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# ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function

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bei der Entstehung dieser Arbeit

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# 1. Introduction

The study of physiologic adaptation and pathophysiologic response to hypoxia is presently an area of intense investigation. Recent reports suggest that both transcriptional and non-transcriptional hypoxia pathways may contribute to a broad range of diseases, and that a number of parallels exist between tissue responses to hypoxia and to acute inflammation (1). Past studies have revealed a central role of extracellular nucleotide phosphohydrolysis and nucleoside signalling in innate immune responses during conditions of limited oxygen availability (hypoxia) or during acute inflammation (2). For example, metabolic enzymes and vascular nucleotide levels are consistently increased during hypoxia (3-7). The contribution of individual nucleosides (ATP, ADP, AMP) to these innate responses remain unclear.

Polymorphonuclear granulocytes (PMN) function as a first line of cellular response during an acute inflammatory episode (8). Previous reports have suggested that PMN may release ATP during conditions of inflammation or hypoxia (9). Such extracellular ATP may either signal directly to vascular ATP receptors (7), or may function as a metabolite following conversion via ecto-apyrase (CD 39, conversion of ATP to adenosine monophosphate (AMP)) and ecto-5`-nucleotidase (CD 73, conversion of AMP to adenosine). Under such conditions, adenosine is available to activate adenosine receptors on the endothelial cell surface (10). As such, emigration of PMN through the endo- and epithelial barrier may lead to a disruption of such tissue barriers (11-13) and such a setting creates the potential for extravascular fluid leakage and subsequent edema formation (14, 15). However, with some exceptions, most episodes of hypoxia and/or ischemia-reperfusion are self-limiting, suggesting that endogenous protective mechanisms may exist to fortify the vascular barrier during such insults.

## 1.1. Structural and functional elements of the vascular barrier

The predominant barrier (~90%) to movement of macromolecules across a blood vessel wall is presented by the endothelium (16, 17). Passage of macromolecules across a cellular monolayer can occur via either a paracellular route (i.e., between cells) or a transcellular route (i.e., through cells). In non-pathologic endothelium, macromolecules such as albumin (molecular weight ~40 kD) appear to cross the cell

monolayer by passing between adjacent endothelial cells (i.e., paracellular) although some degree of transcellular passage may also occur (18, 19). Endothelial permeability is determined by cytoskeletal mechanisms that regulate lateral membrane intercellular junctions. Tight junctions, also known as zona occludens, comprise one type of intercellular junction (20, 21). Transmembrane proteins found within this region which function to regulate paracellular passage of macromolecules include the proteins occludin, and members of the junctional adhesion molecule (JAM) and claudin families of proteins (22). Tight junctions form narrow, cell-to-cell contacts with adjacent cells and comprise the predominant barrier to transit of macromolecules between adjacent endothelial cells (23). Endothelial macromolecular permeability is inversely related to macromolecule size. Permeability is also dependent on the tissue of origin. For example, endothelial cells in the cerebral circulation (i.e., blood-brain barrier) demonstrate an exceptionally low permeability (24, 25). Endothelial permeability may increase markedly upon exposure to a variety of inflammatory compounds (e.g., histamine, thrombin, reactive oxygen species, leukotrienes, bacterial endotoxins) or adverse conditions (e.g., hypoxia, ischemia) (16, 26). Reversible increases in endothelial permeability are produced by administration of cytochalasin or other agents that disrupt cytoskeletal microfilaments (16, 27). Likewise, increases in endothelial permeability are accompanied by disruption of peripheral actin microfilaments and formation of gaps between adjacent endothelial cells (16, 27). Administration of compounds that decrease endothelial permeability result in an irregular endothelial cell contour, greater convolution of cell margins, closer cell-to-cell contact, and increased surface area and cell perimeter (27). These changes in cell morphology are accompanied by a loss of F-actin in stress fibers, “ruffling” of dense peripheral bands of F-actin, and increase in the polymerized actin pool without significant changes in total F-actin endothelial cell content (21, 22). Interestingly, these changes in intracellular actin are similar to those observed during PMN transendothelial migration (28). By comparison, thrombin-induced increases in permeability result in a centralization (and peripheral loss) of F-actin. Both of these changes (permeability and F-actin distribution) are inhibited by isoproterenol (29). Phallacidin, an F-actin-stabilizing compound, also markedly attenuates thrombin-induced increases in permeability and accompanying morphologic changes (Figure 1).

In addition to the above components of the vascular barrier, the glycocalyx may play a role in determining movements of fluid and macromolecules across the endothelium. The endothelial glycocalyx is a dynamic extracellular matrix composed of cell surface proteoglycans, glycoproteins, and adsorbed serum proteins, implicated in the regulation and modulation of capillary tube hematocrit, permeability, and hemostasis (30). As such, increased paracellular permeability of such molecules as water, albumin and hydroxyethyl starch can be observed following experimental degradation of the functional components of the glycocalyx (31), and functional components of this glycocalyx may be dynamically regulated by endogenous mediators such as adenosine (32).

### 1.2. Vascular barrier during inflammation

Ongoing inflammatory responses are characterized by dramatic shifts in tissue metabolism. These changes include large fluctuations in energy supply and demand and diminished availability of oxygen (8) . Such shifts in tissue metabolism result, at least in part, from profound recruitment of inflammatory cell types, particularly myeloid cells such as neutrophils (PMN) and monocytes. The majority of inflammatory cells are recruited to, as opposed to being resident at, inflammatory lesions, and myeloid cell migration to sites of inflammation are highly dependent on hypoxia-adaptive pathways (8, 33). Consequently, much recent attention has focused on understanding how metabolic changes (e.g. hypoxia) relate to the establishment and propagation of the inflammatory response.

As outlined above, many parallels exist between hypoxic and inflamed tissues (1). For example, during episodes of hypoxia, polymorphonuclear leukocytes (PMN) are mobilized from the intravascular space to the interstitium, and such responses may contribute significantly to tissue damage during consequent reperfusion injury (3, 36). Moreover, emigration of PMN through the endo- and epithelial barrier may lead to a disruption of such tissue barriers (11-13) and such a setting creates the potential for extravascular fluid leakage and subsequent edema formation (14, 15). In contrast, transcriptional pathways mediated by hypoxia-inducible factor (HIF) may serve as a barrier-protective element during inflammatory hypoxia. For example, experimental studies of murine inflammatory bowel diseases have revealed extensive mucosal hypoxia and concomitant HIF-1 activation during colitis (34). Mice

engineered to express decreased intestinal epithelial HIF-1 exhibit more severe clinical symptoms of colitis, while increased HIF levels were protective in these parameters. Furthermore, colons with constitutive activation of HIF displayed increased expression levels of HIF–regulated barrier-protective genes (multidrug resistance gene-1, intestinal trefoil factor, CD73), resulting in attenuated loss of barrier during colitis in vivo. Such studies identify HIF as a critical factor for barrier protection during mucosal inflammation and hypoxia (35).

### *1.2.1. Barrier disruptive pathways*

Macromolecule transit across blood vessels has evolved to be tightly controlled. Relatively low macromolecular permeability of blood vessels is essential for maintenance of a physiologically optimal equilibrium between intravascular and extravascular compartments (36, 37). Endothelial cells are primary targets for leukocytes during episodes of infection, ischemic or traumatic injury, which all together can result in an altered barrier function. Disturbance of endothelial barrier during these disease states can lead to deleterious loss of fluids and plasma protein into the extravascular compartment. Such disturbances in endothelial barrier function are prominent in disorders such as shock and ischemia-reperfusion and contribute significantly to organ dysfunction (3, 38-41).

Previous studies have indicated that activated PMN release a number of soluble mediators, which dynamically influence vascular permeability during transmigration. As such, PMN have been shown to liberate factors that can either disrupt or protect the endothelial barrier: For example, it was recently shown that activation of PMN through $\beta_2$ integrins elicits the release of soluble factor(s) which induce endothelial cytoskeletal rearrangement, gap formation and increased permeability (42). This PMN-derived permeabilizing factor was subsequently identified as heparin-binding protein (HBP, also called azurocidin or CAP37 (42), a member of the serprocidin family of cationic peptides (43). HBP, but not other neutrophil granule proteins (e.g. elastase, cathepsin G), was shown to induce $Ca^{2+}$-dependent cytoskeletal changes in cultured endothelia and to trigger macromolecular leakage in vivo. Interestingly, HBP regulation of barrier may not be selective for PMN, and in fact, endothelial cells themselves are now a reported source of HPB (44). It is therefore possible that endothelia may self-regulate permeability through

HBP under some conditions, and that mediators found within the inflammatory milieu may also increase endothelial permeability.

Similarly, PMNs were observed to significantly alter endothelial permeability by release of glutamate, following FMLP activation. This crosstalk pathway appears to be of particular importance for the regulation of the vascular barrier of the brain ("blood brain barrier"). In fact, treatment of human brain endothelia with glutamate or selective, mGluR group I or III agonists resulted in a time-dependent loss of phosphorylated vasodilator-stimulated phosphoprotein (VASP) and significantly increased endothelial permeability. Glutamate-induced decreases in brain endothelial barrier function and phosphorylated VASP were significantly attenuated by pretreatment of human brain endothelia with selective mGluR antagonists. Even in an *in vivo* hypoxic mouse model, the pretreatment with mGluR antagonists significantly decreased fluorescein isothiocyanate-dextran flux across the blood-brain barrier, suggesting that activated human PMNs release glutamate and that endothelial expression of group I or III mGluRs function to decrease human brain endothelial VASP phosphorylation and barrier function..

A recently described gene regulatory pathway revealed a critical role for BMK1/ERK5 in maintaining the endothelial barrier and blood vessel integrity: A targeted deletion of big mitogen-activated protein kinase1 gene (BMK1) (also known as ERK5, member of the MAPK family), in adult mice leads to disruption of the vascular barrier. Histological analysis of these mice reveals that, after BMK1 ablation, hemorrhages occurred in multiple organs in which endothelial cells lining the blood vessels became round, irregularly aligned, and, eventually, apoptotic. In vitro removal of BMK1 protein also led to the death of endothelial cells partially due to the deregulation of transcriptional factor MEF2C, which is a direct substrate of BMK1. Additionally, endothelial-specific BMK1-KO leads to cardiovascular defects identical to that of global BMK1-KO mutants. Taken together, these studies identify the BMK1 pathway as critical for endothelial function and for maintaining blood vessel integrity (45).

### *1.2.2. Barrier protective pathways*

Acute increases in vascular permeability to macromolecules closely coincide with tissue injury of many etiologies, and can result in fluid loss, edema, and organ dysfunction (16, 46, 47). Previous studies have indicated that extracellular

nucleotide metabolites may function as an endogenous protective mechanism during hypoxia and ischemia (48-50). One important factor may be increased production of endogenous adenosine, a naturally occurring anti-inflammatory agent (50-52). Several lines of evidence support this assertion. First, adenosine receptors are widely expressed on target cell types as diverse as leukocytes, vascular endothelia, and mucosal epithelia and have been studied for their capacity to modulate inflammation (53). Second, murine models of inflammation provide evidence for adenosine receptor signaling as a mechanism for regulating inflammatory responses *in vivo*. For example, mice deficient in the $A_{2A}$-adenosine receptor ($AdoRA_{2A}$) show increased inflammation-associated tissue damage (54). Third, hypoxia is a common feature of inflamed tissues (12) and is accompanied by significantly increased levels of adenosine (55-57). At present, the exact source of adenosine is not well defined, but likely results from a combination of increased intracellular metabolism and amplified extracellular phosphohydrolysis of adenine nucleotides via surface ecto-nucleotidases.

The vascular endothelium is the primary interface between a hypoxic insult and the surrounding tissues. At the same time, the endothelium is central to the orchestration of leukocyte trafficking in response to chemotactic stimuli. This critical anatomic location places vascular endothelial cells in an ideal position to coordinate extracellular metabolic events important to endogenous anti-inflammatory responses. We recently identified a neutrophil-endothelial cell crosstalk pathway that is coordinated by hypoxia. This pathway utilizes extracellular nucleotide substrates, liberated from different cell types. Extracellular ATP release has been shown from endothelial cells, particularly under sheer stress, hypoxia and inflammation. In addition, fMLP activated neutrophils can release ATP. Activated platelets comprise an additional source for extracellular adenine nucleotides (59, 60). The role of endothelial CD39 (Ecto-apyrase, conversion of ATP/ADP to AMP) has been viewed as a protective, thromboregulatory mechanism for limiting the size of the hemostatic plug (60, 61). Metabolism of adenine nucleotides derived from activated platelets is crucial in limiting excessive platelet aggregation and thrombus formation (62, 63). Similarly, excessive platelet accumulation and recruitment can be treated with the use of soluble forms of CD39 (64, 65). Moreover, a thromboregulatory role could be demonstrated in a model of stroke, where *cd39*-null mice showed increased sizes of infarction that could be reduced by treatment with soluble CD39 (66). Surprisingly,

targeted disruption of *cd39* resulted in prolonged bleeding and increased vascular leak and fibrin deposition in hypoxemia (67), suggesting a dual role for ATP metabolism by CD39 in modulating hemostasis and thrombotic reactions. Moreover, this observation may be related to an activation and desensitization of the purinergic type $P2Y_1$ receptor. Activation of the $P_2Y_1$-platelet receptor appears to be crucial in the activation process of platelets. As such, $P2Y_1$ deficient mice exhibit signs of prolonged bleeding time and resistance to thromboembolism (68). In contrast to these studies, we observed a barrier-protective influence during hypoxia that was not related to the activation of PX receptors, but to a downstream metabolism and signaling of ATP metabolites (esp. adenosine).

Extracellular ATP is readily converted on the endothelial surface to adenosine, due to the enzymatic function of CD39 and CD73 (5'-Ecto-nucleotidase, conversion of AMP to adenosine).

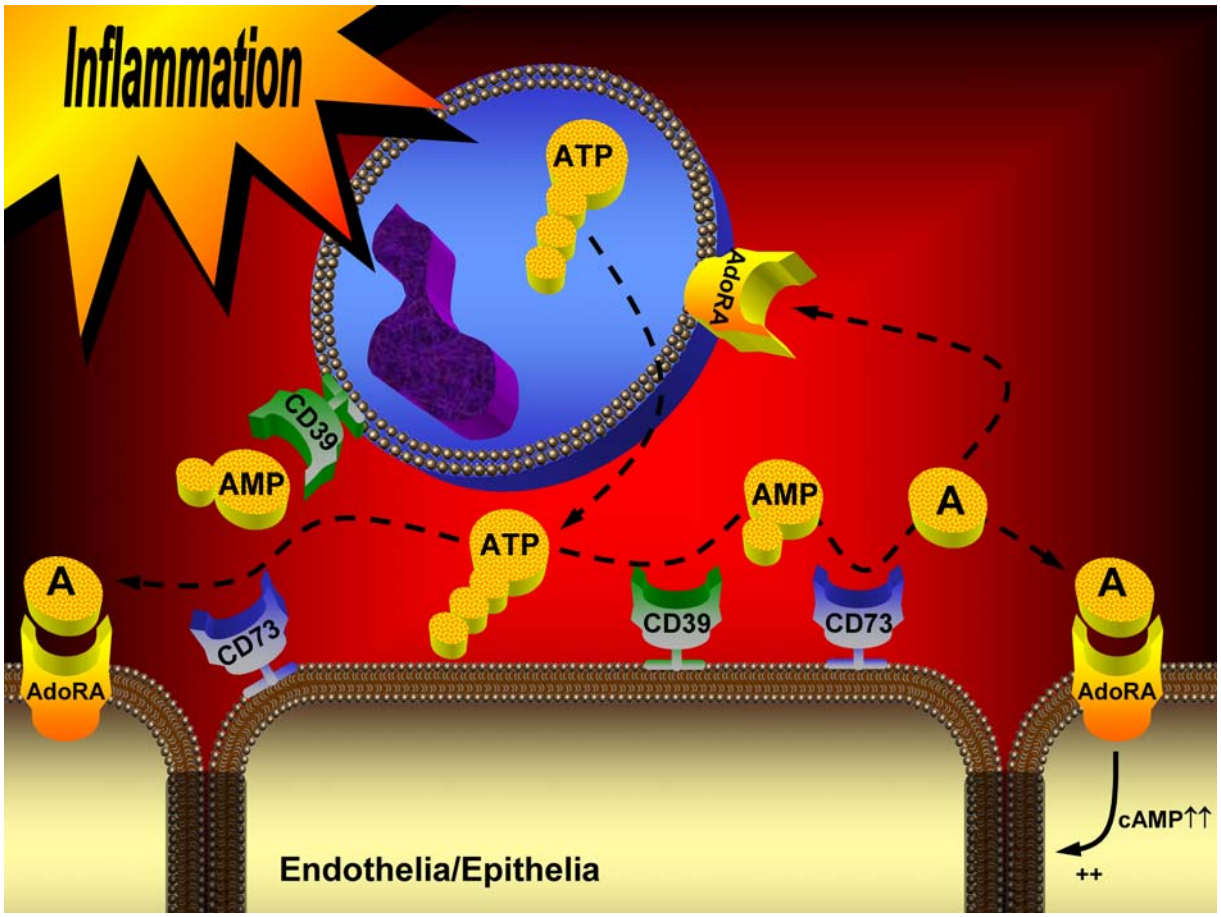


***Figure 9: Model of coordinated nucleotide metabolism and nucleoside signaling in hypoxia and during inflammation cells***

Such adenosine binds to surface expressed PMN adenosine receptors to limit excessive accumulation of PMN within tissues, and as such, functions as a feedback loop to attenuate potential tissue injury (58). With regard to this latter point, it was recently shown that hypoxia coordinates both, transcriptional and metabolic control of the surface ecto-nucleotidases CD39 and CD73 (69-71), and as such, significantly amplifies the extracellular production of adenosine from adenine nucleotide precursors. In fact when using cd39- and cd73-null animals we found that extracellular adenosine produced through adenine nucleotide metabolism during hypoxia is a potent anti-inflammatory signal for PMN *in vitro* and *in vivo*. These findings identify CD39 and CD73 as critical control points for endogenous adenosine generation and implicate this pathway as an innate mechanism to attenuate excessive tissue PMN accumulation (58).

In addition to a role in limiting excessive neutrophil tissue accumulation, CD39 and CD73 are also critical control points for vascular permeability. For the purpose of investigating overall organ vascular permeability, we used Evan's blue dye, which tightly binds to plasma albumin (72). To do this, mice were administered 0.2% Evan's blue dye (0.5% in PBS) by intravenous tail vein injection, subjected to room air (normoxia) or normobaric hypoxia (8% $O_2$/92% $N_2$). At the end of exposure, animals were anesthetized, heparinized (50U by i.p. injection) and fluid overloaded (3 c.c. normal Ringer's solution i.p.). Animals were then exsanguinated by femoral cut-down to flush all vascular beds, and tissues were harvested. Tissues were rinsed in PBS, and Evan's blue was extracted with formamide at 56$^{o}$C for 2hr, and quantified at 610nm with subtraction of reference absorbance at 450nm. This model entails the quantification of formamide-extractable Evans blue (73) from tissues of mice as a readout for overall vascular permeability of different organs. In fact we found that vascular permeability in tissues derived from animals subjected to normobaric hypoxia (8% $O_2$/92% $N_2$) ranged from 2 - 4-fold more permanent to Evans blue than normoxic controls (9).

In order to identify the role of CD73 in vascular permeability, we used this model in mice that were administered with the CD73 inhibitor 5'-alpha, beta-methylenediphosphate (APCP) or in mice following targeted deletion of CD73. In fact, we found dramatic increase of hypoxia-elicited dysfunction of the vascular barrier in different organs (lung, heart, intestine, kidneys) following CD73 inhibition or deletion. Vascular leak associated with hypoxia was, at least in part, reversed by

reconstitution with soluble 5'-nucleotidase and adenosine receptor agonists in the cd73-null mice. Histological examination of lungs from hypoxic *cd73*$^{-/-}$ revealed perivascular interstitial edema associated with inflammatory infiltrates surrounding larger pulmonary vessels (74). Taken together, these studies identify CD73 as a critical mediator of vascular permeability *in vivo*. When measuring vascular permeability during hypoxia in mice with targeted deletion of CD39, similar increases in vascular barrier function could be observed in different organs (9). Taken together, these studies have identified adenosine generation of the hypoxic vasculature via nucleotide-phosphohydrolysis as a critical cellular strategy to generate adenosine and maintain vascular barrier function.

### *1.2.3. Effect of adenosine receptor activation on endothelial barrier function*

*In vitro* studies of endothelial permeability suggested, that activation of a specific endothelial adenosine receptor, the $AdoRA_{2B}$, leads to a barrier resealing response following PMN transmigration (75). Thus we were able to show that of the four different adenosine receptors that are expressed by endothelia, only the $AdoRA_{2B}$ is selectively induced by hypoxia (9). Activation of the $AdoRA_{2B}$ is associated with increases in intracellular cAMP concentration due to the activation of the adenylate cyclase (50). By inhibition of cAMP formation, the resealing of the endothelial barrier during PMN transmigration can be obviated (75). Such increases in cAMP following activation of the $AdoRA_{2B}$ lead to an activation of protein kinase A (PKA) (22). Further studies revealed a central role of PKA-induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a protein responsible for controlling the geometry of actin-filaments (76). Adenosine-receptor mediated phosphorylation of VASP is responsible for changes in the geometry and distribution of junctional proteins, thereby affecting the characteristics of the junctional complex and promoting increases in barrier function (77, 78).

## 1.3. Increased Adenosine Production during hypoxia

At present, the exact metabolic steps for generation of extracellular adenosine in hypoxia are not well characterized, but likely involve increased enzymatic phosphohydrolysis from precursor adenine nucleotides (ATP, ADP and AMP). For instance, we recently demonstrated that hypoxia coordinates both transcriptional and

metabolic control of the surface ecto-nucleotidases CD39 and CD73 (69-71), and thereby amplifies extracellular accumulation of adenosine. Additional mechanisms also exist to amplifying adenosine signaling during hypoxia include coordinate changes at the adenosine receptor level. For instance, the vascular endothelial adenosine receptor subtype $AdoRA_{2B}$ is selectively induced by hypoxia and such increases in receptor density are associated with increased vascular barrier responses to adenosine (71).

Once generated into the extracellular milieu, adenosine is rapidly cleared through passive or active uptake through nucleoside transporters, termed equilibrative nucleoside transporters (ENT) and concentrative nucleoside transporters (CNT), respectively, expressed on a variety of cell types (79). The predominant nucleoside transporters of the vascular endothelium are ENT1 and ENT2 (80), bi-directional transporters functioning as diffusion-limited channels for transmembrane adenosine flux. Previous studies have suggested that vascular adenosine flux during hypoxia is predominantly inward (81), thereby terminating extracellular adenosine signaling. However, more recent studies indicate that the expression of ENT1 may be transcriptionally regulated by hypoxia (82, 83), thereby functioning to fine tune extracellular levels of adenosine.

Therefore, considering that endothelial adenosine uptake could influence endothelial cell function during hypoxia, as well as a recent study suggesting that ENT1 and ENT2 gene-regulation are influenced by hypoxia in murine cardiomyocytes (82, 83), we examined the influence of hypoxia on vascular endothelial adenosine transport. Results from these studies revealed that endothelial ENT1 and ENT2 gene expression and function are attenuated by hypoxia, and that this regulatory circuit maps, at least in part, to hypoxia inducible factor 1 (HIF-1)-mediated repression of ENT expression. These studies provide new molecular insight into endogenous mechanisms of tissue protection during hypoxia (84).

### 1.4. Role of Adenosine Deaminase in vascular inflammation during hypoxia

As outlined above, the physiologic adaptation and pathophysiologic response to hypoxia are currently areas of intense investigation and several reports suggest that both transcriptional and metabolic pathways may contribute to a broad range of diseases. For example, during episodes of hypoxia / ischemia, polymorphonuclear

leukocytes (PMN) are mobilized from the intravascular space to the interstitium, and such responses may contribute significantly to tissue damage during consequent reperfusion injury (3, 58). Emigration of PMN through the endothelial barrier is associated with a disruption of tissue barriers creating the potential for vascular fluid leakage and subsequent edema formation (9, 74). Among others, such “hypoxia-associated” disorders include the systemic inflammatory response syndrome, sepsis, acute respiratory distress syndrome and acute myocardial infarction (3).

At the same time, studies have indicated that extracellular nucleotide metabolites (particularly adenosine) may function as an endogenous anti-inflammatory metabolite during hypoxia (7, 9, 58, 74, 84-86). Vascular adenosine signaling during hypoxia has been implicated to dampen pathophysiologic changes related to increased tissue permeability, accumulation of inflammatory cells, and transcriptional induction of pro-inflammatory cytokines during hypoxia (2, 53). Several lines of evidence support this assertion (7). First, adenosine receptors are widely expressed on vascular endothelial cells, and have been studied for their capacity to modulate inflammation (53, 58, 74). Second, murine models of inflammation and/or hypoxia provide evidence for adenosine receptor signaling as a mechanism for regulating hypoxia responses *in vivo*. Indeed, mice genetically deficient in surface enzymes necessary for adenosine generation (ecto-apyrase, CD39 [conversion of ATP to AMP] and 5’-ectonucleotidase, CD73 [conversion of AMP to adenosine]) show increased hypoxia-associated tissue damage and vascular leak syndrome during hypoxia (9, 74). Third, hypoxia accompanies the normal inflammatory response (87) and is associated with significantly increased levels of adenosine (53). The exact source(s) of adenosine are not well defined, but likely result from a combination of increased intracellular metabolism and amplified extracellular phosphohydrolysis of adenine nucleotides via surface ecto-nucleotidases (9, 74). In addition, recent studies have also shown that hypoxia-inducible-factor (HIF) dependent transcriptional repression of equilibrative nucleoside transporters (ENT) results in decreased capacity of the vascular endothelium to transport extracellular adenosine, thereby providing an additional mechanism to elevate vascular adenosine levels during hypoxia (84).

Despite of the central role of adenosine in innate inflammatory responses, chronically increased levels of adenosine may be detrimental (88). For example, levels of adenosine are increased in the lungs of asthmatics (89), in which elevations

correlate with the degree of inflammatory insult (90), suggesting a provocative role of adenosine in asthma or chronic obstructive pulmonary disease (91). In addition, adenosine-deaminase (ADA)-deficient mice develop signs of chronic pulmonary injury in association with elevated pulmonary adenosine levels. ADA-deficent mice, in fact, die within weeks after birth from severe respiratory distress (92), and recent studies suggest that attenuation of adenosine signaling may reverse the severe pulmonary phenotypes in ADA-deficient mice, suggesting that chronic adenosine elevations can affect signaling pathways that mediate aspects of chronic lung disease (93-94). Likewise, human ADA deficiency is a well characterized severe combined immunodeficiency syndrome associated with T cell cytotoxicity by deoxyadenosine (95).

Given the biological necessity to balance extracellular adenosine levels with potential chronic toxicity, we sought to define whether mechanisms exist to degrade extracellular adenosine in models of increased adenosine (hypoxia). As guided by initial microarray analyses, studies in endothelial cells, murine models of hypoxia, as wells as chronically hypoxic human subjects revealed parallel induction of extracellular ADA and CD26 by hypoxia, thereby increasing the capacity for extracellular adenosine catabolism. These studies provide new molecular insight into innate adaptation to hypoxia and identify ADA as a potential therapeutic target in the treatment of vascular leak syndrome or excessive inflammation associated with acute hypoxia (96).

# 2. Materials and Methods

## 2.1. Materials

FITC-Dextran
Percoll
Acid citrate
fMLP
Chrono-Lume
18-a-gylcyrrhetinic
Connexin mimetic peptides
E-ATP
E-AMP
CD39
Cd73
FACS lysing solution
Cellfix
BCECF-AM
Leukotriene B4

## 2.2. Methods

### *2.2.1. Isolation of Human PMN*

After approval by the Insitutional Review Board and obtaining written informed consent from each individual, PMN were freshly isolated from whole Blood obtained by venipuncture from healthy, volunteer donors. The blood was anticoagulated with acid citrate (10 ml monovets with Na-citrate, Sarstedt, Nümbrecht, Germany) and the platelets, plasma, mononuclear cells and erythrocytes were removed by double-density centrifugation using the Percoll system, which consists of colloidal silica particles coated with polyvinyl pyrrolidone. In short, a double density gradient with 4 ml Percoll denisty 72,13 % at the bottom and 4 ml Percoll density 63,11 % on top was prepared. Then, 4 ml anticoagulated whole blood was layered carefully on top. The tubes were centrifuged at 500g for 30 min at room temperature, no break and

the band with PMN was carefully separated with a Pasteur-pipette. From then on, the PMN were maintained at 4°C until activation. Residual erythrocytes were removed by lysis in cold $NH_4Cl$ buffer (400g, 10 min, 4°C, break on), followed by two careful washes in HANKs minus (400g, 10 min, 4°C, break on). The supernatant is discarded. Remaining cells were greater than 99% PMN as demonstrated by microscopic evaluation. PMN were studied within 2h of their isolation. In general, this technique yielded about 0.5 – 1 x $10^8$ PMN from 50 ml of fresh blood.

### *2.2.2. Preparation of Activated PMN Supernatants and Measurement of ATP or myeloperoxidase (MPO) content*

To measure nucleotide release from activated PMN, freshly isolated human neutrophils were activated with N-formyl-methionyl-leucyl-phenylalanine (fMLP), which stimulates neutrophils and monocytes but not lymphocytes and platelets. Samples from the supernatant were taken at different time points after activation and analyzed by HPLC. In short, freshly isolated human PMN were transferred from cold (4°C) calcium free buffer (HANKS balanced salt solution without calcium, HANKS minus) into HANKS plus with 100 nM fMLP at a concentration of 107 cells/ml, and rotated end-over-end at 37°C. At 1, 5, 10 and 15 min, 200 µl samples were transferred into ice-cold Eppendorf cups and immediately pelleted (500g for 4 min, 4°C). The resultant cell-free supernatants were assessed by HPLC (model 1050; Hewlett-Packard, Palo Alto, California, USA) with an HP 1100 diode array detector by reverse-phase on an HPLC column (Luna 5-µm C18, 150 x 4.60 mm; Phenomenex, Torrance, California, USA) with 100% H2O mobile phase. ATP was thus identified by its chromatographic behaviour (retention time, UV absorption spectra, and coelution with standards). To exactly quantify the ATP content within the supernatant, a highly sensitive luciferase based technique was used (CHRONO-LUME reagent, Chrono-log Corp, Haverton, PA). Luciferase activity was assessed on a luminometer (Turner Designs Inc., Sunnyvale, California, USA) and compared with internal ATP standards. In subsets of experiments, the granular marker MPO was assessed. In short, MPO was quantified from the cell-free supernatant after adjustment of the pH to 4.2 with 1.0 M citrate buffer (pH 4.2) and color development was assayed at 405 nm on a microtiter plate reader after mixing equals parts of the supernatant with a solution containing 1mM 2,2`-azino-bis (3-ethylbenzothiazoline-6-

sulfonate) (ABTS, Sigma-Aldrich) and 10nM hydrogen peroxide ($H_2O_2$) in 100 nM citrate buffer (pH 4.2). After appropriate color development the reaction was terminated by the addition of SDS to a final concentration of 0.5% (3, 97). In subsets of experiments, PMN were preincubated and fMLP stimulated in the presence of brefeldine A, verapamile, dipyridamole, 18-α-glycyrrhetinic acid (18-α-GA), which inhibited transjunctional currents, and anandamide (Sigma Aldrich). In addition, the effects of connexin-mimetic peptides were tested (for connexin 43: SRPTEKTIFII; for connexin 40: SRPTEKNVFIV, Biosource, Solingen, Germany) (98).

### *2.2.3. PMN Granule isolation*

The granule fraction from PMN was purified from resting neutrophils as previously described (99) Briefly, neutrophils were subjected to nitrogen cavitation followed by centrifugation to remove nuclei and non-disrupted cells. The resulting postnuclear supernatant was applied to the top of a discontinuous, 3-layer Percoll gradient (1.050/1.09/1.12 g/mL) and centrifuged at 37000g for 30 min at 4°C. Gradients were aspirated from the bottom through a peristaltic pump attached to a fraction collector set to deliver 1 mL in each fraction. The granular fraction was pooled and Percoll was removed by centrifugation, and the biological material was resuspended in 1 mL HANKS minus. Aliquots were assayed for the presence of the marker protein MPO. ATP content of the granular fraction was quantified as above and compared with the ATP content of the cytosolic fraction.

### *2.2.4. Measurement of endothelial surface enzyme activity of the ecto-apyrase (CD39) and the 5'-ectonucleotidase (CD73)*

We assessed surface enzyme activity as described previously (9, 70) by qunatifying the conversion of etheno-ATP (E-ATP, Molecular Probes Inc.) to etheno-AMP (E-AMP, Sigma Aldrich; CD 39 activity) or e-AMP to etheno-adenosine (CD 73 activity). Briefly, HBSS with or without ab-methylene ADP (APCP) was added to freshly isolated PMN (107/ml). After 10 min, E-ATP/E-AMP (final concentration 1µM) was added and samples were taken at indicated time points, removed, acidified to pH 3.5 with HCl, spun /10,000g for 20 sec, 4°C), and frozen (-80°C) until analysis via HPLC. This was done with an HPLC pump P680 and a Hitachi Fluorescence Detector L-

7480 on a reverse-phase column (Grom-Sil 120-ODS-ST-5µ; 150 x 3 mm Grom) using a mobile phase gradient from 0 to 33 % acetonitrile/0.3 mM Kh2PO4 (pH5) in 10 min. CD 39/CD73 activity was expressed as percent E-ATP/E-AMP conversion in this time frame.

*2.2.5. Endothelial Cell Isolation and Culture*

Human microvascular endothelial cells (HMEC-1) were a kind gift of Francisco Candal, Centers for Disease Control, Atlanta, GA (104) and were harvested and cultured by a modification of methods previously described (3, 72). In brief, HMECs were harvested with 0.1% trypsin and incubated at 37°C in 95% air/5% $CO_2$. Culture medium was supplemented with heat-inactivated fetal bovine serum, penicillin, streptomycin, L-glutamine, epidermal growth factor, and hydrocortisone. For preparation of experimental HMEC monolayers, confluent endothelial cells were seeded at ~1x$10^5$ cells/ $cm^2$ onto either permeable polycarbonate inserts or 100 mm Petri dishes. Endothelial cell purity was assessed by phase microscopic "cobblestone" appearance and uptake of fluorescent acetylated low-density lipoprotein.

*2.2.6. Endothelial Macromolecule Paracellular Permeability Assay*

Using a modification of methods previously described (72, 74), HMECs on polycarbonate permeable inserts (0.4-µm pore, 6.5-mm diam; Costar Corp., Cambridge, MA) were studied 7-10 d after seeding (2-5 d after confluency). Inserts were placed in HBSS-containing wells (0.9 ml), and HBSS (alone or with PMN, PMN supernatant, or ATP) was added to inserts (100 µl). At the start of the assay (t = 0), FITC-labeled dextran 70 kD (concentration 3.5 µM) was added to fluid within the insert. The size of FITC-dextran, 70 kD, approximates that of human albumin, both of which have been used in similar endothelial paracellular permeability models. Fluid from opposing well (reservoir) was sampled (50µl) over 60 min (t = 20, 40 and 60 min). Fluorescence intensity of each sample was measured (excitation, 485 nm; emission, 530 nm; Cytofluor 2300; Waters Chromatography, Bedford, MA) and FITC-dextran concentrations were determined from standard curves generated by serial dilution of FITC-dextran. Paracellular flux was calculated by linear regression of

sample fluorescence. Consistent with observations of other investigators, control experiments demonstrated decreased paracellular permeability with forskolin and 8-bromo-cAMP and increased paracellular permeability with thrombin and hydrogen peroxide.

*2.2.7. Immunoblotting experiments*

PMN were freshly isolated from human donors and lysed for 10 min in lysis buffer (107 PMN/500µl; 150 M NaCl, 25 mM Tris, pH 8.0, 5mM EDTA, 2% Triton X-100, and 10 % mammalian tissue protease inhibitor cocktail; Sigma Aldrich), and collected into microfuge tubes. After spinning at 14,000g to remove cell debris, the pellet was discarded. Proteins were solublized in reducing Laemmli sample buffer and heated to 90°C for 5 min. Samples were resolved on a 12% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature in PBS supplemented with 0.2% Tween 20 (PBS-T) and 4% BSA. The membranes were incubated in 10 µg/ml polyclonal rabbit phospho-connexin 43 (ser368) antibody (Cell Signaling Technology, Danvers, MA USA) for 1h at room temperature, followed by 10 min washes in PBS-T. The membranes were incubated in 1:3,000 goat anti-rabbit IgG ( ICN Biomedicals/Cappel), and conjugated to horseradish peroxidase for 1 h at room temperature. The wash was repeated and proteins were detected by enhanced chemiluminscence.

*2.2.8. Flowcytometric analysis of PMN surface expression of CD 39 and CD 73*

Whole blood was obtained by venipuncture from human volunteers and anticoagulated with acid citrate (10 ml monovets with Na-citrat, Sarstedt, Nümbrecht, Germany). 100 µl of whole blood were stained with fluorescine labelled monoclonal antibodies against CD 73 (Serotec) and PE-labelled anti CD 39 (Becton Dickinson) and their IgG subclass specific isotypes respectively a

Ccording to the instructions of the manufacturer. After 30 minutes of incubation at room temperature, erythrocytes were lysed using FACS lysing solution (Becton Dickinson) and spun (1200 RPM for 5 min, 4°C). Cells were washed two times in HANKS minus, fixed (CellFix, Becton Dickinson) and analysed within less than half an hour in a Becton Dickinson FACSort equipped with CellQuest software. Forward

and right-angle light scatter were used for gating granulocytes, monocytes and lymphocytes. Isotypes were set within the first decade of the 4-decade scale.

*2.2.9. PMN adhesion assay*

Freshly isolated PMNs were labelled for 30 minutes at 37°C with 5 µM BCECF-AM (2`,7`-bis(carboxyethyl)-56-carboxyfluorescein-acetoxymethyl ester; 5 µM final concentration; Calbiochem, San Diego, CA) and washed three times in calcium free HBSS. Labelled PMN (1x $10^5$/ monolayer) were activated with 100 nM fMLP and added to washed normoxic or hypoxic monolayers of confluent HMEC-1. Plates were centrifuged at 150g for 2 minutes to uniformly settle PMN, and adhesion was allowed for 10 minutes at 37°C. Monolayers were gently washed three times with HBSS, and fluorescence intensity (485-nm excitation, 530-nm emission) was measured on a fluorescent plate reader (Cytofluor 2300, Millipore, Bedford, MA). Adherent cell numbers were determined from standard curves generated by serial dilution of known PMN numbers diluted in HBSS. All data were normalized for background fluorescence by subtraction of fluorescence intensity of samples collected from monolayers incubated in buffer only, without addition of PMN (3). To test the influence of connexin mimetic peptides, fMLP activation of PMN was performed after 10 minutes of pre-incubation and in the presence of indicated concentrations of connexin mimetic peptides (for connexin 43: SRPTEKTIFII; for connexin 40: SRPTEKNVFIV, 0-1000 µM). As a control, the non-specific adenosine receptor antagonists 8PT was used (both PMN and HMEC-1 monolayers were (pre-)incubated with 10 µM 8-PT).

*2.2.10. Isolation and activation of murine PMN*

In subsets of experiments**,** PMN were isolated from mice with induced ablation of ablation of Cx43. this was achived using adult Cx43Cre-ER(T)/fl mice that received intraperitoneal injections of 3 mg 4-hydroxytamoxifen (4-OHT) once per day on five consecutive days as previously described (100). The animals were sacrificed at day 12 after the first injection. For control, Cx43fl/fl mice were used. In other experiments, PMN isolated from heterozygote Cx43+/- (101) and cd39-/- mice were used (102). In short, age and gender matched knockout mice and littermate controls

received intraperitoneal heparin (300 i.E./kg) and pentobarbital (100 mg/kg). After induction anesthesia, whole blood was obtained by cardiac puncture (500-800 µl per animal) and the animals were sacrificed. PMN were isolated with a double density gradient using 4 ml Percoll 73% at the bottom and 4 ml Percoll 63% above, with the heperanized blood of one animal carefully layered on top. The tubes were centrifuged at 500g for 30 minutes at room temperature, no break and the band with PMN was carefully separated. PMN were studied within 2h of their isolation. In general, this yielded about 1-5 x 106 PMN per animal. Due to low expression rates of fMLP receptors on murine PMN, activation was performed with leukotriene B4 (LTB4 100nM, Calbiochem) (103). In short, freshly isolated PMN were transferred from cold (4°C) calcium free buffer (HANKS minus) into HANKS plus with 100nM LTB4 at a concentration of 106 cells/ml, and rotated end-over-end at 37°C. At 1, 5, 10, 15 minutes, 200 µl samples were transferred into ice-cold Eppendorf cup ans immediately pelleted (500g for 30s, 4°C). The resultant cell-free supernatants were assessed for ATP content with a standard luciferase based technique (Chrono-Lume reagent, Chrono-log Corp, Haverton, PA) as above (n=4-6 animals per condition). Cx43 expression was assessed by western blot analysis from cardiac tissue. In short, mouse myocardial extracts were snap frozen, homogenized with a mortar in liquid nitrogen and transferred to 1 x Cell lysis buffer (Cell Signaling, Beverly, MA, containing in mM: Tris 7.5 20, NaCl 150, EDTA 1, EGTA 1, sodium pyrophosphate 2.5, b-glycerolphophate1, Na3VO4 1, Triton X-100 1%, Leupeptin 1µg/ml, Complete Protease Inhibitor Cocktail 1x (Roche, Basel, Switzerland)). Subsequently, the samples were sonicated for 20s and centrifuged at 14000g for 10 minutes. The supernatants were collected and the protein concentrations were determined using Dc protein assay (Biorad, Hercules, CA). 25 µg total proteins were electrophortically separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Cx 43 was detected usig a rabbit polyclonal antibody against rat total Cx43 (Zymed, Berlin, Germany, dilution 1:1000) and GAPDH was detected using a monoclonal antibody against rabbit GAPDH (HyTest, Turku, Finland, dilution 1:2500). Immunoreactive signals were detected by chemiluminescence (LumiGLO, Cell Signaling) and quantified using Scion Image software. These protocols were in accordance with National Institutes of Health Guidelines for use of live animals and were approved by the Institutional Animal Care and Use Committee at Brigham and Women´s Hospital and of the University of Essen, Germany.

# 3. Results

## 3.1. PMN release ATP upon activation

We have previously shown that PMN have the capacity to release adenine nucleotides in the form of ATP (7, 9), though the molecular details of nucleotide release from PMN remain largely unknown. Here, we sought to understand details of ATP release from PMN. Initially, we determined whether ATP release was activation-dependent. For these purposes, we distinguished extracellular ATP levels in the presence and absence of the potent PMN activator fMLP.

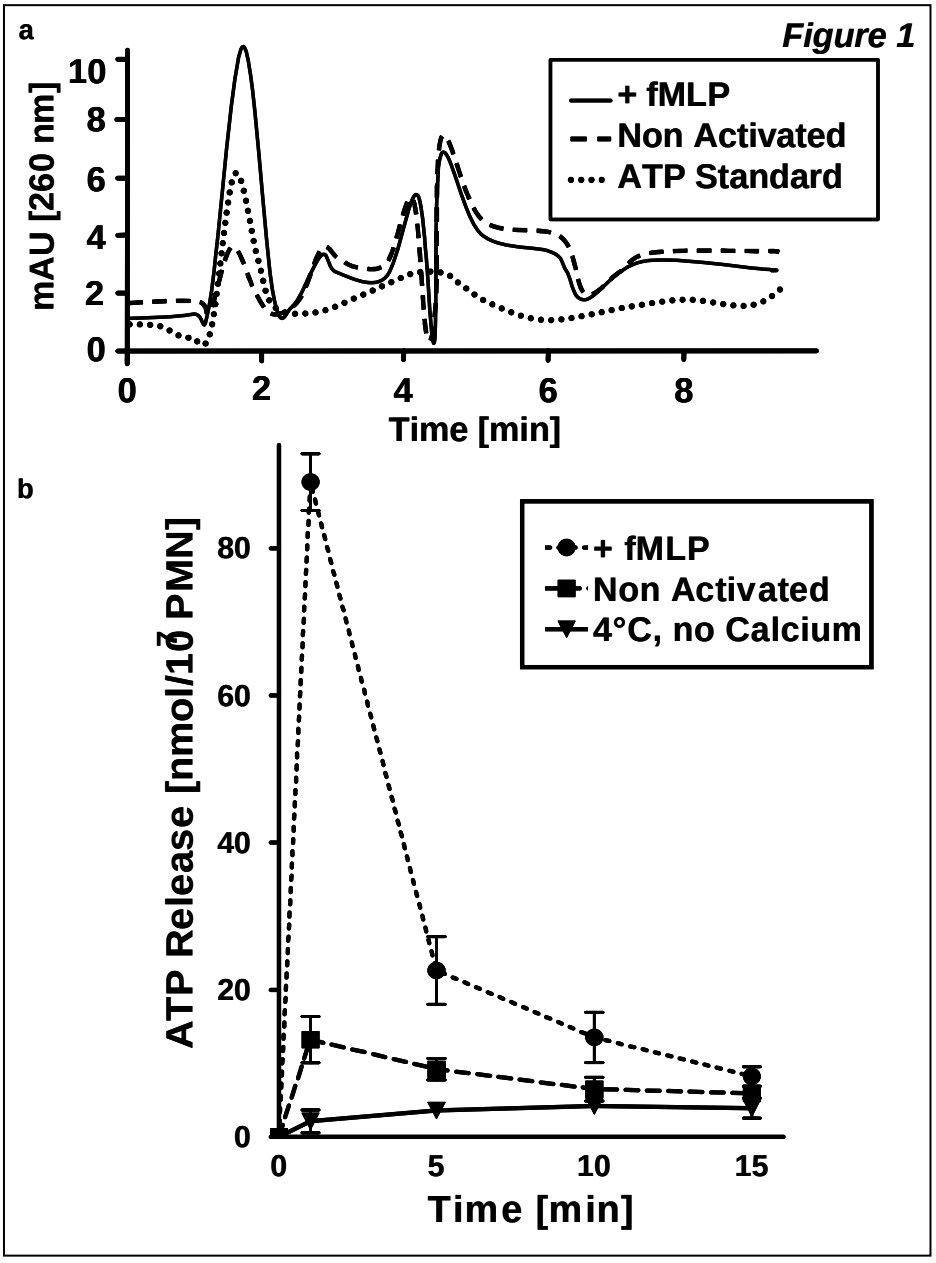


***Figure 1: 5`-Adenosintriphosphate (ATP) release from PMN is activation dependent***

As shown in Figure 1, ATP was readily detected in supernatants of freshly isolated PMN (based on biophysical criteria such as retention time, coelution with internal ATP standards (Figure 1A) and UV absorption spectra (data not shown)) and ATP release increased by greater than approximately six-fold upon fMLP activation (area under curve of the HPLC tracing). These findings from HPLC-based detection were confirmed using a luminometric ATP detection assays. As shown in Figure 1B, ATP release from freshly isolated PMN was 4.2 ± 1.6 nmol/$10^7$ PMNs without activation at 4°C in $Ca^{2+}$-free HBSS. Higher ATP levels were observed at 37°C in $Ca^{2+}$ containing buffer (maximal levels 13.2 ± 6.3 nmol/$10^7$ PMNs; $p < 0.001$ by ANOVA), and progressively dissipated to control levels within 15 min. These results indicate a metabolic and activation-dependent release of ATP from human PMN.

### 3.2. Mechanism of extracellular ATP metabolism

In the course of these studies, we addressed the rapid loss of extracellular ATP following PMN activation (Figure 1). In our experimental setting of the $10^7$ PMN/ml, extracellular ATP concentrations were as high as 100nM, while cytoplasmic concentrations were as high as 5mM (see later), thereby resulting in a 50,000-fold transmembrane ATP gradient, making passive ATP reuptake highly unlikely. As second possibility, we considered extracellular ATP phosphohydrolysis by PMN. A primary source of extracellular ATP phosphohydrolysis is cell surface CD 39 (67), and therefore, we determined whether PMN express surface CD 39. For these purposes, we used a non-native exogenous substrate (etheno-ATP) which could be distinguished from endogenous ATP via HPLC analysis (9, 70). To measure CD 39 activity, we quantified etheno-ATP conversion to etheno-AMP by intact PMN ($10^7$ PMN/ml) in the presence and absence of the CD 73 inhibitor alpha-beta-methylene-ADP (APCP) (10 μM).

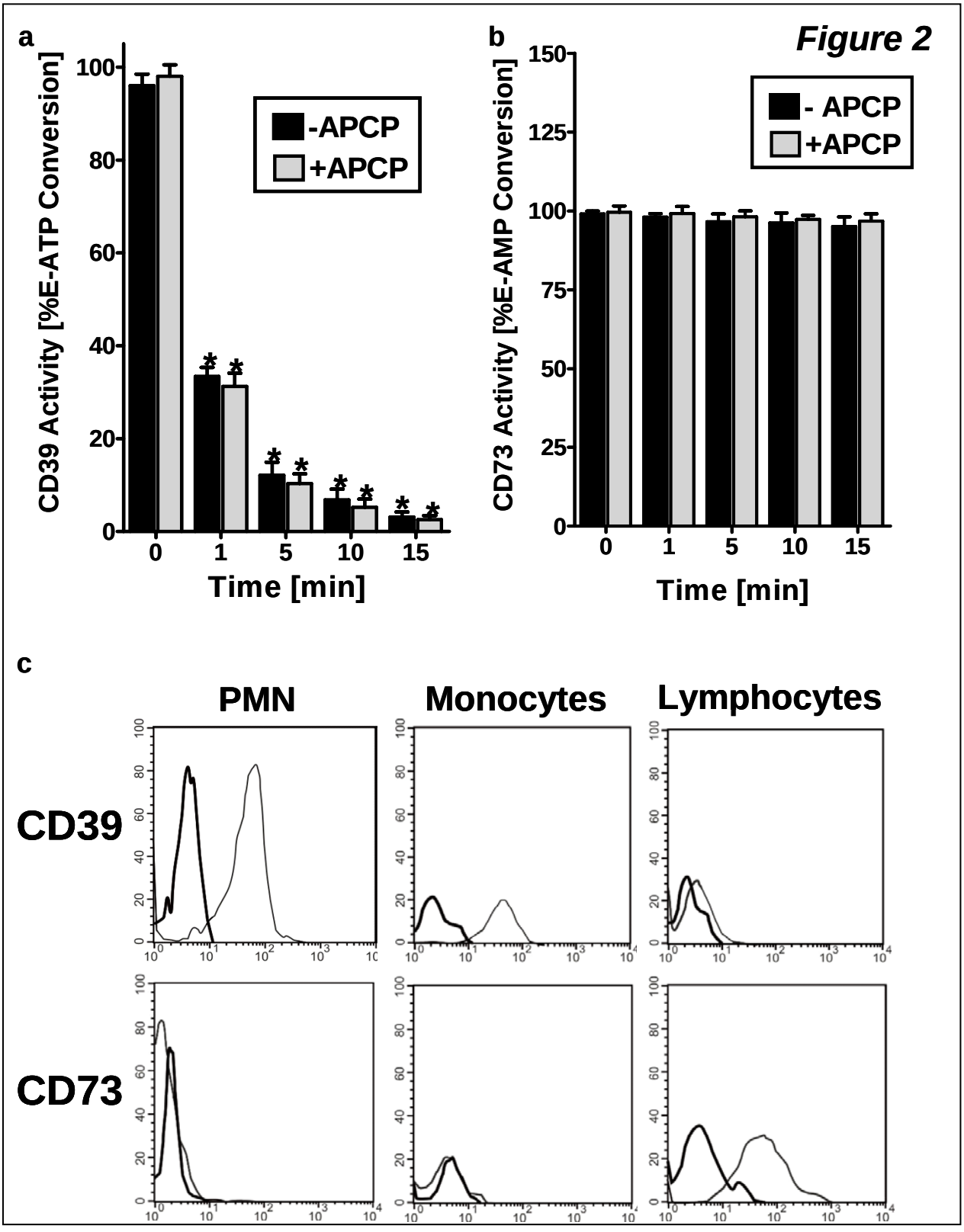


***Figure 2: Expression and function of the ecto-apyrase (Cd 39) and 5`-nucleotidase (CD 73) on the surface of PMN***

As shown in Figure 2A, isolated PMN rapidly metabolized etheno-ATP to etheno-AMP, suggesting high levels of CD 39 activity. Surprisingly, etheno-AMP was stable in the supernatant independent of the presence of APCP (10 µM), suggesting that PMN express little or no CD 73. To confirm this hypothesis, we measured CD 73 activity on PMN (conversion of etheno-AMP to etheno-adenosine (70)). This analysis confirmed our inhibitor experiments and revealed no detectable CD 73 on intact PMN (Figure 2B). To confirm these results, we utilized FACS-analysis for C D 39 and CD 73 on various leukocyte populations. As shown in Figure 2C, PMN and monocytes express no detectable CD 73, whereas CD 73 is highly expressed on lymphocytes. These experiments in PMN were repeated following fMLP stimualtion, and did not influence the pattern of CD 39 and CD 73 expression (data not shown).

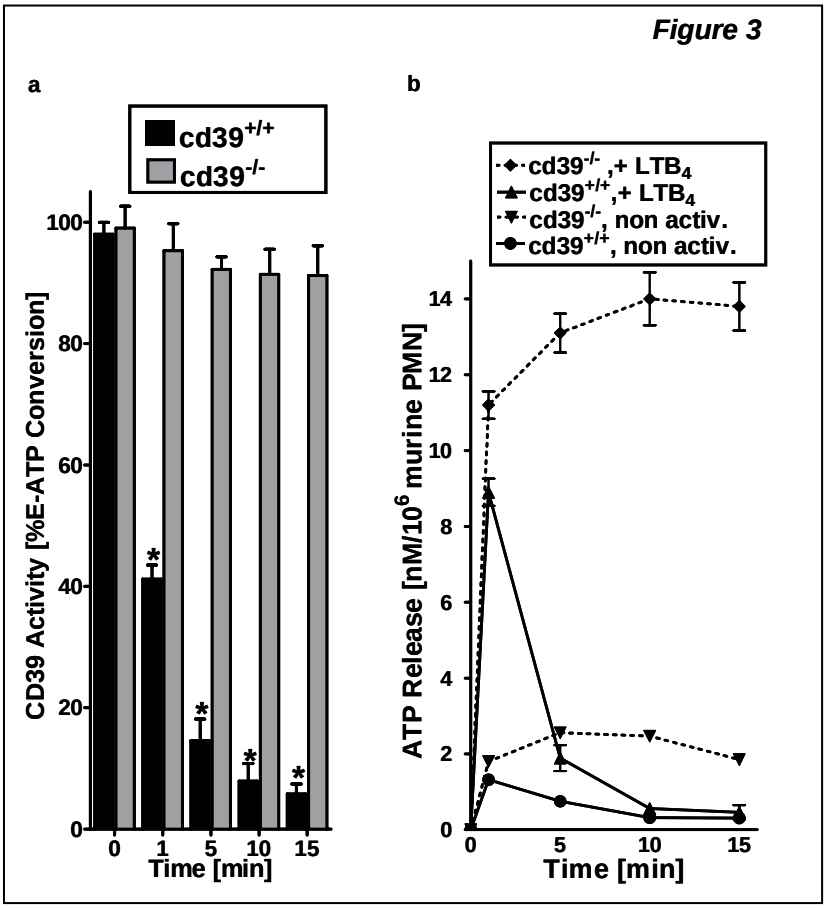


***Figure 3: ATP is stable in the supernatant of activated cd39$^{-/-}$ mice***

### 3.3. Different kinetics of ATP-levels within the supernatant of activated PMN derived from cd39-null-mice

We next extended the above findings with human PMN to murine PMN. For these purposes, we compared CD 39 activity on isolated PMN from cd39-null mice (67) and littermate controls.

As shown in Figure 3A, while PMN from littermate controls readily converted etheno-ATP to etheno-AMP, such activity was completely absent on PMN from cd39$^{-/-}$ mice (Figure 3A, $p < 0.01$ by ANOVA). As next step, we measured ATP concentrations in the supernatant of activated PMN from cd 39$^{-/-}$ mice and compared them to littermate controls. Since murine PMN express little or no surface fMLP receptors, we used leukotriene B4 (LTB4, 100nM) for activation of PMN (103). As shown in Figure 3B, freshly isolated PMN from wild-type mice released ATP in an activation-dependent manner (maximal 6.7 ± 0.57 fold increase), with similar kinetics as human PMN (see Figure 1B). Similar to wild-type mice, PMN from cd 39$^{-/-}$ mice also released ATP in an activation-dependent manner. Moreover, the lack of extracellular metabolism through surface CD 39 resulted in accumulation of ATP (1.6 ± 0.09-fold increase in maximal ATP levels compared to PMN from wildtype mice, $p < 0.05$). Similarly, ATP levels in the supernatant of unactivated PMN from cs 39$^{-/-}$ mice were higher and stayed close to their peak concentration throughout the experiment compared to wildtype PMN (Figure 3B, $p < 0.05$ by ANOVA). Taken together, these experiments suggest that PMN surface CD 39 contributes significantly to the rapid metabolism of ATP following PMN activation.

### 3.4. Biologically active adenosine liberated via PMN CD 39 and endothelial CD 73

Based on the above observation that PMN express CD 39 but not CD 73 on their surface, and that ATP in the presence of PMN is rapidly hydrolyzed to AMP, we hypothesized that an additional cell-type is necessary to contribute CD 73 dependent AMP conversion and establish an adenosine dependent signaling pathway (3). Due to the close special relationship of PMn to the endothelium during transendothelial migration (13), its pivotal role to orchestrate PMN invasion into the underlying tissues during inflammatory hypoxia (3, 33), and the fact that Cd 73 is induced by hypoxia on the endothelial surface (9, 70), we examined effects of supernatants from

activated PMN on normoxic or post-hypoxic endothelial cell function as a model for neutrophil-endothelial crosstalk. To pursue these experiments, we activated PMN with fMLP and exposed HMEC-1 to ddifferent concentrations of the supernatant and measured paracellular barrier function, using a previously described in vitro model (9, 75). Consistent with previous studies (75), endothelial exposure to the supernatant of PMN resulted in a concentration-dependent decrease in paracellular permeability ($p < 0.01$ by ANOVA, with maximal 71 ± 5 % decrease in flux, data not displayed). Such changes in paracellular permeability were inhibited by as much as 93 ±6 % by the non-specific adenosine receptor antagonist 8-phenyl-theophylline (3 μM), thereby significantly implicating adenosine in this response. These results define a biochemical crosstalk pathway involving PMN expressed CD 39 and endothelial expressed CD 73.

### 3.5. Mechanisms of PMN ATP release

ATP exists in the cytoplasm at millimolar concentrations (104) and can be released extracellularly by several mechnisms, including exocytosis of ATP containing vesicles (105-107), transport via connexin hemichannels (108), through nucleoside transporters (109, or direct transport through ATP-binding cassette (ABC) proteins (110-111). As first step, we considered exocytosis of ATP containing granular vesicels as possible mechnaism. To inhibit vesicular secretion, we used the general secretion inhibitor brefeldin A (BFA) (107).

As shown in Figure 4A, BFA (5 μg/ml) did not influence the kinetics or the absolute amount of ATP liberated from activated human PMN. Consistent with previous studies in human PMN (112), BFA significantly inhibited the activated release of the granule-bound enzyme myeloperoxidase (MPO, Figure 4B). Consistent with these findings, isolated granules from resting PMNs contained greater than 95 % of MPO activity (data not shown), but nearly undetectable levels of ATP (Figure 4C). Cytosolic ATP concentrations were higher than 5 mM (Figure 4C). Taking together, these studies suggest that activation dependent ATP release by neutrophils is not via granular exocytosis.

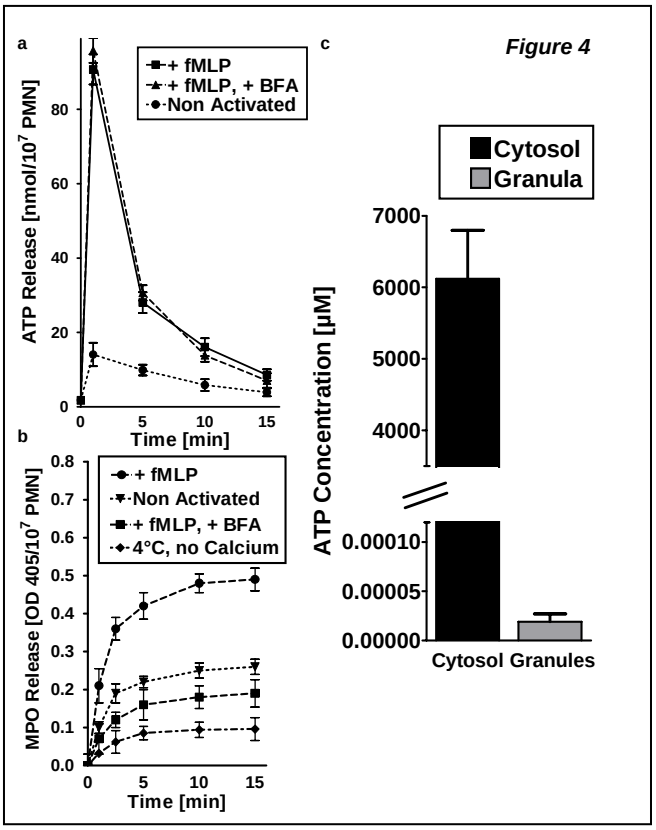


***Figure 4: ATP release from PMN is not vesicular***

### 3.6. The role of Cx 43 in ATP release from PMN

In view of these results that ATP is not granule-bound in PMN, we attempted pharmacological approaches to define mechanisms of ATP release. Based on reports suggesting a role of nucleoside transporter function in cellular ATP release (109), we examined the influence of nucleoside transport inhibitor dipyridamole (1, 10 and 100 µM) (84, 109, 113) on PMN ATP release. In fact, no effect on stimulated ATP release could be demonstrated (data not displayed). Similarly, verapamil, an inhibitor of several ABC proteins and the multi drug resistance gene product (110-111) had no influence on ATP release.

As shown in Figure 5A, no differences in stimulated ATP release was detectable between controls and samples treated with 1, 10 or 100 µM verapamil.

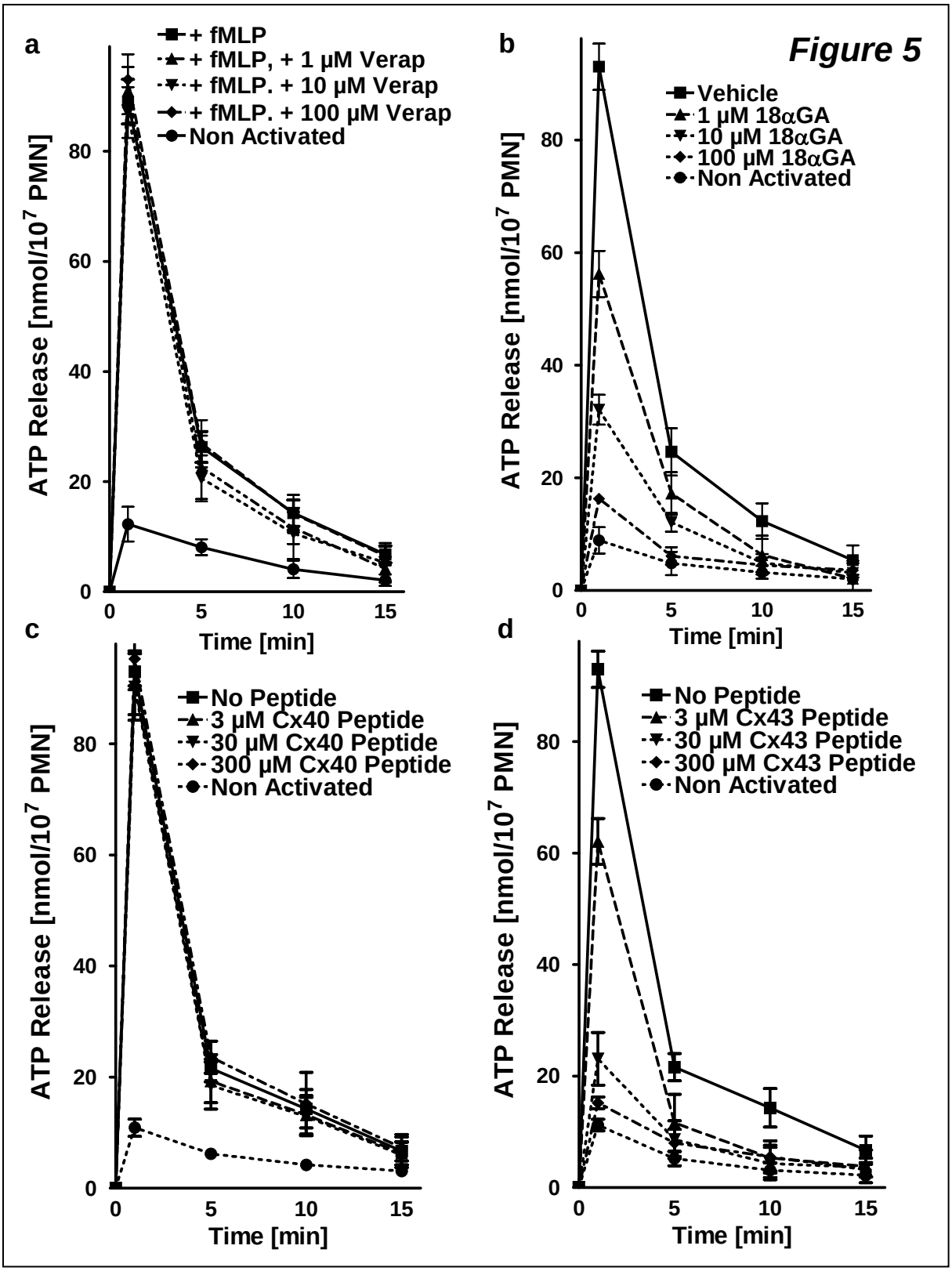


***Figure 5: fMLP stimulated ATP release from PMN is via connexin 43***

Based on previous reports suggesting that connexin hemichannels may serve as ATP release channels in glial cells (108) and the observation that PMN express surface connexins (114-115), we measured ATP release of PMN in the presence of the non-specific gap junction inhibitor 18αGA (116). As shown in Figure 5B, addition of 18αGA resulted in a concentration dependent inhibition of ATP release from fMLP-activated PMN (Figure 5B, $p < 0.01$ by ANOVA). Additional experiments with the non-specific gap junction inhibitor anandamide (117) confirmed the above results, revealing a 4.6 ± 0.62 fold decrease in stimulated ATP release in the presence of 100 μM anandamide ($p < 0.01$ by ANOVA, date not shown).

We extended these findings to define specific connexin contributions to PMN ATP release. For these purposes, we next used connexin peptides specifically directed against Cx 40 and Cx 43 (114-115, 118). As shown in Figure 5C, peptides specific for Cx 40 did not significantly influence ATP liberation from activated PMN. By contrast, the peptides which block Cx 43 showed a concentration-dependent inhibition of ATP liberation ($p < 0.01$ by ANOVA), with an over 6-fold reduction of maximal ATP release at 1 min after fMLP stimualtion. These results significantly implicate Cx 43 in activated ATP release from human PMN.

### 3.7. Activation-dependent PMN Cx 43 dephosphorylation

It is known that hexameric assemblies of connexin 43 molecules (so called connexons) from hemichannels connnecting the intracellular with the extracellular space (119). The conductance and permeability of such Cx 43 hemichannels is regulated by modification of their cytoplasmic domain, with phosphorylation of Ser368 causing a conformational change resulting in decreased connexon permeability (120, 121). Therefore, we examined the influence of fMLP on Cx 43 Ser-238 phosphorylation in intact PMN.

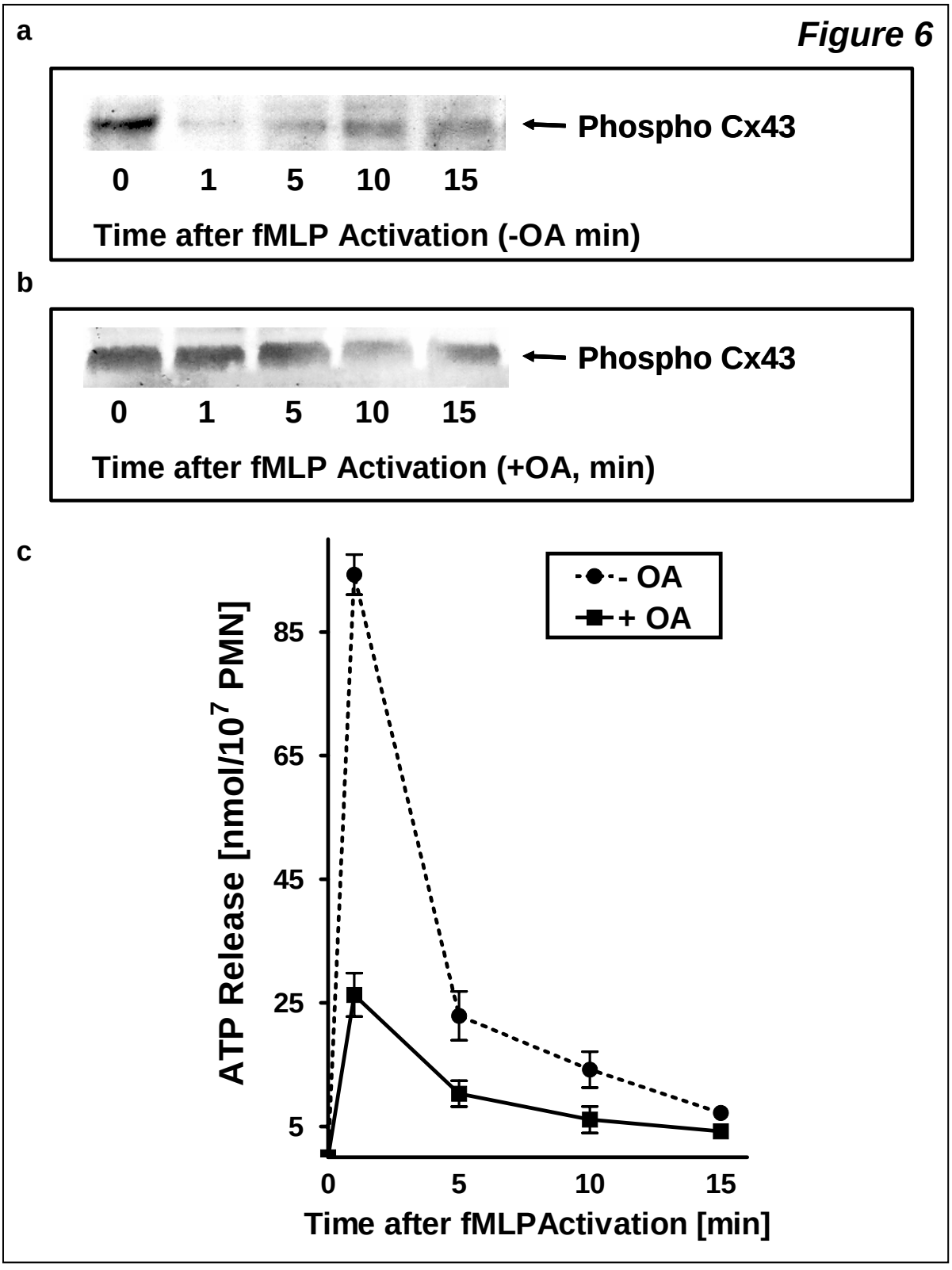


***Figure 6: Effects of phosphatase A2 inhibitor okadaic acid (OA) on Cx 43 phosphorylation and ATP release during fMLP activation of PMN***

As shown in Figure 6A, Cx 43 is prominently phosphorylated in resting PMN (Figure 6A, 0 min). Within 1 min of fMLP activation, phosphorylation of Cx 43 precipitously decreases, and slowly recovers over 15 minutes. These results are consistent with fMLP-dependent dephosphorylation of Cx 43, conformational opening of Cx 43 hemichannels.

Previous reports have implicated protein phosphatase 2A in Cx 43 dephosphorylation (122). Therefore, we performed the above experiment in the presence of the protein phosphotase inhibitor okadaic acid (100nM). As shown in Figure 6B, fMLP induced dephosphorylation of Cx 43 was attenuated in the presence of 100 nM OA. Based on this observation, and previous reports suggesting that fMLP activation of PMN may be modulated by OA (123-124), we assessed ATP release of PMN in the presence of OA. As shown in Figure 6C, ATP of PMN was decreased 4.1 ± 0.3-fold in the presence of 100 nM OA. Taken together, these results reveal activation-dependent dephosphorylation of Cx 43 via protein phosphatase and resultant activation of ATP release in human PMN.

### 3.8. Role of Cx 43 dependent ATP release by PMN in modulating endothelial cell function

To investigate the role of Cx 43 dependent ATP release, we next generated supernatants from fMLP-activated PMN that were preincubated (10 min) and activated in the presence of 18αGA or connexin mimetic peptides alone did not result in a change of endothelial flux rates (data not shown), the barrier protective effects of the supernatant was absent if PMN were activated in the presence of 18αGA (10 μM) or the connexin mimetic peptide specific for Cx 43 (100 μM). This suggests that connexin-dependent ATP release is required for the observed barrier effects of the supernatant (Figure 7A).

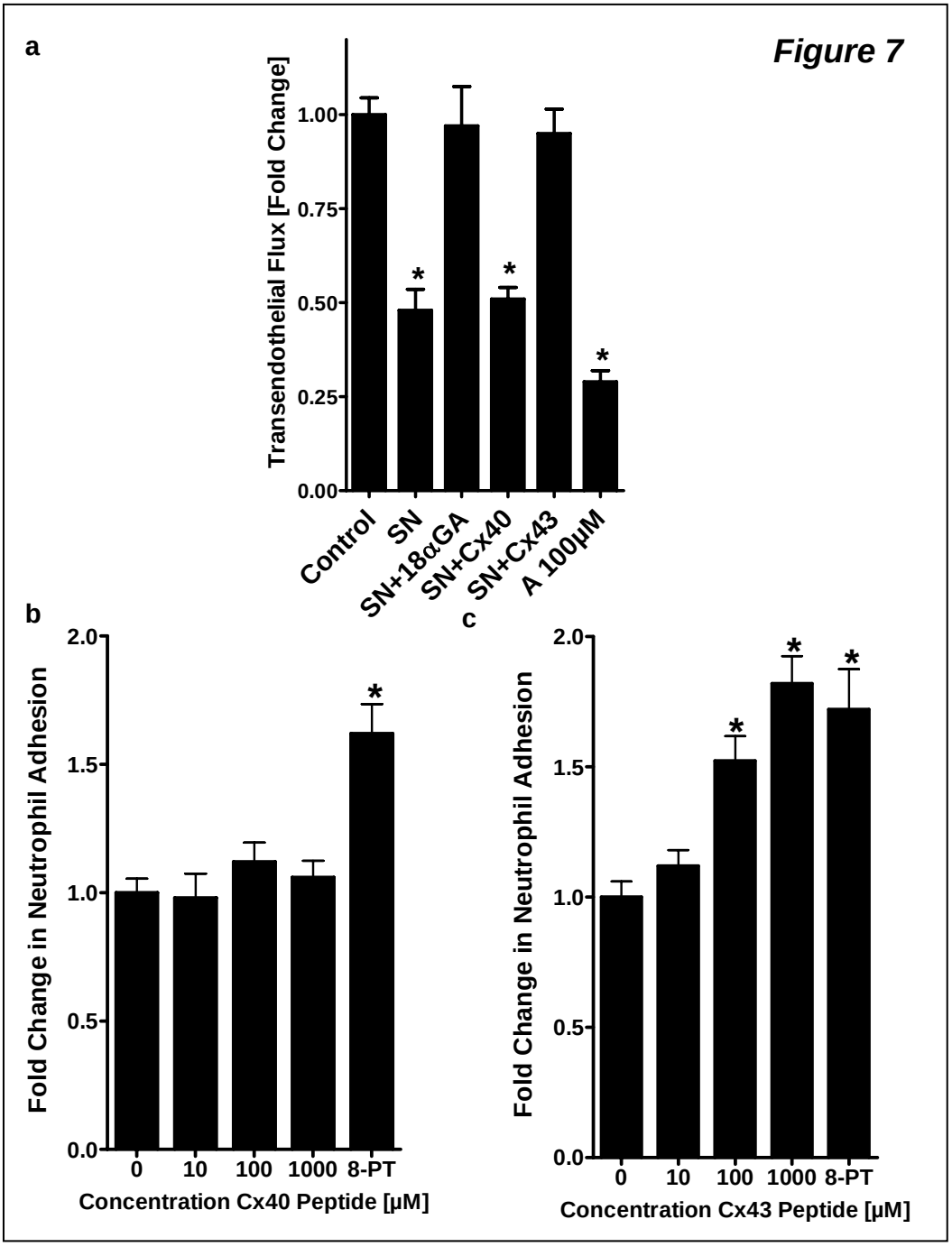


***Figure 7: Role of PMN-dependent ATP release in modulating endothelialcell functions***

Taken together, these results suggest that the known barrier protective effects of supernatants from activated PMN require Cx 43 dependent ATP liberation from PMN. Moreover, these experiments also highlight the role of PMN and endothelia as crosstalk-partners in an adenosine dependent signaling pathway, with PMN liberating ATP and CD 39-dependent phosphohydrolysis to AMP, while endothelial CD 73 activity results in the generation of adenosine and activation of endothelial adenosine receptors.

As a second model of crosstalk between PMN and endothelia, we invetigated the role of ATP release from PMN for neutrophil adhesion to normoxic or post-hypoxic endothelia. Consistent with previous studies, adhesion of fMLP activated PMN was increased by addition of the non-specific adenosine receptor antagonist 8-PT (3 µM), suggesting that such increases in PMN to endothelia are dependent on adenosine signaling (Figure 7B) (3). As next step, we measured PMN adhesion to endothelia in the presence of the connexin mimetic peptide specific for Cx 40 (Figure 7B) and for Cx 43 (Figure 7C). Similar to using different concentrations of the peptides alone (data not shown), the addition of the Cx 40 specific peptide did not alter PMN adhesion to a measureable degree. In contrast, inhibition of ATP release from fMLP activated PMN with the Cx 43 specific connexin mimetic peptide resulted in a concentration dependent increase in neutrophil-endothelial adhesion. These results demonstrate that Cx 43-dependent release of ATP controls PMN-endothelial adhesion through metabolic crosstalk at the endothelial surface.

### 3.9. Activated PMN from mice with induced deletion of Cx 43 show decreased ATP release

As proof of principle for biologically-relevant PMN Cx 43 activity, we isolated PMN from age and gender matched mice with induced deletion of Cx 43 (Cx $43^{Cre\text{-}ER(T)/fl}$ + 4-OHT, further referred to as Cx $43^{-/-}$) and the corresponding floxed control animals (Cx $43^{fl/fl}$), as well as heterozygote Cx 43-null mice (Cx $43^{+/-}$). As depicted from western blot analysis from cardiac tissue in Figure 8A and 8B, administration of tamoxifen resulted in nearly complete deletion of Cx 43 in the Cx $43^{-/-}$ mice, and a corresponding 50 % decrease in Cx $43^{+/-}$ mice. Floxed control animals (Cx $43^{fl/fl}$) had similar cardiac Cx 43 expression to that of wildtype animals. Consistent with our results from phyrmacological inhibition of Cx 43, isolated PMN ATP release upon activation was nearly completely abolished in Cx $43^{-/-}$ mice ($p < 0.001$ by ANOVA compared to wildtype mice and compared to floxed controls, Figure 8C). By contrast, Cx $43^{-/-}$ mice had higher ATP levels than Cx $43^{-/-}$ knockout mice, but lower than wildtype animals or floxed controls ($p < 0.05$ compared to wildtype, floxed controls or Cx $43^{-/-}$ by ANOVA). The floxed control mice had similar ATP levels than wildtype controls. As shown in Figure 8D, the total amount of PMN ATP release was closely correlated with the degree of Cx 43 expression (Figure 8B).

These studies provide genetic evidence that ATP release from activated PMN occurs in a Cx 43-dependent fashion.

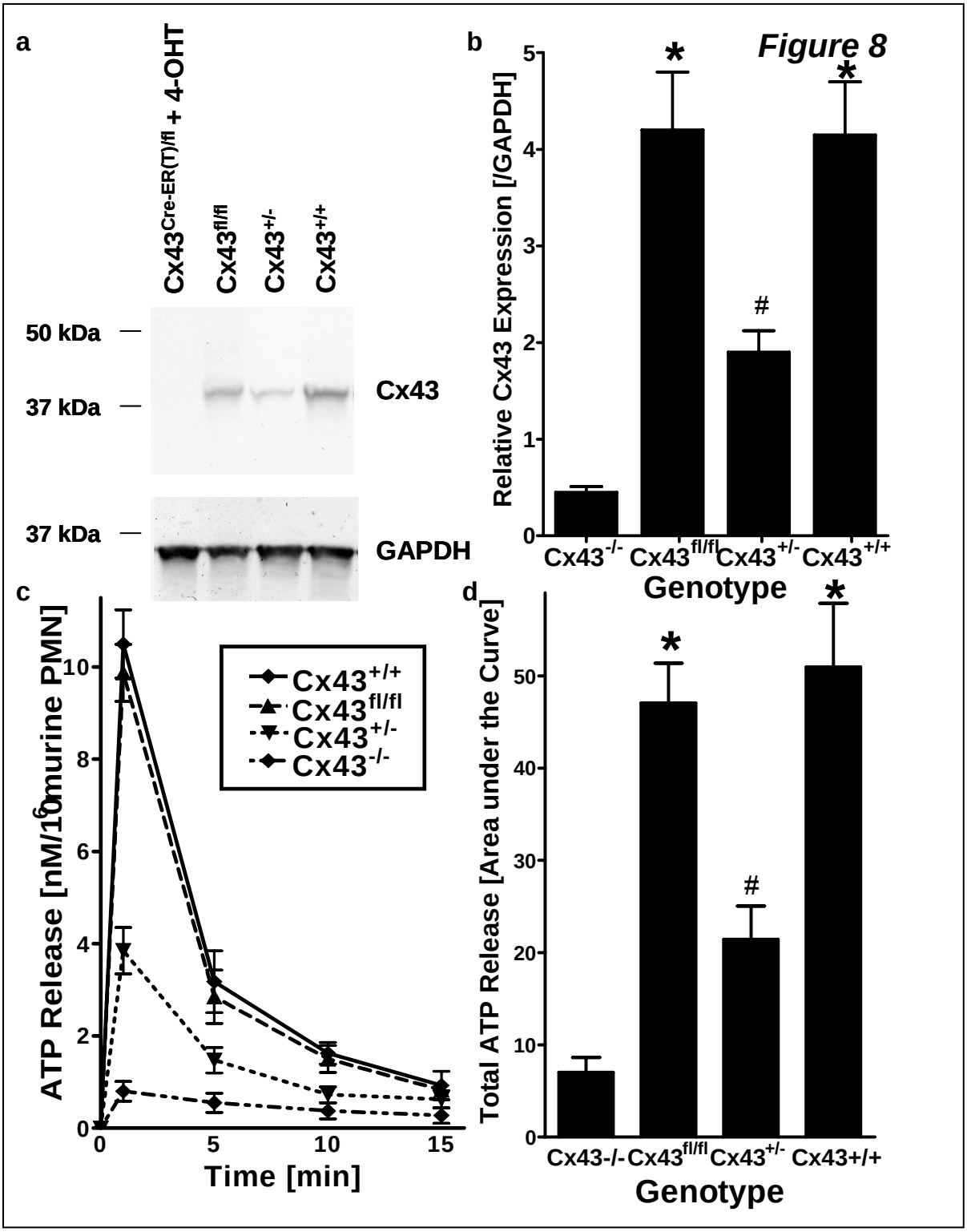


***Figure 8: ATP release from murine PMN in genetic models of Cx 43 expression***

## 4. Discussion

Metabolic and transcriptional responses to inflammation are common denomiantors of multiple cardiovascular (125) and pulmonary diseases (94). In particular, adaptation to “inflammatory hypoxia” has become an area of intense investigation (2, 87, 96). Important in this regard, a consistent finding in hypoxic tissues is increased extracellular nucleotide levels (3-6, 126). In addition to platelets, PMN may comprise an important source for increases in extracellular ATP concentrations. Due to their role as first line of cellular response to inflammatory hypoxia, here we pursued mechanisms and functional consequences of ATP release from aktivated PMN. We identified a novel role for Cx 43 in activation-dependent ATP release from PMN. Further studies revealed that in the presence of PMN, ATP is rapidly metabolized to AMP through catalytic activity involving PMN surface CD 39. Confirmatory studies in inducible Cx 43-deficient murine revealed that Cx 43 expression correlated with PMN ATP release. Take together, these studies demonstrate nucleotide loberation at sights of acute inflammation by PMN, and identify Cx 43 dependent ATP release as a central part of an innate inflammatory response controlling adenosine-dependent endothelial function (Figure 9).

Historically, activated platelets were thought to serve as the primary source for extracellular adenine nucleotides (59-60). From this perspective, endothelial CD 39 has been viewed as a protective, thromboregulatory mechanism for limiting the size of the hemostatic plug (60-61). Metabolism of adeine nucleotides derived from activated platelets is crucial in limiting excessive platelet aggregation and thrombus formation (62-63). Similarly, excessive platelet accumulation and recruitement can be treated with the use of soluble forms of CD 39 (64-65). Moreover, a thromboregulatory role could be demonstrated in a model of stroke, where cd 39-null mice showed increased sizes of infarction that could be reduced by treatment with soluble CD 39 (66). Surprisingly, targeted disruption of CD 39 resulted in prolonged bleeding and increased vascular leak and fibrin deposition in hypoxemia (67), suggesting a dual role for ATP metabolism by CD 39 in modulating hemostasis and thrombotic reactions. Most likely, this observation is related to an activation and desensitization of the prinergic type $P_2Y_1$ receptor on platelets. Activation of the $P_2Y_1$-platelet receptor appears to be crucial in the activation process of platelets. As such, $P_2Y_1$ deficient mice exhibit signs of prolonged bleeding time and resistance to

thromboembolism (68). In contrast to these studies, we observed a barrier-protective and antiinflammantory role of ATP released from PMN, due to rapid metabolim to adenosine, apparently unrelated to the activation of P2 receptors.

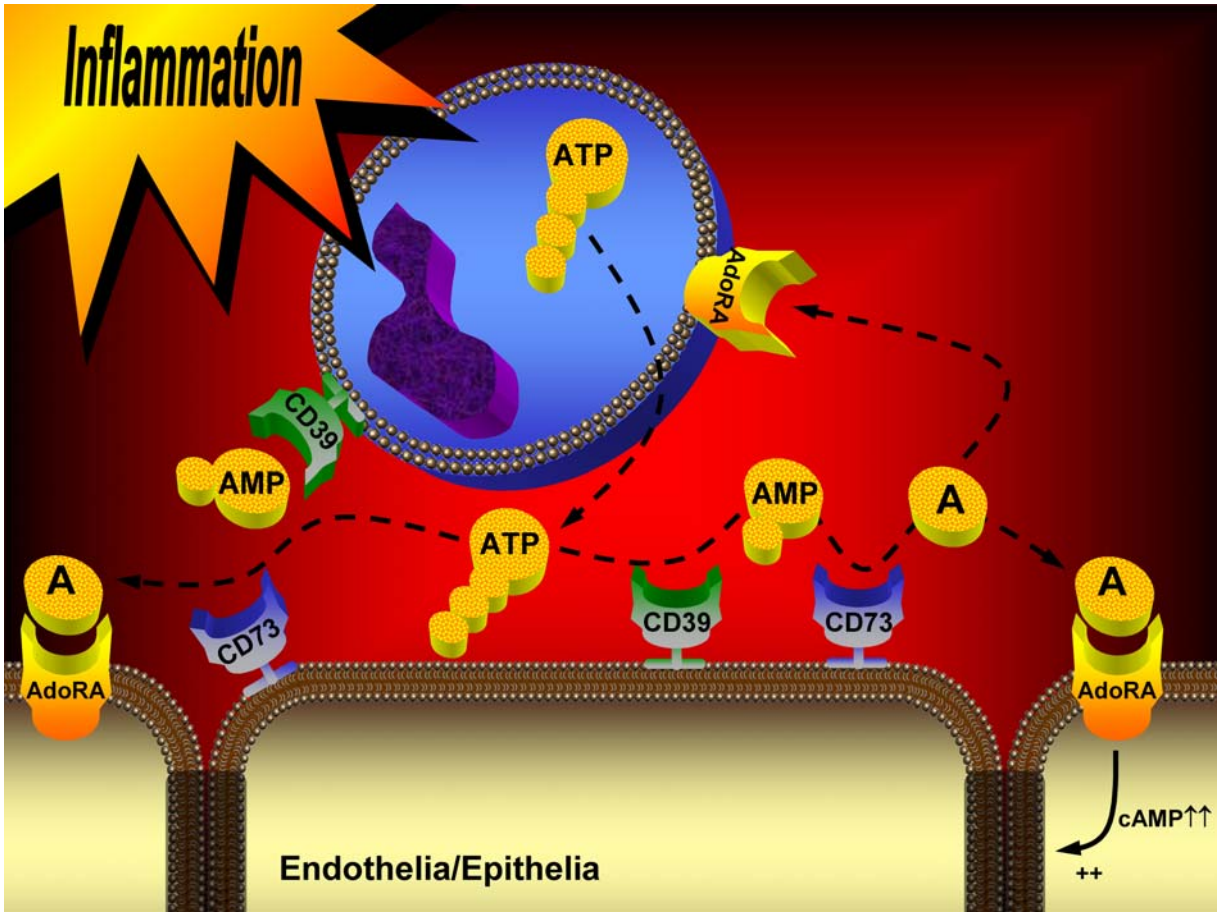


***Figure 9: Model of coordinated nucleotide metabolism and nucleoside signaling in hypoxia and during inflammation cells***

Consistent with previous studies (3, 75), our results highlight extracellular nucleotide-phosphohydrolysis and nucleoside signaling through biochemical crosstalk involving more than one cell type (Figure 9), As demonstrated here, PMN release ATP in an activation-dependent fashion and such ATP is "auto-hydrolyzed" to AMP through PMN surface CD 39. Further metabolism of AMP to adenosine requires an additional cell type to contribute CD 73 activity in order to generate adenosine. As such, PMN CD 39 may function as an immuno-modulatory control point, requiring close special relationship to CD 73-positive cells (such as endolthelia, epithelial, or lymphocytes). Conversely, the fact that PMN express high

levels of CD 39 on their extracellular surface may be critically important in their role of limiting ADP dependent activation of platelets during excessive thrombosis or inflammation. Such a role is consistent with studies in transgenic expression of human CD 39 (hCD39) in mice. These mice display no overt spontaneous bleeding tendency, but exhibit impaired platelet aggregation, prolonged bleeding times, and resistance to systemic thromboembolism. By contrast, donor hearts transgenic for hCD39 appear to be substantially protected from thrombosis and survived longer in murine models of cardiac transplantation (127). However, the contribution of different cell types (endothelial, cardiac myocytes, myeloid cells) to the observed antithrombosis remains unclear. It seems not unreasonable to hypothesize a contribution of PMN-dependent CD 39 to this role, as PMN are among the first cell types recruit during cardiac transplantation and during rejection. However, convincing evidence for the individual contribution to different cell types and different tissues for the observed anti-inflammatory and anti-thrombotic effects of CD 39 will require tissue/organ specific deletion of the gene.

Consistent with our findings, previous studies have shown that Cx 43 phosphorylation may be modulated by activation as occurs during inflammation and hypoxia, resulting in an alteration in cellular functions. For instance, some studies suggest dephosphorylation of Cx 43 and uncoupling of myocardial gap junctions occur during myocardial ischemia. Under such circumstances, Cx 43 may be reversibly dephosphorylated and rephosphorylated during hypoxia and reoxygenation dependent on fluctuations in intracellular ATP content (128). Moreover, several studies have implicated a role of Cx 43 in cardioprotection by ischemic preconditioning (129), insomuch as protection by ischemic preconditioning is lost in cardiomyocytes and hearts of heterozygous connexin 43 deficient mice (Cx $43^{+/-}$)(130). Additional studies demonstarte that the absence of cardioprotection in mice with genetic modulation of Cx 43 expression does not involve intercellular communication through gap junctions (131), but appears to be related to a more specific deficit in reactive oxygen species formation in response to diazoxide and accordingly less protection (132). In view of the results from the present study showing a critical role of Cx 43 as a phosphorylation dependent ATP release channel may also be important in cardioprotection by preconditioning. In fact, previous studies have demonstrated a critical role of extracellular adenosine generation by CD 73 in cardiac ischemic preconditioning (133-134). Thus, ATP

released from cardiac cells through dephosphorylated Cx 43 may be a critical source for extracellular adenosine generation via CD 73 and cardioprotection by cardiac ischemic preconditioning. In conjunction, a reduction of cardiac Cx 43 expression may thus abolish cardioprotective effects of ischemic preconditioning by a reduced substrate availability for extracellular adenosine generation.

## 5. Summary

In summary, our results highlight for the first time a critical role of Cx 43 on the surface of PMN in releasing ATP from inflammatory cells during activation. Such ATP is rapidly hydrolyzed to adenosine via close association with CD 73 expressing cell types. thus, PMN dependent release of ATP may play a critical role in the metabolic control of innate inflammatory pathways.

## 6. References


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