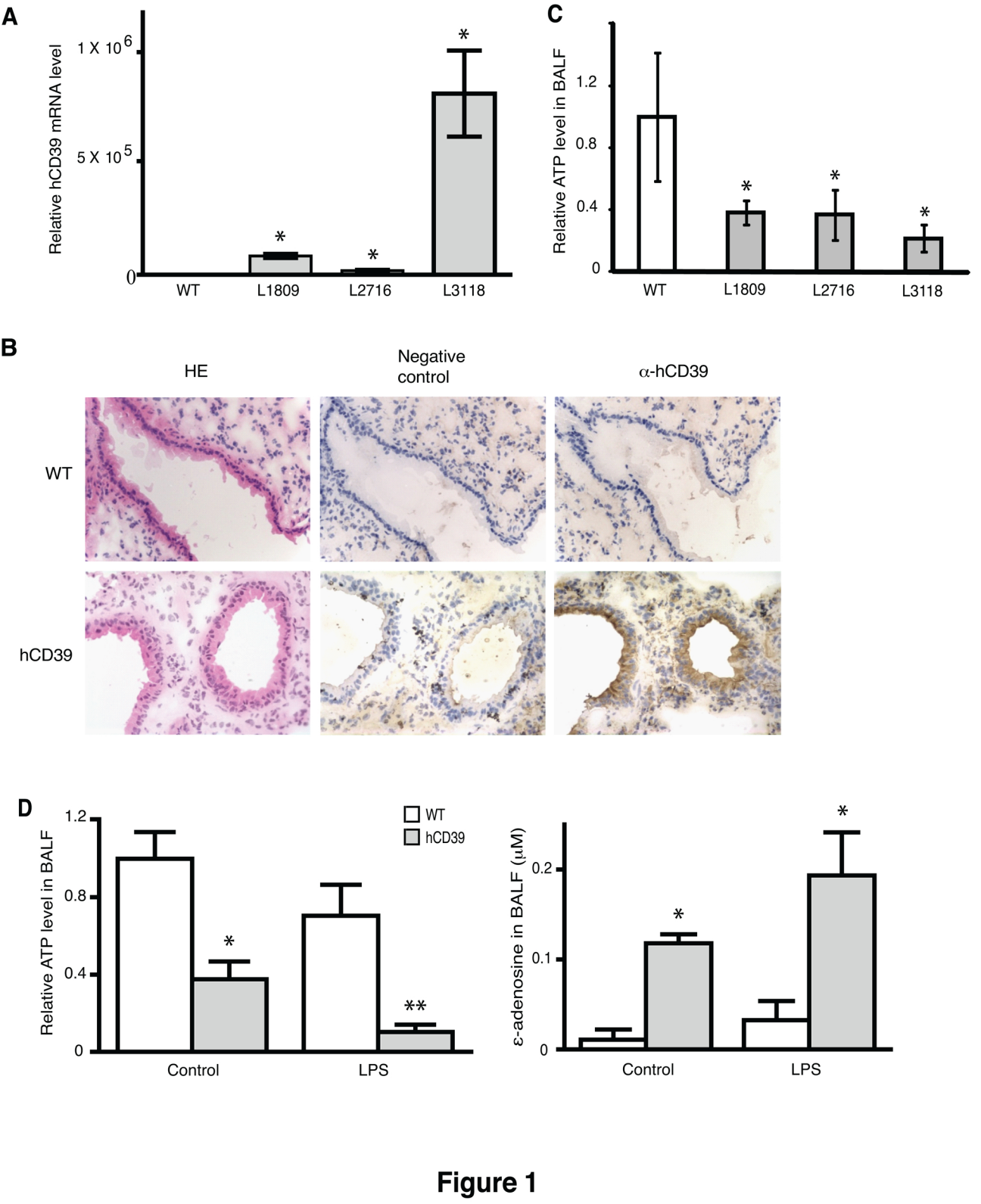

A
Relative hCD39 mRNA level
1 X 10^6
5 X 10^5
0
WT
L1809
L2716
L3118
C
Relative ATP level in BALF
1.2
0.8
0.4
0
WT
L1809
L2716
L3118
B
HE
Negative control
α-hCD39
WT
hCD39
D
Relative ATP level in BALF
WT
hCD39
Control
LPS
ε-adenosine in BALF (μM)
Control
LPS

Figure 1

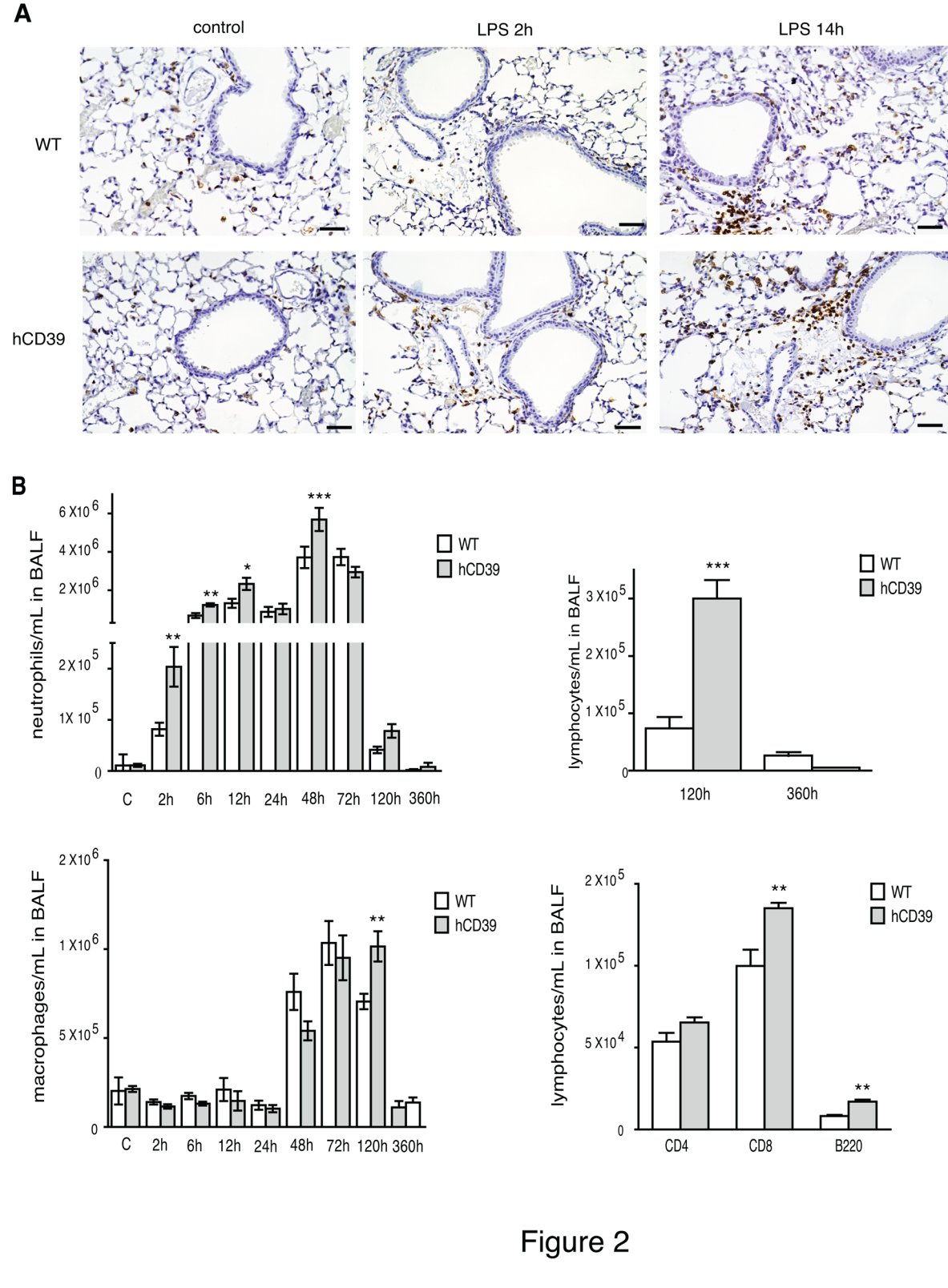

A
control
LPS 2h
LPS 14h
WT
hCD39
B
neutrophils/mL in BALF
6 X10^6
4 X10^6
2 X10^6
2 X10^5
1X 10^5
0
C
2h
6h
12h
24h
48h
72h
120h
360h
WT
hCD39
lymphocytes/mL in BALF
3 X10^5
2 X10^5
1 X10^5
macrophages/mL in BALF
2 X10^6
1 X10^6
5 X10^5
5 X10^4
CD4
CD8
B220

Figure 2

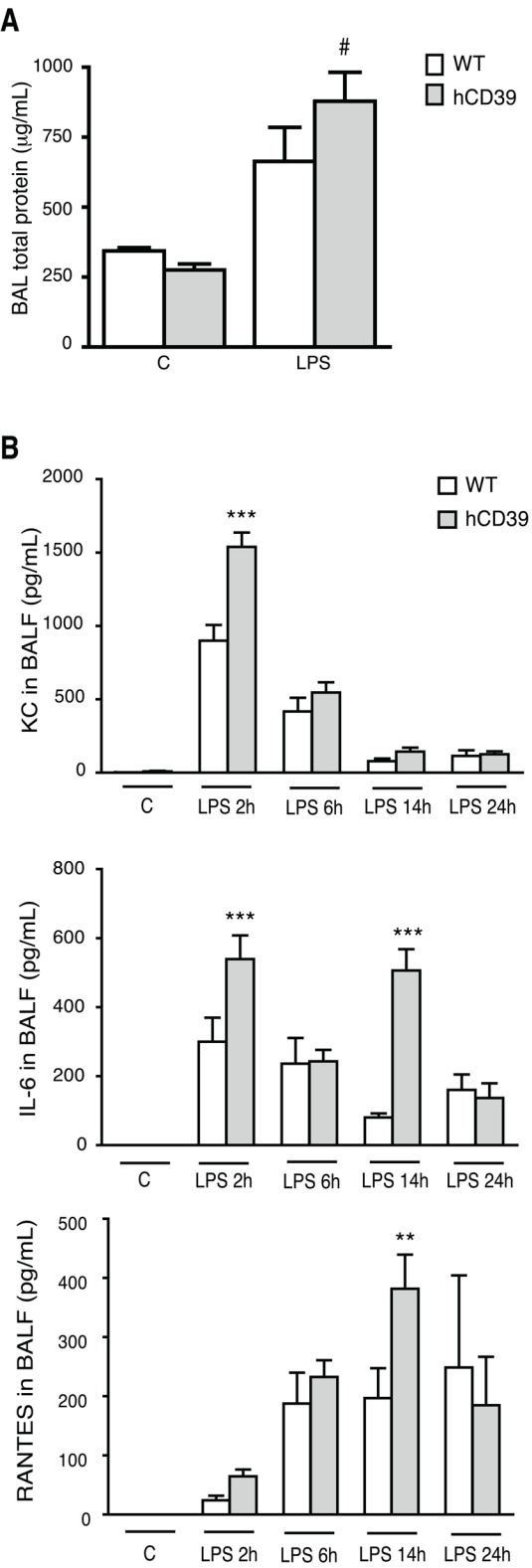

A
BAL total protein (μg/mL)
1000
750
500
250
0
#
WT
hCD39
C
LPS
B
KC in BALF (pg/mL)
2000
1500
1000
500
0
***
WT
hCD39
C
LPS 2h
LPS 6h
LPS 14h
LPS 24h
IL-6 in BALF (pg/mL)
800
600
400
200
0
***
***
C
LPS 2h
LPS 6h
LPS 14h
LPS 24h
RANTES in BALF (pg/mL)
500
400
300
200
100
0
**
C
LPS 2h
LPS 6h
LPS 14h
LPS 24h

Figure 3

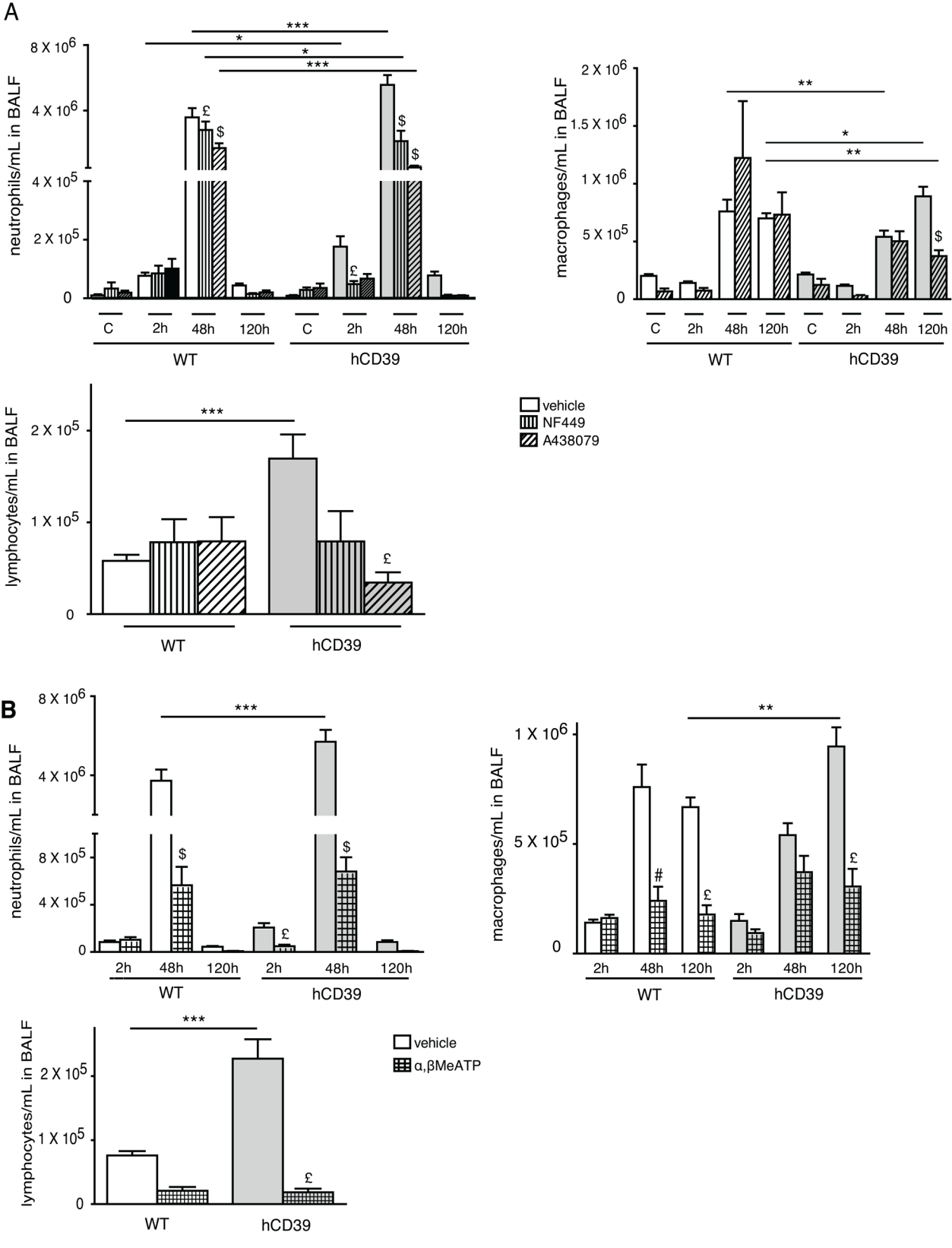
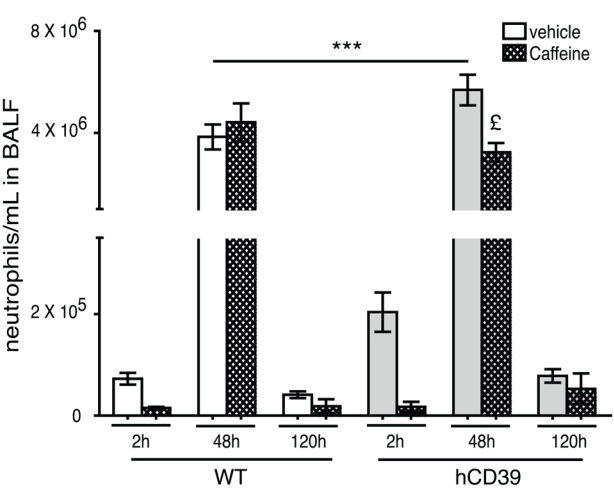
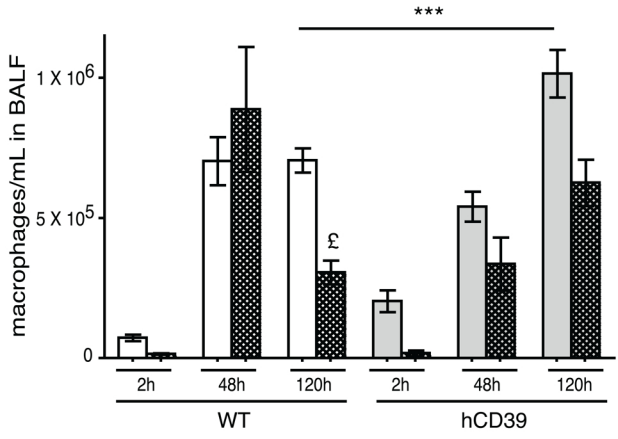

A
neutrophils/mL in BALF
8 X 10^6
4 X 10^6
4 X 10^5
2 X 10^5
0
C
2h
48h
120h
WT
hCD39
macrophages/mL in BALF
2 X 10^6
1.5 X 10^6
1 X 10^6
5 X 10^5
lymphocytes/mL in BALF
2 X 10^5
1 X 10^5
vehicle
NF449
A438079
B
α,βMeATP

Figure 4


8 X 10^6
4 X 10^6
2 X 10^5
0
neutrophils/mL in BALF
vehicle
Caffeine
***
£
2h
48h
120h
2h
48h
120h
WT
hCD39


1 X 10^6
5 X 10^5
0
macrophages/mL in BALF
***
£
2h
48h
120h
2h
48h
120h
WT
hCD39

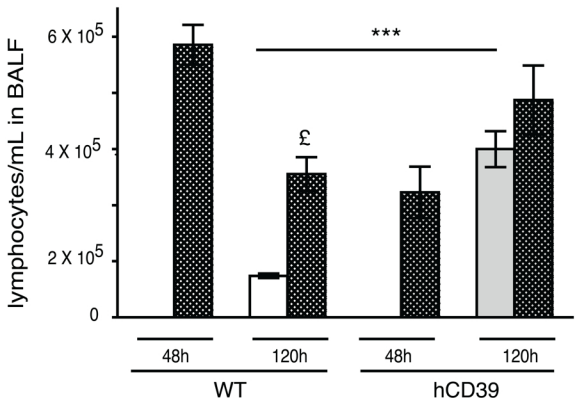

6 X 10^5
4 X 10^5
2 X 10^5
0
lymphocytes/mL in BALF
***
£
48h
120h
48h
120h
WT
hCD39

Figure 5

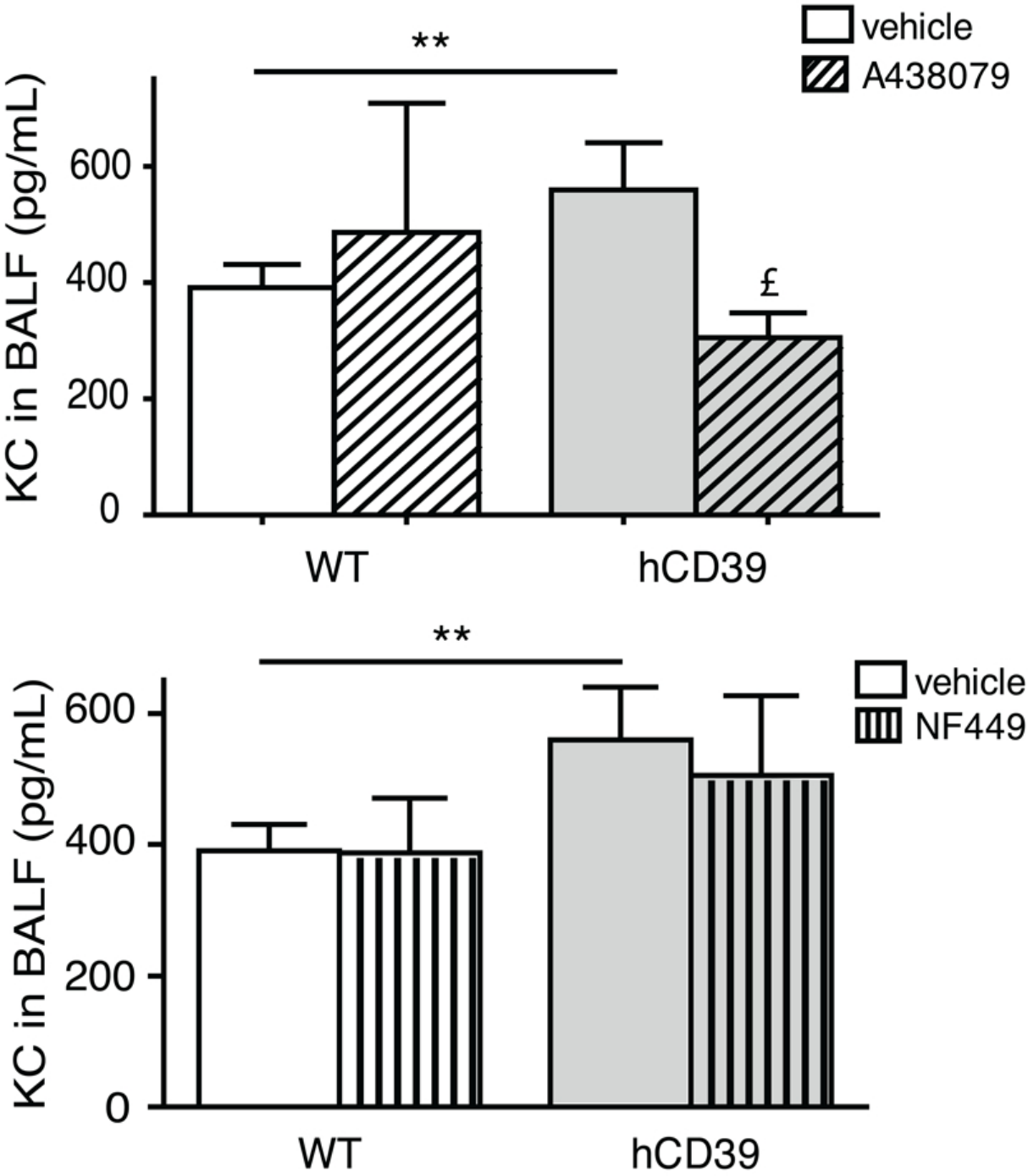

vehicle
A438079
**
KC in BALF (pg/mL)
600
400
200
0
£
WT
hCD39
**
vehicle
NF449
KC in BALF (pg/mL)
600
400
200
0
WT
hCD39

Figure 6

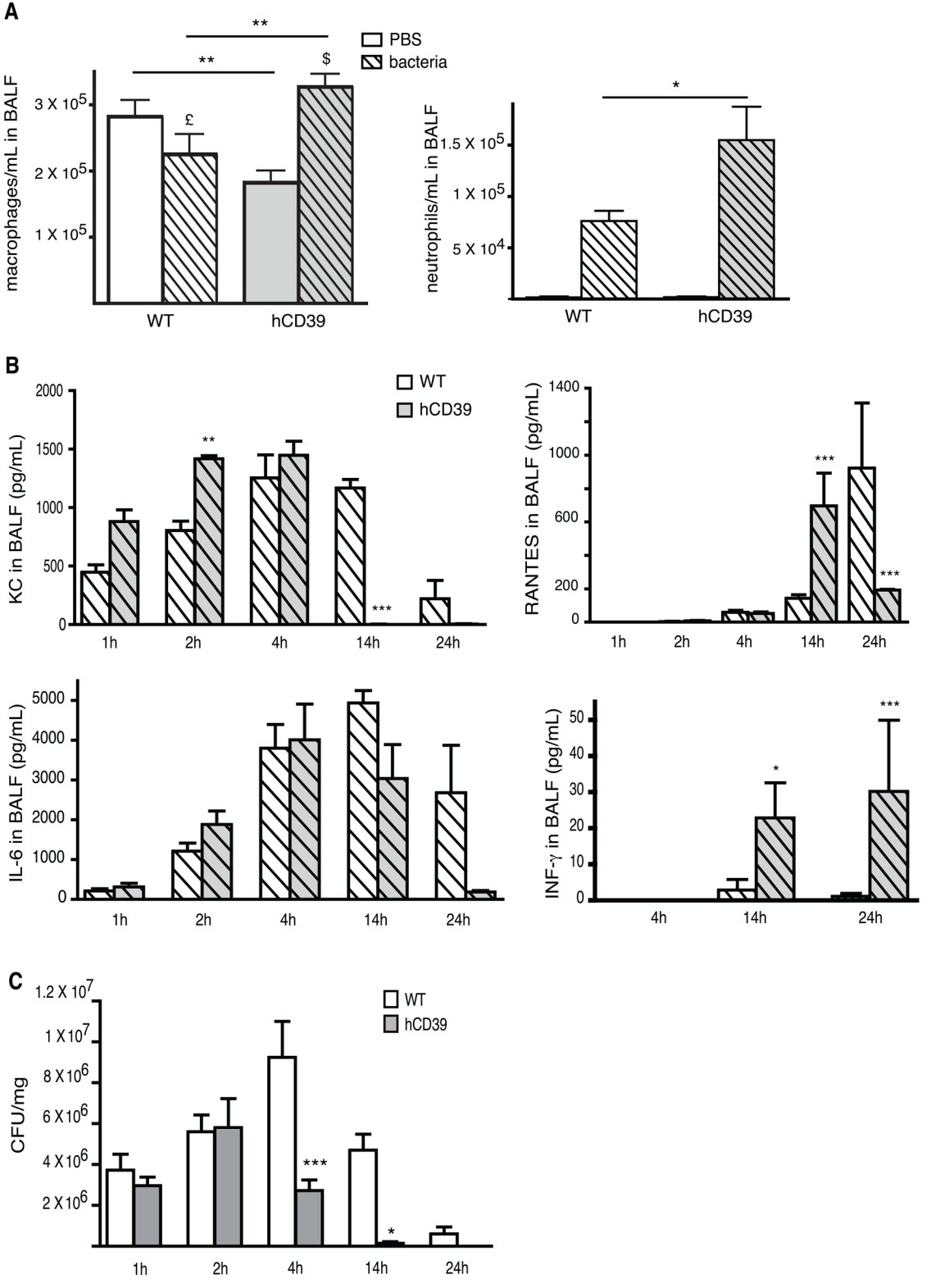

A
PBS
bacteria
macrophages/mL in BALF
3 X 10^5
2 X 10^5
1 X 10^5
WT
hCD39
neutrophils/mL in BALF
1.5 X 10^5
1 X 10^5
5 X 10^4
B
KC in BALF (pg/mL)
RANTES in BALF (pg/mL)
IL-6 in BALF (pg/mL)
INF-γ in BALF (pg/mL)
1h
2h
4h
14h
24h
C
CFU/mg
1.2 X 10^7
1 X10^7
8 X10^6
6 X10^6
4 X10^6
2 X10^6

Figure 7

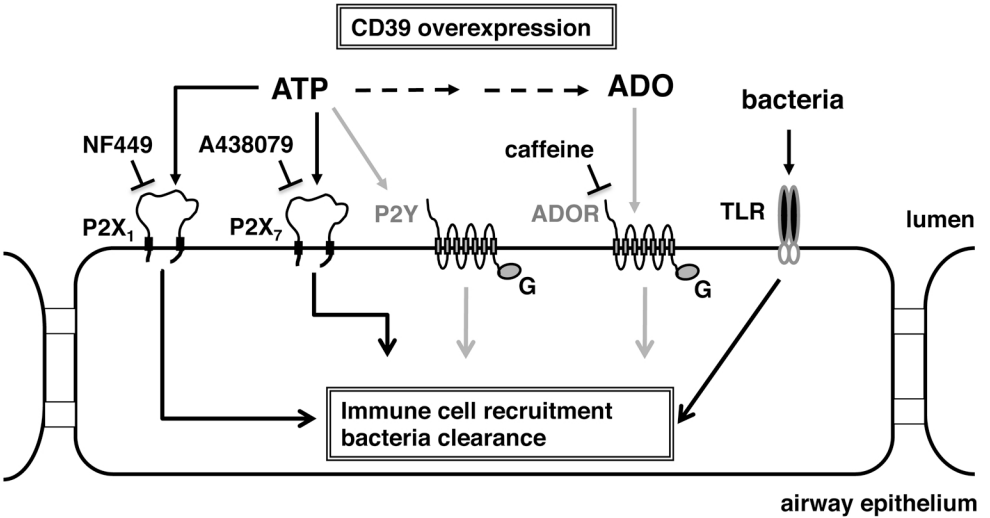

CD39 overexpression
ATP
ADO
bacteria
NF449
A438079
caffeine
P2X1
P2X7
P2Y
ADOR
TLR
lumen
G
G
Immune cell recruitment
bacteria clearance
airway epithelium

Figure 8