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Ph.D. Thesis:

Adenosine signalling pathway as modulator of the inflammatory response

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Dedicated to my first teachers…my parents!

Only the pursuit of happiness is guaranteed. The rest is up to you.

David T. Fagan

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A_1AR, A_1 adenosine receptor

$A_{2A}AR$, A_{2A} adenosine receptor

$A_{2B}AR$, A_{2B} adenosine receptor

A_3AR, A_3 adenosine receptor

AC, adenyl cyclase

ADA, adenosine deaminase

ADP, adenosine 5'-diphosphate

AK, adenosine kinase

ALT-146e, 4-{3-[6-Amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester

AMP, adenosine 5'-monophosphate

AMPDA, AMP deaminase

APCP, adenosine 5'-(α,β-methylene) diphosphate

APCs, antigen-presenting cells

ARs, adenosine receptors

ATP, adenosine 5'-triphosphate

BSA, bovine serum albumin

cAMP,cyclic adenosine monophosphate

CBL, casitas B-lineage lymphoma

CCPA, 2-chloro-N^6- cyclopentyladenosine

$CD4^+$, cluster of differentiation 4

$CD8^+$,cluster of differentiation 8

CD39, ectonucleoside triphosphate diphosphohydrolase

CD73, ecto-5′nucleotidase (e-5′NT)

CF101, N^6-(3-iodobenzyl)-adenosine-5′-N-methyl-uronamide

CF102, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-N-methyl-uronamide

CF502, [(1′R; 2′R; 3′R; 4′R; 5′S)-4-{2-chloro-6-[(3 chlorophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo[3.1.0]hexane-2,3-diol]

CF602, N- (3,4- dichloro-phenyl)-2-cyclohexyl-IH- imidazo [4, 5-c] quinoline 4-amine

CGS 21680, 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine

cN-I, cytoplasmic 5'-nucleotidase

cN-II, cytoplasmic 5'-nucleotidase-II

COX, cyclooxygenase

COX-1, cyclooxygenase-1

COX-2, cyclooxygenase-2

COX-3, cyclooxygenase-3

CREB, cyclic AMP response element binding

CTLA-4, cytotoxic T-lymphocyte antigen 4

DAPI, 4',6-Diamidino-2-phenylindole

DCs, dendritic cells

DMSO, dymethysulfoxide

DPCPX, 8-cyclopentyl-1,3-dipropylxanthine

DTT, dithiothreitol

ECL, enhanced chemiluminescence

ELISA, enzyme-linked immunosorbent assay

EDTA, ethylenediaminetetraacetic acid

EIA, enzymatic immune assay

E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase

e-5'NT, ecto-5'nucleotidase

ERK1, extracellular signal-regulated kinase 1

ERK2, extracellular signal-regulated kinase 2

E3KARP, exchanger type 3 kinase A regulatory protein

ε-adenosine, 1, N^6- Etheno-adenosine

ε-AMP, 1, N^6- Etheno-adenosine-5'-O-monophosphate

FGF-2, fibroblast growth factor-2

FGF-R, fibroblast growth factor receptor

FGFRs, fibroblast growth factor receptors

FGFR-L1, FGFR-like 1

FK-838, 6-oxo-3-(2-phenylpyrazolo[1,5-α]pyri- din-3-yl)-1(6H)-pyridazinebutanoic acid)

fMLP, N-Formyl-L-methionyl-L-leucyl-L-phenylalanine

FoxP3, Forkhead box protein 3

GPI, glycosyl phosphatidylinositol

HBSS, Hank's Balanced Salt Solution

HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HMW, high molecular weight

HPSGs, heparin sulphate proteoglycans

HRP, horseradish peroxidase

HUVECs, Human Umbilical Vein Endothelial Cells

ICAM-1, Intercellular Cell Adhesion Molecule Type 1

IFN-γ, interferon gamma

IL-1β, Interleukin-1 beta

IL-2, Interleukin-2

IL-4, Interleukin-4

IL-6, Interleukin-6

IL-10, Interleukin-10

IL-12, Interleukin-12

IL-18, Interleukin-18

IMP, inosine 5'-monophosphate

iNKT, invariant natural killer

LMW, low molecular weight

LPS, lipopolysaccharide

LTB$_4$, leukotriene B$_4$

MAPK, mitogen-activated protein kinase

MCP-1, monocyte chemotactic protein 1

MT, methyltransferase

MTT, 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide

NAD, nicotine amide dinucleotide

NDP, nucleoside-diphosphate kinase

NF-kB, nuclear factor-kappaB

NHERF-2, sodium-hydrogen exchange regulatory cofactor-2

NK, natural killer

NO, nitric oxide

NOS, nitric oxide synthases

NSAID, non-steroidal anti-inflammatory drug

NT, nucleoside transporter

NTPDase, nucleoside triphosphate diphosphohydrolase

PAF, platelet activating factor

Pi, inorganic phosphates

PBS, phosphate buffer saline

PGE$_2$, prostaglandin E$_2$

(PG)G$_2$, prostaglandin G$_2$

PGH$_2$, prostaglandin H$_2$

PGJ$_2$, prostaglandin J$_2$

PGs, prostaglandins

PI3-k/PKB, phosphoinositide 3-kinase/protein kinase B

PIP$_2$, phosphatidylinositol 4,5-bisphosphate

PKA, protein kinase A

PKC, protein kinase C

PLA$_2$, phospholipase A$_2$

PLC, phospholipase C

PMNs, Polymorphonuclear leukocytes

PMSF, phenylmethylsulphonyl fluoride

PNP, purine nucleoside phosphorylase

RAF, Rapidly Accelerated Fibrosarcoma

RAS, Rat sarcoma

RNS, reactive nitrogen species

ROS, reactive oxygen species

SDS, sodium dodecyl sulphate

Ser, serine

siRNA, small interfering RNA

SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor

SPRY, Sprouty

STAT, Signal Transducer and Activator of Transcription

Tc, cytotoxic T

TCA, trichloroacetic acid

Th, T helper

TCR, T-cell receptor

Thr, threonine

TGF, transforming growth factor

TGF-β, transforming growth factor β

TNF-α, tumor necrosis factor alpha

UPLC, Ultra-Performance Liquid Chromatography

VCAM-1, vascular cell adhesion molecule 1

XO, xanthine oxidase

ZM 241385, 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol

ABSTRACT

Adenosine is an endogenous nucleoside that has been recognised to be a molecule with autocrine/paracrine functions, acting as a signal molecule to preserve host defence and tissue integrity during inflammation and trauma in addition to its important role as homeostatic regulator. The physiological activities of adenosine involve its interactions with four types of receptors, designed as A_1, A_{2A}, A_{2B} and A_3. Adenosine mediates its anti-inflammatory activity primarily through the A_{2A} receptor ($A_{2A}AR$). The ecto-5'nucleotidase/CD73 degrades adenosine 5'-monophosphate (AMP) to adenosine and represents a key enzyme for adenosine accumulation at the site of injury. Aim of this research work was to explore different aspects of adenosine signalling pathway in inflammation.

Several findings implicate the adenosine signalling pathway as an innate mechanism to attenuate excessive tissue damage and identify CD73 as critical control points for endogenous adenosine generation. It has been shown that CD73 plays an important role in regulating vascular permeability and leukocyte trafficking in inflammatory disease; and a crucial role in the regulation of immune/inflammatory cell function. A better understanding of the role of CD73 enzyme in the development of inflammatory processes can help to identify new therapeutic strategies aimed at strengthening the endogenous anti-inflammatory mechanisms. For this reason, we sought to investigate the role of CD73, the key enzyme in "switching on" adenosine signalling, in the development of inflammation through its pharmacological blockade by using the selective inhibitor, adenosine 5'-(α,β-methylene) diphosphate (APCP; 400 μg/site), in an *in vivo* model of acute inflammation represented by carrageenan-induced pleurisy in rats. We found that local inhibition of CD73 significantly increased cell accumulation, exudate formation and pro-inflammatory cytokine

content into the pleural cavity in the acute phase of inflammation with no differences in the sub-acute phase. The *in vivo* treatment with APCP induced cells recruited into the pleural cavity to change in a phenotype with increased ability to migrate *in vitro* either in presence or in absence of a chemotactic stimulus. In parallel, these cells showed a reduced CD73 expression and activity compared to cells collected from control group. In addition, APCP, *in vitro*, strongly increased the ability of cells from control groups to migrate in presence of a chemotactic stimulus. Local inhibition of CD73 increased also the infiltration of the lung with polymorphonuclear leukocytes (PMNs) and the degree of lung injury 4 hours following carrageenan injection.

The interest to explore the role of adenosine signalling pathway in the control of inflammation has been growing further following evidence that adenosine signalling is also involved in the mechanism of action of some well-known anti-inflammatory drugs. With regard to this, we focused our interest on the possible involvement of adenosine signalling in the anti-inflammatory mechanism of nimesulide, *in vivo* (rat paw oedema) and *in vitro* (J774A.1cell line); indeed, there is evidence that nimesulide anti-inflammatory effect is the consequence of regulation of the production and actions of a wide range of inflammatory mediators, independently from the sole cyclooxygenase-2 (COX-2) enzyme inhibition. To date, the molecular mechanisms at the basis of nimesulide peculiar pharmacological effects are still unclear. *In vivo*, in the model of carrageenan-induced rat paw oedema, we found that the anti-inflammatory effect of nimesulide (5 mg/kg i.p.) was inhibited by pre-treatment with the adenosine A_{2A} receptor antagonist, ZM 241385 (3 mg/kg i.p.), and by local administration of the CD73 inhibitor, APCP (400 µg/paw). Furthermore, we observed increased activity of 5'-nucleotidase/CD73 in plasma and paws of nimesulide-treated rats, 4 h following oedema induction that represented the inflammatory peaking point. *In vitro*, the inhibitory effect of nimesulide on nitrite and prostaglandin (PG)E_2 (PGE$_2$) production by

lipopolysaccharide (LPS)-activated J774 macrophage cell line was again reverted by ZM 241385 and APCP. Furthermore, nimesulide increased CD73 activity in J774 macrophages while it did not inhibit nitrite accumulation by LPS-activated small interfering RNA (SiRNA) CD73 silenced J774 macrophages. Our data demonstrate that the anti-inflammatory effect of nimesulide is, in part, mediated by CD73-derived adenosine acting on A_{2A} receptors.

There is evidence that $A_{2A}AR$ activation beside anti-inflammatory effects promotes wound healing and extracellular matrix production; given that extracellular matrix and fibroblasts take an active part in the modulation of inflammation beside wound healing, we investigated whether and how extracellular matrix was involved in the anti-inflammatory effect of A_{2A} receptor. Specifically, we evaluated changes in tissue fibroblast growth factor-2 (FGF-2), an important growth factor for fibroblasts that has been shown to facilitate not only tissue regeneration but also to dampen inflammation, following systemic administration of the A_{2A} agonist, CGS 21680, in a rat model of acute inflammation (paw oedema). We observed that CGS 21680 prevented oedema development and inflammation, confirming an anti-inflammatory effect of $A_{2A}R$. The effect of CGS 21680 was specific, through A_{2A} adenosine receptor stimulation, as revealed by co-administration with ZM 241385 that reverted CGS 21680 inhibitory effect. On the basis of histological analysis showing an increased matrix deposition following rat treatment with CGS 21680, we evaluated whether the beneficial effect of A_{2A} agonist, CGS 21680, was paralleled by changes in FGF-2 expression. Interestingly, we found that the expression of FGF-2 in rat paws, evaluated at each hour following carrageenan injection, was increased following rat treatment with CGS 21680. Immunofluorescence analysis confirmed data obtained by western blotting and also showed spots of co-localization between $A_{2A}R$ and FGF-2.

In conclusion, in this research work we demonstrate that CD73 regulates cell migration in the acute phase of inflammation and that the anti-inflammatory effects mediated by $A_{2A}R$ activation are paralleled by changes in extracellular matrix morphology. These findings suggest the important role of CD73/adenosine/A_{2A} signalling in the control of the acute phase of inflammation, characterised by PMNs infiltration, but also in the control of a late phase, characterised by re-arrangement of extracellular matrix. In addition, we also demonstrate that CD73/adenosine/A_{2A} axis is involved in the mode of action of nimesulide. Our study may open a path to re-evaluate the mechanism of action of nimesulide and to identify new therapeutic opportunities in COX-2 inhibitors which display a more potent activity on adenosine signalling. Furthermore, these results may give the cue to project an innovative anti-inflammatory strategy based on the manipulation of endogenous anti-inflammatory pathways.

1.1 ADENOSINE AND INFLAMMATORY RESPONSE

1.1.1 Inflammatory response

Inflammation is a physiological response to disrupted tissue homeostasis caused by endogenous and exogenous stimuli that involves a complex biological cascade of molecular and cellular signals. The end-point of inflammation is to rapidly destroy or isolate the underlying source of the disturbance, to remove damaged tissue and, then, to restore tissue homeostasis (Medzhitov, 2008). Classically, inflammation is characterized by *rubor* (redness), *tumor* (swelling), *calor* (heat), *dolor* (pain), and *functio laesa* (loss of function), symptoms caused by increased blood flow to the affected area, leakage of fluid into tissues and the accumulation of activated leukocytes.
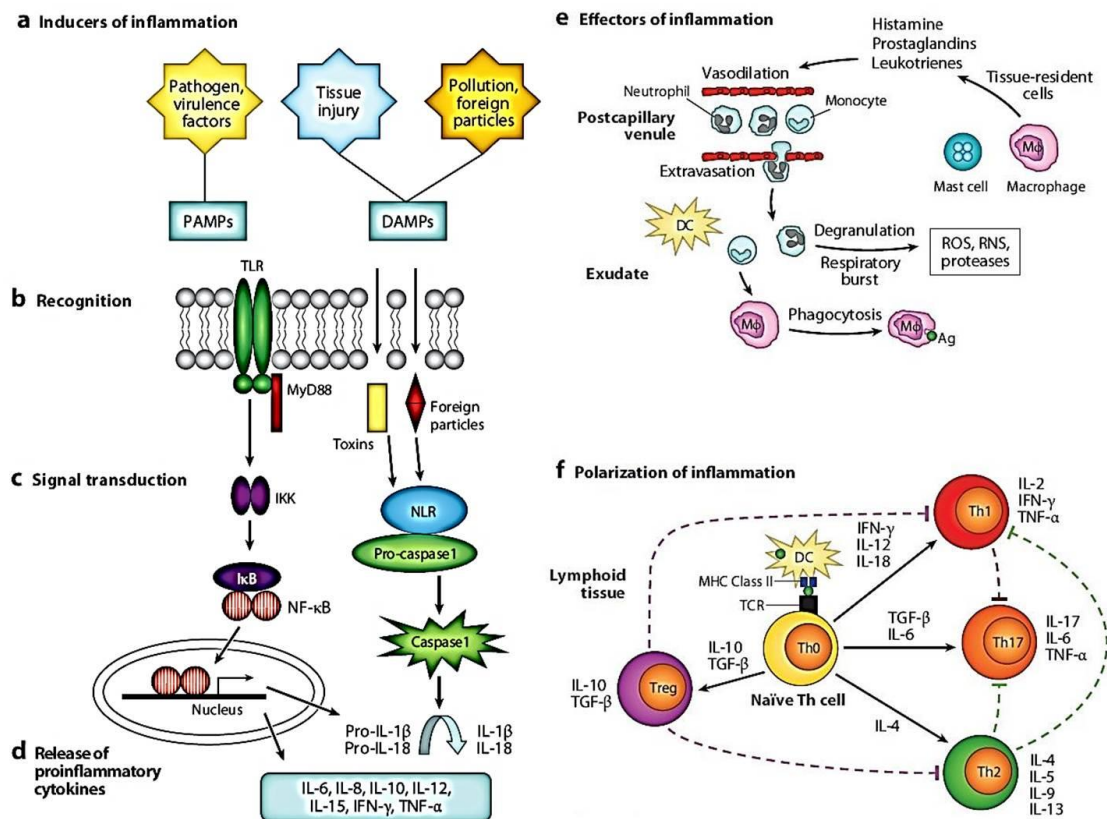
Figure 1. A primer of the inflammatory cascade. Adapted from Noah TA, Zachary M, Weil, Randy JN. (2012) Annu Rev Ecol Evol Syst. 43:385–406. Inflammation is induced by pathogens, tissue injury, and foreign particles (a). The first step of the inflammatory cascade is the recognition of infection or damage (b) that is generally accomplished by the detection of pathogen-associated molecular patterns (PAMPs), or by endogenous molecules such as alarmins and damage-associated molecular patterns (DAMPs). Many damage signals are recognized by germ-line encoded receptors, such as transmembrane Toll-like receptors (TLRs) and intracellular nucleotide-binding domain and leucine-rich-repeat containing receptors (NOD-like receptors or NLRs). Once recognition of ligands occurs, TLRs activate of common signalling pathways that culminate in the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells; **c**). Transcription and translation of target genes lead to the third stage of the inflammatory cascade, which is the inducible expression of pro-inflammatory cytokines, such as interleukin-1-beta (IL- 1β), Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and others (**d**). NLRs signal the inflammasome, which activates caspase-1 to convert cytokines into active forms (IL-1β and Interleukin-18 (IL-18), which then elicit inflammation after being released from the cell (**d**). Therefore, a variety of pro-inflammatory cytokines and chemokines are produced and released to promote effector functions of inflammation. Chemokines (attractants) and various costimulatory molecules, facilitate the recruitment of effector cells (**e**), such as monocytes and neutrophils, to the site of disturbance; blood-borne neutrophils and monocytes migrate to the site of disturbance by chemotaxis selectively pass through endothelial cells to reach target sites (extravasation). Mast cells and tissue-resident macrophages promote this migration by releasing histamine, leukotrienes, and prostaglandins, which have rapid effects upon the vasculature, including vasodilation and increased vascular permeability. Neutrophils create a cytotoxic environment by releasing noxious chemicals from cytoplasmic granules (a process called degranulation). Rapid release of these chemicals requires consumption of both glucose and oxygen, known as the respiratory burst. Toxic chemicals released include highly reactive oxygen and nitrogen species (ROS and RNS, respectively) and various proteinases. These substances are destructive to both pathogens and hosts. The effector functions of inflammation are further regulated by the adaptive immune system (**f**). When stimulated by cells presenting antigen, naïve Th cells (Th0; never exposed to antigen) can differentiate into several different types of effector and regulatory cells: Th1 cells (pro-inflammatory), Th2 cells (anti-inflammatory), regulatory T-cells (Tregs), and Th17 cells (pro-inflammatory).

Inflammation resolves when the injurious agent is eliminated and mediators produced are demolished or missing. Although inflammatory response is usually beneficial, it can also be potentially harmful to the host. Thus, regulatory endogenous anti-inflammatory mechanisms that limit damage from an excessive immune response have evolved. It has become clear that the resolution of inflammation is an active phenomenon that is orchestrated by endogenous "pro-resolving" mediators in a highly coordinated way, acting as a "metabolic switch" necessary to preserve the organism defense and tissue integrity (Gilroy *et al.*, 2004). During the past years, a large number of cellular and molecular anti-inflammatory and pro-resolution mediators have been identified including annexin-1, lipoxins, resolvins, protectins, maresins, adenosine (Buckley *et al.*, 2013). When the regulatory mechanisms of the inflammatory response are defective or the ability to clear damaged tissue and foreign substances is impaired, a prolonged and damaging inflammatory response occurs. (Nathan, 2002; Serhan and Savill, 2005; Lawrence and Gilroy, 2007). Inappropriate or prolonged

inflammation is the main cause of many disorders including asthma (Barnes, 1999), cancer (Coussens and Werb, 2002), atherosclerosis (Libby, 2002), autoimmune disease, Alzheimer's and Parkinson's diseases (Amor *et al.*, 2014); therefore, it is important to understand the physiological mechanisms that sense excessive damage and contribute to terminate inflammation.
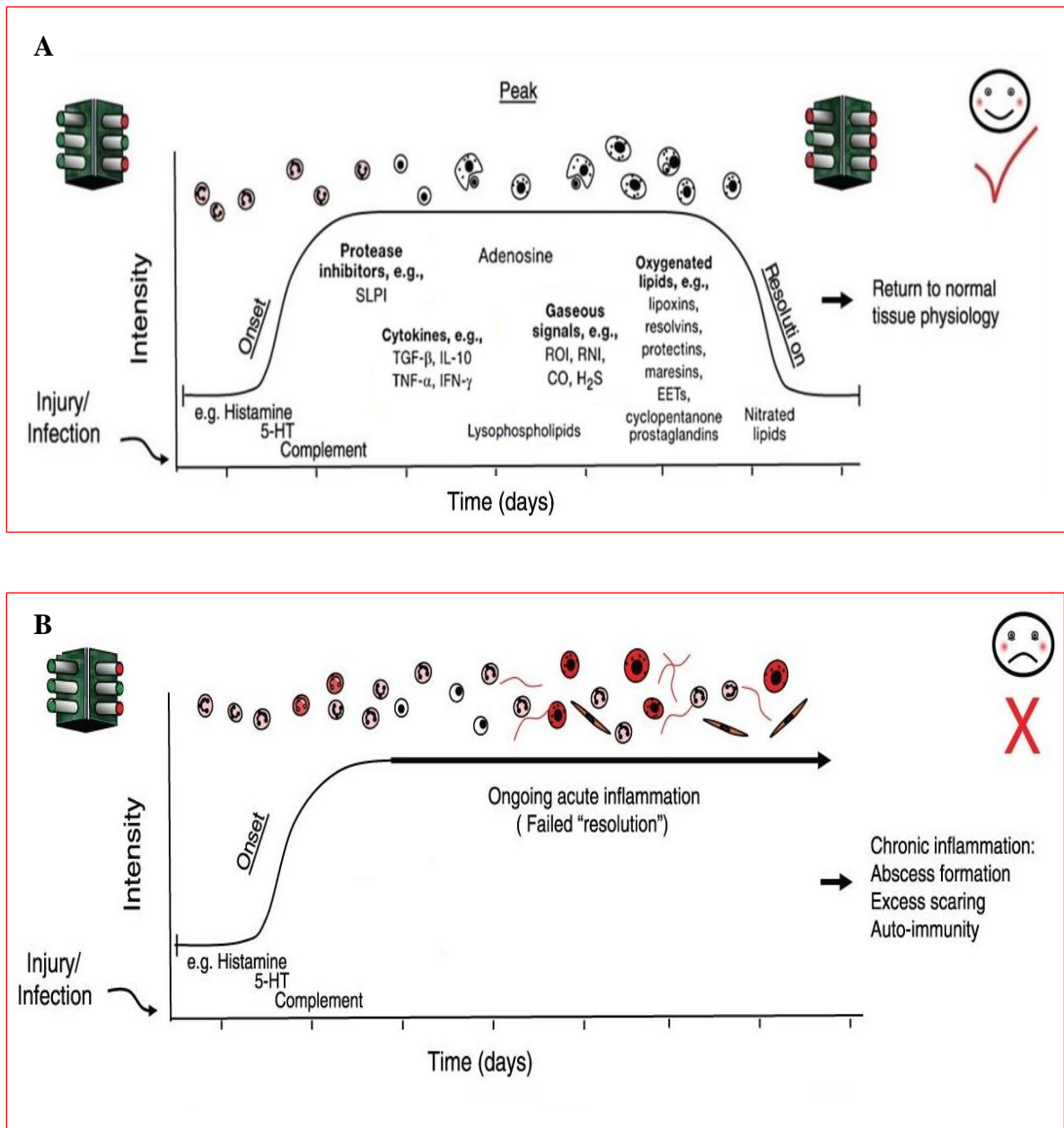
Figure 2. Cellular and molecular components of the inflammatory response and the requirements for resolution. Adapted from Serhan CN, Brain SD, Buckley CD, Gilroy DW, Haslett C, O'Neill LA, Perretti M, Rossi AG, Wallace JL. (2007) FASEB J. 21:325-32. At the beginning of the inflammatory response, pro-inflammatory mediators are mainly released, which initiate and augment the acute phase of the response (**A**). However, this is counterbalanced by endogenous anti-inflammatory signals such as corticosterone, which

serve to temper the severity and limit the duration of the early onset phase. As inflammation progresses, certain "stop signals" at appropriate "checkpoints" prevent further leukocyte traffic into tissue. These stop signals include the lipoxins, resolvins, prostaglandins (PGs) of the D series and adenosine and pave the way for monocyte migration and their differentiation to phagocytosing macrophages, which remove dead cells and then exit the site of inflammation. Stromal cells such as fibroblasts also contribute to the resolution of inflammation by the withdrawal of survival signals and the normalization of chemokine gradients, thereby allowing infiltrating leukocytes to undergo apoptosis or leave the tissue through the draining lymphatics. This sequential set of responses leads to complete resolution and, importantly, the restoration of the inflamed tissue to its prior physiological functioning. This is the ideal sequence of events in physiological inflammation, which contrast to the situation in pathological inflammation (**B**) where some of the factors that initiate the resolution program lead to the inappropriate accumulation of leukocytes in the wrong place at the wrong time.

Current anti-inflammatory therapy is largely based on the blocking of key pro-inflammatory mediator pathways that are elicited on the initiation of an acute inflammatory response, an innovative strategy to manage inflammation could be the pharmacological manipulation of endogenous anti-inflammatory mechanisms.

1.1.2 Adenosine

Adenosine is an endogenous ubiquitous purine nucleoside, composed of adenine linked to a ribose via a β-N^9-glycosidic bond (Figure 3).
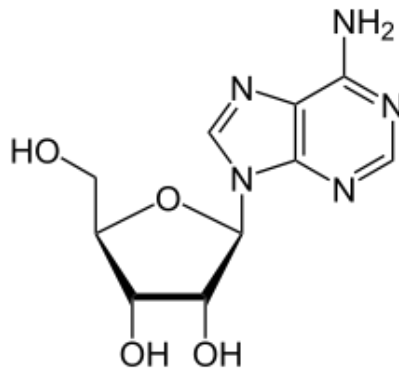
Figure 3. Chemical structure of adenosine

Adenosine was first recognized to reduce heart rate, blood pressure and to induce coronary vasodilatation by Drury and Szent-Györgyu in 1929. Since then, it has become clear that in addition to functioning as central factor in the biochemistry of energy production, adenosine regulates numerous cellular functions. There is evidence that adenosine acts as a key

regulatory molecule, mostly protective but in certain scenarios injurious, in the pathophysiology of inflammatory diseases (Haskó and Pacher, 2008). For this reason, adenosine has been variously called as "signal of life," "retaliatory metabolite," and "body's natural defense" (Engler, 1991; Cohen and Downey, 2008) as a consequence of its ability to mediate an auto-regulatory loop, whose function is to protect organs from injury following the initiating stressful stimuli such as during inflammation (Newby, 1984; Haskó and Cronstein, 2004).

1.1.3 Adenosine formation and metabolism

Adenosine is constitutively present at low concentrations intracellularly as well as extracellularly and it is a product of complete dephosphorylation of adenine nucleotides. Adenosine intracellular production is mediated either by an intracellular $5'$-nucleotidase, cytoplasmic 5'-nucleotidase (cN-I), that breaks down AMP to adenosine (Phillips and Newsholme, 1979; Zimmermann *et al*., 1998; Sala-Newby *et al*.,1999) or by hydrolysis of S-adenosyl-L-homocysteine (SAH) by the activity of the S-adenosyl-L-homocysteine hydrolase (SAHH) (Broch and Ueland, 1980). Within cells, adenosine is an intermediate for the synthesis of nucleic acids and adenosine 5'-triphosphate (ATP). Adenosine generated intracellularly is transported into the extracellular space mainly via specific bi-directional transporters through facilitated diffusion that efficiently evens out the intra- and extracellular levels of adenosine. In some tissues such as the kidney, there is a concentrative nucleoside transport protein capable of maintaining high adenosine concentrations against a concentration gradient. These transport proteins are termed "equilibrative nucleoside transporter" (ENT1 and ENT2), for the equilibrative transport, and "concentrative nucleoside transporter" (CNT1 and CNT2), for the concentrative types (Jennings *et al*., 1998).

On the other hand, after intracellular reuptake, adenosine can be phosphorylated to AMP by adenosine kinase (AK) (Spychala *et al*., 1996) or deaminated to inosine by adenosine deaminase (ADA) (Fox and Kelley, 1978). ADA, but not AK, is also present in the extracellular space (Lloyd and Fredholm, 1995). Inosine produced can be expelled from the cell or be degraded to hypoxanthine, xanthine and ultimately to uric acid (Kovács *et al*., 2011). These pathways ensure the maintenance of intracellular adenosine concentrations through a strict enzymatic control.
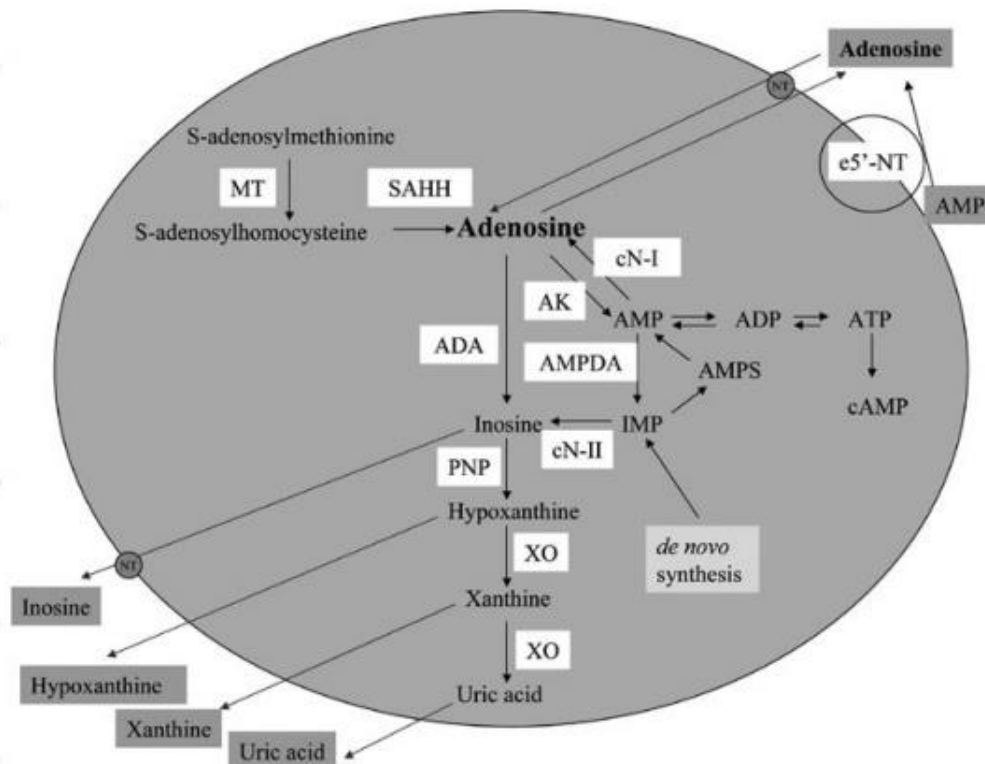
Figure 4. **Adenosine formation and metabolism in the cell.** From Borowiec, Lechward, Tkacz-Stachowska, Składanowski. (2006) Acta Biochim Pol. 53:269-78. For abbreviations, see abbreviation list.

Under basal conditions, adenosine extracellular levels are in nanomolar range, from 30 to 200 nM (Ballarín *et al*., 1991) and the maintenance of these concentrations depends on a balance between the release/removal of the nucleoside by membrane transporters/enzymes and its formation from ATP due to the activity of ecto-nucleotidases.

In the extracellular space, adenosine is produced via enzymatic hydrolysis of extracellular ATP in a two-step enzymatic process regulated by coupled cell ectoenzymes. In this cascade, the ecto-nucleoside triphosphate diphosphohydrolase 1 (E-NTPD1, also known as CD39) dephosphorylates ATP to adenosine 5'-diphosphate (ADP) and AMP whereupon ecto-5' nucleotidase (e-5'NT, also known as CD73) hydrolyses AMP to adenosine (Zimmermann, 2000). Minor producers of extracellular adenosine include alkaline phosphatases and members of the nucleotide pyrophosphatase and phosphodiesterase family, which hydrolyse pyrophosphate bonds and phosphodiester bonds in nucleotides (Yegutkin, 2014).

The half-life of adenosine in the extracellular space is about of 10 s. Under physiological conditions, most of the extracellular adenosine appears to re-enter cells through equilibrative transporters. A small fraction can be irreversibly converted into inosine and its derivatives (hypoxanthine, xanthine, uric acid) by ADA and xanthine oxidase. Extracellular adenosine can also be metabolized by ecto-kinases to regenerate AMP, ADP, and ATP (Yegutkin, 2014).

Extracellular levels of adenosine can rise from low nanomolar to micromolar concentrations in response to increased metabolic demand, injury or stress, such as ischemia and inflammation, mainly by the breakdown of ATP released either by lysis or non-lytic mechanisms from multiple cell types (Fredholm, 2007). The termination of extracellular adenosine signalling occurs by uptake or degradation of this nucleoside, achieved by nucleoside transporters and ecto-adenosine deaminase, respectively. Under pathological conditions, there is a parallel marked induction of enzymes that are responsible for ATP-dependent adenosine signalling as well as of adenosine receptors and, in contrast, the suppression of enzymes involved in adenosine metabolism, such as AK (Chen *et al*., 2013). The reason for the adenosine extracellular increase is related to the activation of an auto

regulatory loop, whose function is to protect organs from injury following the initiating stressful stimuli (Bours *et al*., 2006).
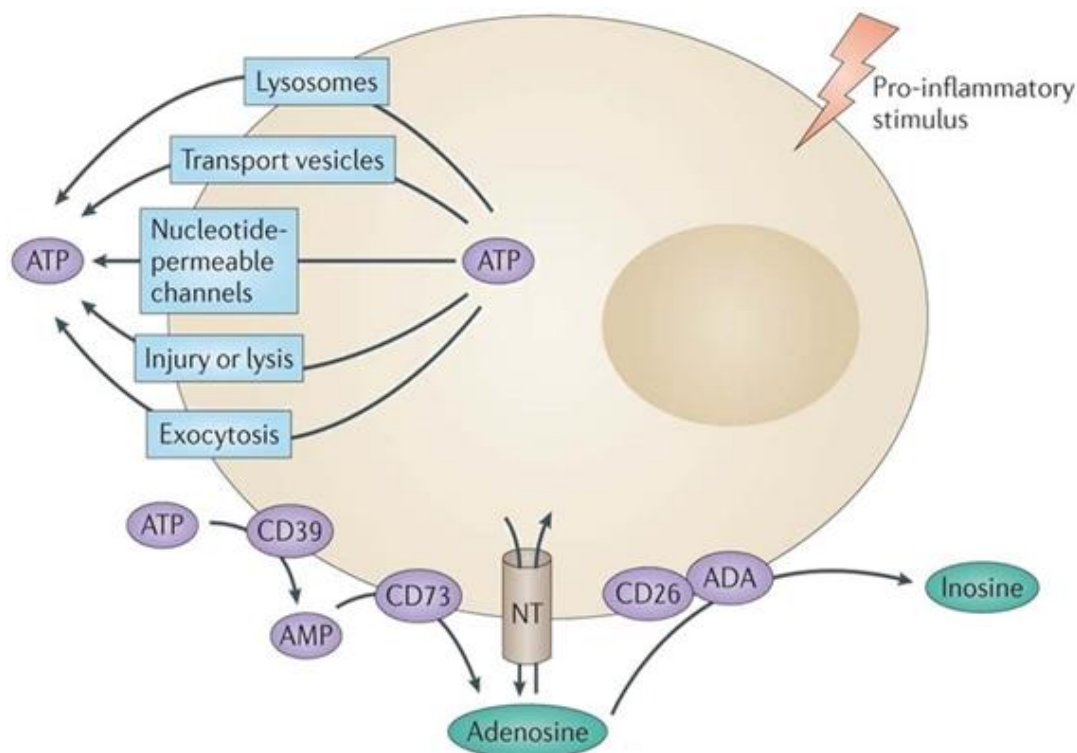
Figure 5. Adenosine synthesis and metabolic pathways outside of a cell. Adapted from Antonioli L, Blandizzi C, Csóka B, Pacher P, Haskó G. (2015) Nat Rev Endocrinol. 11:228-41. For abbreviations, see abbreviation list.

1.1.4 Adenosine receptors and signal transduction mechanism

Adenosine signalling is evoked through activation of four distinct and widely expressed receptors: A_1, A_{2A}, A_{2B} and A_3. All four subtypes, known as purinoceptors P1, are members of the superfamily of G-protein-coupled receptors (GPCRs) which transfer signals by activating heterotrimeric G proteins (Ralevic and Burnstock, 1998). Adenosine receptors display the typical topology of GPCRs, characterized by a common central core domain consisting of seven transmembrane helices numbered from 1 to 7 that are composed of 20-27 amino acids and that are largely α-helical, with an extracellular amino-terminus and an intracellular carboxy-terminus (Ralevic and Burnstock, 1998).

First evidence for the existence of adenosine receptors was in 1965 when DeGubareff and Sleator observed that adenosine action on heart could be antagonized by caffeine; subsequently, in 1970, Sattin and Rall showed that adenosine regulates cell function via occupancy of specific receptors on the cell surface. To date, adenosine receptors have been cloned, and the signal transduction mechanisms have been described. Classically, the first effector molecule of adenosine receptors is the enzyme adenyl cyclase (AC) which is either stimulated or inhibited, increasing or decreasing cyclic adenosine monophosphate cAMP levels according to the receptor subtype triggered (van Calker *et al*., 1979).

Adenosine receptors have been implicated in several biological functions, both physiological and pathological. These receptors have distinct localization, signal transduction pathways and different regulation upon exposure to agonists (Ralevic and Burnstock, 1998). Moreover, the expression levels of all the adenosine receptors are transcriptionally regulated and can change rapidly and dramatically in response to various stimuli (Murphree *et al*., 2005). A_1 and A_{2A} adenosine receptors possess high affinity for adenosine ($K_m < 30$ nM) whereas A_3 and, in particular, the A_{2B} adenosine receptors are low affinity receptors (K_m 1–20 μM). Thus, A_{2B} and A_3 adenosine receptors are likely only activated under high metabolic and stressful cellular conditions (Fredholm *et al*., 2001).

1.1.4.1 A_1 adenosine receptor

A_1 adenosine receptor (A_1AR) is coupled to G proteins belonging to the family of the G_i/G_q. Activation of A_1AR inhibits AC activity through activation of pertussis toxin-sensitive G_i proteins, it decreases PKA activity and phosphorylation of the cyclic AMP response element binding (CREB) protein (Van Calker *et al*., 1979). In cardiac muscle and neurons, A_1AR can activate pertussis toxin-sensitive K+ channels, as well as K_{ATP} channels, and inhibit Q-, P- and N-type Ca^{2+} channels. Furthermore, activation of A_1AR leads to increased intracellular Ca^{2+} levels due to the stimulation of phospholipase C (PLC), which in turn

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promotes the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (Stiles, 1992). Moreover, the enhancement of intracellular calcium can activate certain enzymes, such as protein kinase C (PKC), phospholipase D (PLD), phospholipase A_2 (PLA_2). Activation of A_1AR can also increase mitogen-activated protein kinase (MAPK pathway in cells of the Chinese hamster ovary and COS-7 fibroblast-like cells by βγ subunit of $G_{i/o}$ protein. A_1AR is widely distributed and is expressed in the central nervous system (neocortex, cerebellum, hippocampus and the dorsal horn of the spinal cord); adipose tissue, skeletal muscle, liver, kidney, salivary glands, esophagus, colon, eyes, heart muscle (sinoatrial and atrioventricular nodes, ventricles); lungs, pancreas and inflammatory cells such as neutrophils (Polosa, 2002).

1.1.4.2 A_{2A} adenosine receptor

A_2 adenosine receptors are subdivided into the A_{2A} and A_{2B} receptors, based on high and low affinity for adenosine, respectively. High levels of the A_{2A} receptor are found in brain striatum, immune cells of the spleen, thymus, leukocytes and in blood platelets; intermediate levels are found in the heart, lung and blood vessels. A_{2A} adenosine receptor ($A_{2A}AR$) couples to G_s protein in peripheral tissues or G_{olf} protein in the brain (Kull et al., 2000). Increased cAMP level stimulates cAMP-dependent kinase (protein kinase A, PKA) which, in turn, activates several pathways through calcium channels, potassium channels, CREB, MAPK and PLC activation. Activation of $A_{2A}AR$ can also promote activation of PKC in a cAMP-dependent and independent mechanisms (Sheth *et al*., 2014). There is evidence that the $A_{2A}AR$ can occur as receptor homodimer as well as heterodimers with other receptors such as dopamine D_2 receptor (Fredholm *et al*., 2007). Moreover, recent studies have shown that there are several proteins interacting with the cytoplasmic tail of the activated $A_{2A}AR$ resulting in G-protein-independent signalling pathways (Fredholm *et al*., 2007).

1.1.4.3 A_{2B} adenosine receptor

A_{2B} adenosine receptor ($A_{2B}AR$) is widely expressed in the brain, gastrointestinal tract, bladder, lung and on endothelial cells, muscle cells, mast cells, fibroblasts (Polosa, 2002). Activation of $A_{2B}AR$ can stimulate AC and PLC through activation of G_s and G_q proteins, respectively (Feoktistov and Biaggioni, 1995). The $A_{2B}AR$ has also been described to be involved in the extracellular signal-regulated kinase (ERK) ERK1, ERK2 (Schulte and Fredholm, 2003) and p38 MAPK in mast cells.

1.1.4.4 A_3 adenosine receptor

A_3 adenosine receptor (A_3AR) couples to classical second-messenger pathways such as inhibition of AC, stimulation of PLC and D and calcium mobilization (Zhou *et al*., 1992; Abbracchio *et al*., 1995). A_3AR can regulate the activity of PLC via a pertussis toxin-sensitive G protein in rat basophilic leukemia cells or by direct coupling to G_q protein. It has been widely identified in several tissues and cell type, including kidney, testis, lung, mast cells, eosinophils, neutrophils, heart and in brain cortex (Polosa, 2002). In the heart, A_3AR mediates cardioprotective effects through the activation of K_{ATP} channels that are coupled to RhoA–phospholipase D signalling, mediating the protection of cardiac myocytes from ischemia. Like other adenosine receptors, A_3 receptors are coupled to MAPK and lead to stimulation of ERK1/2.
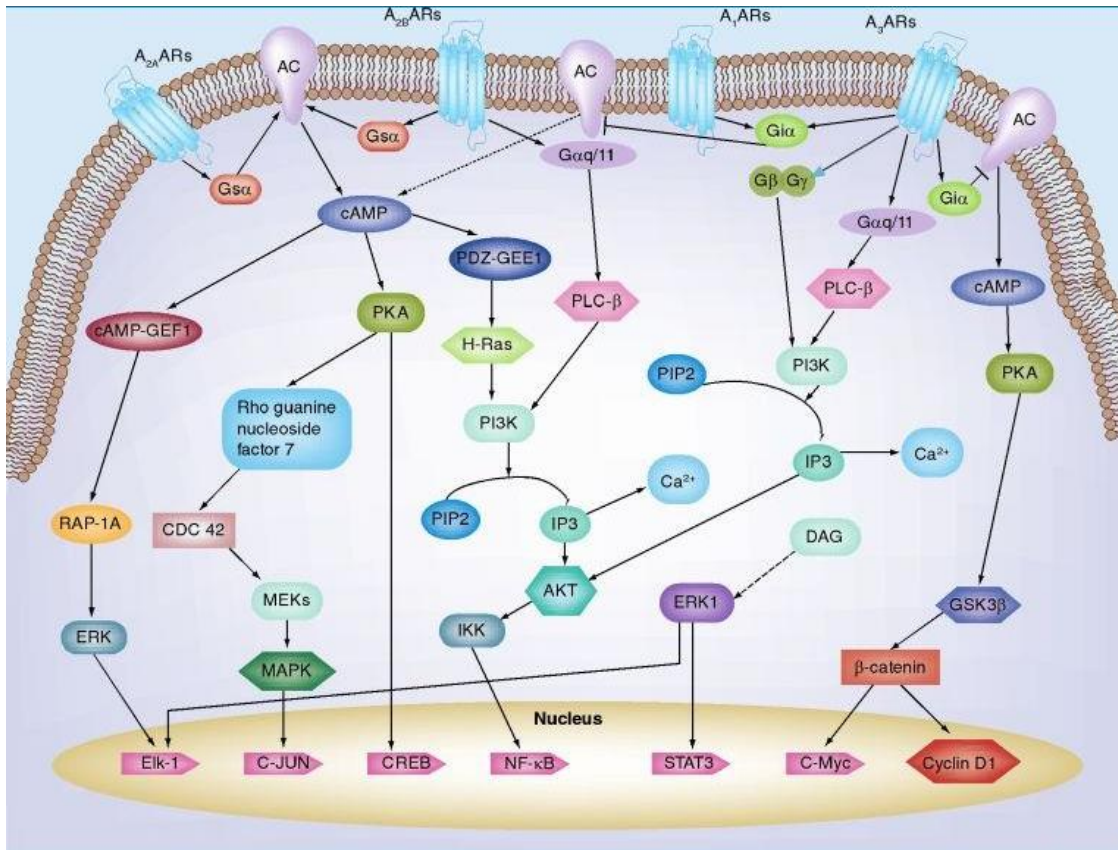
Figure 6. Principal signalling pathways activated by adenosine receptors. From Padovan M, Vincenzi F, Govoni M, Bortoluzzi A, Borea PA; Varani K. (2013) Int J Clin Rheumatol. 8:13-25. For abbreviations, see abbreviation list.

1.1.5. Overall effect of adenosine and its receptors on inflammation

Adenosine has been recognised as an endogenous signalling molecule that is able to signal inflammation as well as modulate the inflammatory response (Haskó and Cronstein, 2013). Adenosine is one of the many biomolecules that accumulate in the inflammatory milieu conferring pleiotropic effects which can be beneficial or harmful (Driver *et al*., 1993). Adenosine effects are the outcome of receptor activation and may be pro-inflammatory or anti-inflammatory depending 1) on the tissue or cells where receptor are expressed, 2) on the affinity between adenosine and a specific receptor and 3) on the extent of tissue receptor expression under pathological conditions (Bours *et al*., 2006). Generally, at the early stage of the inflammatory process, high level of extracellular adenosine directs toward an immune response more specific. At the late stage of inflammation, adenosine resolves the

inflammation by downregulating immune cells. Extracellular adenosine regulates the function of the innate and adaptive immune systems through targeting virtually every cell type that is involved in orchestrating an immune/inflammatory response (Haskó and Pacher, 2008).

1.1.5.1 Adenosine and neutrophils

Neutrophils are the most abundant circulating leukocytes, and are among the first cells to respond to injury; they represent the cellular hallmark of an acute inflammation. Neutrophils release adenosine but also respond to it via expression of all four adenosine receptor subtypes. The response of neutrophils to adenosine is highly dependent on the inflammatory microenvironment and, in part, regulated by the extent of expression of adenosine receptors on neutrophils and the affinity. Neutrophils are recruited to inflamed sites by a combination of chemokines and adhesive interactions with the vascular endothelium. Adenosine acts on both neutrophils and endothelial cells to control neutrophil adhesion and transmigration. At early stages of inflammation, nanomolar concentrations of adenosine enhance neutrophil adhesion to the vascular endothelium by stimulation of A_1 receptors on both neutrophils (Cronstein *et al*., 1992; Felsch *et al*., 1995) and endothelial cells (Zahler *et al*., 1994). Once the inflammatory reaction is in progress, adenosine micromolar concentrations inhibit the shedding of L-selectin and integrins (mainly CD11b/CD18) on neutrophils (Thiel *et al*., 1996), the release of the neutrophil chemoattractant Interleukin-8 (IL-8) from endothelial cells and reduce expression of adhesion molecules endothelial-selectin (E-selectin) and vascular cell adhesion molecule 1 (VCAM-1) on the endothelial cell surface (Bouma *et al*., 1996). These adenosine inhibitory effects are mediated trough A_{2A} and A_{2B} receptors. Also the A_3AR has been implicated in the regulation of neutrophil adhesion since it reduces platelet activating factor (PAF)-stimulated neutrophil adherence to coronary endothelium (Jordan *et al*., 1999). Adenosine acting via A_2 receptors, inhibits pro-inflammatory

mediator release from activated neutrophils such as TNF-α and leukotriene B$_4$ (LTB$_4$) while promotes the release of anti-inflammatory mediators such as PGE$_2$ (Pouliot *et al*., 2002; Cadieux *et al*., 2005). Similar to regulation of neutrophil chemotaxis, low concentrations of adenosine promote phagocytosis via A$_1$ receptor binding, while elevated concentrations of adenosine inhibit phagocytosis via activation of A$_{2A}$ receptors (Salmon and Cronstein, 1990). Adenosine appears to inhibit neutrophil granule release by binding A$_{2A}$ and A$_3$ receptors (Anderson *et al*., 2000) and to inhibit the neutrophil oxidative burst by activating A$_{2A}$ and A$_{2B}$ receptors (van der Hoeven *et al*., 2011; Sun *et al*., 2007). In contrast, A$_1$ receptor activation enhances superoxide generation during FcRγ-mediated stimulation of human neutrophils. Cell death is essential to both homeostatic turnover of neutrophils in the resting state and during tissue inflammation. It has been demonstrated that adenosine analogues delay apoptosis of resting human neutrophils in culture (Walker *et al*., 1997a).

1.1.5.2 Adenosine and mononuclear phagocyte system

The mononuclear phagocyte system (MPS) is a family of cells comprising bone marrow progenitors, circulating monocytes, resident macrophages and dendritic cells (DCs) that have in common the property of phagocytosis. These cells synthesize and release into the environment cytokines and other proteins that play a central role in the development of acute and chronic inflammation and wound healing (van Furth and Cohn, 1968). A variable expression of adenosine receptors has been observed on monocytes (Thiele *et al*., 2004) and macrophages (Nemeth *et al*., 2005) which is based, at least in part, on their maturation phase. A$_1$, A$_2$ and A$_3$ adenosine receptors are barely expressed on quiescent monocytes but their density increases during the differentiation of monocytes into macrophages (Thiele *et al*., 2004). Adenosine receptor activation can influence macrophage function depending on the receptor subtypes expressed and the environment in which macrophages are exposed to extracellular adenosine. Endogenous adenosine is capable of preventing monocyte

maturation as demonstrated by the fact that ADA activity is increased during early monocyte differentiation and that ADA inhibition during this period delayed the maturation process (Fischer *et al*., 1976). High concentrations of exogenous adenosine seem to prevent monocyte development into macrophages and to arrest monocyte development at a stage with high accessory function, a phenotype that is similar to dendritic cells (Najar *et al*., 1990). Adenosine influences monocyte maturation also by promoting the formation of multinucleated giant cells via A_1 receptor stimulation, whereas A_2 receptor activation prevents the generation of giant cells (Merrill *et al*., 1997). Adenosine suppresses macrophage colony-stimulating factor (MCF)-induced proliferation of mouse bone marrow macrophages through A_{2B} receptors (Xaus *et al*., 1999). Regarding adenosine effect on monocyte/macrophage phagocytosis, it has been shown that the stimulatory effect of A_1 adenosine receptors in undifferentiated monocytes is overcome by an A_2 adenosine receptor-mediated suppression of phagocytosis in mature macrophages (Salmon *et al*., 1993).

The activation of A_{2A}, A_{2B} and A_3 adenosine receptors suppresses the production of several pro-inflammatory cytokines, such as TNF-α, IL-6, Interleukin-12 (IL-12), macrophage inflammatory protein 1 alfa (MIP-1α) by monocytes and macrophages thus promoting a polarization of monocyte and macrophage response toward an anti-inflammatory phenotype (Csóka *et al*., 2012, Haskó *et al*., 1996; Haskó *et al*., 1998). In contrast, adenosine increases the production of the anti-inflammatory cytokine such as Interleukin-10 (IL-10) (Németh *et al*., 2005; Csóka *et al*., 2007). Adenosine has also shown to be a broad inhibitor of NO production by M1 macrophages (Haskó *et al*., 1996). Adenosine can affect monocyte adhesion during inflammation modulating the adhesion molecule expression on endothelium and also regulating the interactions between monocytes and the vascular endothelium

(Bours *et al*, 2006). Moreover, adenosine affects both RNS and ROS production by monocytes/macrophages (Si *et al*.,1997).

Adenosine has also shown to modulate the functions of DCs, that are specialized antigen-presenting cells characterized by their ability to migrate into target sites, to process antigens, and to activate naïve T cells. All four adenosine receptors are present on both immature myeloid and mature DCs (Panther *et al*., 2001). The modulation of DCs functions by adenosine depends on the expression of adenosine receptors, which is related to the maturation status of these cells. Immature human dendritic cells express mainly A_1 and A_3 adenosine receptors which, once activated, stimulate immature dendritic cell recruitment to sites of inflammation via an increase in intracellular calcium. In contrast, dendritic cell maturation is associated with the upregulation of A_{2A} adenosine receptor-mediated signalling responses, culminating in a decrease in pro-inflammatory cytokine release such as IL-6, IL-12, TNF-α (Panther *et al*.,2003; Schnurr *et al*., 2004). Adenosine also inhibits the expression of C-C chemokine receptor type 5 (CCR5), macrophage inflammatory protein 3 beta MIP-3 β/chemokine (C-C motif) ligand 19 (CCL19), and multidrug resistance protein 1 (MDR-1), molecules involved in dendritic cells migration, and slows the *in vivo* and *in vitro* migration of mature dendritic cells (Hofer *et al*., 2003). It has been demonstrated the involvement of A_3 adenosine receptors in addition to A_{2A} adenosine receptors in the inhibitory effect of adenosine on TNF release in DCs (Dickenson *et al*., 2003). It has been shown that adenosine enhances IL-10 secretion by mature DCs reducing their capacity to promote Th1 cell differentiation (Panther *et al*., 2003). In the presence of toll-like receptor signalling, $A_{2B}AR$ activation inhibits DC-mediated T cell activation, indeed, its stimulation reduces lipopolysaccharide-induced surface expression of major histocompatibility complex class II (MHCII) molecules and cluster of differentiation 86 (CD86) which results in decreased Interleukin-2 (IL-2) expression by T cells. However, in the absence of toll-like

receptor signalling, the activation of the $A_{2B}AR$ can be pro-inflammatory causing the increases of pro-inflammatory IL-6, which together with transforming growth factor- β (TGF-β) can deviate naïve $CD4^+T$ cells to a Th17 phenotype that favors chronic inflammation.

1.1.5.3 Adenosine and lymphocytes

Chronic inflammation is often accompanied by recruitment of cell types participating in an immunological response, the lymphocytes. Adenosine has also been implicated in the regulation of lymphocyte function. It is well recognized that adenosine is an important endogenous immunosuppressing regulator; it has been demonstrated that the lack of the enzyme ADA is responsible for severe immunodeficient disease (Hirschhorn, 1995). Adenosine can regulate lymphocyte function indirectly by the activation of adenosine receptors on macrophages, dendritic cells, neutrophils, mast cells, in this way, by altering the production of several cytokines, such as TGF-β and IL-10 it produces an immunosuppressive environment. Adenosine can also regulate lymphocyte function directly by binding and activating adenosine receptors on lymphocytes (Bours *et al*., 2006). A_{2A}, A_{2B} and A_3 adenosine receptors are expressed on human and mouse T lymphocytes and their activation appears to represent a potent endogenous immunosuppressive pathway that regulates the excessive immune response against potent external insults. Adenosine receptor subtype expression by T lymphocytes can be modulated by T cell receptor signalling, indeed activated human T lymphocytes exhibit elevated $A_{2A}AR$, as well as $A_{2B}AR$ and A_3AR (Mirabet *et al*., 1999; Gessi *et al*., 2004). A_{2A} receptor is the major adenosine receptor influencing the function of lymphocytes (Huang *et al*., 1997). $CD4^+$ rather than $CD8^+$ T cells express A_{2A} adenosine receptors. Adenosine potently inhibits a wide range of T lymphocyte responses to antigenic stimulation, including cellular proliferation (Ohta *et al*., 2009), synthesis of IL-2 by $CD4^+$ T cells (Naganuma *et al*., 2006) and also by both

polarized T_C1, T_C2 CD8$^+$ cytotoxic cells (Erdmann *et al*., 2005) and synthesis of pro-inflammatory cytokines such as TNF-α and interferon gamma (IFN-γ), by both naïve CD4$^+$ T cells (Lappas *et al*., 2005; Naganuma *et al*., 2006) and polarized Th1 and Th2 cells (Csóka *et al*., 2008). In addition, both A_{2A} and A_{2B} adenosine receptors have been found on antigen-presenting cells (APCs) and strongly influence T cell activation. Moreover, adenosine by signalling primarily through A_{2A} and A_3 adenosine receptors on the surface of T cells provokes up-regulation of CD25 (IL-2 receptor α chain) (Huang *et al*., 1997; Butler *et al*., 2003), expression of cytotoxic effector molecules such as perforin and Fas ligand (Koshiba *et al*., 1997; Hoskin *et al*., 2002), and granule exocytosis by cytotoxic T lymphocyte (CTL) (Koshiba *et al*., 1997). A_{2A}AR stimulation also upregulates the expression of negative costimulatory molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and down regulates expression of the positive costimulatory molecule CD40 ligand (CD-40L) (Sevigny *et al*., 2007). Adenosine signalling has generally inhibitory effects on lymphocyte migration into the inflamed tissues; indeed, the extravasation of lymphocytes through blood vessels is influenced by A_{2A} receptors-mediated suppression of intercellular adhesion molecule 1 expression on lymphocytes and diminished production of IFN-γ and IFN-γ-inducible chemokines that are chemotactic to activated lymphocytes (Henttinen *et al*., 2003). Adenosine also decreases the barrier function of vascular endothelium by activating A_{2B}ARs. Exposure to adenosine during T cell activation or in naïve T cells through A_{2A}AR signalling promotes long-term T-cell anergy and the development of a regulatory phenotype (Treg) characterized by high expression of CD25, CTLA-4, and Forkhead box protein 3 (FoxP3) (Zarek *et al*., 2008). Next, Tregs not only utilize adenosine as one of their immunosuppressive mechanisms but also receive positive regulation from adenosine to enhance their number and immunosuppressive activity (Ohta *et al*., 2012). Thus, adenosine can not only directly and instantly inhibit the immune

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response through its receptor on the effector cells, but also can recruit other immunoregulatory mechanisms, including Tregs.

There is evidence that adenosine can regulate the differentiation and function of Th17 cells. It has been shown that A_{2A} receptor activation limits the production of Th17 cells through suppression of IL-2 production (Zarek *et al*., 2008). In contrast, Wilson and co-workers (2011) have demonstrated that adenosine represents a biological mediator that can enhance the development of Th17 cells acting at A_{2B}Rs.

Natural killer (NK) cells express A_1, A_{2A} and A_3 adenosine receptors. Adenosine acting at the A_{2A} receptor of NK cells limits their capacity to produce pro-inflammatory cytokines such as TNF and IFN-γ while increases their production of Interleukin-4 (IL-4) and TGF (Lappas *et al*., 2006). A_{2A}R activation also strongly inhibits the production of IFN-γ by invariant natural killer (iNKT) cells (Lappas *et al*., 2006; Nowak *et al*., 2010). Unlike conventional T cells, iNKT cells can be rapidly activated early during the inflammatory responses and produce copious amounts of cytokines shortly after T-cell receptor (TCR) engagement. Adenosine also inhibits both perforin and FasL cytotoxic molecules. In contrast, NK cell cytolytic function is enhanced through A_1 and A_3 receptor activation (Priebe *et al*., 1990; Harish *et al*., 2003; Raskovalova *et al*., 2005).

B lymphocytes express all of four adenosine receptors (Sakowicz-Burkiewicz *et al*., 2012). It is likely that adenosine exerts its effect on B lymphocytes primarily through A_{2A} receptors. It has been demonstrated that adenosine suppresses the proliferation and the antibody production of B cells (Seegmiller *et al*., 1977) and that adenosine affects B cells functions by inhibiting the activation of NF-kB in response to B cell antigen receptor (BCR) triggering (Minguet *et al*., 2005). Moreover, adenosine protects human B cells from B cell receptor-induced apoptosis mostly via A_{2A} and A_{2B} receptors and by downregulating caspase-3 activation (Sakata *et al*., 2000).

1.1.5.4 Adenosine and mast cells

Mast cells are bone marrow-derived inflammatory cells known for their involvement in allergic and inflammatory diseases; these cells can release mediators that have both immediate and chronic effects on airway constriction and inflammation. Adenosine receptors are expressed on mast cells with a different pattern depending on the species (human, murine, rat, etc.). Studies using pharmacological reagents and antisense DNA suggest that the A_1 receptor is responsible for the mast cell-independent component of adenosine-induced bronchoconstriction (Ali *et al*., 1994). Adenosine can modulate mast cell degranulation but which receptor is involved is uncertain. For example, *in vitro* studies employing a human mast cell line and a dog mastocytoma line have shown a role for the A_{2B} receptor in adenosine-induced mast cell activation (Feoktistov and Biaggioni, 1995; Auchampach *et al*., 1997) whereas *in vitro* and *in vivo* studies in rodent mast cells indicate that A_3 adenosine receptor plays a primary role in mediating adenosine responses (Fozard *et al*., 1996; Reeves *et al*., 1997). However, these differences could be attributed to the selectivity of the adenosine analogs used, to different signalling pathways among mast cells from various tissues (or that have been generated *in vitro* using different methods), and to differences among species. It is possible that at low concentrations of adenosine, that are likely insufficient to activate the A_{2B} and A_3 adenosine receptors, only the high affinity A_{2A} receptors signal prevails, downregulating mast-cell degranulation. It has been shown that basal levels of adenosine produced in the lung are insufficient to activate the A_{2B} and A_3 adenosine receptors; conversely, in asthamatics, levels of adenosine into inflamed airway increase dramatically, and are sufficient to activate the A_3AR and $A_{2B}AR$ (Driver *et al*., 1993; Feoktistov *et al*., 1998). The infiltration of airway smooth muscle cells with mast cells in the asthmatic lung (Bradding and Brightling, 2007) coupled with this elevation in adenosine levels and subsequent activation of these cells via the A_3 and/or A_{2B} receptor may

play an important role in the pathogenesis of asthma as well as of other diseases in which

coordinate increases in adenosine and mast cells is observed (Feoktistov *et al*., 1998; Tilley

et al., 2003).
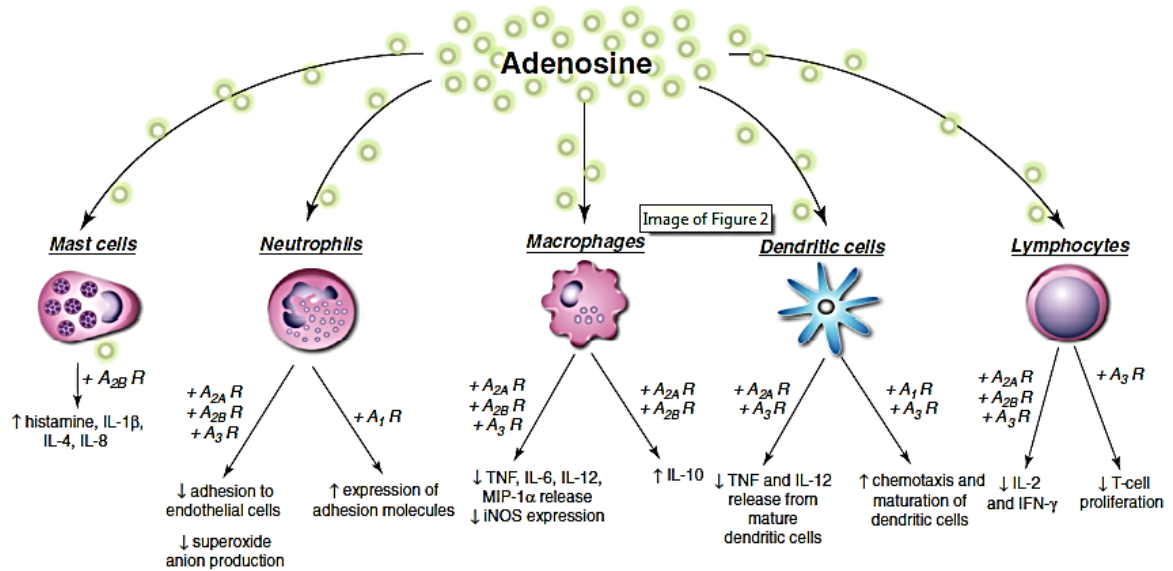
Figure 7. Some representative actions mediated by adenosine in human immune cells. From Antonioli L, Csóka B, Fornai M, Colucci R, Kókai E, Blandizzi C, Haskó G. (2014) Drug Discov Today. 19:1051-68.

1.1.6 Adenosine receptors and inflammation

It has been well documented that adenosine receptors play an important role in the

regulation of inflammation, they may exert anti- or/and pro-inflammatory effects.

1.1.6.1 A_1AR

The role of A_1 receptor in inflammatory response is controversial. Several studies with

selective agonists and antagonists have demonstrated pro-inflammatory effects of A_1

receptors in different inflammatory models. For example, in acute pancreatitis induced with

cerulein or taurocholate in rats, the selective A_1 receptors agonist CCPA produced an

increase in leukocyte infiltration and interstitial edema in pancreatic tissue attenuated by

FK-838, a selective A_1 receptor antagonist (Satoh *et al*., 2000). In addition, treatment with

DPCPX, an A_1 receptor antagonist, has prevented endothelial damage, neutrophil migration

and alveolar injury in a model of ischemia reperfusion in the lungs (Neely and Keith, 1995). On the other hand, studies using selective A_1 receptor agonist or antagonists and knockout animals have shown that activation of the A_1 receptor can promote anti-inflammatory effects. For instance, in a mouse model of renal ischemia reperfusion injury, it has been shown a protective effects of the A_1 receptor, by using A_1 receptor agonist CCPA, and antagonist, DPCPX (Lee *et al*., 2004a), further confirmed in studies performed with A_1 receptor deficient mice (Lee *et al*., 2004b). Moreover, the absence of the A_1 receptor has demonstrated to promote pro-inflammatory effects in the lungs, enhancing leukocyte migration and levels of cytokines, including IL-4 and IL-13 (Sun *et al*., 2005).

In summary, the A_1 receptor can activate intracellular signalling pathways that result in tissue injury or protection, through pro-inflammatory or anti-inflammatory effects, respectively, because the activated pathways depend on the species/tissue/organ and the stage/progression of injury, on the predominant inflammatory cell type as a function of species, on the intracellular signalling and desensitization mechanisms (da Rocha Lapa *et al*., 2014).

1.1.6.2 $A_{2A}AR$

Unlike A_1 receptors, there is a substantial consensus regarding the effects of A_{2A} adenosine receptors on the inflammatory response. The central role of the $A_{2A}AR$ in counteracting inflammation has been demonstrated in numerous studies using $A_{2A}AR$ selective agonists to inhibit the inflammatory responses in a variety of cell types *in vitro* as well as *in vivo* models of inflammation and in studies using $A_{2A}AR$ deficient mice (Fredholm *et al*., 1996; Link *et al*., 2000; Ohta and Sitkovsky, 2001; Sitkovsky, 2003; Antonioli *et al*., 2006; Haskò and Pacher, 2008). The anti-inflammatory properties of $A_{2A}AR$ receptors are mediated, at least in part, by A_{2A} adenosine receptors signalling on immune cells as described above. The anti-inflammatory effects of adenosine A_{2A} receptor activation is due to the activation of

protein kinases which interfere with the IκB kinase complex that selectively inhibits the NFκB pathway, to the activation of CREB protein which mediates gene expression directly and indirectly by competing with the NFκB pathway and also through the activation of the exchange factor activated by cAMP which has a well-known immunosuppressive effect on immune cells (Huang *et al*., 1997; Haskò and Cronstein, 2004). The tissue-protecting properties of A_{2A} receptors have been well shown in different models of inflammatory damage and systemic inflammation such as ischemia reperfusion liver injury in mice (Day *et al*., 2004), inflammatory bowel disease (Odashima *et al*., 2005), in mouse models of endotoxemia and sepsis (Sullivan *et al*., 2004), in models of myocardial infarction, acute lung injury and spinal cord compression injury (Li *et al*., 2006; Yang *et al*., 2006; Reutershan *et al*., 2007). Furthermore, it has been demonstrated that $A_{2A}AR$ activation also protects from stress–induced gastric lesion in the rat that are dependent upon neutrophil infiltration (Odashima *et al*., 2006). On the other hand, in A_{2A} receptor knockout mice has been shown the exacerbation of various types of inflammation, compared to wild-type mice, supporting the $A_{2A}AR$ dependent control of inflammation (Day *et al*., 2003; Nadeem *et al*., 2007; Hussey *et al*., 2012). Moreover, experiments performed on knockout mouse models, have shown that $A_{2A}AR$ together with A_3AR mediates the anti-inflammatory effect of methotrexate, which is used as a treatment of arthritis (Montesinos *et al*., 2003).

It is well established that inflammatory tissue damage is accompanied by the accumulation of extracellular adenosine in inflamed sites due to its release from non-immune and immune cells. Because endogenous adenosine levels are elevated during an inflammatory process and endogenous adenosine can activate A_{2A} receptors to attenuate inflammation and tissue damage, strategies that aim to raise adenosine production and its availability to activate A_{2A} receptors present great anti-inflammatory potential.

1.1.6.3 A$_{2B}$AR

It has been found that A$_{2B}$ARs may mediate opposite responses in different types of inflammatory conditions in various tissues and/or at different stages of their progression (Feoktistov and Biaggioni, 2011). Several pharmacological studies have suggested that activation of A$_{2B}$AR by adenosine has pro-inflammatory effects, mainly including the production of pro-inflammatory cytokines and chemokines (Feoktistov and Biaggioni, 1995; Zhong *et al.*, 2004; Sun *et al.*, 2006). However, Yang D. and co-workers (2006) have found that A$_{2B}$AR knockout mice have increased inflammation at baseline and after LPS exposure, indicating that the A$_{2B}$AR mediates anti-inflammatory responses. A$_{2B}$AR knockout mice have also shown enhanced mast cell activation (Hua *et al.*, 2007), confirming that the A$_{2B}$AR plays an anti-inflammatory role at baseline. However, these studies have not investigated whether these phenotypes in mice lacking A$_{2B}$AR result from loss of direct agonist activation of A$_{2B}$AR or from loss of an unidentified function of the A$_{2B}$AR. Ryzhov and colleagues (2008a, 2008b) concluded that activation of A$_{2B}$AR by adenosine results in inflammatory effects, consistent with their and others' previous pharmacological studies. However, they also found inflammatory exacerbation in A$_{2B}$AR knockout mice, such as Yang D. (2006) and Hua (2007) did. Therefore, they have supposed that A$_{2B}$AR may exert an adenosine-independent down-regulation of pro-inflammatory cytokines, possibly by the association with other proteins mediated signalling pathways (Ryzhov *et al.*, 2008b). Up to now, several proteins such as netrin-1 (Corset *et al.*, 2000), Ezrin/PKA/NHERF-2/E3KARP (Sitaraman *et al.*, 2002) and SNARE proteins (Wang *et al.*, 2004) have been reported to form a 24 multi-complex with A$_{2B}$AR. However, all these coupled proteins of A$_{2B}$AR cannot explain the anti-inflammatory role of A$_{2B}$AR. The apparent low affinity of adenosine for A$_{2B}$Rs suggests that this receptor may have important roles in pathological environments where adenosine levels are elevated. Taking into account the lack of information about the

A$_{2B}$ receiver and its involvement in several inflammatory diseases, it may be a candidate target for future therapeutic intervention.

1.1.6.4 A$_3$AR

A$_3$AR appears to have a conflicting role in inflammatory responses, since both pro- and anti-inflammatory effects have been demonstrated depending on the cell type and animal species being studied (Gessi *et al*., 2008). Several studies demonstrate that A$_3$AR activation produces pro-inflammatory responses. Evidence for a role of A$_3$AR in increasing inflammation derives by studies in mast cells where its activation has shown to be responsible for release of allergic mediators (Ramkumar *et al*., 1993). In addition, it has been reported that A$_3$AR mRNA is high in lung tissue of patients with airway inflammation and that A$_3$AR activation mediates rapid inflammatory cell influx into the lungs of sensitized guinea pigs (Walker *et al*., 1997b; Spruntulis and Broadley, 2001). On the other hand, several studies have shown that A$_3$AR activation can have an anti-inflammatory effect. For example, in murine BV2 microglial cells, activation of A$_3$AR has suppressed LPS-induced TNF-α production by inhibiting phosphoinositide 3-kinase/protein kinase B (PI3-k/PKB) and NF-κB activation (Lee *et al.*, 2006). In sepsis, mice lacking A$_3$AR have significantly higher levels of plasma TNF-α, increased mRNA encoding pro-inflammatory cytokines, and enhanced nuclear translocation of NF-κB in their renal cortices compared to wild type mice (Lee *et al*., 2006). Similarly, in rheumatoid arthritis patients, the A$_3$AR agonist CF502 mediates an anti-inflammatory effect by inhibiting the PI3K/PKB, and NF-κB signalling pathway (Ochaion *et al*., 2008). Thus, A$_3$AR can have protective or deleterious effect, depending on the cell type involved as well as on the duration of its activation. In fact, it is worth noting that both human and rat A$_3$ adenosine receptors are desensitized within a few minutes after agonist exposure (Trincavelli *et al*., 2002), and A$_3$ARs are very sensitive to prolonged stress *in vitro* (Von Arnim *et al*., 2000). A$_3$AR may

be considered as a specific target to control inflammation even if much is still to be learned

about its role in inflammation.

1.2 EXTRACELLULAR ADENOSINE PRODUCERS, CD39AND CD73, AND INFLAMMATORY RESPONSE

CD39 and CD73 represent the predominant pathways of extracellular adenosine production as confirmed using genetically manipulated mice (Volmer *et al*., 2006; Grenz *et al*., 2007) as well as *in vitro* (Grünewald and Ridley, 2010). Specifically, CD39 catalyses the hydrolysis of ATP and ADP to AMP with the liberation of two molecules of inorganic phosphates (P_i) and the corresponding nucleoside monophosphate product, AMP, is then degraded to adenosine and phosphate by e-5′NT/CD73 (Zimmermann, 2000). Biological actions of NTPDase1/CD39 and e-5′NT/CD73 are mainly a consequence of their enzymatic activity. Importantly, the activity of CD39 is reversible by the actions of nucleoside-diphosphate kinase (NDP) kinase and AK, whereas the activity of CD73 is virtually irreversible. Therefore, e-5′NT/CD73 represents the "peacemaker" enzyme for extracellular adenosine production (Hart *et al*., 2008; Allard *et al*., 2012).

1.2.1 Functions and structures

CD39 belongs to the NTPDase family that consists of eight members (Robson *et al*., 2006). Like NTPDase-2,-3, and -8, NTPDase1/CD39 is located at the surface of the plasma membrane (Robson *et al*., 2006) while NTPDase 4–7 have mainly intracellular localization (NTPDases 5 and 6 can also be present at the surface of the plasma membrane and secreted as soluble enzymes) and do not participate in the metabolism of extracellular nucleotides (Robson *et al*., 2006). Millimolar concentrations of divalent cations such as Mg^{2+} and Ca^{2+} are essential for nucleotide hydrolysis (Wang and Guidotti, 1996) with maximal activity at pH 7.0–8.5 (Kukulski *et al*., 2005). NTPDase1/CD39 exhibits a substantially smaller K_m in comparison to the other members of its family, which allows the hydrolysis of phosphopurines, ATP and uridine 5'-triphosphate (UTP), at concentrations at least 3 times

lower (Kukulski and Komoszynski, 2003; Kukulski *et al*., 2005). NTPDase1/CD39 hydrolyzes ATP almost directly to AMP with the transient production of minor amounts of free ADP (Kaczmarek *et al*., 1996). As consequence, NTPDase1/CD39 can most efficiently terminate the P2-receptor mediated signalling or prevent its inactivation (Kukulski *et al*., 2011). Structurally, NTPDase1/CD39 is highly glycosylated protein characterized by two transmembrane domains, a small cytoplasmic domain comprising the NH_2 and COOH-terminal segments, and a large extracellular hydrophobic domain. In this domain there are five highly conserved sequence domains known as apyrase conserved regions, that are crucial for the catalytic activity of the enzyme. NTPDase1/CD39 is firmly anchored to the membrane via two transmembrane domains that are important for maintaining catalytic activity and substrate specificity (Robson *et al*., 2006). NTPDase1/CD39 becomes catalytically active upon its localization on the cell surface, and its glycosylation is crucial for correct protein folding, membrane targeting, and enzyme activity (Smith and Kirley, 1998). In addition, it has been demonstrated the presence of soluble NTPDase1/CD39 freely circulating in the human and murine bloodstream and contributing to the metabolism of intravascular ADP (Coade and Pearson, 1989; Yegutkin *et al*., 2007, 2012). Moreover, NTPDase1/CD39 can be released from pancreatic acini (Sorensen *et al*., 2003), platelets, endothelial cells (Banz *et al*., 2008; Visovatti *et al*., 2012), and some cancer cells (Clayton *et al*., 2011) as exosome- or microparticle-embedded enzyme.
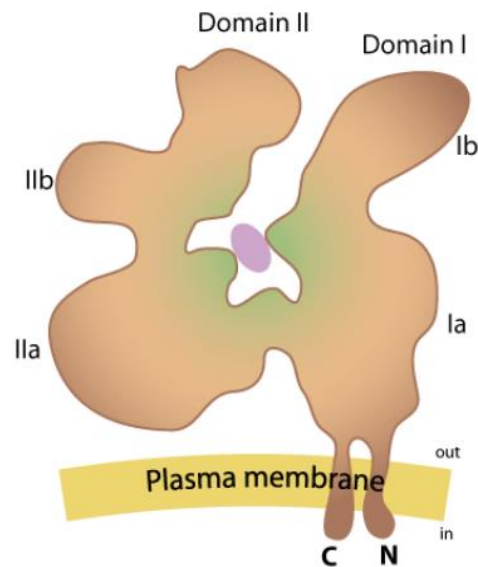
Figure 8. Schematic representation of the NTPDase1/CD39 structure. Modified from Robson SC, Sevigny J, Zimmermann H. (2006) Purin signal. 2:409–30.

NTPDase1/CD39 was first described as a surface marker of Epstein Barr virus (EBV)-transformed B cells (Rowe *et al*., 1982) and proposed to be a cell-surface signalling molecule playing a role in the regulation of effector functions of activated lymphocytes (Dombrowski *et al*., 1998). It is expressed in a large variety of tissues including heart, placenta, lung, liver, skeletal muscle, thymus, kidney, pancreas, testis, ovary, prostate, colon and brain (Zimmermann, 1999). It has been suggested that NTPDase1/CD39 may have functions beyond apyrase activity, its localization within lipid rafts implies that this ecto-enzyme may be involved in cell-cell contacts and signalling. For instance, NTPDase1/CD39 can act as an ATP channel (Bodas *et al*., 2000) or directly interact with Ran-binding protein M, a membrane scaffolding protein with guanosine 5'-triphosphate (GTP)ase activity so playing a role in intracellular signalling (Wu *et al*., 2004).

NTPDase1/CD39 is the dominant NTPDase of the vasculature. Endothelial CD39, by converting ADP to adenosine, play an important role in limiting the extent of platelet aggregation (Robson *et al*., 2005, 2006; Yegutkin, 2008). Accordingly, CD39 knockout mice have shown disordered thromboregulation and increased infarct volumes upon experimental stroke (Enjyoji *et al*., 1999; Pinsky *et al*., 2002). Recombinant soluble and

catalytically active CD39 has demonstrated to block ADP-induced platelet aggregation *in vitro* and to inhibit collagen-induced platelet reactivity (Gayle *et al.*, 1998).

It has been also reported that NTPDase1/CD39 plays important role in modulating the inflammatory responses during host–pathogen interactions (Knowles, 2011; Sansom *et al*., 2008) and tumorigenesis (Bastid *et al*., 2012). Several pro-inflammatory cytokines, oxidative stress, and hypoxia can affect CD39 expression through the transcription factors specificity protein 1, signal transducer and activator of transcription 3, and zinc finger protein growth factor independent-1 transcription factor (Eltzschig *et al*., 2009; Chalmin *et al*., 2012). Moreover, CD39 is upregulated in several solid tumors (colorectal cancer, head and neck cancer, pancreatic cancer) as well as in chronic lymphocytic leukemia (Bastid *et al*., 2012).

e-5'NT/CD73 is the key enzyme in the extracellular adenosine production, indeed catalyzing the hydrolysis of AMP to adenosine, it plays a crucial role in "switching on" adenosine signalling. Structurally, e-5'NT/CD73 is a glycosyl phosphatidylinositol (GPI) anchored membrane protein via a C-terminal serine residue (Ser 523), which belongs to the 5'-nucleotidase family that includes seven members; other forms of 5'NT exist in the cytoplasm and lysosomes and can be distinguished from e-5'NT/CD73 by their substrate affinities, requirement for divalent magnesium ion, activation by ATP, and inhibition by inorganic phosphate. The enzyme exists as a noncovalent dimer and each subunit (60-74 kDa) consists of two distinct domains linked by a single α-helix (residues 318–336): an N-terminal domain (residues 27–317), which binds two divalent metal ions important for catalysis, and a C-terminal domain (residues 337–549), responsible for binding the nucleotide substrate and by which the enzyme is anchored to the cell membrane (Misumi *et al*., 1990). The active site is created at the interface between the two domains and is formed from residues of both domains (Knapp *et al*., 2012). e-5'NT/CD73 exists in both open and

closed conformations. The single α helix comprises a small region, which makes able the enzyme to undergo large domain movements and switch between the open and closed conformations. Crystal structures of the dimeric human e-5'NT/CD73 show an extensive 114° conformational switch between the open and closed forms of the enzyme. The dimerization interface is formed by the C-terminal domains and exhibits interchain motions of up to 13°. The large domain motion, is required for catalysis (Knapp *et al*., 2012). This enzyme binds Zn^{2+} and other divalent cations which are required for the enzymatic activity and 5'-AMP is the preferred physiological substrate (Zimmermann, 1992). The fact that the main function of e-5'NT/CD73 is the generation of extracellular adenosine from AMP has been confirmed in CD73 knockout mice that show markedly impaired production of extracellular adenosine that is not compensated in vivo by other ectoenzymes such as alkaline phosphatases (ALP) and acid phosphatases (ACP) or by the release of adenosine via ENT. The hydrolysis of 5'-AMP is stereoselective since the L-enantiomer is not a substrate. K_m values for AMP are in the range of 3-50 µM (Zimmermann, 1992). ATP and specially ADP, which are the most common substrates of NTPDases, are potent inhibitors of this enzyme (Zimmermann, 1992). Soluble forms of the enzyme have been described (Coade and Pearson, 1989; Zimmermann *et al*., 2012) which are derived from the membrane-bound form by hydrolysis of the GPI anchor by phosphatidylinositol-specific phospholipase or by proteolytic cleavage (Yegutkin, 2008; Heuts, 2012). A subcellular expression of CD73 has been studied intensively in rat liver tissue where the molecule is expressed both intracellularly and on the surface of hepatocytes. A similar localization has been observed in rat fibroblasts, guinea pig neutrophils, and capillary endothelial cells (Widnell *et al*., 1982; Robinson and Karnovsky, 1983; Zimmermann, 1992).

e-5'NT/CD73 was recognized for the first time 82 years ago for its activity in the heart and skeletal muscle (Reis, 1934). Independently of its enzymatic role, e-5'NT/CD73 can function as a co-stimulatory molecule for T cells, mediate cell-cell adhesion, or may regulate cell interaction with extracellular matrix components and migration on them (Airas *et al*., 1995, Airas and Jalkanen, 1996; Airas, 1998; Loertscher and Lovery, 2002; Andrade *et al*., 2011). It is also considered to be related to the transport process, cellular growth, synthesis of fibrous protein and calcification, neurotransmission, and oxygen sensing mechanism (Moriwaki *et al*., 1999).
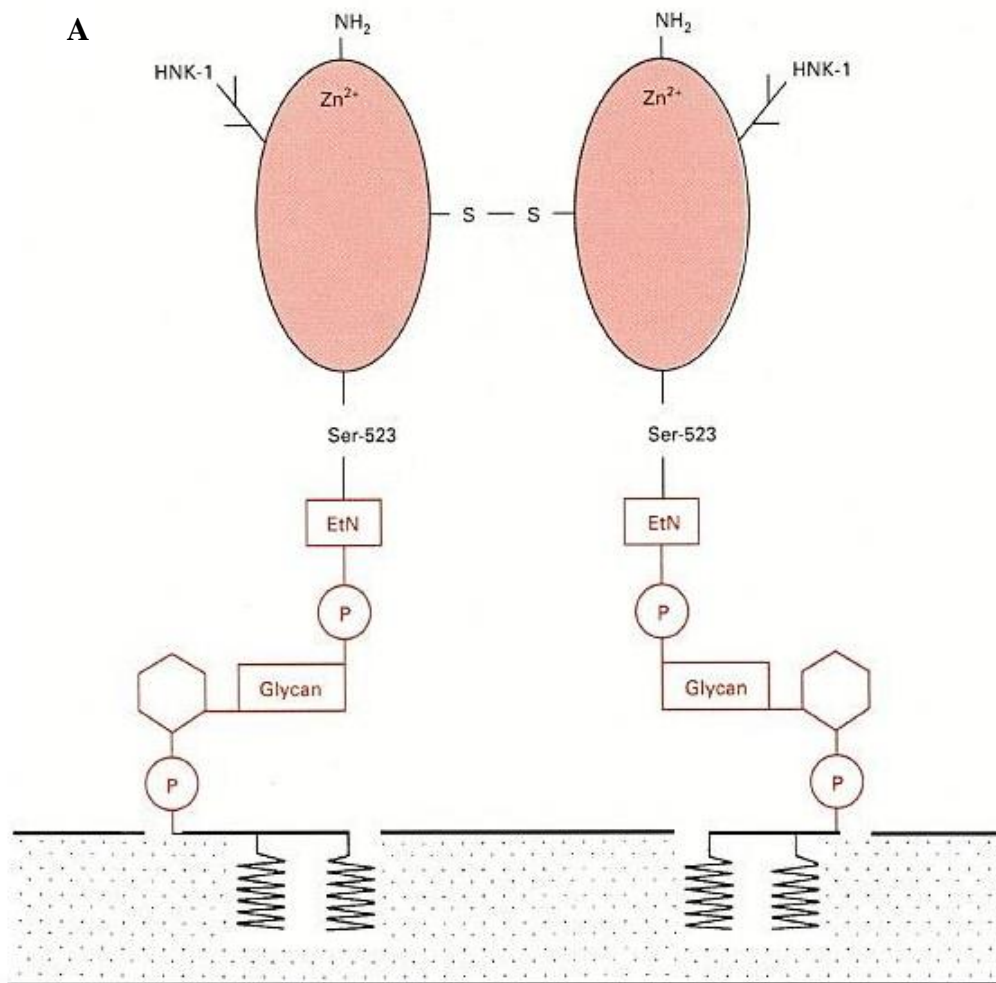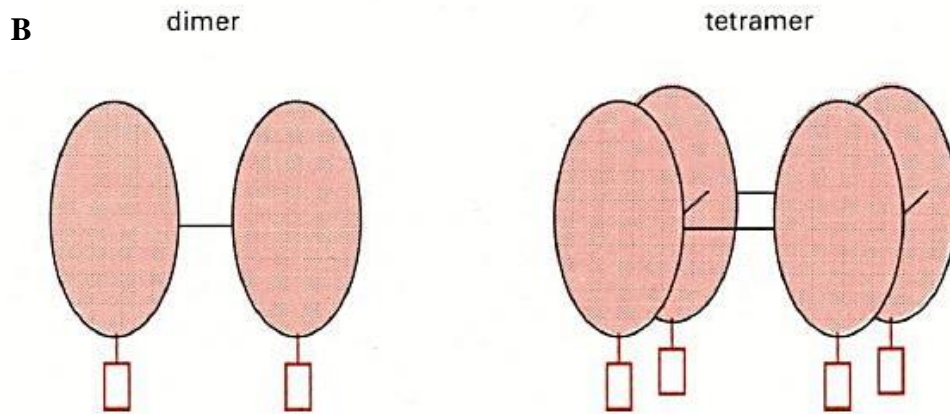
B   dimer   tetramer

Figure 9. A) Molecular model of e-5'NT/CD73 and B) the soluble forms derived from it. e-5'NT/CD73 exists as a dimer of the two subunits linked by at least one disulphide bridge. Soluble forms can exist as dimers or tetramers. From Zimmermann H. (1992) Biochem J. 285:345-65.
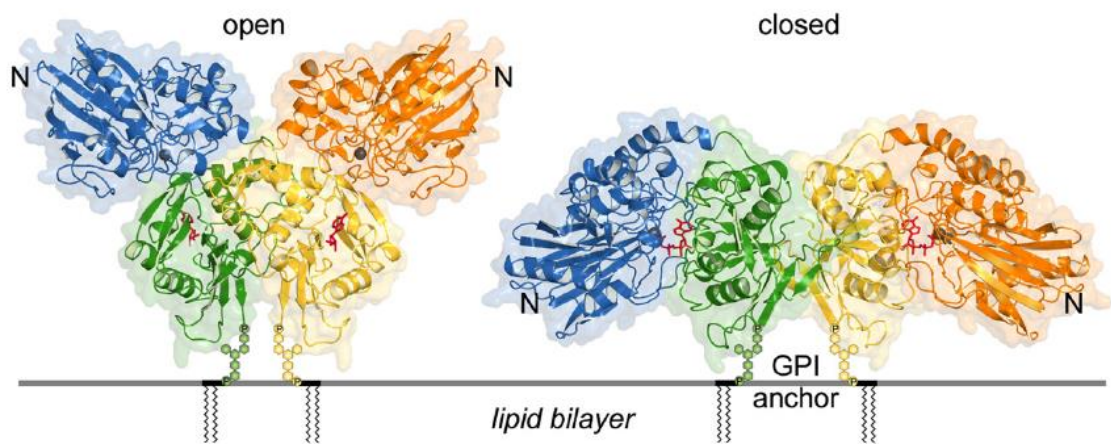
Figure 10. Human e-5'NT/CD73 crystal structure. From Knapp K, Zebisch M, Pippel J, El-Tayeb A, Müller CE, Sträter N. (2012) Structure. 20:2161-73.

CD73 is expressed in different tissues, including colon, pancreas, salivary glands, brain, kidney, liver, lung, heart (Zimmermann, 1992; Zimmermann *et al*., 2012). It has been suggested the importance of CD73 in the diagnosis of inflammatory muscle disease, malignancies and hepatobiliary diseases (Sunderman, 1990; Moriwaki *et al*., 1999) and as a marker of both general and local inflammation in rheumatoid arthritis patients (Johnson *et al*., 1999). Hypoxic conditions, as well as interferons, TGF-β1, IL-1β, PGE$_2$ and triiodothyronine are capable of inducing cell surface expression of CD73 (Savic *et al*., 1990;

Synnestvedt, 2002; Carneiro-Ramos *et al*., 2004; Niemelä *et al.*, 2004; Regateiro *et al*., 2011). Inflammatory cytokines, such as IL-4, IL-12, Interleukin-21 (IL-21) and IFN-γ, counteract CD73 expression induced by TGF-β1 (Regateiro *et al*., 2011). This suggests that inflammatory and anti-inflammatory milieu is important determinant of e-5'NT/CD73 expression and, therefore, of adenosine levels in the tissues. e-5'NT/CD73 is also up regulated in several neoplastic tissues (Beavis *et al*., 2012). Besides host cells, many pathogens (i.e. *Toxoplasma gondii*, *Escherichia Coli*) have CD39-CD73-like machinery, which helps the pathogen subvert the host inflammatory response.

1.2.2 CD39 and CD73 in inflammation

As above mentioned, several studies indicate that the generation of adenosine during the inflammatory response is protective functioning as a feedback loop to attenuate potential tissue injury. The role of adenosine in "switching off" inflammation has been an area of active research; more recently, researchers have focused on the importance of CD39/CD73 axis during the inflammatory response. Little is known of how NTPD1/CD39 and the e-5'NT/CD73 are themselves regulated during inflammation. Animals lacking these enzymes have revealed their importance in contributing to extracellular adenosine in different organs and situations showing circulating nucleotide levels and related pathology (Enjyoji *et al*., 1999). In agreement with a lack of their catalytic activity, amplified inflammatory response has been observed in CD39 and CD73-deficient mice (Eltzschig *et al*., 2004; Friedman *et al*., 2009; Reutershan *et al*., 2009; Haskó *et al*., 2011). Interestingly, NTPD1/CD39 and e-5'NT/CD73 are expressed by a broad range of immune cells, including monocytes, neutrophils, fibroblasts, dendritic cells, myeloid-derived suppressor cells, B lymphocytes, and some T-cell subsets. CD39 and CD73 can be considered as "immunological switches" by removing pro-inflammatory ATP and promoting an anti-inflammatory and immune

suppressive milieu (Antonioli *et al.*, 2013). It has recently been observed that CD73 and CD39 may also contribute in host inflammatory responses to microbial infection (Yegutkin *et al.*, 2010; Haskó *et al.*, 2011; Théâtre *et al.*, 2012).

1.2.3 Overall effect of CD39 and CD73 on cells of the immune system

1.2.3.1 CD39/CD73 and neutrophils

Neutrophils widely express CD39 (Pulte *et al.*, 2007) and, to some extent, CD73 (Flögel *et al.*, 2012) that play an important role in regulating their functions (Linden, 2006). There is evidence that NTPD1/CD39 and e-5'NT/CD73 limit neutrophil activation, chemotactic functions and neutrophil adhesion to the endothelium (Eltzschig *et al.*, 2004; Corriden *et al.*, 2008; Reutershan *et al.*, 2009; Kukulski *et al.*, 2011).

1.2.3.2 CD39/CD73 and mononuclear phagocyte system

It has been established that monocytes and macrophages express CD39 and CD73. CD39 has found on >90% of human monocytes (Pulte *et al.*, 2007) while only a small percentage (about 5%) of human monocytes (CD14$^+$CD16$^+$) express CD73 on their surface (Sciaraffia *et al.*, 2014). This CD14$^+$CD16$^+$ population of monocytes is increased in some disease conditions and is considered to be pro-inflammatory (Ziegler-Heitbrock, 2007). However, about 30% of monocytes and macrophages retrieved from the synovial fluid of arthritic mice do so (Flögel *et al.*, 2012). Furthermore, in CD73-deficient mice augmented tumor immunity has been linked to decreased numbers of intratumoral anti-inflammatory macrophages (Yegutkin *et al.*, 2011). Upregulation of IL-1β, IL-18, IL-6, TNF-α, tissue factor (TF) by these cells, alterations in regulatory macrophage development that leads to lethal inflammatory responses and septic shock have been observed in CD39-deficient mice (Lévesque *et al.*, 2010). Zanin and co-workers (2012) have found that, in mice, the pro-inflammatory phenotype M1 macrophages show reduced expression and activity of both

CD39 and CD73, leading to reduced ATP degradation. By contrast, the anti-inflammatory phenotype M2 macrophages, show an increased expression and activity of both catabolic enzymes, followed quickly by the conversion of ATP into adenosine. Thus, M2 macrophages generate an adenosine-rich environment, which in turn can augment the anti-inflammatory and tissue remodeling activities of these cells.

CD39 and CD73 are also expressed by human monocyte-derived DCs (Berchtold *et al*., 1999) and are involved in regulating the recruitment and activation of lymphocytes. Extracellular ATP is very rapidly hydrolyzed by DCs thus protecting the cells from ATP-induced apoptosis (Girolomoni *et al*., 1993). Differently to other myeloid leukocytes where the expression is developmentally regulated (Clifford *et al*.,1997), DCs express both enzymes during all maturation stages. The expression of CD73 and CD39 by DCs at all stages of their differentiation might be important to protect these crucial cells at their immature antigen processing stage in the peripheral tissues as well as at their mature, T cell sensitizing stage, in the lymphoid organs (Berchtold *et al*., 1999). TGF induces CD73 expression in DCs, enabling these cells to generate adenosine within an immune-regulatory microenvironment. Mizumoto and colleagues (2002) have established that Langerhans cells, DCs residing in the skin, from CD39 knockout mice do not hydrolyze ATP and ADP, unlike wild-type cells and that CD39 is required for optimal stimulation of hapten-reactive T cells in mice. Moreover, it has been demonstrated that the suppressive action of IL-27 on Th1 and Th17 responses is mediated, at least in part, by the induction of CD39 in DCs and the resulting accumulation of adenosine (Mascanfroni *et al*., 2013).

1.2.3.3 CD39/CD73 and endothelial cells

CD39 and CD73 are also expressed on endothelial cells. It is well known that CD39 and CD73 on endothelial cells regulate leucocyte trafficking (Jalkanen and Salmi, 2008) and play a role in the regulation of hemostasis by converting the local environment from a

prothrombotic ATP/ADP state, to an antithrombotic, adenosine rich environment (Atkinson *et al.*, 2006). Accordingly, alterations in the expression and activity of CD39 or CD73 result in disordered hemostasis and thromboregulation (Enjyoji *et al.*, 1999; Koszalka *et al.*, 2004). CD39 and CD73 are known to play an important role in leukocyte trafficking (Linden, 2006; Eltzschig *et al.*, 2004). In areas of ongoing inflammation, CD39 and CD73 expressed on the surface of endothelial cells, coordinate the metabolism of ATP to adenosine. Activated PMNs are a source of extracellular ATP that through two enzymatic steps results in the liberation of adenosine. Activated platelets constitute an additional source of extracellular adenosine. Adenosine generated, can activate surface endothelial adenosine receptors, particularly the $A_{2B}AR$ that enhances endothelial barrier function. These findings identify CD39/CD73 pathway as a protective innate mechanism to preserve the vascular integrity and to prevent intravascular fluid loss (Eltzschig *et al.*, 2004). It has been shown that leukocyte binding on endothelial cell triggers the inhibition of CD73 activity, thereby reducing local adenosine production which favors vascular permeability and leukocyte transmigration (Henttinen *et al.*, 2003). The importance of CD73 and CD39 on endothelial cell function has been largely evidenced by studies performed on mice lacking these enzymes. CD73 and CD39-deficient mice present an increased accumulation and adhesion of leukocytes to the vascular endothelium (Eltzschig *et al.*, 2004, Petrovic-Djergovic *et al.*, 2012; Takedachi *et al.*, 2008). In contrast to its role in vascular endothelium, CD73 does not affect the permeability of lymphatic endothelium (Ålgars *et al.*, 2011). It has been demonstrated that CD73 normally suppresses pro-inflammatory responses in human endothelial cells, indeed its depletion induces a similar response to pro-inflammatory stimuli such as the cytokine TNF-α, consisting in high levels of the leukocyte adhesion molecules Intercellular Cell Adhesion Molecule Type 1 (ICAM-1), VCAM-1 and E-selectin (Grünewald and Ridley, 2010). interferons (IFNs) I and II increase CD73

expression at the surface of endothelial cells, thus promoting adenosine generation and subsequent enhancement of endothelial cell barrier (Niemelä *et al*., 2004, 2008). In contrast, TNF-α may reduce the capacity of human endothelial cells to produce adenosine by reducing CD73 surface expression and activity; probably this could be an important mechanism of endothelial activation during inflammation (Kalsi *et al*., 2002).

1.2.3.4 CD39/CD73 and lymphocytes

Originally, CD39 and CD73 have been identified as markers of B cell activation (Rowe *et al*., 1982; Resta *et al*., 1998). Subsequently, their expression has also been demonstrated on resting B cells (Saze *et al*., 2013). On lymphocytes, CD73 and CD39 represent a maturation marker, being absent from the surface of both immature B and T cells (Thompson *et al*., 1986; Heilbronn and Zimmermann, 1995) and increasing their expression during T and B cell development. It has been demonstrated that human circulating B cells co-express CD39 and CD73 and adenosine receptors (ARs). Interestingly, in human B cell line, it has been observed a coordinated expression of $A_{2A}AR$ and CD73 (Napieralski *et al*., 2003). Recently, Schena and co-workers (2013) have shown that ATP is stored in secretory vesicles in human B cells and that may be released upon B cell receptor stimulation. This ATP may be hydrolyzed to adenosine by CD39 and CD73 expressed on B cells, inducing an autocrine adenosine signalling which facilitates immunoglobulin diversification via class switch recombination, an essential process in mounting a humoral immune response. Accordingly, patients with impaired class switching antibody responses present B cells deficient in CD73 expression (Schena *et al*., 2013). Moreover, CD39 and CD73 are highly expressed in murine $CD4^+$ $Foxp3^+$ Treg cells and have been widely used as markers of this T-cell subpopulation (Kobie *et al*., 2006; Deaglio *et al*., 2007). CD73 and CD39 expression is augmented upon TCR engagement. Human Treg cells express CD39 on their surface, but in contrast to murine Treg cells, CD73 is mainly localized in the cytosol (Mandapathil *et al*.,

2010). It has been suggested that CD73 is secreted from human Tregs and that in this form is able to convert AMP to adenosine (Antonioli *et al*., 2013). It is thought that CD39 allows the entrance of Treg cells into inflamed regions, where high levels of ATP are present and subsequently it confers protection against ATP-induced apoptosis. Adenosine produced in tandem by CD39 and CD73 locally by Tregs inhibits the activation of effector T cells and APCs through A_{2A} receptors (Deaglio *et al*., 2007; Romio *et al*., 2011). In addition, adenosine stimulating A_{2A} receptors, expressed on Tregs, elicits their expansion and increases their immunoregulatory activity (Ohta *et al*., 2012). Thus, dual expression of CD39 and CD73 not only serves as a phenotypic marker for Tregs, but also provides an autocrine feedback loop to enhance the anti-inflammatory functions of this subset of T cells. CD73 is also expressed by Th primed precursor (Thpp) cells (Yang *et al*., 2005; Kobie *et al*., 2006) that can produce adenosine and suppress the proliferation of $CD4^+$ or $CD8^+$ T cells (Kobie *et al*., 2006).

Although Th17 cells are known to promote tissue inflammation and autoimmunity, *in vitro* Th17 cells generated with the cytokines IL-6 and TGF-β, that are crucial for Th17 cell development, express CD39 and CD73 which, by producing adenosine, suppress T cell activation (Chalmin *et al*., 2012). Interestingly, such a function has been found only in Th17 cells induced by TGF-β *plus* IL-6, whereas those induced in the absence of TGF-β are not immunosuppressive. TGF-β - induced CD73 activity may be responsible for this difference. Additionally, in human and mice, CD39 is also expressed on pre-existing memory T cells of Th1-, Th2- and Th17-types with heightened alloreactivity (Moncrieffe *et al*., 2010; Zhou *et al*., 2009). It has been proposed that CD39 expression by memory T cells may contribute to "switch off" the ongoing inflammation and/or to save these cells from ATP-induced apoptosis/necrosis (Zhou *et al*., 2009).

Murine NK cells abundantly express CD39 mRNA, but very little CD73 mRNA; their presence suggests that extracellular adenosine can be produced during inflammation and modulate immune function. It has been reported that mice lacking CD39 are protected from acute vascular injury after hepatic ischemia/reperfusion injury because CD39 loss and alteration in ATP receptors activation abolish secretion of IFN-γ by NK cells in response to inflammatory mediators, therefore limiting tissue damage (Beldi *et al*., 2010). In the liver, CD39 is not expressed by quiescent sinusoidal endothelial cells (Beldi *et al*., 2010). In contrast, in organs, such as the kidney, where CD39 is highly expressed by the endothelium, it has been shown to be protective in ischemia/reperfusion injury. In resting human NK cells, the expression of CD39 is reported to be very low (~5%) (Pulte *et al*., 2007). NKT cells co-express CD39 and CD73 and it has been shown that knockout mice for CD39 are protected from Con A-induced hepatitis by increasing NKT apoptosis and modulating cytokine secretion *in vivo* (Beldi *et al*., 2008).

1.3 ADENOSINE SIGNALLING AND ANTI-INFLAMMATORY DRUGS

Interestingly, adenosine signalling pathway has also been shown to be required for the biological effect of several drugs that modulate the inflammatory/immune response. Indeed, there is evidence that CD73 is required for the anti-inflammatory effect of methotrexate (Montesinos *et al*., 2007); furthermore, the beneficial effect of IFN-β in patients affected by multiple sclerosis has been associated to CD73 upregulation at the blood-brain barrier (Niemelä *et al*., 2008); evidence shows that the anti-inflammatory effects of aspirin involves also adenosine accumulation, independently from cyclooxygenase (COX) inhibition (Cronstein *et al*., 1999). It has also revealed CD73 involvement in the beneficial effects exerted by statins (Meijer *et al*.; 2009, 2010) that, originally developed to improve lipid profile, have demonstrated a *surplus* of beneficial pleiotropic effects, including reduced inflammation. Very recently, it has been shown that in mice the anti-arthritic effect of exogenously administered fructose 1,6–biphosphate, an endogenous intermediate of glycolysis, involves CD39/CD73/adenosine signalling pathway (Veras *et al*., 2015).

1.3.1 Nimesulide

Nimesulide (*N*-(4-Nitro-2-phenoxyphenyl)-methane sulphonamide; Figure 11) is a Non-steroidal anti-inflammatory drug (NSAID), with a unique chemical structure, belonging to the sulphonamides class.
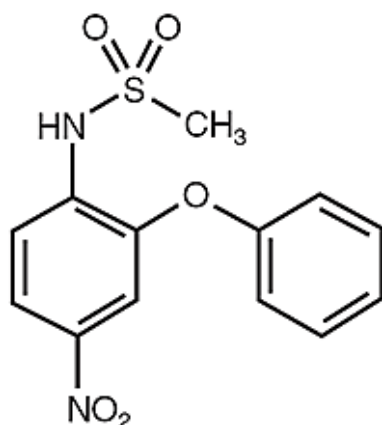
Figure 11. Chemical structure of nimesulide

Nimesulide has anti-inflammatory as well as antipyretic and analgesic activities. Like all NSAIDs, nimesulide blocks the biosynthesis of PGs starting from arachidonic acid, by inhibiting the enzyme COX. The clinical use of NSAIDs far preceded the characterization of their molecular target, the COX enzyme, that was identified by Vane Jr in 1971. In the early 1990s, a second isoform (COX-2) was discovered, distinct from the first one, then renamed cyclooxygenase-1 (COX-1) (Xie *et al*., 1991). COX-1 is constitutively expressed in most cells and elaborates beneficial "housekeeping" prostaglandins involved in various physiological processes, i.e. maintaining gastromucosal integrity, renal function and platelet aggregation, whereas COX-2 is induced by pro-inflammatory cytokines, tumor promoters and mitogens, at sites of inflammation/tissue injury; prostaglandins formed via COX-2 enzyme mediate pain, inflammation, fever (Seibert *et al*., 1994). However, recent discoveries call this paradigm into question and reveal unappreciated functions for both enzymes; for instance, recent studies have shown that COX-1 may contribute to the inflammation processes whereas COX-2 is constitutively expressed in several tissues and organs such as the brain, kidneys, endothelium and reproductive tissues. In addition, using a model of carrageenan-induced pleurisy in rats, Gilroy and colleagues (1999) have shown that COX-2 has a pro-inflammatory role in the early phase but an anti-inflammatory role in

the later phase of inflammation. The anti-inflammatory role has been associated with the production of cyclopentenone prostaglandins (prostaglandin D_2 (PGD_2) and 15 deoxy$\Delta^{12-14}PGJ_2$) and the absence of PGE_2 (Gilroy *et al*., 1999).
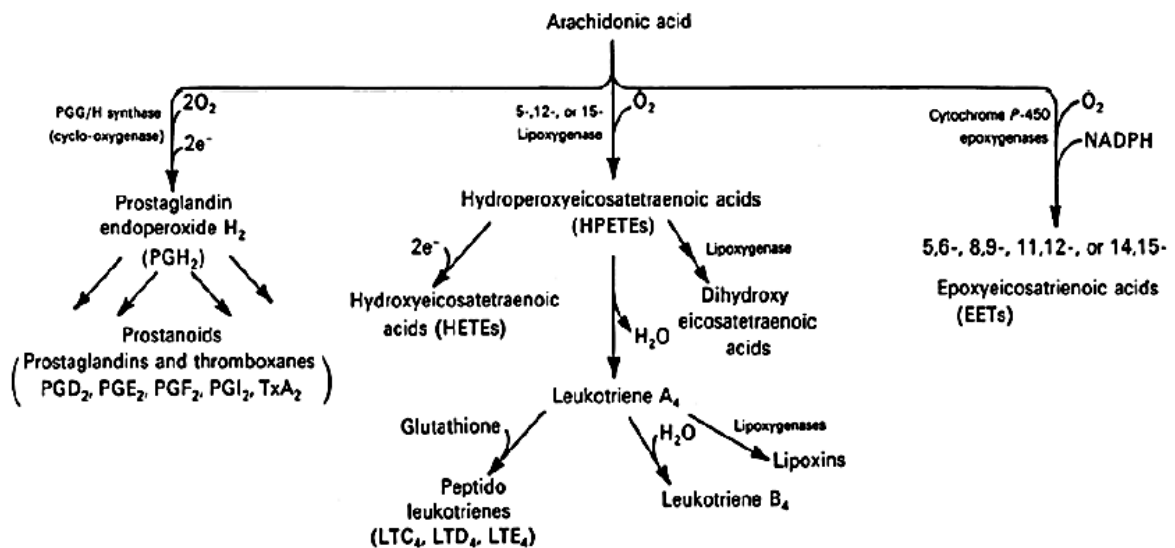
Figure 12. The Arachidonic Acid cascade. From Nathan C. (2002) Nature. 420:19-26. Various pathways lead to the formation of eicosanoids. The first step in their biosynthesis is the production of free arachidonic acid in tissues from membrane phospholipids upon stimulation of the enzyme phospholipase A_2 or from the hydrolysis of diacylglycerol by diacylglycerol lipase. There are then three main enzymatic cascades for eicosanoid formation, involving cyclooxygenases, lipoxygenases, and enzymes of the cytochrome P-450 family.

Human COX-1 and COX-2 are heme-dependent enzymes, membrane bound in the nucleus and endoplasmic reticulum in all cells except the erythrocytes. They are homodimers of 576 and 581 amino acids respectively, that show a high sequence homology (60%). Possessing two separate but linked active sites, the COXs catalyze the bis-deoxygenation and subsequent reduction of arachidonic acid (AA) to (PG) G_2 and PGH_2. Each subunit of the dimer consists of three domains, the epidermal growth factor domain (residues 34–72), the membrane binding domain (residues 73– 116), and the catalytic domain comprising the bulk of the protein, which contains the cyclooxygenase and peroxidase active sites on either

side of the heme prosthetic group. The cyclooxygenase active site is created by a long hydrophobic channel that is the site of NSAIDs binding. The arachidonate binding site is located in the upper half of the channel, from Arginine (Arg)-120 to near Tyrosine (Tyr)-385. The most significant difference between the isoenzymes is the substitution of isoleucine at position 523 in COX-1 with valine in COX-2. The smaller Val523 residue in COX-2 allows access to a hydrophobic "side-pocket" resulting in a larger (about 20%) and more accessible channel, in COX-2 (which Ile523 sterically hinders) that can accommodate bulky groups such as the methyl sulphonyl moiety of nimesulide. Indeed, nimesulide is a preferential COX-2 inhibitor (Famaey, 1997; Bennett and Villa, 2000).
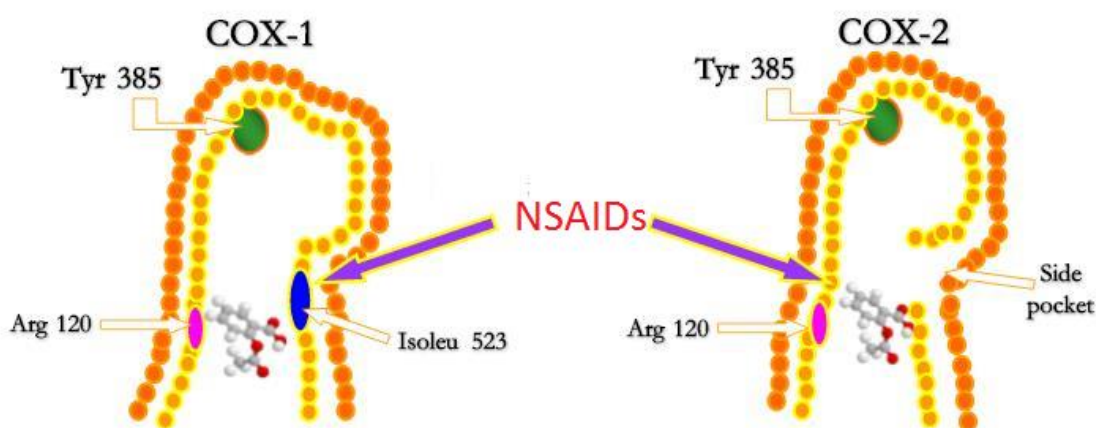
Figure 13. Differences in the NSAIDs binding sites of COX-1 and COX-2.

The therapeutic action of NSAIDs is produced by the inhibition of COX-2, while the undesired side effects occur from inhibition of COX-1 activity. It has been also characterized a COX enzyme, designated as cyclooxygenase-3 (COX-3), in dog brain which, unlike COX-1 and COX-2, is sensitive to inhibition with paracetamol. COX-3, is a 65-kDa membrane protein whose cyclooxygenase activity is about 80% lower than that of COX-1. Preferential expression of COX-3 in the brain and heart has been reported. COX-3 is considered to play a key role in the biosynthesis of prostanoids known to be important

mediators in pain and fever. Drugs that preferentially block COX-1 also appear to act on COX-3.

Nimesulide was invented by Dr. George Moore (a medicinal-organic chemist), Dr. Karl F Swingle (a pharmacologist), Dr. Bob Scherrer (a medicinal chemist) and their colleagues at Riker Laboratories Inc (Northridge, California, US, later part of the 3M Company at St Paul, Minnesota, US) in 1971. They aimed at finding a molecule to achieve oxyradical scavenging on the concept that free radicals are critical factors in chronic inflammatory disease. Since its first launch in 1985 in Italy as Aulin® and Mesulid®, nimesulide has been extensively used in Europe, South America and Asia; it has never been approved in USA, Japan, UK, Germany and Canada. It is available in a variety of forms: capsules, tablets, powder or granules for oral suspension, suppositories, mouth dissolving tablets and topical gel. Nimesulide has a relatively rapid onset of action, with significantly reductions in pain and inflammation observed within 15 minutes from drug intake, it has been the primary treatment choice for a rapid anti-inflammatory and analgesic effect (Rainsford, 2006). Nonetheless, in Spain, Finland, Belgium and Ireland nimesulide has been withdrawn from the market following reports of serious liver damages. A recent evaluation from the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMA) has concluded that the overall benefit/risk profile of nimesulide is favourable and in line with that of the other NSAIDs such as diclofenac, ibuprofen, and naproxen; but that there is a need to limit the duration of use to minimize the risks associated with nimesulide as hepatotoxicity (Franchi *et al*., 2015). The mechanism subtended to nimesulide hepatotoxicity is not known, it is thought that is an example of idiosyncratic drug toxicity. Most cases resolve in few days after stopping therapy. However, multiple instances of acute liver failure with death or need for emergency liver transplantation have been described. Current indications limit nimesulide use with a

prescription as a second line treatment of acute pain where acute inflammation is the most predominant component and primary dysmenorrhea for which the recommended dose is 100 mg twice daily for no more than 15 days in adolescents and adults above 12 years old. The use of nimesulide is contraindicated in patients with known hepatic impairment, during third trimester of pregnancy and in breast feeding women. Unlike other classical NSAIDs, it has high gastro tolerability due to its relatively high pK_a value (6.5) and preferential COX-2 selectivity (COX-2/COX-1=0.19; Famaey, 1997; Bennett and Villa, 2000). Nimesulide is generally well tolerated, adverse effects are mainly gastrointestinal (diarrhea, heartburn, nausea, loose motions); dermatological (rash, pruritus); and central (somnolence, dizziness) (Bjarnason *et al*., 2005). Nimesulide is well absorbed from the gastrointestinal tract following oral administration. The presence of food did not reduce either the rate or extent of nimesulide absorption. When nimesulide is administered in the suppository form, the C_{max} is lower and occurs later than after oral administration; the bioavailability of nimesulide via suppository ranged from 54 to 64%, relative to that of orally administered formulations. Around 99% is bound to plasma protein. Nimesulide undergoes extensive hepatic biotransformation, mainly to 4-hydroxynimesulide (which also appears to be biologically active). Minor metabolites have been detected in urine and faeces. The elimination half-life varies from 2 to 5 hours (Bernareggi, 1998). Since nimesulide is extensively bound to plasma proteins it can be displaced from binding sites by concurrently administered drugs such as fenofibrate, salicylic acid and tolbutamide. Nimesulide causes enzymatic induction of theophylline when administered concomitantly with it and reduces the diuretic effect of concomitantly administered furosemide (Perucca, 1993).

1.3.2 Nimesulide as atypical non-steroidal anti-inflammatory drug (NSAID)

Nimesulide is a unique drug, different from both non-selective and selective COX-2 inhibitors, not only due to its chemical structure. Indeed, while selective COX-2 inhibitors increase the risk for cardiovascular diseases, nimesulide does not exert significant cardiotoxicity. In addition, nimesulide has been shown to inhibit cancer cell proliferation (Shaik *et al*., 2004; Liang *et al*., 2009), to protect rats from cerebral ischemia (Candelario-Jalil, 2008) and to protect from NSAIDs-induced ulcers (Süleyman *et al*., 2002). Nimesulide has potent anti-inflammatory activity. Its mechanism of action is multifactorial with a unique and broad action on the inflammatory process which targets different pathways. Like all NSAIDs, nimesulide inhibits the synthesis of prostaglandins, chemical messengers that mediate inflammation. In addition, nimesulide has shown a variety of effects that are not due to COX-2 inhibition including:

➢ Reduction of toxic oxygen metabolites generation by stimulated PMNs (Ottonello *et al*., 1993; Bevilacqua *et al*., 1994).

➢ Inhibition of histamine release and action (Rossoni *et al*., 1993).

➢ Inhibition of collagenase and other metalloproteinases activity(Pelletier *et al*., 1993).

➢ Inhibition of LTB_4 and C_4 release (Tool *et al*., 1996).

➢ Inhibition of PAF production by activated cells (Tool *et al*., 1996).

➢ Inhibition of urokinase synthesis (Pelletier *et al*., 1997).

➢ Inhibition of cytokine release such as TNF-α, IL-6 (Pelletier *et al*., 1997).

➢ Phosphorylation and activation of glucocorticoid receptors (Pelletier *et al*., 1999).

➢ Reduction of neutrophils adherence and receptor expression (Rainsford and Members of the Consensus Report, 2006).

- Inhibition apoptotic process in connective tissue cells (Rainsford and Members of the Consensus Report, 2006).

- Inhibition of nitric oxide synthases (NOS) enzyme (Rainsford and Members of the Consensus Report, 2006).

Thus, the ability of nimesulide to affect so many relevant mediators in inflammation confers its potent anti-inflammatory activity with a biochemical mechanism that has not been elucidated at all, but it goes beyond COX-2-dependent prostaglandin synthesis inhibition. However molecular mechanisms at the basis of nimesulide peculiar pharmacological effects are still unclear (Rainsford, 2006).

1.4 ADENOSINE/A$_{2A}$ SIGNALLING AND FGF-2

1.4.1 Adenosine: a link between inflammation and wound healing

Inflammation is regarded as a key step in wound healing. Wound healing is a complex process involving soluble mediators, blood cells, extracellular matrix and parenchymal cells (Singer and Clark, 1999 ; Bullers *et al*., 2012). The process of inflammation to the wound healing is divided into three phases: 1) inflammation process that includes also hemostasis phase that represents the immediate response; 2) tissue formation and 3) tissue remodeling (Eming *et al*., 2007). Inflammatory cells are present during each of the phases of wound repair. Neutrophils, circulating monocytes that differentiate into mature tissue macrophages and mast cells are predominant in the early-stage. Cytokines, chemokines, growth factors, NO and large amounts of ROS produced by infiltrating immune cells during early inflammatory phase set the stage for tissue repair. In the late inflammatory phase of wound repair, T lymphocytes appear in large number in the wound with M2 macrophages. As inflammation resolves and the number of leukocytes diminishes, the wound undergoes a lengthy period of remodeling and resolution (Koh and Di Pietro, 2011). Persistent or exacerbated inflammation may interfere with wound healing by impairing the quality of healing. Complete resolution of an acute inflammatory response and the return of the local tissues to homeostasis are necessary for activation of subsequent steps of wound healing process. The overall balance of the pro and anti-inflammatory signals determine if the wound progresses to a chronic state.

A
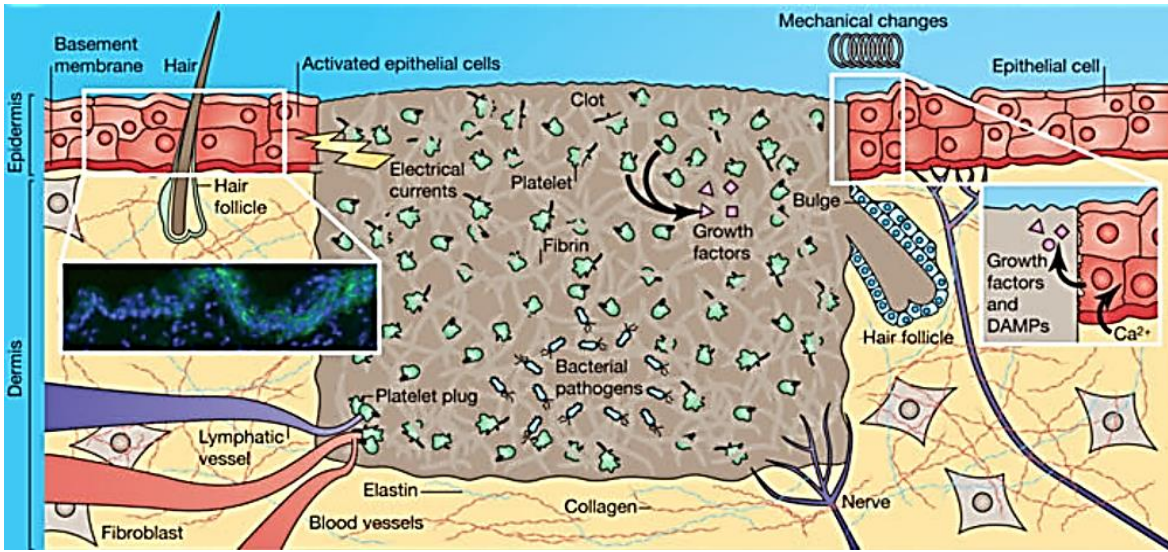
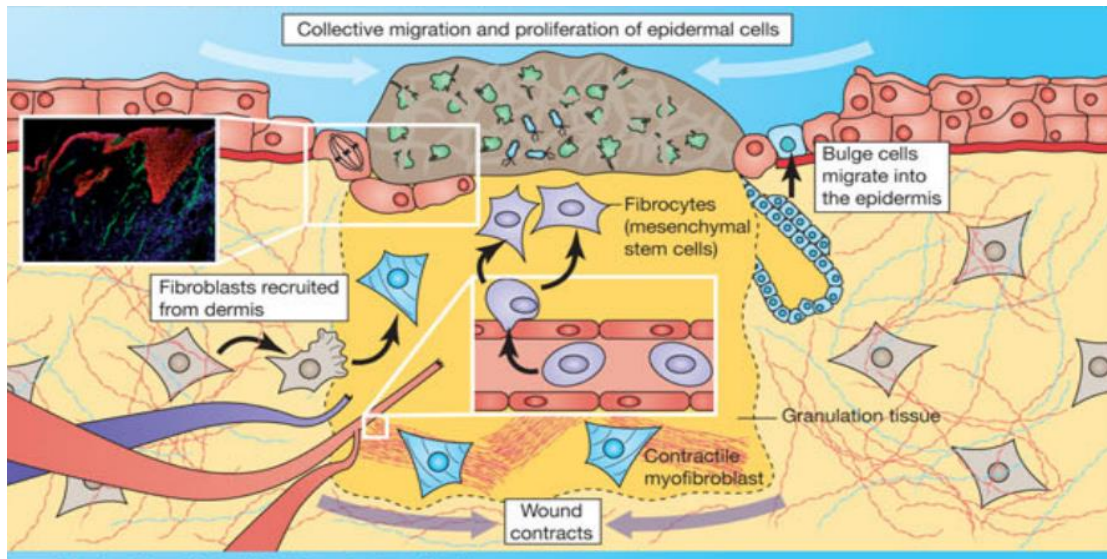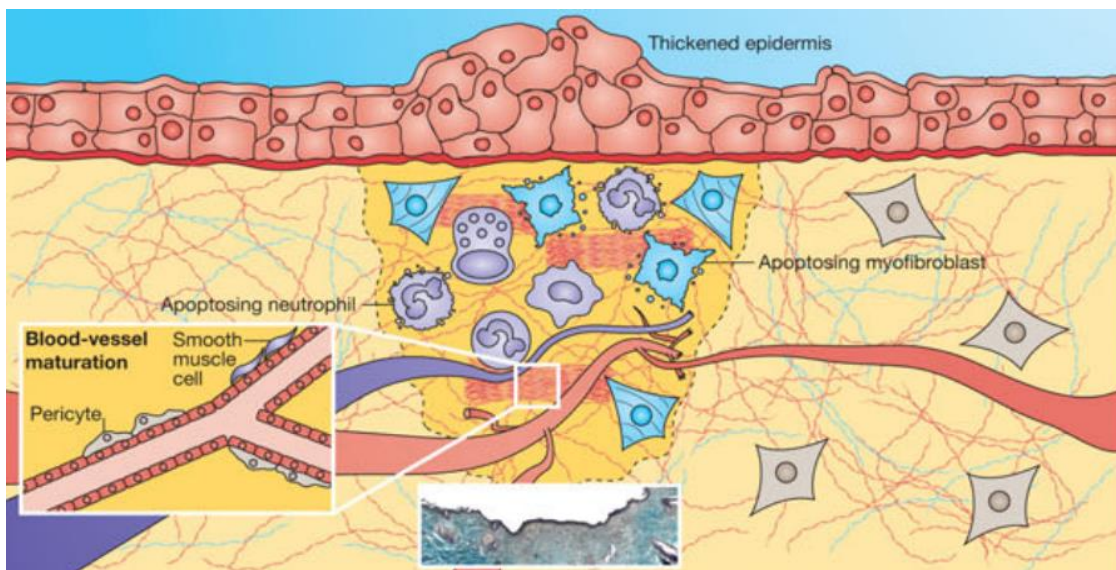**B**
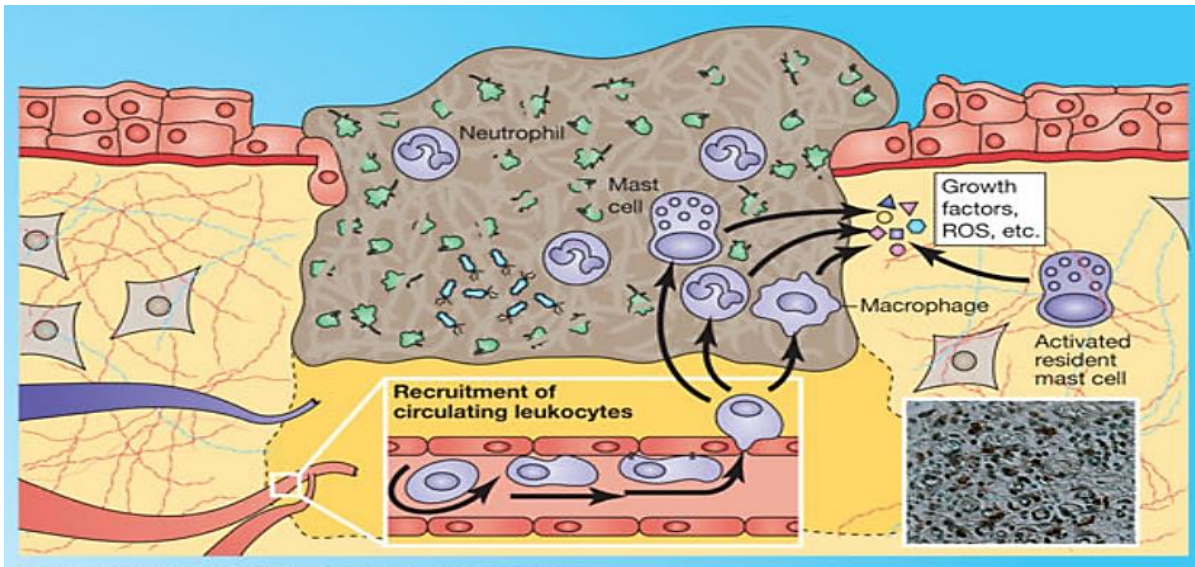
C

D

Figure 14. **Different Phases of Wound Healing.** From Shaw T, Martin P. (2009) J of Cell Sci 122:3209-13.
A) Immediate response **B**) Inflammatory response **C**) Proliferative phase **D**) Remodeling/Maturation Phase.

As already reported, adenosine during inflammation and tissue destruction is released into the extracellular space and acts as a negative regulator of both inflammation and immune-mediated tissue destruction. A_{2A} receptor has been described to be anti-inflammatory in several models of acute and chronic inflammation. Furthermore, the same receptor is also involved in adenosine-mediated wound healing. Montesinos and co-workers (1997) first reported that topical application of adenosine A_{2A} receptor agonists promotes more rapid wound healing in full-thickness dermal wounds in both normal mice and streptozotocin-induced diabetes mellitus rats, this effect is reversed by selective $A_{2A}AR$ antagonists. Later studies using mice lacking $A_{2A}AR$ and selective A_{2A} agonists confirmed that A_{2A} is the adenosine receptor most involved in promoting wound healing (Montesinos and Cronstein, 2001; Victor-Vega *et al*., 2002). It has been demonstrated that adenosine A_{2A} receptor activation promotes fibroblast migration *in vitro* and increases matrix production and fibroblast infiltration into the wounds (Montesinos *et al*., 1997). Moreover, adenosine promotes angiogenesis *in vitro* and *in vivo* both directly and indirectly by stimulating vascular endothelial growth factor (VEGF) production by macrophages and endothelial cells and by suppressing production of the antiangiogenic factor thrombospondin 1 (Leibovich *et al.,* 2002; Montesinos *et al*., 2002; Desai *et al*., 2005). In addition to directly stimulate collagen production by fibroblasts (Chan *et al*., 2006), adenosine also favors collagen production by promoting recruitment of bone marrow-derived fibrocytes from the circulation. In some situations, collagen production following adenosine A_{2A} receptor stimulation can be a two-edged sword; mice treated with subcutaneous bleomycin develop diffuse dermal fibrosis and both $A_{2A}AR$ knockout mice and mice treated with a selective $A_{2A}AR$ antagonist are protected from the development of bleomycin-induced dermal fibrosis (Chan *et al*., 2006); in addition, mice lacking adenosine deaminase show diffuse dermal fibrosis, which is blocked by adenosine A_{2A} receptor blockade (Fernández *et al*.,

2008). It is interesting to note that $A_{2A}AR$ activation modulates the functions of all the inflammatory cells observed at the site of a wound; adenosine, acting at its receptors, can suppress the pro-inflammatory functions of these cells so decreasing their deleterious effects and transform a potentially destructive response to regeneration response by increasing local vascularization and extracellular matrix deposition.

1.4.2 Fibroblast growth factor-2 (FGF-2)

Fibroblast growth factor-2 (FGF-2) also termed basic FGF (bFGF), is a small peptide belonging to the FGF family of heparin-binding growth factors (FGFs) that consists of 23 members. FGF-2 contains four cysteine residues at amino acids 26, 70, 88 and 93 with no intramolecular disulfide bonds, a large number of basic residues, and two sites (Ser 64 and Thr 112) that can be phosphorylated by PKA and C, respectively (Bikfalvi *et al*., 1997). Cysteines at 26 and 93 are conserved while those at 70 and 88 are absent or located elsewhere in other FGFs (Arakawa *et al.*, 1989). FGF-2 consists of 12 anti-parallel β-sheets organized into a trigonal pyramidal structure. FGF-2 exists in five different molecular weight isoforms: 18-kDa or low molecular weight (LMW) FGF-2, and 22-, 22.5-, 24- and 34-kDa or high molecular weight (HMW) FGF-2. The HMW forms derive from alternative translation initiation (CUG) codons and contain the complete LMW sequence in addition to an NH_2-terminal extension of varying length (Moscatelli *et al*., 1987; Sommer *et al*., 1987; Florkiewicz and Sommer, 1989; Prats *et al*., 1989; Okada-Ban *et al*., 2000). These isoforms are equally active, but while the HMW isoforms are predominantly localized to the nucleus and are not secreted, the 18-kDa form is mainly cytoplasmic and can also be released by cells (Bugler *et al*., 1991; Florkiewicz *et al*., 1991; Mignatti *et al*., 1991; Renko *et al*., 1990). 18-kDa is released from cells through an unclear mechanism that is independent of the endoplasmic reticulum (ER)-Golgi system lacking a classical signal peptide and involves exocytosis and requires ATP (Mignatti *et al*., 1992; Florkiewicz *et al*., 1995). The

release seems to be mediated by vesicle shedding at the plasma membrane or following cell damage (Taverna *et al*., 2003). Moreover, while 18-kDa form acts through activation of cell surface FGF-receptors, the HMW (22, 22.5, 24 and 34 kDa) FGF-2s signal independently of FGFR. In addition to inducing FGF receptor signal transduction, bound 18 kDa FGF-2 can be internalized, either in association with heparan sulfate or its receptor. Once internalized, FGF-2 can be degraded, possibly into 4-10 kDa bioactive fragments, or translocated to the nuclear fraction of various cell types as a receptor-ligand complex (Hawker and Granger, 1992; Clarke *et al*., 2001). As above mentioned, HMW forms of FGF-2 are associated with nuclear translocation. Entry into the nucleus is associated with FGF-induced effects such as casein kinase II activation, which is necessary for cell-cycle progression and proliferation. FGF-2 is generally readily sequestered to the extracellular matrix, as well as the cell surface, by heparin sulphate proteoglycans (HPSGs). FGF-2 is released from the extracellular matrix by heparinases, proteases or specific FGF-binding proteins, and the liberated FGF-2 subsequently bind to cell surface HPSGs. The biological activity of extracellular FGF-2 is mediated through a dual receptor system consisting of five high-affinity, tyrosine kinase receptors (FGFR-1 (flg), FGFR-2 (bek), FGFR-3, and FGFR-4, FGFR-6) and low-affinity, heparin and heparan sulfate proteoglycans located at the cell surface (Moscatelli, 1987; Flaumenhaft *et al*., 1989; Lee *et al*., 1989; Dionne *et al*., 1990; Keegan *et al*., 1991; Partanen *et al*., 1991; Okada-Ban *et al*., 2000). A sixth receptor, FGFR-5 (also known as FGFRL1), can bind FGF-2, but has no tyrosine kinase domain, and might negatively regulate signalling (Wiedemann and Trueb, 2000). However, it has been suggested that also intracellular FGF-2 might have a direct biological role, particularly within the nucleus. Heparin and cell surface heparan sulfate seem to dimerize monomeric FGF-2 that is subsequently able to dimerize and activate FGF receptors (Ornitz, 2000). It has been shown that heparin and heparan sulfate can also inhibit FGF-2 receptor binding and activity. Cells

that do not express heparin-like glycosaminoglycans show reduced receptor binding affinity and reduced biological response. However, it has been reported that FGF-2 may be able to signal in the absence of heparin-like glycosaminoglycans (Padera *et al*., 1999). Several cell types express fibroblast growth factor receptors (FGFRs) including fibroblasts, monocytes, macrophages, endothelial cells, keratinocytes, vascular smooth muscle, hematopoietic progenitors and epithelial cells. The FGFRs consist of three extracellular immunoglobulin (Ig) domains, a single transmembrane helix, and an intracellular split tyrosine kinase domain. The second and third Ig domains form the ligand-binding pocket and have distinct domains that bind both FGF-2 and HSPGs (Turner and Grose, 2010). FGF receptors signal as dimers and ligand-dependent dimerization leads to a conformational shift in receptor structure that activates the intracellular kinase domain, resulting in intermolecular transphosphorylation of the tyrosine kinase domains and the intracellular tail. Phosphorylated tyrosine residues on the receptor function as docking sites for adaptor proteins, which themselves may also be directly phosphorylated by FGFRs, leading to the activation of multiple signal transduction pathways including RAS–RAF–MAPK, PI3K–PKB, signal transducer and STAT and PLCγ (Turner and Grose , 2010). FGFRs have also been shown to bind and directly phosphorylate ribosomal S6 kinase. Signalling can be negatively regulated at several levels by receptor internalization or by the induction of negative regulators, including FGFR-L1, SPRY, (CBL), MAPK phosphatase 1 and 3 (Turner and Grose, 2010). Therefore, FGF-2 is a molecule with autocrine, paracrine and intracrine signalling mechanisms.

First discovered to stimulate the proliferation of fibroblasts (Holley and Kiernan, 1968) and isolated from bovine pituitary extracts by Armelin (1973) and then found in a cow brain extract by Gospodarowicz and colleagues (1975), subsequently, FGF-2 has been isolated from many tissues and cell types including visceral and vascular smooth muscle cells,

cardiac muscle cells, lining epithelium of the colon and bronchus, neurons, plus cerebellar Purkinje cells, megakaryocytes and platelets, endothelial cells, mast cells, glomerular parietal epithelial cells and podocytes, astrocytes, $CD4^+$ and $CD8^+$ T cells, fibroblasts (*plus* extracellular matrix), and numerous embryonic mesodermal and neuroectodermal tissues. The HMW FGF-2 expression may be absent or restricted even in tissues that produce FGF-2 protein. For example, in human epiphyseal growth plate cartilage, HMW FGF2 is expressed only in proliferating chondrocytes in contrast to LMW FGF-2. For a long time, FGF-2 has been known to only initiate fibroblasts proliferation. It is now proved that FGF-2 induces proliferation of many other cells including endothelial cells, cortical neurons, mesenchymal stem cells that can differentiate into mesenchymal lineages, including osteoblasts, chondrocytes, and adipocytes, smooth muscle cells (Raballo *et al*., 2000; Ahn *et al*., 2009). The function of FGF-2 is not restricted to cell growth. Recently, FGF-2 has been shown to affect differentiation, mobility, and survival of several cell types including fibroblasts, osteoblasts, smooth muscle cells, and neuroblasts. There is now evidence that FGF-2 is implicated in many biological processes including angiogenesis, embryonic development (brain, limb, lung, heart, muscle, bone, blood, eye and skin) wound healing and inflammation (Dono *et al*., 1998; Ortega *et al*., 1998; Montero *et al*., 2000; Böttcher and Niehrs, 2005; Jeon *et al*., 2007; Presta *et al*., 2009).

1.4.3 FGF-2 and inflammation

Inflammation and wound healing are closely integrated processes. As already mentioned, FGF-2 is a powerful mitogen for fibroblasts and plays a crucial role in wound healing. Fibroblasts are actively involved in the resolution of inflammation. Beside fibroblasts, other inflammatory cell types can express FGF-2, including mononuclear phagocytes, $CD4^+$ and $CD8^+$ T lymphocytes, and mast cells. Several studies have shown that there is an increased expression of FGF-2 in inflamed tissues; high levels of FGF-2 have been found in

bronchoalveolar lavage fluids from asthmatic subjects, in inflammatory bowel disease and in synovial fluid in severe rheumatoid arthritis (Manabe *et al*., 1999; Kanazawa *et al*., 2001; Redington *et al*., 2001). It has been proved that interferon IFN-α *plus* IL-2, IL-1β , and NO increase FGF-2 production and release (Cozzolino *et al*., 1993; Walford and Loscalzo, 2003; Lee *et al*., 2004c). Therefore, it has been suggested that FGF-2 is involved in the modulation of the inflammatory processes. Griffioen and colleagues (1996) have demonstrated the inhibition of cell adhesion molecules expression on Human Umbilical Vein Endothelial Cells (HUVECs) pre-treated with FGF-2 for 3 days. Zhang and Issekutz (2001) have confirmed these data showing that treatment for 24 hours or longer downregulates the expression of ICAM-1, VCAM-1, and monocyte chemotactic protein 1 (MCP-1) protein production, in addition, they have found that FGF-2 treatment of HUVECs for 18 hours temporarily increases expression of ICAM-1 and VCAM-1, but not E-selectin, in response to a variety of stimuli including cytokines. Therefore, they have suggested that FGF-2 may be an important positive regulator of leukocyte recruitment in acute inflammation and an anti-inflammatory regulator during chronic inflammation. Human recombinant basic fibroblast FGF-2 has shown to inhibit croton oil-induced ear swelling and carrageenan-induced paw oedema in mice and to reduce peritonitis induced by carrageenan in mice and rats by inhibiting NOS activity, by reducing NO, malondialdehyde, and PGE_2 content and by increasing superoxide dismutase (SOD) activity (Hu and Wu, 2001). Moreover, in a murine model of asthma, recombinant FGF-2 has been shown to reduce airway responsiveness, mucus production, and lung inflammation and also to reduce allergen-induced proliferation of T cells (Jeon *et al*., 2007). Although different studies suggest FGF-2 involvement in the inflammatory process, its role in inflammation is still unclear.

1.5 AIM OF THE STUDY

On the basis of a large amount of literature, as described, the CD39/CD73/adenosine axis is an important pathway that sense inflammation. Adenosine affects several cellular functions through the interaction with its receptors; for a long time the attention of researchers has been focusing on this molecule and its receptors and to date some functions of adenosine receptors have been clear revealed while for others remains a certain ambiguity. More recently, scientists have shifted their focus toward the two enzymes leading adenosine generation from ATP, but without losing sight of adenosine and its receptors; thus moving from the role of "adenosine in inflammation" toward the role of "CD39/CD73/adenosine axis in inflammation". Thus, it is clear as such a change of point of view confers importance to this pathway in the control of the inflammatory/immune response. In this respect, it is very important to keep in mind that inflammation is not a "self-confined" pathology but it is closely related to tumour, thrombosis, fibrosis and other diseases. Thus, it is easy to understand as CD39/CD73/adenosine axis has a place in several disorders. This has been further corroborated by evidence that several drugs, targeting different diseases all related to inflammation, affect CD39/CD73/adenosine axis.

To date, the anti-inflammatory therapy is mainly based upon an approach directed to inhibit inflammatory mediators. However, it is worth considering that inflammation, being a self-limiting process, is regulated by pro- and anti- inflammatory mediators, among the latter mediators, adenosine plays a role. On this basis, a better knowledge on the role of CD39/CD73/adenosine signalling within the network of molecules orchestrating the inflammatory response will help to consider different approaches to face inflammation, based on the stimulation of an endogenous anti-inflammatory pathway.

The aim of the present research work was to explore different aspects of adenosine signalling pathway in inflammation. We have first considered the role of CD73, the key enzyme in "switching on" adenosine signalling, in the development of inflammation using an *in vivo* model of inflammation represented by carrageenan-induced pleurisy in rats, a useful model to study the acute and the subacute phase of the inflammatory process.

As already described, the role of adenosine signalling pathway in the control of inflammation has been growing further following evidence that adenosine signalling is also involved in the mechanism of action of some well-known anti-inflammatory drugs. With regard to this, we focused our interest on the possible involvement of adenosine signalling on nimesulide anti-inflammatory effect. Indeed, nimesulide, among all COX-2 selective inhibitors, shows some peculiar effects that cannot only be ascribed to COX-2 inhibition. In addition, it is worth noting that nimesulide, as already described above, is not only devoid of gatrolesivity, such as all COX-2 selective inhibitors, but it may protect by FANS-induced gastric ulcer and, highly important, in contrast to the other COX-2 inhibitors, nimesulide does not increase the risk of cardiovascular disease. There is evidence that no single mechanism is sufficient to account for all the anti-inflammatory activities of nimesulide and its molecular mechanisms are not well defined (Süleyman *et al*., 2008). An early work has demonstrated that the inhibitory effect of nimesulide on neutrophil function *in vitro* is mediated by adenosine (Capecchi *et al*., 1993); furthermore, several nimesulide cell functions have been attributed to the increased cAMP production as the consequence of phosphodiesterase IV (PDEIV) inhibition. Recently, it has been shown that nimesulide potentiates the anti-rheumatic profile of methotrexate in collagen–induced arthritis in mice. Authors have hypothesised that both drugs might share a common mechanism involving adenosine release or adenosine receptor activation on immune cells (Al-Abd *et al*., 2010). Thus, for these findings, nimesulide has piqued our interest and we tested the hypothesis of

the involvement of CD73/adenosine/A_{2A} signalling pathway in the anti-inflammatory effect of nimesulide *in vivo* (rat paw oedema) and *in vitro* (J774A.1 cell line).

In the end, as already reported, physiological inflammation is a dynamic process, controlled by endogenous anti-inflammatory signals which are implicated in its development and resolution, adenosine is among them. It is well known that fibroblasts and extracellular matrix take an active part in the modulation of inflammation (Sorokin, 2010). There is evidence that fibroblasts, main producers of extracellular matrix components, can determine the nature and the duration of leukocyte infiltration (Buckley *et al*., 2001) and contribute to resolution of inflammation (Serhan *et al*., 2007). It has been shown that A_{2A}AR activation beside anti-inflammatory effects promotes wound healing and extracellular matrix production (Montesinos *et al*., 1997), suggesting a role for adenosine signalling either in the acute phase of inflammation, mainly characterized by vascular events and leukocyte trafficking, or in the late phase, characterized by cell proliferation and tissue regeneration. Thus, we investigated whether and how the extracellular matrix was involved in the anti-inflammatory effect mediated by A_{2A} receptor activation. For this purpose, we evaluated changes in tissue FGF-2 (18 kDa), an important growth factor for fibroblasts that has been shown to facilitate not only tissue regeneration but also to dampen inflammation, following systemic administration of the A_{2A} agonist, CGS 21680, in a model of acute inflammation, *in vivo* (rat paw oedema).

2. EXPERIMENTAL SECTION

2.1 CD73 AND ACUTE INFLAMMATION

2.1.1 MATERIALS AND METHODS

2.1.1.1 Reagents

APCP was purchased from Tocris Bioscience, (Bristol, UK); Malachite green phosphate assay kit was obtained from Sciencell, Research Laboratories (Carlsbad, USA); dithiothreitol (DTT), phenylmethylsulphonyl fluoride (PMSF) and leupeptine were purchased from ICN Pharmaceuticals (Italy). Bradford reagent was obtained from Bio-Rad Laboratories (Italy). enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D systems (Abingdon, UK). All salts and Giemsa reactive were purchased from Carlo Erba (Italy). Paraffin, Hematoxylin of Mayer solution and eosin 0.5% alcoholic solution were obtained from Kaltek (Padova, Italy). Polyclonal goat antibody to CD73 and monoclonal mouse antibody to β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-goat IgG and anti-mouse IgG conjugated to horseradish peroxidase were obtained from Dako (Denmark). enhanced chemiluminescence (ECL) system was purchased from Amersham Pharmacia Biotech (Milan, Italy). All other reagents and compounds used were obtained from Sigma-Aldrich (Milan, Italy).

2.1.1.2 Animals

Male Wistar rats, body weight 220-260 g, were purchased from Harlan Nossan, (Milan, Italy). All animals were allowed to access to food and water *ad libitum*. The light cycle was automatically controlled and the room temperature was thermostatically regulated to $22 \pm 1°$ C. Before the experiments, rats were housed and acclimatized under these conditions for

four days. All the *in vivo* procedures were performed according to the Italian and European regulations (DL/2014) on the protection of animals used for experimental and other scientific purposes and were approved by Italian Ministry of Health. Each experimental group consisted of five rats.

2.1.1.3 Carrageenan-induced pleurisy in rats

Male Wistar rats were slightly anaesthetized with 4% enflurane mixed with 0.5 L/min O_2, 0.5 L/min N_2O; following a small skin incision at the level of the left sixth intercostal space and underlying muscle dissection, pleurisy was induced by injecting 200 µL of λ-carrageenan solution 1% w/v (dissolved in distilled water) into the pleural cavity. Groups of rats were treated locally with the CD73 inhibitor (APCP, 400 µg/site), or with the respective vehicle (distilled water) immediately before carrageenan injection. The skin was then sutured and animals were returned to their cages and allowed to have food and water *ad libitum*. 4 and 72 hours following pleurisy induction, rats were sacrificed by CO_2 inhalation, the chest was carefully opened, and exudate was harvested by washing each pleural cavity with 2 mL of sterile saline containing 10 U/mL heparin. Any exudate with blood contamination was discarded. The total volume was measured, next aliquots were taken to analyze the content in cells, cytokines, and AMPase activity. The leukocytes in the exudate were suspended in saline and counted with TC20™ Automated Cell Counter (Bio-Rad, Italy) after vital trypan blue staining and centrifugation (1200 rpm for 10 min); supernatants and cell pellets were frozen at -80°C, whereas differential cell count was determined in smears by May-Grunwald-Giemsa staining.

2.1.1.4 Western blotting analysis

The inflammatory exudates recovered from the pleural cavity were washed twice in saline and the resulting suspensions were pelleted by centrifugation at 1200 rpm for 10 min at 4°C. The cell pellets were resuspended in 50 μL of ice-cold lysis buffer (200 mM HEPES, 400 mM NaCl, 1 mM DTT, 1 % igepal, 20% glycerol, *plus* the protease inhibitor cocktail Roche) and maintained in constant agitation for 45 min at 4°C. Then the suspension was centrifuged at 14000 rpm for 10 min in a 4° C pre-cooled centrifuge and the supernatant (whole extract) was collected and stored at -80° C until analysis.

Lung tissue was immediately transferred into a tube preloaded with one (6.35 mm) diameter zirconium oxide coated ceramic grinding sphere together with ice-cold lysis buffer (Tris-HCl ,50 mM pH 7.5; NaCl, 150 mM; sodium orthovanadate, 1 mM; β glycerophosphate, 20 mM; ethylenediaminetetraacetic acid (EDTA) , 2 mM; PMSF, 1 mM; leupeptin, 5 μg/mL; aprotinin, 5 μg/mL; pepstatin, 5 μg/mL) and put into a FastPrep®-24 homogenizer (MP Biomedicals, Santa Ana, California, USA) for lysis. The settings were 6.5 (speed) for 2x20 s. In between the runs, the tube was cooled on ice for 5 min. The sample was then centrifuged for 15 min at 12000 rpm at 4° C and the supernatant (whole extract) was collected and stored at -80 °C until analysis. Protein concentration was determined by Bradford assay according to manufacturer's instructions. Protein samples (35 μg) were mixed with 4X gel loading buffer (250 mM Tris, 8% sodium dodecyl sulphate (SDS), 40% glycerol, 20% 2-mercaptoethanol, 10 mg /mL of bromophenol blue) and heated for 5 min at 95°C. Samples were then electrophoresed in an 8% discontinuous polyacrylamide gel and then transferred onto nitrocellulose membranes. Correct gel loading and transfer of proteins to the nitrocellulose membrane were confirmed by Ponceau staining. Afterwards, nitrocellulose membrane was blocked using a blocking solution containing 5% (w/v) non-fat dry milk in phosphate buffer saline (PBS) supplemented with 0.1% (v/v) tween 20, for 30

minutes at room temperature. It was then incubated overnight at 4° C on a shaker with an anti CD73 polyclonal goat antibody (1:200 dilution). The nitrocellulose membrane was washed three times for 10 minutes and then incubated for 2 h at room temperature with the secondary antibody anti-goat IgG (1:2000 dilution) conjugated with peroxidase. After three washes, protein bands were detected using the ECL detection kit and Image Quant 400 GE Healthcare software (GE Healthcare, Italy). Protein bands were quantified using Quantity One® software (Bio-Rad, Italy). Successively, to confirm the equal protein loading, membranes were stripped and incubated with anti β-actin monoclonal antibody (1:2000 dilution) and subsequently with anti-mouse IgG conjugated to peroxidase (1:2000 dilution), both for 3 h at room temperature.

2.1.1.5 Migration assay

Chemotaxis was evaluated on cells obtained from pleural exudate collected 4 h after carrageenan injection in a 48-well modified Boyden chamber (AP48, Neuro Probe, USA). In brief, exudates were centrifuged at 1200 rpm for 10 min and cell pellets were suspended in saline and following trypan blue staining, were counted with TC20™ Automated Cell Counter (Bio-Rad, Italy). To evaluate chemotaxis, 50 μL of cell suspension (250 x 10^3 cells in Roswell Park Memorial Institute (RPMI-1640) medium containing 0.1% bovine serum albumin (BSA) were placed on top of the polycarbonate filter (8 μm pore size, Neuro Probe, USA) whereas 25 μL of chemoattractant N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 1ng/mL in RPMI-1640 medium containing 0.1% BSA) were added to the lower wells. In another set of experiments, cells obtained from control group of rats were also pretreated with APCP 1 and 5 μM. Spontaneous migration was determined using RPMI-1640 medium (containing 0.1% BSA) alone. Each condition was set up in triplicate. Chambers were incubated for 1.30 h at 37°C in humidified air containing 5% CO_2. Following incubation, the chamber was disassembled and the filter was carefully removed

and washed with sterile PBS. Cells that failed to migrate through the filter were wiped off the top surface of the filter. Migrated cells on the filter were fixed and stained with DAPI (Vector Laboratories, Burlingame, Ontario, Canada), and migration was quantified as the total pixel count of DAPI stained nuclei under the fluorescence microscope using Fiji software. Migration index was determined by dividing the number of cells migrated in the experimental condition by the number of cells migrated in the negative control cultures (medium only).

2.1.1.6 Cytokine measurement

TNF-α, IL-1β, IL-6, MCP-1, IFN-γ, IL-17 levels were evaluated in the exudate collected 4 and 72 h after the induction of pleurisy. The assay was conducted by using ELISA kits according to the manufacturer's instructions.

2.1.1.7 Morphological analysis

Morphological analysis was performed on tissue samples from sham rats, APCP treated rats and control group of rats, to evaluate the effect of CD73 inhibition on lung morphology following carrageenan-induced pleurisy. Lung biopsies were taken at 4 and 72 h after carrageenan injection and fixed for 1 week in formaldehyde solution (4% v/v, in distilled water) at room temperature, afterwards, tissues were processed and embedded in paraffin. Successively, in order to perform morphological analysis, tissue sections (7 μm thick) were deparaffinized with xylene and stained with haematoxylin and eosin. A minimum of 5 sections *per* animal was analyzed by using a standard light microscope (original magnification: x 20) and photographed by a Leica DFC320 video camera (Leica,Milan, Italy) connected to a Leica DM RB microscope by using the Leica Application Suite software V 4.1.0.

2.1.1.8 AMPase activity

AMPase activity was assessed in cells and lungs collected from rats 4 and 72 h following pleurisy induction, as a measure of e-5'NT activity, by colorimetric measurement of the Pi released following incubation with the substrate, as described by Nedeljkovic $et\ al$., (2006). In brief, on the day of analysis, protein extraction from inflamed cells and lungs were performed as described above. To initiate the enzymatic reaction, samples (50 μg of proteins) were incubated with 200 μL of medium containing MgCl$_2$ (10 mM), NaCl (120 mM), KCl (5 mM), glucose (60 mM), Tris-HCl (50 mM), pH 7.4. After 10 min, AMP (2 mM) was added as substrate and samples kept at 37°C for 40 min. The reaction was then stopped by the addition of trichloroacetic acid (final concentration 5% w/v). Following sample centrifugation at 3000 rpm for 10 min, at 37°C, the release of Pi was measured using malachite green as a colorimetric reagent and KH$_2$PO$_4$ as standard (Chan $et\ al$., 1986). To have the net value of Pi produced following enzymatic reaction, aspecific Pi released in the absence of AMP in each sample was evaluated and the value obtained was subtracted from the value obtained following incubation with AMP. Protein concentration was measured by Bradford assay, using BSA as standard and results were expressed as Pi released (pmol/min/ μg protein). AMPase activity was also assessed in exudates collected from rats 4 and 72 h following pleurisy induction as a measure of soluble-5'NT activity by performing the same procedure described above.

2.1.1.9 Statistical analysis

All data are presented as means ± standard error (S.E.); statistical analysis was performed on raw data by means of Student's t-test for unpaired data or by one-way ANOVA followed by Bonferroni post-hoc test. A p value < 0.05 was considered statistically significant.

2.1.2 RESULTS

2.1.2.1 Leukocyte accumulation

Local treatment with CD73 inhibitor, APCP (400 μg/site), significantly increased cell accumulation into the pleural cavity 4 h following carrageenan injection (Figure 15 A). There was no difference in the leukocyte number from exudates collected at 72 h after carrageenan injection from APCP-treated and control rats. Differential cell count of leukocytes migrated into the pleural cavity showed that PMNs dominated the early phase (4 h) of the reaction and were replaced by monocytes (MCs) at 72 h (Figure 15 B; C).
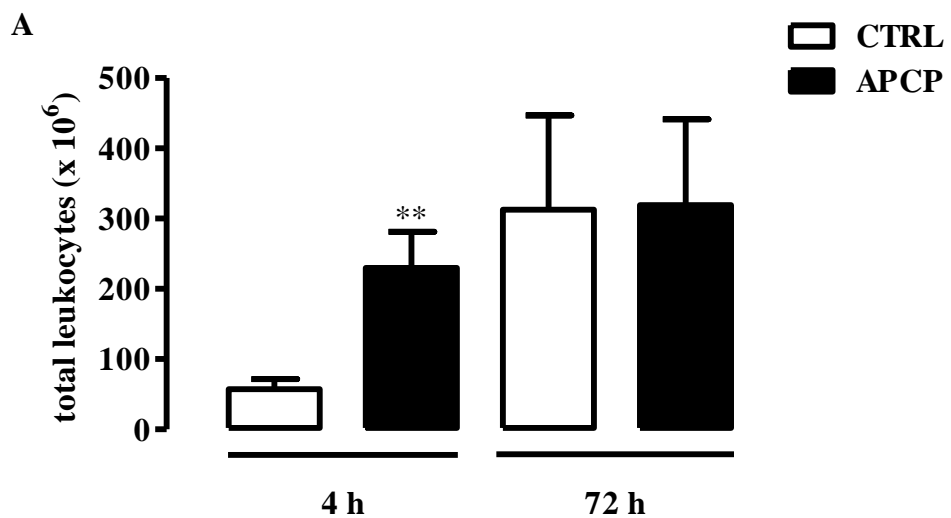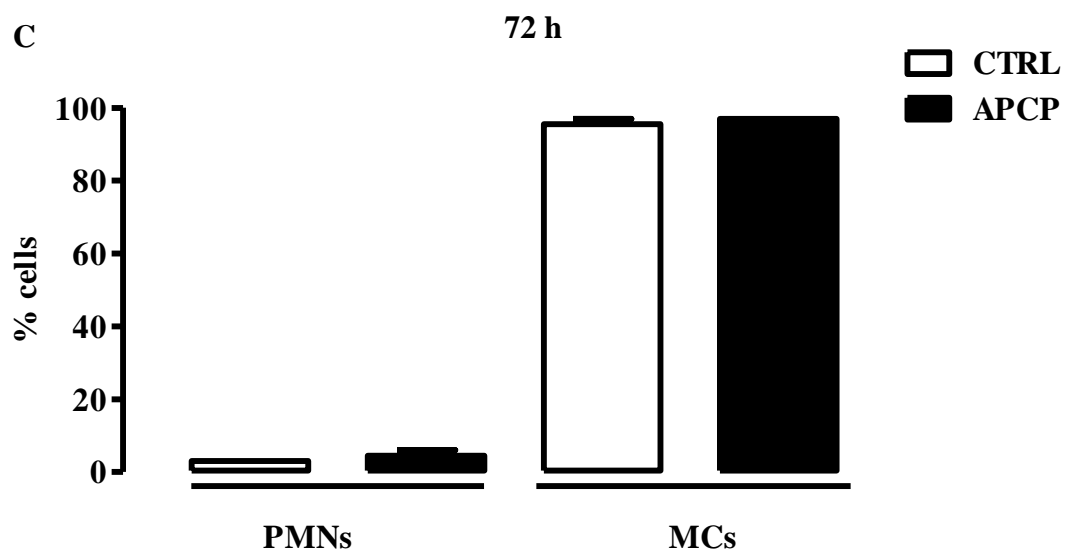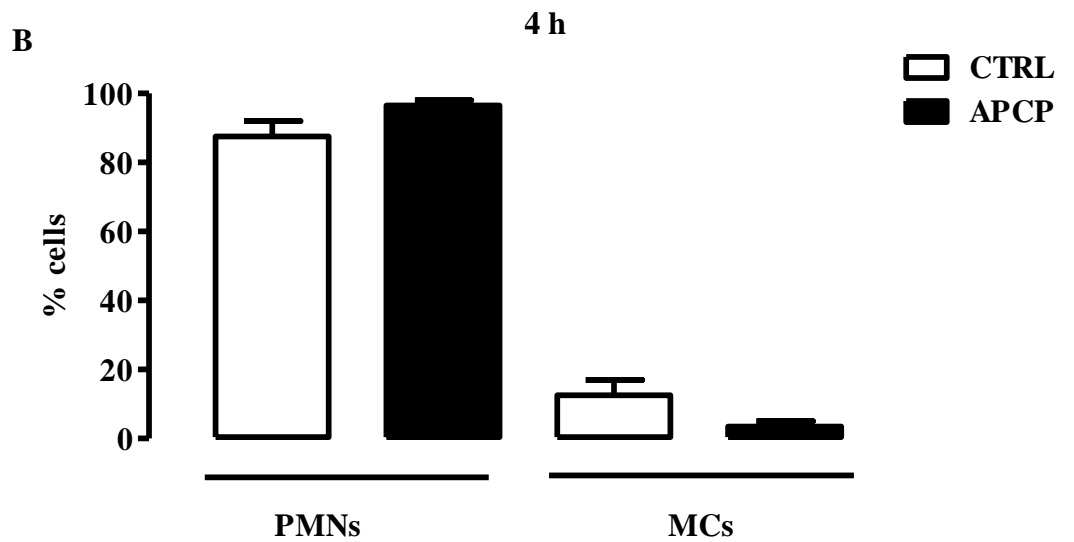
Figure 15. Effect of APCP on leukocyte infiltration into the pleural cavity 4 and 72 h following carrageenan-induced rat pleurisy. A) Total leukocytes; B) and C) differential cell count. **p<0.01 *vs* ctrl. Student's t-test (n=8).

2.1.2.2 Exudate formation

Local treatment with APCP (400 μg/site) significantly increased the volume of exudate into the pleural cavity 4 h following carrageenan injection. There was no difference in exudate volume collected at 72 h after carrageenan injection from APCP-treated and control rats. (Figure 16).
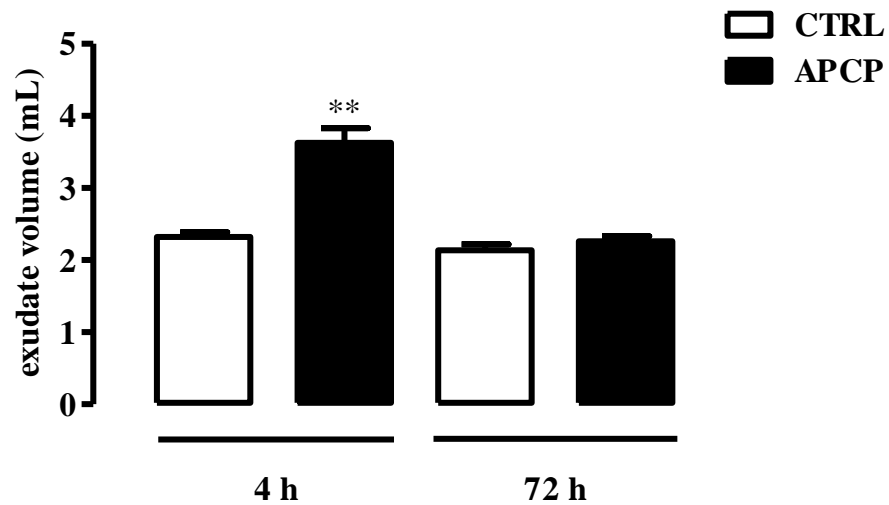
Figure 16. Effect of APCP on exudate volume collected from the pleural cavity at 4 and 72 h after carrageenan injection. **p<0.01 *vs* ctrl. Student's t-test (n=8).

2.1.2.3 Cytokine measurement

Following local treatment with APCP there was a significant increase in TNF-α, IL-6, IL-1β and MCP-1 levels evaluated in exudates collected from the pleural cavity 4 hours following pleurisy induction (Figure 17). In contrast, cytokines levels in exudate were not increased 72 h following carrageenan-induced pleurisy except for MCP-1 whose levels were still significantly increased by local treatment with APCP (400 μg/site) (Figure 18).
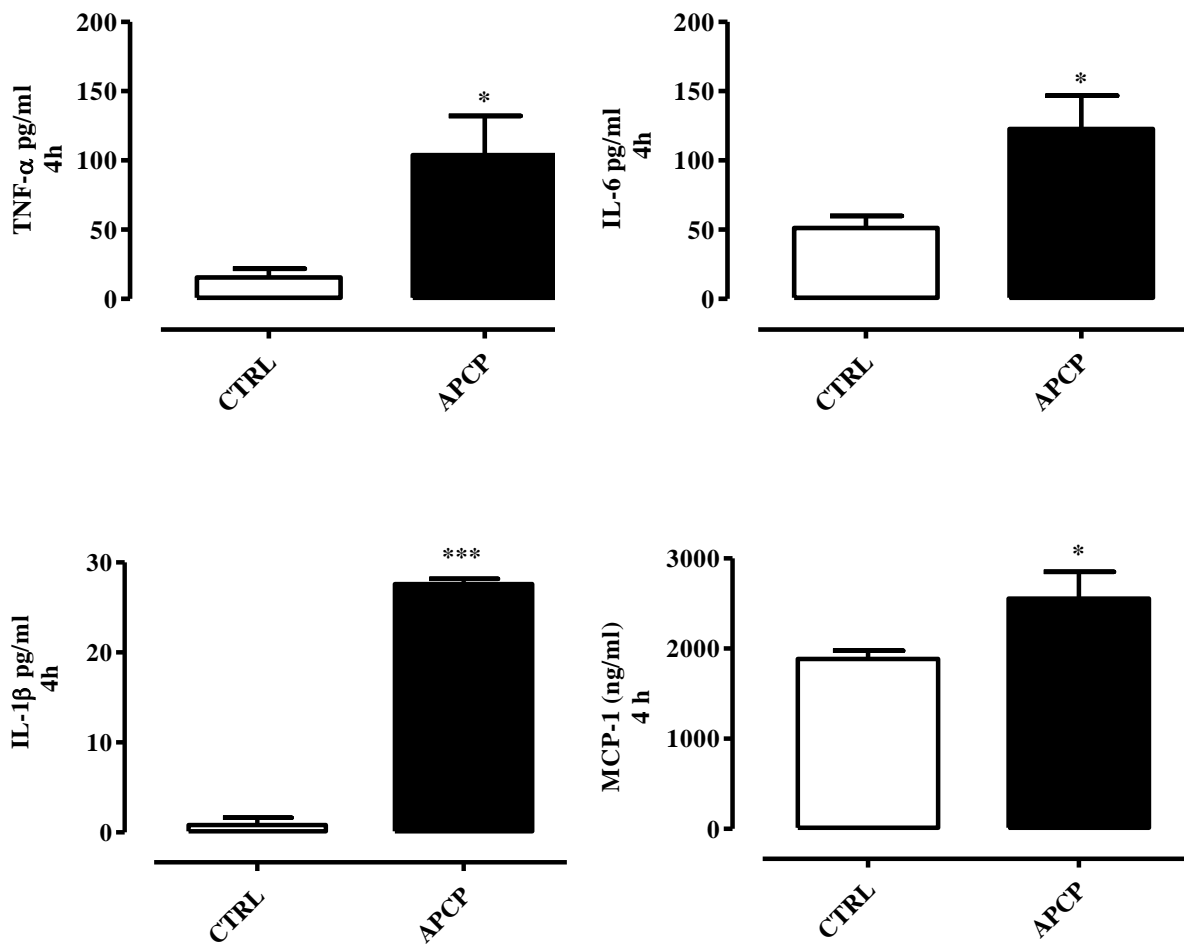
Figure 17. Effect of APCP on levels of TNF-α, IL-6, IL-1β and MCP-1 in exudates recovered from the pleural cavity 4 h following carrageenan-induced rat pleurisy . *p< 0.05;***p<0.001 *vs* ctrl. Student's t-test (n=3).

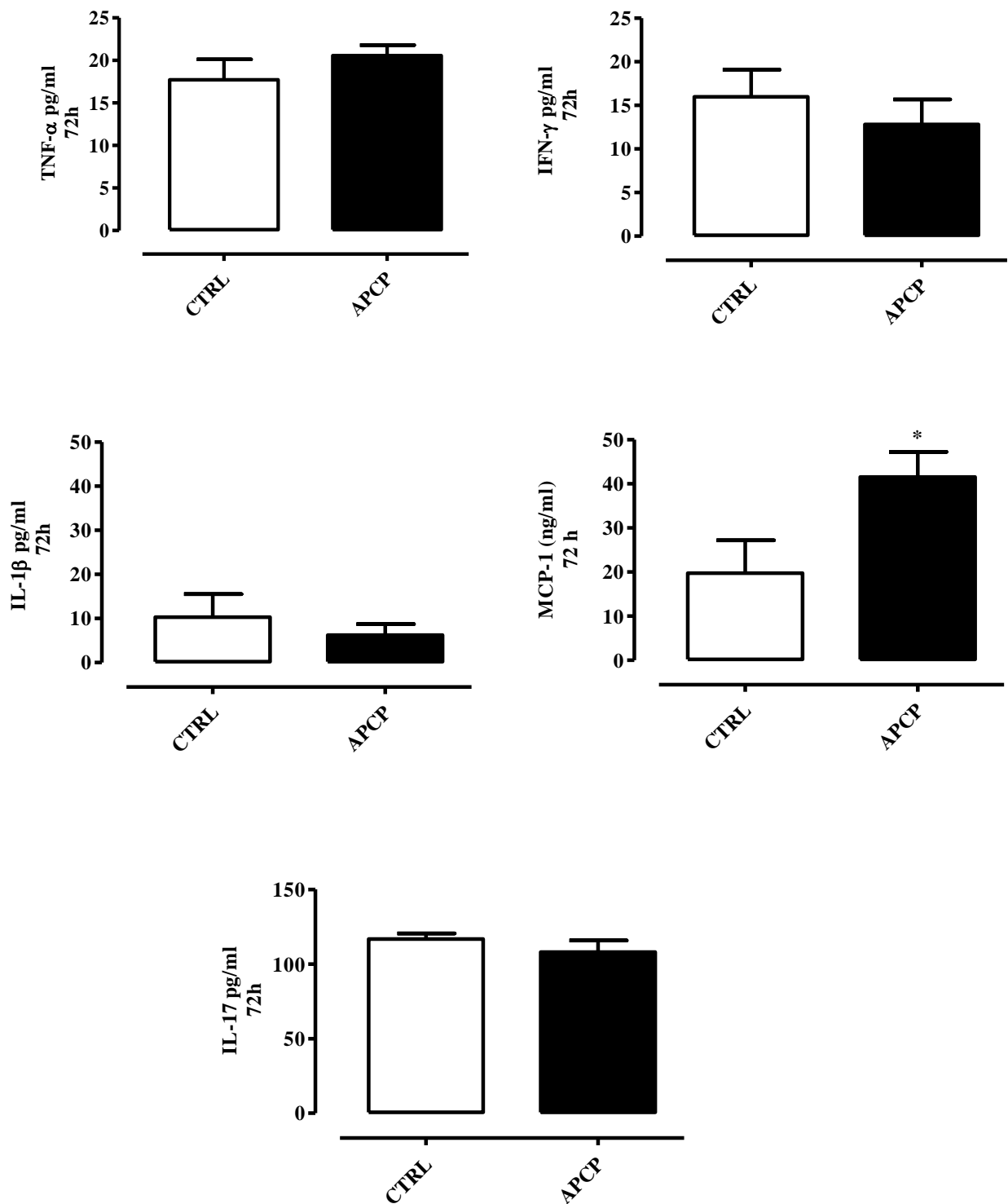**Figure 18. Effect of APCP on levels of TNF-α, IFN-γ, IL-1β, MCP-1, IL-17 in exudates recovered from the pleural cavity 72 h following carrageenan-induced rat pleurisy.** *p< 0.05 *vs* ctrl. Student's t-test (n=3).

2.1.2.4 Western blotting analysis

Western blotting analysis performed on cells recruited into the pleural cavity at 4 after carrageenan injection revealed a reduced CD73 expression from APCP treated group of rats (Figure 19 A). In contrast, there was no difference on cells from exudates collected 72 h following pleurisy induction from APCP-treated and control rats (Figure 19 B).
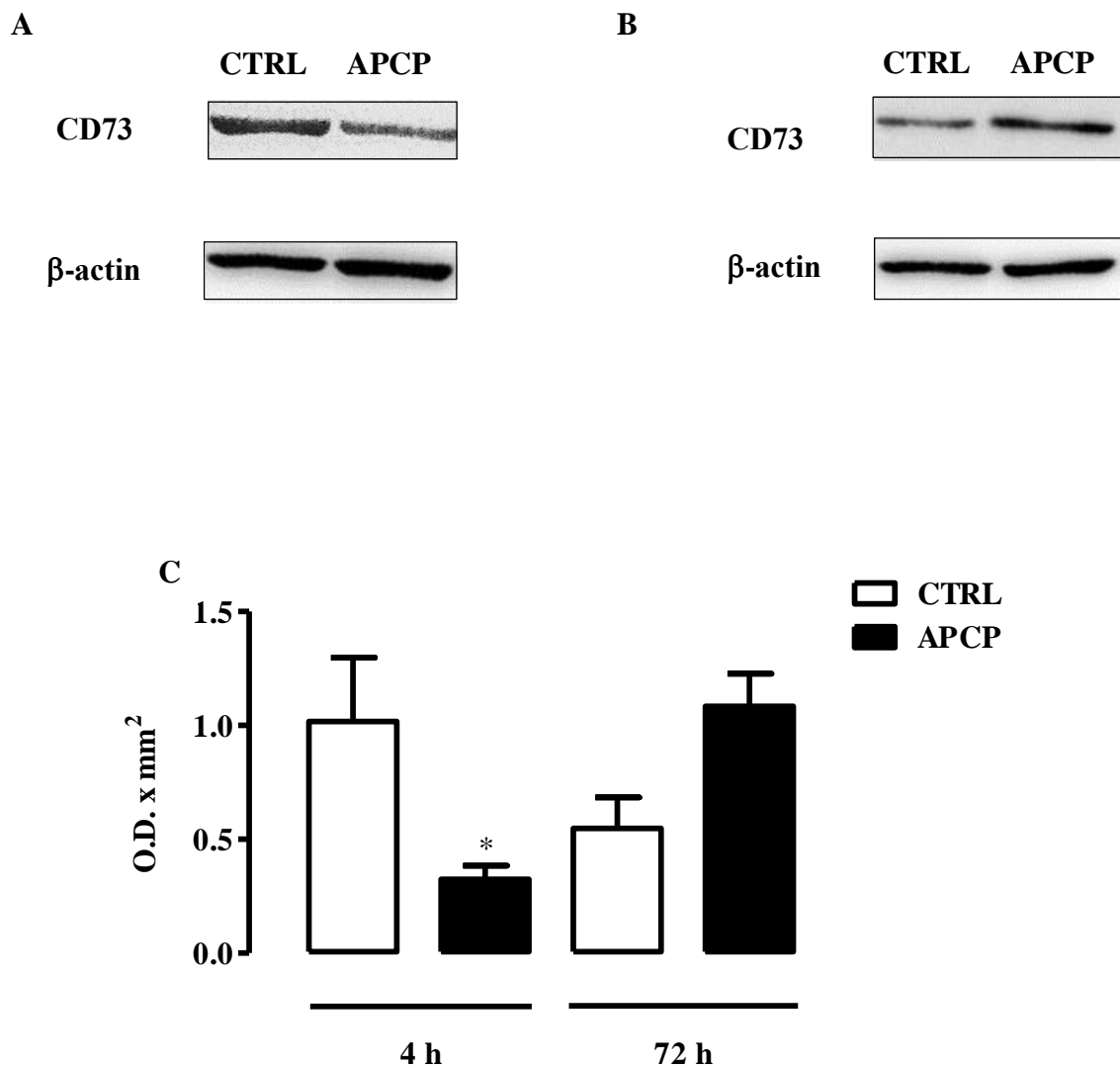
Figure 19. Detection of CD73 by western blotting analysis on cells collected from pleural exudates 4 h (A) and 72 h (B) following carrageenan-induced rat pleurisy and C) densitometric analysis (O.D. optical density). *p< 0.05 *vs* ctrl. Student's t-test (n=5).

Western blotting analysis performed on lungs collected 4 and 72 h following carrageenan-induced pleurisy from APCP treated group of rats and ctrl did not show any significant difference (Figure 20).
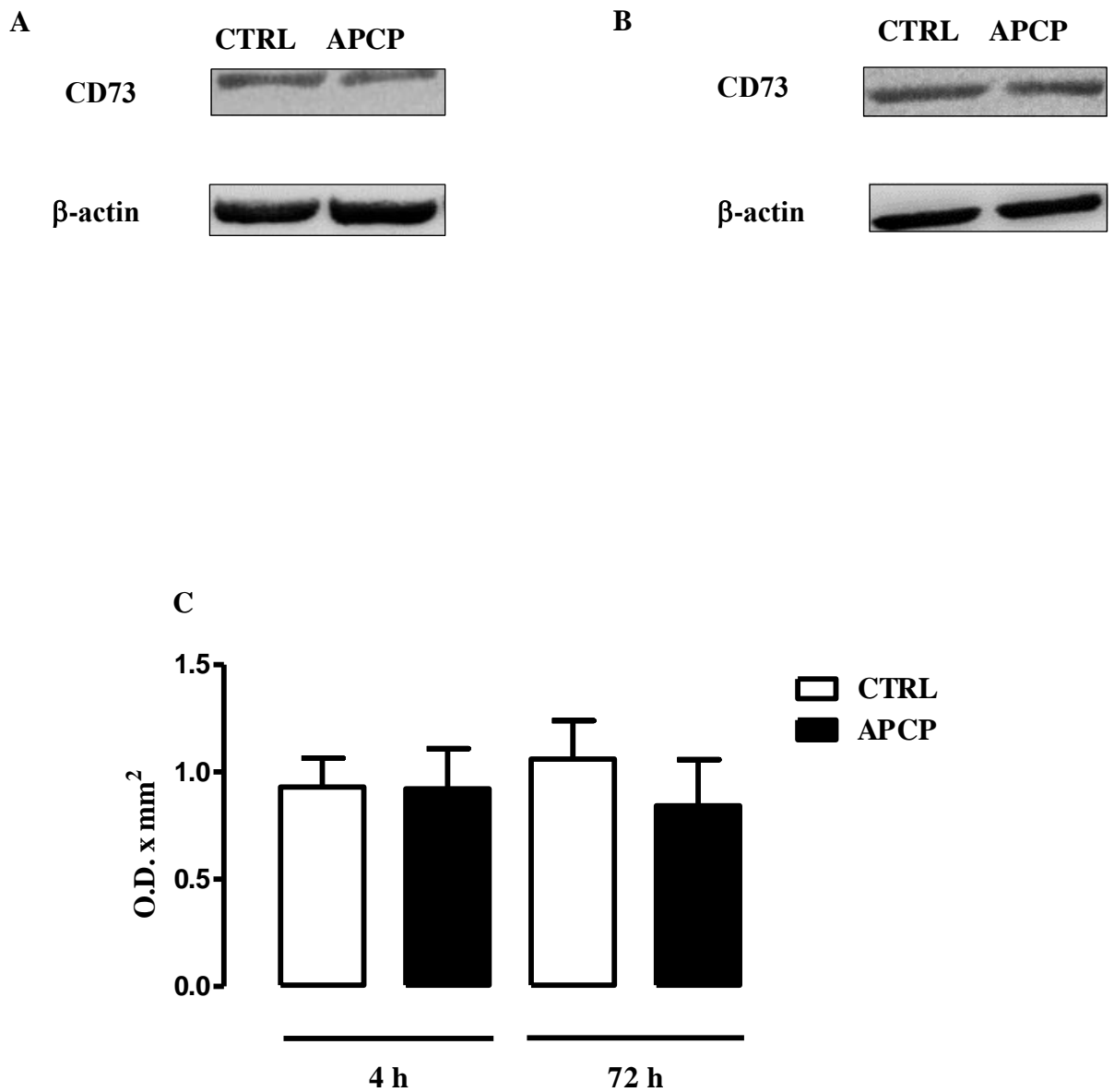

Figure 20. Detection of CD73 by western blotting on lung tissues collected 4 h (A) and 72 h (B) following carrageenan-induced rat pleurisy and C) densitometric analysis (O.D. optical density).

2.1.2.5 AMPase activity

Local treatment with APCP significantly reduced AMPase activity in exudate, cell lysate, and lung homogenates 4 h following carrageenan injection, demonstrating CD73 specific inhibition, whereas at 72 h AMPase activity was not different from control values, in any of the tissues considered (Figure 21).
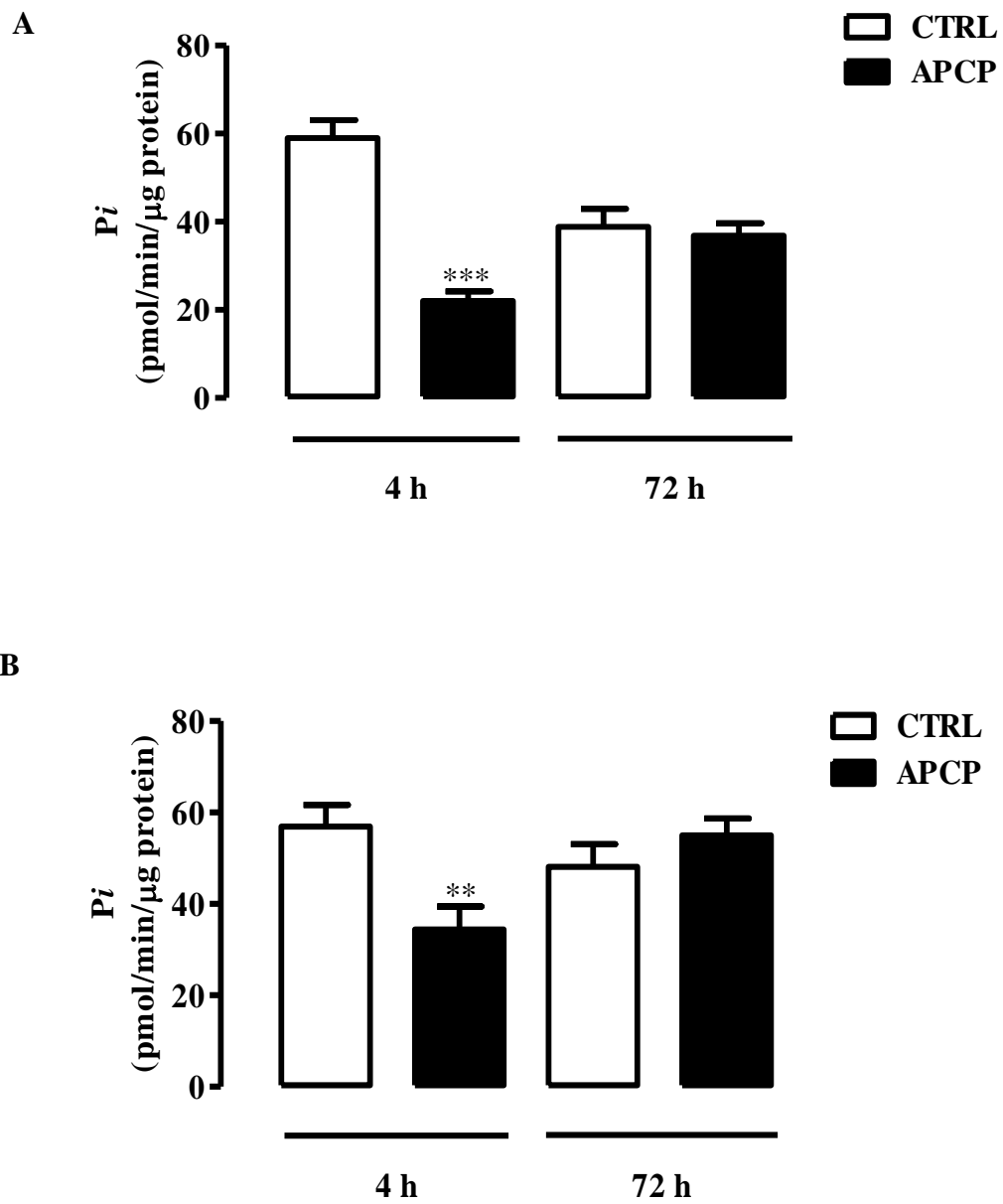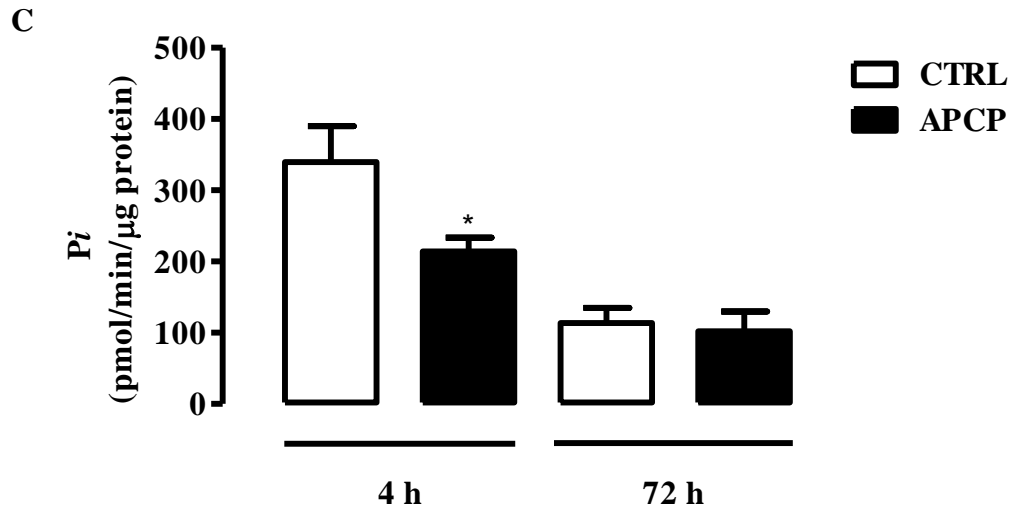
Figure 21. AMPase activity in cells (A), lungs (B), exudates (C) collected 4 and 72 h following carrageenan-induced pleurisy. *p<0.05; **p<0.01; p<0.001 *vs* ctrl. Bonferroni's Multiple Comparison Test (n=5).

2.1.2.6 Chemotaxis assay

Cells obtained from exudates collected from APCP treated group of rats 4 h after carrageenan injection showed increased ability to migrate *in vitro* either in presence (Figure 22, A) or in absence (Figure 22 B) of a chemotactic stimulus (fMLP), compared to cells from control groups (Figure 22 C and D). The ability to migrate of cells obtained from exudates collected from control group of rats 4 h after carrageenan injection was strongly increased following *in vitro* treatment with APCP (1 and 5 µM; Figure 22, E and F) in a concentration-dependent manner.

A
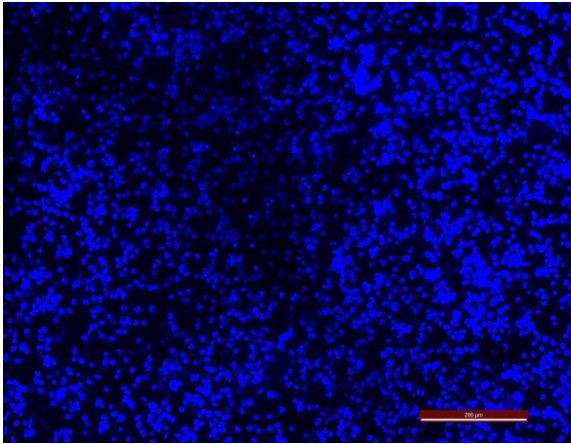
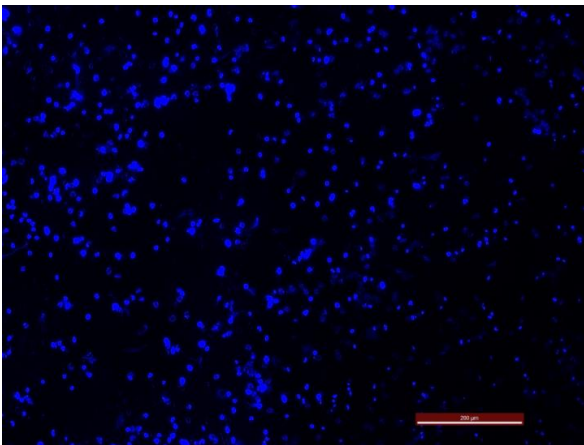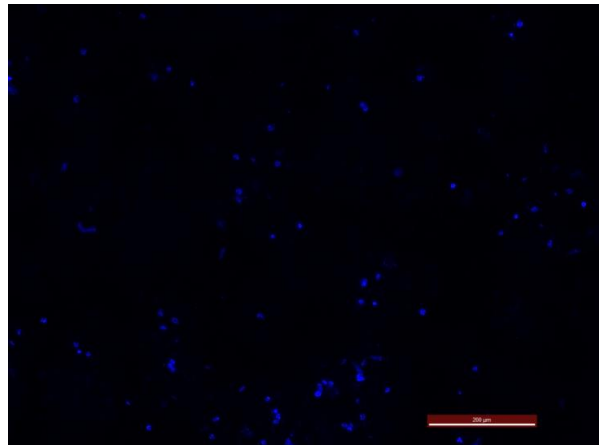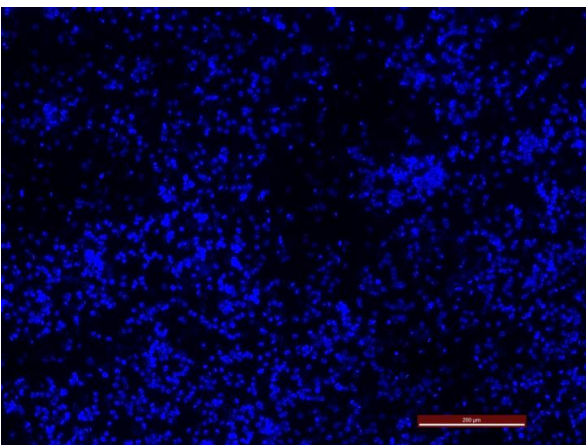**B**
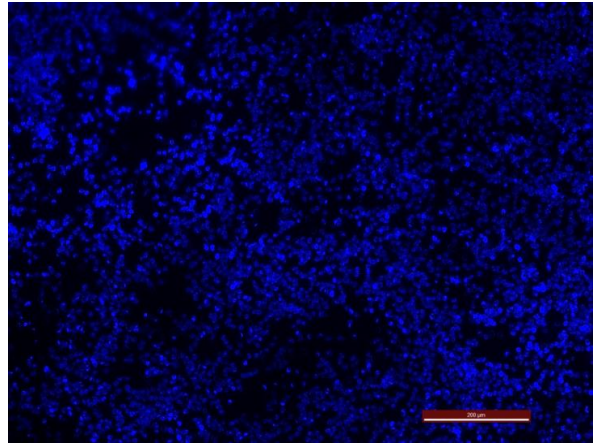
C

D

E

F
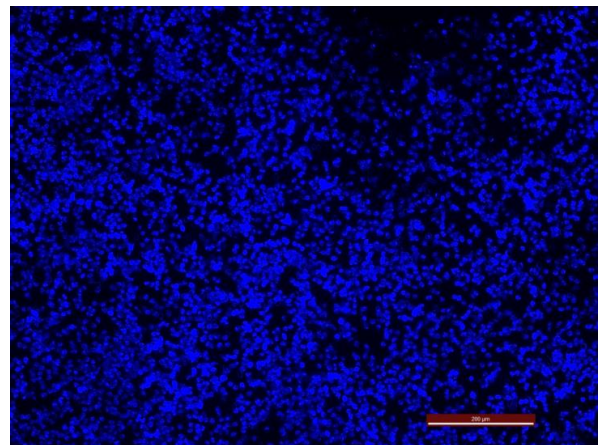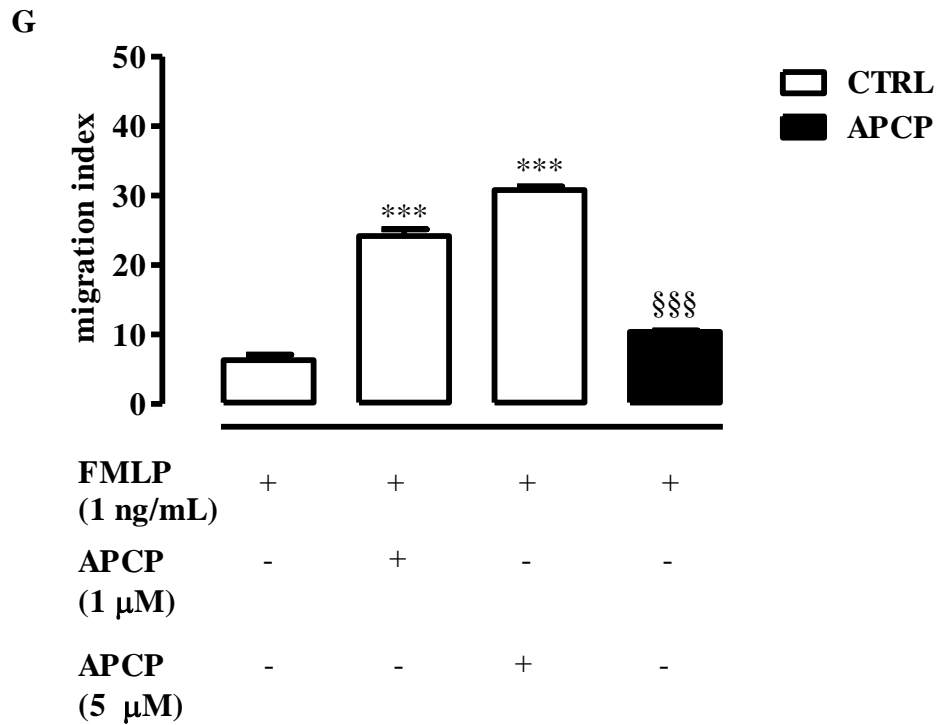
Figure 22. Effect of APCP on chemotaxis of leukocytes collected 4 h after carrageenan injection. Representative immunofluorescence showing DAPI-stained nuclei from **A**) APCP treated group + fMLP; **B**) APCP treated group – fMLP; **C**) CTRL group + fMLP; **D**) CTRL group – fMLP; **E**) CTRL group + fMLP + APCP 1 μM *in vitro*; **F**) CTRL group + fMLP + APCP 5 μM *in vitro*. Original magnification: x10. **G**) migration index. *** and §§§ $p<0.001$ *vs* ctrl. Bonferroni's Multiple Comparison Test (n=8).

2.1.2.7 Morphological analysis

Morphological analysis of lung sections of carrageenan-treated rats showed cell infiltration of the bronchial and perivascular space as well as lung injury (Figure 23 B, C, D, E). No histological alterations were found in sham-rats (Figure 23 A). Local treatment with APCP increased both lung cells infiltration and lung injury 4 h following carrageenan-induced pleurisy (Figure 23 C). No histological alterations between ctrl and APCP treated group of rats were found at 72 h (Figure 23 D and E).

A

B

C

D

E
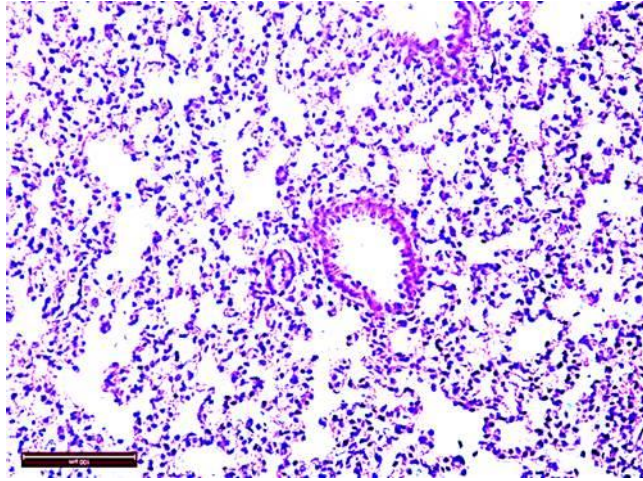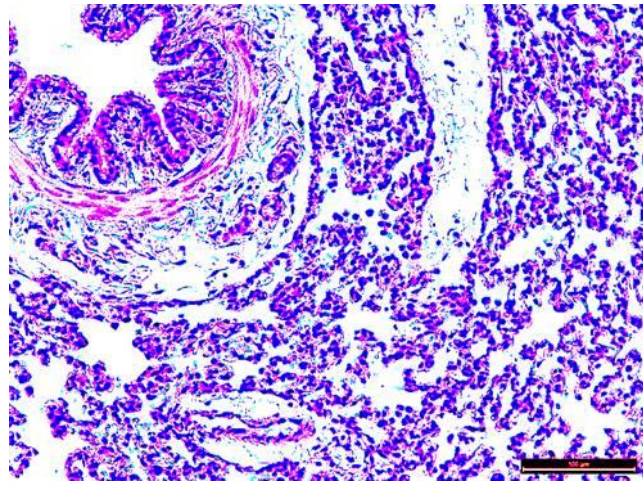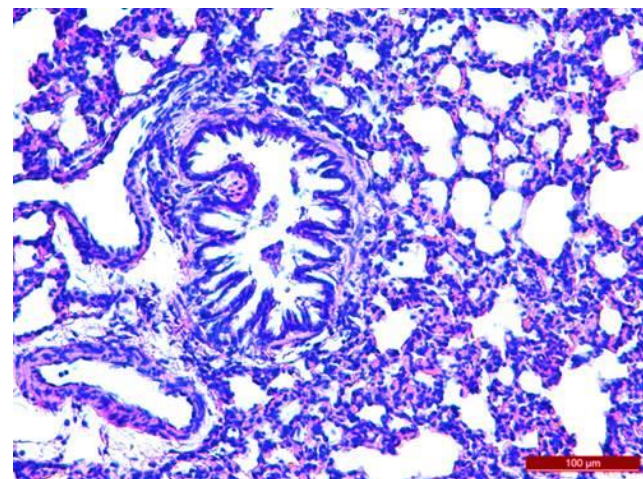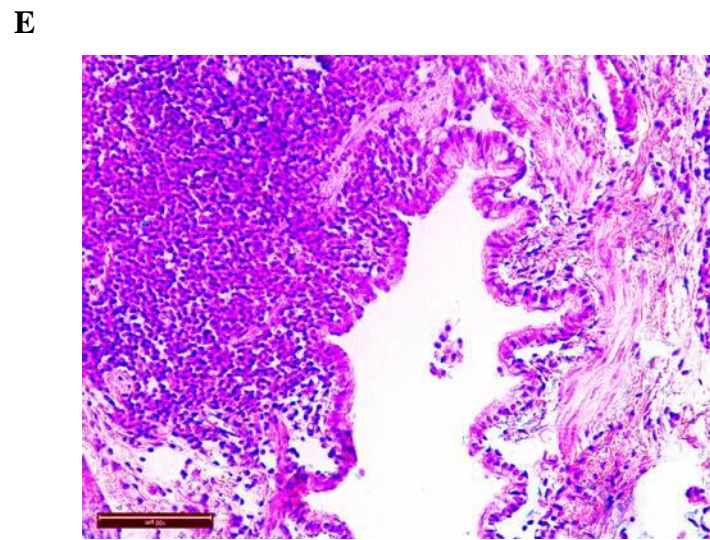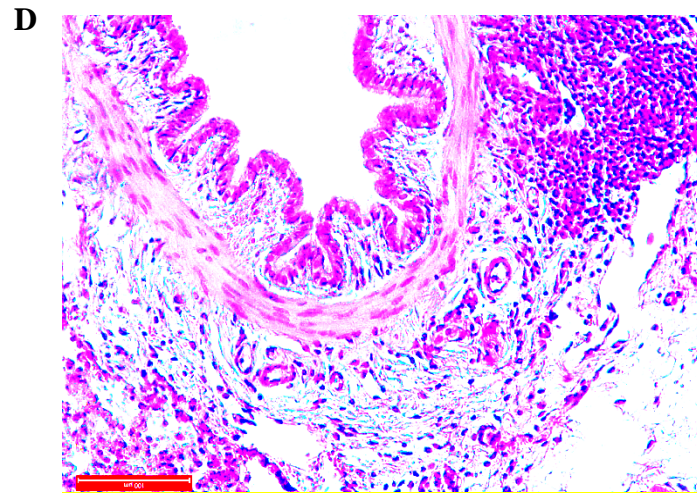
Figure 23. Effect of APCP on lung injury 4 and 72 h following carrageenan-induced pleurisy. Representative morphological analysis of hematoxylin and eosin (H&E)-stained lung sections, from A)sham rats; B) ctrl group at 4 h; C)APCP group at 4 h; D)ctrl group at 72 h; E) APCP group at 72 h. Original magnification: x20. n=5.

2.2 CD73/ADENOSINE/A$_{2A}$AR SIGNALLING PATHWAY AND NIMESULIDE ANTI-INFLAMMATORY EFFECT

2.2.1 MATERIALS AND METHODS

2.2.1.1 Reagents

Nimesulide, dymethysulfoxide (DMSO), AMP, λ-carrageenan, celecoxib, LPS from *Escherichia Coli* (serotype 011:B4) and MTT were purchased from Sigma-Aldrich (Milan, Italy); CGS 21680, ZM 241385, APCP were obtained from TOCRIS Bioscience (Bristol, UK); PGE$_2$, enzymatic immune assay (EIA) assay kit was purchased from Cayman (Tallinn, Estonia); Malachite Green kit was purchased from ScienCell, Research Laboratories (Carlsbad, USA). Polyclonal goat antibody to CD73 and monoclonal mouse antibody to β-actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibody for COX-2 was purchased from BD Biosciences (San Jose, USA). Anti-goat IgG and anti-mouse IgG conjugated to horseradish peroxidase were obtained from Dako (Denmark). ECL system was obtained from Amersham Pharmacia Biotech (Milan, Italy). All salts were purchased from Carlo Erba (Italy). All reagents for cell culture were obtained from Sigma-Aldrich (Milan, Italy), Amersham Biosciences Inc. (UK), Bio-Rad Laboratories (USA) and Microtech (Naples, Italy). All other salts and reagents were purchased from Carlo Erba (Milan, Italy).

2.2.1.2 *In vivo* experiments

2.2.1.3 Animals

Male Wistar rats, body weight 200–250 g, were purchased from Harlan Nossan, (Milan, Italy). Animals were kept under standard conditions with food and water *ad libitum* and maintained in a 12 h/12 h light/dark cycle at 22 ± 1° C. Before the experiments, rats were

housed and acclimatized under these conditions for four days. All the *in vivo* procedures were performed according to the Italian and European regulations (DL/2014) on the protection of animals used for experimental and other scientific purposes and were approved by Italian Ministry of Health.

2.2.1.4 Carrageenan-induced paw oedema

Rats, slightly anesthetized with 4% enflurane mixed with 0.5 L/min O_2, 0.5 L/min N_2O, received in the left hind paw a subplantar injection of 100 µL of λ-carrageenan (suspension 1% w/v). Paw volume was measured at the time zero and each hour for 6 h by a hydropletismometer (Ugo Basile, Comerio, VA, Italy).

2.2.1.5 Drug treatments

Rats were assigned to groups of 5 and treated intraperitoneally (i.p.), just before oedema induction, with the A_{2A} agonist, CGS 21680 (2 mg/kg), or with the A_{2A} antagonist, ZM 241385 (3 mg/kg), given alone or in combination. In another series of experiments, animals were treated with nimesulide (5 mg/kg i.p) given alone or co-administered with ZM 241385 (3 mg/kg i.p.), just before carrageenan injection. Control groups received the vehicle, DMSO (0.5 ml/kg). The effect of CD73 inhibitor, APCP, injected into the rat paw (400 µg/paw) 1 h following carrageenan injection, was also evaluated in both controls and nimesulide-treated animals. Animals were then sacrificed and inflamed tissues were excised and immediately frozen in liquid nitrogen and stored until further analysis.

2.2.1.6 Blood collection and plasma preparation

In some experiments, blood was withdrawn 4 h following oedema induction by cardiac puncture and anticoagulated with trisodium citrate (3.8 % w/v). Plasma was then obtained by centrifugation at 3000 rpm for 15 min.

2.2.1.7 Enzymatic assay in rat inflamed tissues and plasma

AMP hydrolysis was evaluated in samples of inflamed tissues and of plasma collected from rats 4 h following oedema induction, as a measure of e-5'NT and soluble-5NT activity, by colorimetric measurement of the Pi released following incubation with the substrate, as described by Nedeljkovic et al.,(2006). Briefly, on the day of analysis, inflamed tissues were homogenized using liquid nitrogen in the following lysis buffer: Tris-HCl (50 mM) pH 7.5; NaCl (150 mM); sodium orthovanadate (1 mM); β-glycerophosphate (20 mM); EDTA (2 mM); PMSF (1 mM); leupeptin (5 μg/ml); aprotinin (5 μg/ml); pepstatin (5 μg/ml). Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Milan, Italy). To initiate the enzymatic reaction, samples of tissue homogenates or plasma (50 μg of proteins) were incubated with 200 μL of medium containing $MgCl_2$ (10 mM), NaCl (120 mM), KCl (5 mM), glucose (60 mM), Tris-HCl (50 mM), pH 7.4. After 10 min, AMP (2 mM) was added as substrate and samples kept at 37°C for 40 min. The reaction was then stopped by the addition of trichloroacetic acid (TCA, final concentration 5% w/v). Following sample centrifugation at 3000 rpm for 10 min, at 37°C, Pi released was quantified using malachite green as a colorimetric reagent and KH_2PO_4 as standard (Chan et al., 1986). To have the net value of Pi produced following enzymatic reaction, aspecific Pi released in the absence of AMP in each sample was evaluated and the value obtained was subtracted from the value obtained following incubation with AMP. All samples were run in triplicate; results were expressed as Pi released (pmol/min/μg protein).

2.2.1.8 Prostaglandin E_2 assay

Levels of PGE_2 were assayed in plasma obtained from inflamed rats 4 h following carrageenan paw oedema by EIA kit according to the manufacturer's instructions and expressed as pg/ml.

2.2.1.9 Western blotting analysis

From nimesulide-treated rats and control animals, at different times following oedema induction, inflamed paws were excised and the soft tissue was removed, frozen in liquid nitrogen and stored. Tissue samples were then defrosted, weighed and homogenized. Protein extraction was performed as described previously and the protein concentration was measured by the Bradford assay using BSA as standard. Denaturated protein samples (35 μg) were subjected to electrophoresis on an SDS 8% polyacrylamide gel and transferred onto a nitrocellulose transfer membrane (Protran, Schleicher & Schuell, Germany). The membranes were saturated by incubation with non-fat dry milk (5% w/v) in PBS supplemented with 0.1% (v/v) Tween 20 for 1 h at room temperature and then incubated with a goat polyclonal anti-CD73 antibody (1:200 dilution), overnight at 4° C. Successively, membranes were washed and then incubated with the secondary antibody conjugated with horseradish peroxidase (HRP), anti-goat IgG-HRP (1:2000 dilution), for 2 h at room temperature. Protein bands were detected using the ECL detection kit and Image Quant 400 GE Healthcare software (GE Healthcare, Italy). Protein bands were quantified using Quantity One® software (Bio-Rad, Italy). Successively, to confirm the equal protein loading, membranes were stripped and incubated with anti β-actin monoclonal antibody (1:2000 dilution) and subsequently with anti-mouse IgG conjugated to peroxidase (1:2000 dilution), both for 3 h at room temperature.

2.2.1.10 *In vitro* experiments

2.2.1.11 Cell culture

The murine monocyte/macrophage cell line J774A.1 (American Type Culture Collection, Rockville, MD) was grown at 37°C, in humidified 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % foetal bovine serum (FBS), 25 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin and 1% sodium pyruvate. The cells

were maintained in 10 cm^2 dishes and for experiments were plated in 6, 24 or 96 culture wells at a density of 2.5×10^5 cells/ml/well for 18 h.

2.2.1.12 Cell treatment

Non-adherent cells were removed by washing with sterile PBS and, immediately before the experiments, the culture medium was replaced by fresh medium. Cells were pre-incubated with the following compounds: the A_{2A} agonist, CGS 21680 (1 µM) alone or in combination with the ZM 241385 (10 µM); nimesulide (100 µM) alone or in combination with the A_{2A} antagonist, ZM 241385 (10 µM), or with the CD73 inhibitor, APCP (5 µM), celecoxib (10 µM) alone or in combination with APCP (5 µM) or with ZM 241385 (10 µM); PGE$_2$, (30–300 µM). Drug concentrations to be used throughout the whole study were established following preliminary experiments. All incubations were performed 1 h before cell activation with LPS from *Escherichia Coli* (1 µg/mL) except for ZM 241385 that in some experiments was added 6 hours following cell incubation with nimesulide or with celecoxib. Controls were performed by cell incubation with DMSO (final concentration 0.05 %) that was the vehicle for all compounds, apart from APCP that was dissolved in the cell medium. Following LPS activation, cells were then maintained for 24 h in a humidified incubator at 37 °C under 5% CO$_2$ atmosphere.

2.2.1.13 Cell viability assay

To exclude that all stimuli used induced cell death, cell viability was determined by evaluating total mitochondrial activity using the MTT assay. Briefly, 25 µL of MTT (5 mg/ml in PBS) solution was added to each well incubated for 3 h. Thereafter, the cells were lysed and the dark blue crystals solubilized with 100 µL of a solution containing 50% (v/v) N,N-dimethylformamide and 20% (w/v) SDS with an adjusted pH of 4.5. The optical

density was read at 570 nm in a microplate reader and the results were expressed as a percentage (%) of cell viability.

2.2.1.14 Nitrite assay

J774A.1 cells were cultured and treated as described above. The nitrite production was measured as described by Green *et al*., (1982). Briefly, 100 µl of the culture medium 24 hours following challenge with LPS was mixed with an equal volume of Griess reagent (1% sulphanilamide and 0.1% naphthyl ethylenediamine in 5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 540 nm was recorded using a microplate reader. The amount of nitrite (as µmol/L) was calculated by extrapolation from a sodium nitrite standard curve.

2.2.1.15 Prostaglandin E_2 assay

Levels of PGE_2 were assayed in the cell culture medium by EIA kit according to the manufacturer's instructions and expressed as pg/ml.

2.2.1.16 Enzymatic assay

AMPase activity was evaluated in 24 well plates containing J774A.1 macrophage cell line (2.5×10^3 cells/well) plated for 18 h, treated as described above and incubated with or without LPS for 24 h. Afterwards, cells were washed three times with incubation medium without AMP. The enzymatic reaction was then started by the addition of 200 µL of incubation medium as described previously, without Tris-HCl, and with some differences: $MgCl_2$ (2 mM); glucose (10 mM) and HEPES (20 mM), pH 7.4. After 10 min of incubation, the reaction was stopped by collecting an aliquot of the incubation medium and transferring it into Eppendorf tubes containing TCA (final concentration, 5 % w/v), previously placed on ice. Controls to determine non-enzymatic P*i* release were performed by incubating the cells in the absence of the substrate, or the substrate in the absence of the cells. To determine

specificity, experiments were also performed in cells in the presence of the CD73 inhibitor, APCP. All samples were run in triplicate. The release of Pi was measured by the malachite green method, using KH_2PO_4 as a Pi standard, as described above. To have the net value of Pi produced following enzymatic reaction, Pi released by AMP into the assay medium without cells was subtracted from the total Pi released by cells during incubation with AMP, and expressed as pmol/min.

2.2.1.17 Ultra-Performance Liquid Chromatography (UPLC)

In another series of experiments, CD73 activity on J774A.1 macrophage cell line treated as described above was also assessed by quantifying the conversion of ε-AMP, a bioactive, fluorescent analog of AMP (BIOLOG, Bremen, Germany) to ε-adenosine (Jamal *et al*., 1988), using Ultra-Performance Liquid Chromatography (UPLC) analysis. The product ε-adenosine is neither a substrate for nor an inhibitor of adenosine deaminase, which catalyzes the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively, decreasing adenosine levels (Jamal *et al*., 1988). Briefly, cells were washed three times with Hank's Balanced Salt Solution (HBSS) (GibcoTM, Thermo Fisher Scientific, Waltham, MA, USA) without ε-AMP. Afterward, the cells were incubated with ε-AMP (50 μM) in HBSS for 10 minutes at 37 °C. To determine specificity, a similar analysis was performed in cells untreated in the presence of the CD73 inhibitor, APCP (50 μM). To stop the reaction an aliquot of 60 μl of the incubation medium was transferred into an Eppendorf tube on ice and centrifuged at 4 °C for 2.5 min at 550 x g. Aliquots of 40 μl were applied to an ACQUITY UPLC® H-Class Bio (WATERS Corp. Milford, MA, USA) and nucleotides and nucleosides were separated by running a linear gradient of buffer A (150 mM KCl/150 mM KH_2PO_4 at pH 6) and buffer B (15% (v/v) solution of acetonitrile in buffer A) with a flow profile of 0,294 ml/min (0-0.54 min. 100% A; 0.54-1.24 min. 97% A and 3% B; 1.24-4.17 min. 91% A and 9% B; 4.17-7.45 min. 50% A and 50% B; 7.45-8.37

min. 50% A and 50% B; 8.37-8.45 min. 100% A) using a high pressure gradient mixing device. The performance was done on an Acquity UPLCTM BEH C18 column (2.1×150 mm, 1.7 μm; WATERS Corp. Milford, MA, USA). The analyte ε-adenosine was detected at 254 nm by using Waters Multi λ Fluorescence detector 2475 (λEM=410 nm, λEx=280 nm) and its concentration was calculated with reference to standards of known concentration (ε-adenosine; BIOLOG, Bremen, Germany) and expressed as pmol/min. UPLC analysis was also used for the simultaneous quantification of ATP, nicotine amide dinucleotide (NAD), ADP, AMP, adenosine, inosine, hypoxanthine, xanthine produced by J774A.1. The EMPOWER 3 software (WATERS Corp. Milford, MA, USA) was used for data analysis. These experiments were performed at Professor Jürgen Schrader's laboratory, Department of Molecular Cardiology, "Heinrich Heine University", Düsseldorf, Germany.

2.2.1.18 Transfection and real time PCR

For silencing of CD73 mRNA by small interfering RNA (siRNA), J774A.1 cells were double shot transfected using HiPerfect (Qiagen, Germany) according to manufacturer's instructions, with a combination of two siRNAs (Sigma-Aldrich, Milan, Italy) specific for the CD73 or one siRNA (Sigma-Aldrich, Milan, Italy) specific for the green fluorescent protein (GFP).

The sequences CD73 targeted by the siRNAs were: 5-CAUUGCAGCCUGAAGUAGA-3 and 5-GACAUUUGACCUCGUCCAA-3. The GFP sequence targeted by the siRNA was 5-CGGCAAGCUGACCCUGAAGUUCAU-3 and analyzed for mRNA expression levels after 96 hours. Total RNA was isolated from J774A.1 cells with TRI Reagent® (Sigma-Aldrich, Milan, Italy) and analyzed spectroscopically. One microgram of RNA was retro-transcribed using Prime Script RT reagent Kit with gDNA eraser (Takara, Otsu, Japan) and amplified with specific primers described below. For CD73 mRNA fw: 5-GCGTGCATCGCTATGGCCAGTCC 3 and rv: 5- CCACCGTTGGCCAGATAGCTTGG-

3 and fw: 5-AAAACCAACCCGGTGAGCTCCCTC-3 and rv: 5-CTCAGGCTCCCTCTCCGGAATCG-3 for 18s, all primers were purchased from IDT (IDT, Germany). PCR amplification was carried out as by means of SYBR Premix Ex Taq (Takara, Otsu, Japan) according to manual instruction. PCR amplification of 18s rRNA was used as the normalizer. Real-time PCR assays were performed using the Rotorgene RG-3000A cycle system (Qiagen, Germany). These experiments were performed in collaboration with Drs. Astrid Parenti, Sara Paccosi, Andrea Lapucci (Department of Health Sciences, Clinical Pharmacology and Oncology Section; "University of Florence", Florence, Italy).

2.2.1.19 Fluorescence-activated cell sorting (FACS) analysis

The expression of the protein CD73 in J774A.1 silenced for 96 hours and in untreated control was evaluated by flow cytometry platform FACS CANTO II (BD, Franklin Lakes, NJ, USA) after the determination of nitrite release. Monoclonal antibody APC anti-mouse CD73 (clone: TY/11.8; Biolegend, San Diego, CA, USA) and the isotype-identical antibody as control were used for this purpose. Dead cells were excluded by 7-ADD (Sigma-Aldrich, St. Louis, MO, USA). Results were analyzed with FCS Express 5.0 software (De Novo, Glendale, CA, USA). These experiments were performed in collaboration with Drs. Astrid Parenti, Sara Paccosi, Andrea Lapucci, (Department of Health Sciences, Clinical Pharmacology and Oncology Section, " University of Florence", Florence, Italy). In another set of experiments, J774 macrophages were cultured in 24-well plates and treated as described above. After 24 hours, the cells were harvested, resuspended in MACS buffer (BSA 0.5%, EDTA 5 mM in PBS) and pre-incubated with FcR Blocking Reagent (Miltenyi Biotech, Auburn, CA, USA) for 10 min at 4 °C. Then the cells were stained with the following antibodies: CD11b APC-conjugated antibody (clone: M1/70.15.11.5; isotype: rat IgG2b; 1:100 dilution; Miltenyi Biotech, Auburn, CA, USA) and with Mouse 5'-

Nucleotidase/CD73 PE-conjugated Antibody (clone:496406; isotype: Rat IgG2a; 1:10 dilution; R&D systems, Abingdon, UK) for 20 min at 4 °C in dark. After two washes, cells were analyzed on Fluorescence-activated cell sorting analysis (FACS). J774A.1 were also DAPI-stained to exclude dead cells. Subsequently, percentage of cells expressing CD73 was determined by FACS after gating CD11b+ living cells. FACS analysis was carried out on an FACS Calibur FACS Canto, or LSR II (BD Biosciences, Heidelberg, Germany). Ten thousand cells were analyzed in each sample. Data analysis was performed using FlowJo 7.6.1 software (Tree Star, Inc., Ashland, OR, USA) and results were expressed as a percentage of cells positive for CD73. These experiments were performed at Professor Jürgen Schrader's laboratory, Department of Molecular Cardiology, "Heinrich Heine University", Düsseldorf, Germany.

2.2.1.20 Western blotting analysis

Cells treated as reported previously were washed three times with ice-cold PBS and were removed from the culture dish. The resulting suspension was pelleted by centrifugation at 1200 rpm for 5 min at 4°C. The cell pellet was resuspended in 50 µL of ice-cold lysis buffer (200 mM HEPES, 400 mM NaCl, 1 mM dithiothreitol DTT, 1 % igepal, 20% glycerol, along with the protease inhibitor cocktail Roche) and maintained in constant agitation for 45 min at 4°C. Then the suspension was centrifuged at 14000 rpm for 10 min in a 4° C pre-cooled centrifuge and the supernatant was collected and stored at -80 °C until analysis. Immunoblotting analysis of COX-2 protein was performed on whole cell extracts. Denatured proteins (35 µg) were electrophoresed in an 8% discontinuous polyacrylamide gel and then transferred onto nitrocellulose membranes. Afterward, nitrocellulose membrane was blocked using non-fat dry milk (5% w/v) in PBS supplemented with 0.1% (v/v) Tween 20 for 30 minutes at room temperature and then incubated overnight at 4° C on a shaker with an anti COX-2 mouse antibody (1:1000 dilution). The nitrocellulose membrane was

washed five times for 30 minutes and then incubated with the secondary antibody anti-mouse IgG (1:2000 dilution), conjugated with peroxidase for 2 h at room temperature. After three washes, the proteins bands were detected using the enhanced chemiluminescence method and analyzed with Quantity One program as described by the manufacturer. Successively, to confirm the equal protein loading, membranes were stripped and incubated with anti β-actin monoclonal antibody (1:2000) and subsequently with anti-mouse IgG conjugated ed to peroxidase (1:2000), both for 3 h at room temperature.

2.2.1.21 Statistical analysis

All results were expressed as mean ± standard error (S.E.); each in vitro experiment was run in triplicate. Data were analyzed by one way ANOVA followed by Bonferroni's test for multiple comparisons or by Dunnett's test, as appropriate. Two ways ANOVA was also utilized when appropriate. A value of $p < 0.05$ was taken as statically significant.

2.2.2 RESULTS

2.2.2.1 *In vivo* experiments

2.2.2.2 Carrageenan–induced oedema

Treatment of rats with the selective A_{2A} agonist, CGS 21680 (2 mg/kg i.p.), significantly inhibited oedema formation monitored over a period of 6 hours (Figure 24 A) and this effect was reversed by co-administration with the A_{2A} antagonist, ZM 241385 (3 mg/kg i.p.). In contrast treatment with ZM 241385 alone did not modify oedema development (Figure 24 A). In animals receiving nimesulide (5 mg/kg i.p.), oedema formation was significantly reduced and this effect was partially reversed by treatment with ZM 241385 (Figure 24 B).
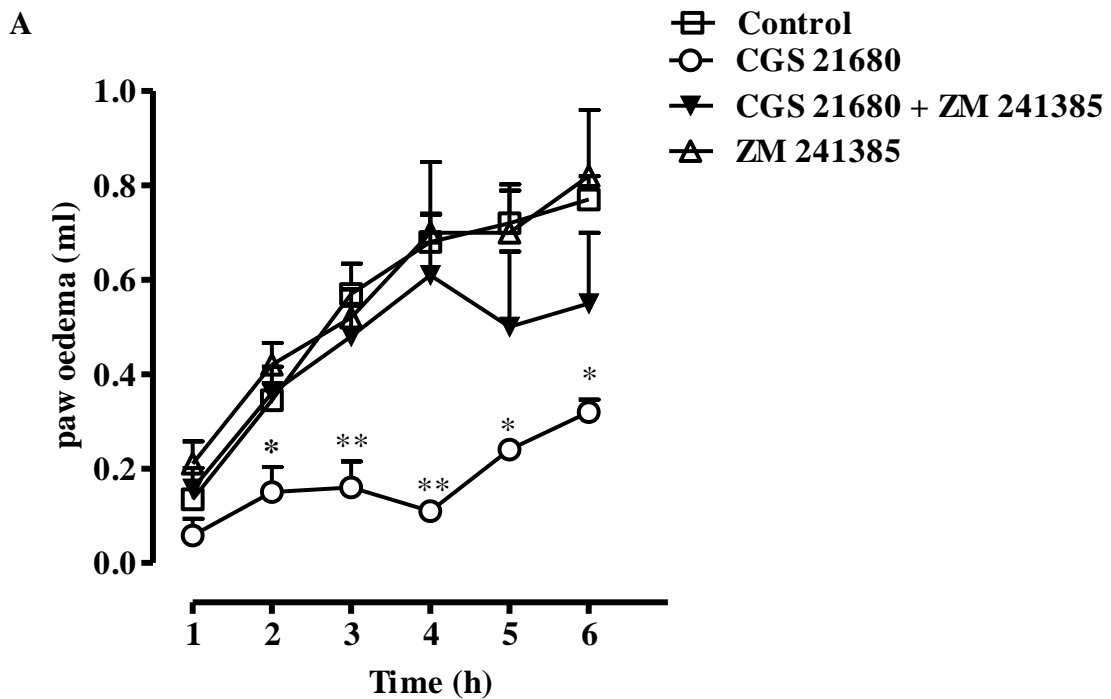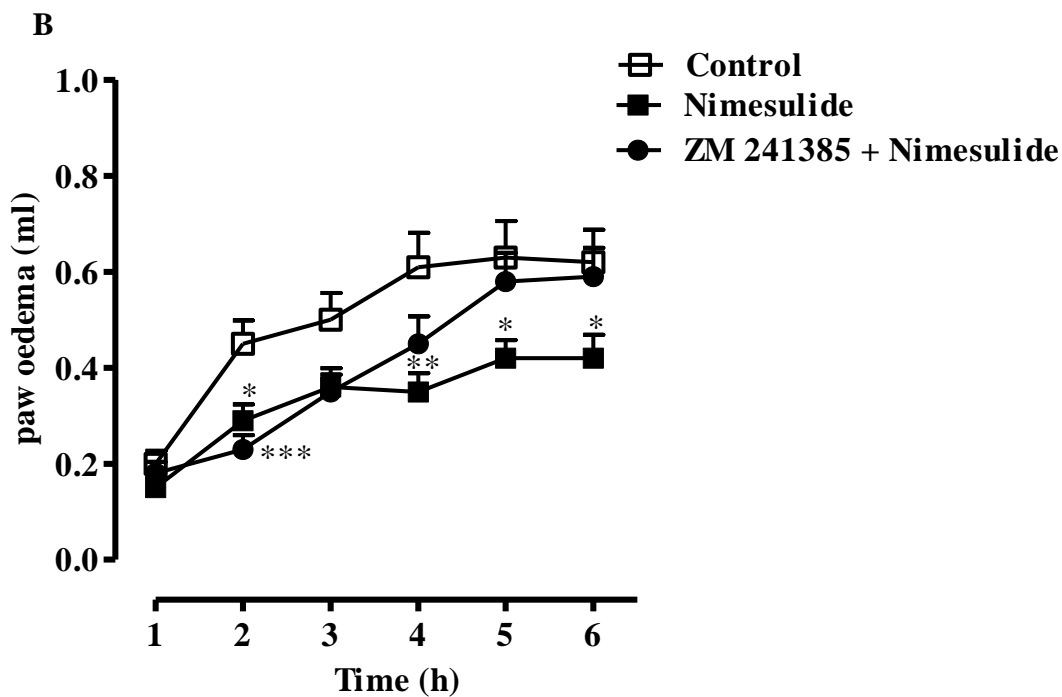
Figure 24. Effect of drug treatments on carrageenan-induced rat paw oedema. *p< 0.05 , ** p< 0.01, ***p< 0.001 *vs*. control.Two ways ANOVA followed by Bonferroni's test (n=10).

2.2.2.3 Prostaglandin E$_2$ assay

The anti-inflammatory effect of nimesulide was associated with a significant reduction of plasma PGE$_2$ levels, evaluated 4 hours following oedema induction, consistent with COX-2 inhibition; however, PGE$_2$ plasma levels were only slightly, but not significantly, reduced by treatment with CGS 21680 (Figure 25).
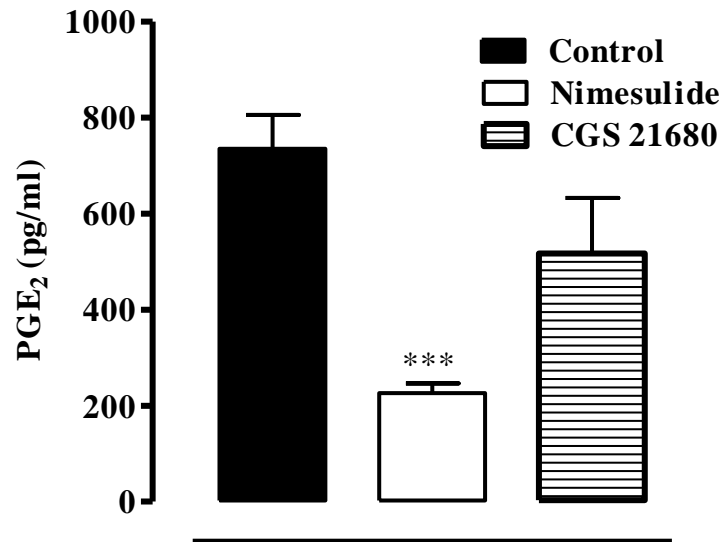
Figure 25. Effect of nimesulide and CGS 21680 on PGE$_2$ plasma levels 4 hours following oedema induction. *** $p < 0.001$ *vs*. control. Bonferroni's test for multiple comparisons (n=5).

2.2.2.4 AMPase activity in inflamed tissues and plasma

The anti-inflammatory effect of nimesulide was accompanied by change in AMPase activity in inflamed tissues and plasma evaluated 4 h following oedema induction, that represented the inflammatory peaking point. In paws obtained from nimesulide-treated rats, AMP hydrolysis was significantly increased compared to the hydrolysis measured in paws from control animals (Figure 26 A). This increased AMPase activity was of functional significance since local injection of the CD73 inhibitor, APCP (400 µg/paw), did not affect oedema development (Figure 26 B) but reverted nimesulide effect (Figure 26 C). AMP hydrolysis was also significantly increased in plasma obtained from animals treated with nimesulide (Figure 26 D).

A

B

C

D
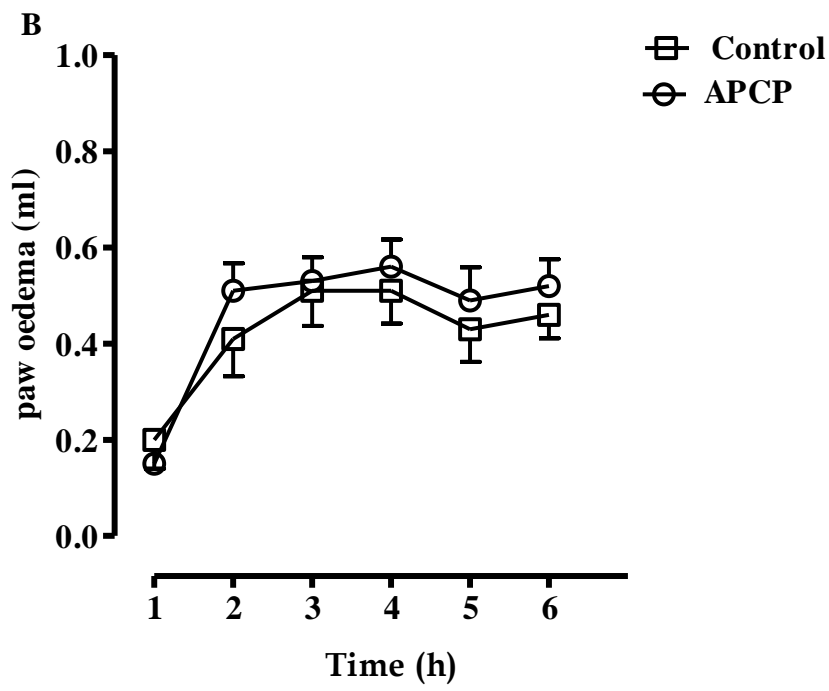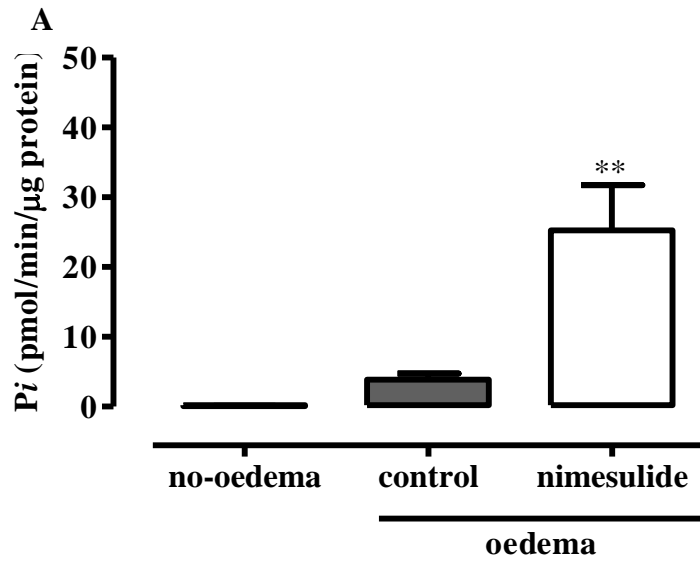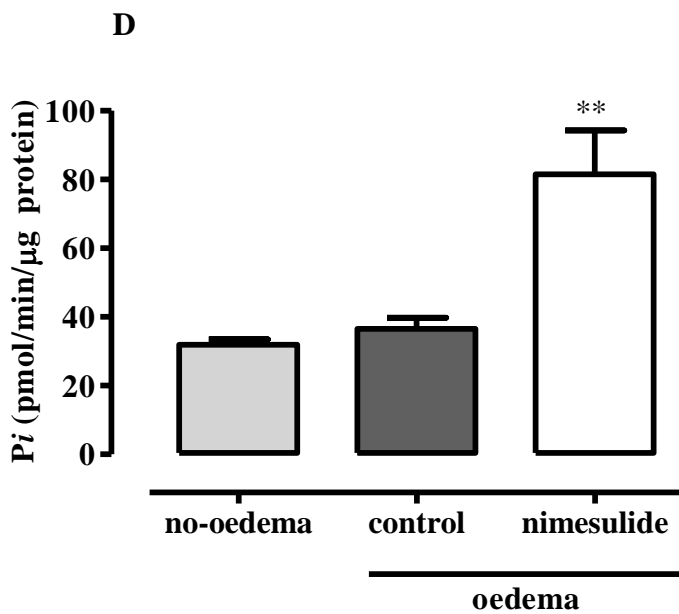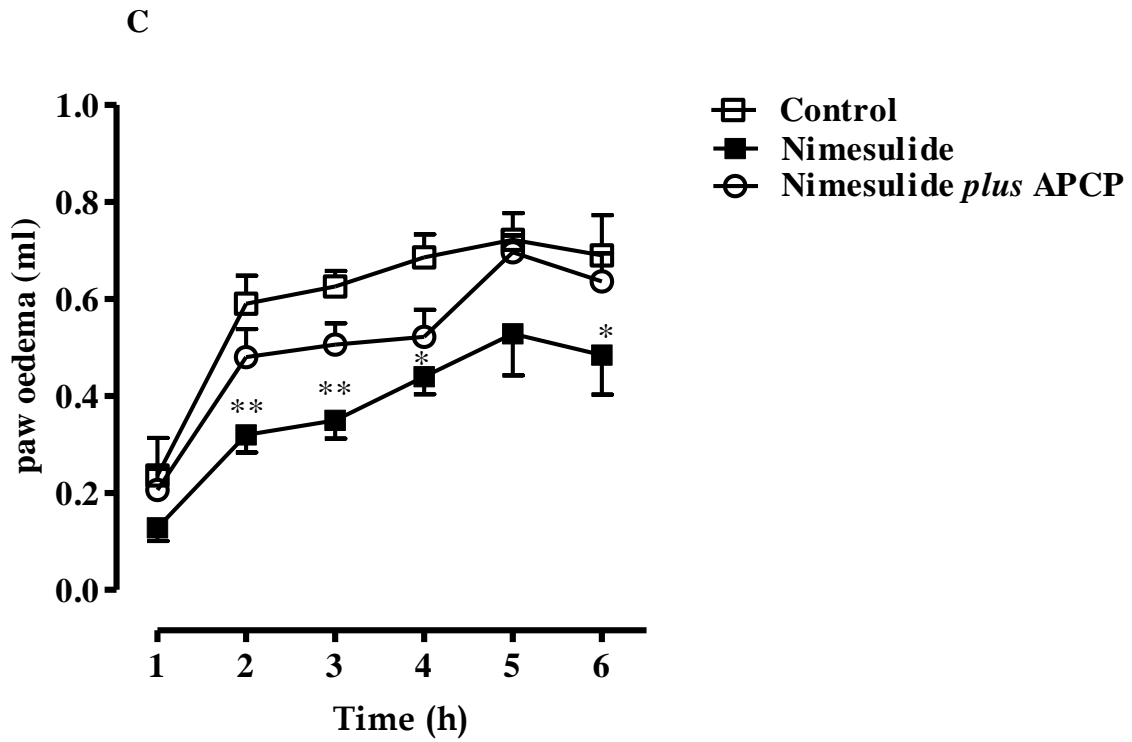
Figure 26. Effect of systemic treatment with nimesulide on AMPase activity in paws (A) and in plasma (D) 4 hours following oedema induction. ** $p < 0.01$ *vs.* control; one way ANOVA followed by Dunnett's test (n=8); **and effect of CD73 inhibitor (APCP, 400 µg/paw) on rat paw oedema and nimesulide anti-inflammatory effect (B, C).** *$p < 0.05$, ** $p < 0.01$ *vs.* control. Two ways ANOVA followed by Bonferroni's test (n = 5).

2.2.2.5 Western blotting analysis

CD73 expression was evaluated by Western blotting analysis on inflamed tissues obtained each hour following oedema induction. There was no difference in the expression of CD73 in inflamed paws obtained from control (Figure 27A) and nimesulide (Figure 27 B) treated rats.

A

B
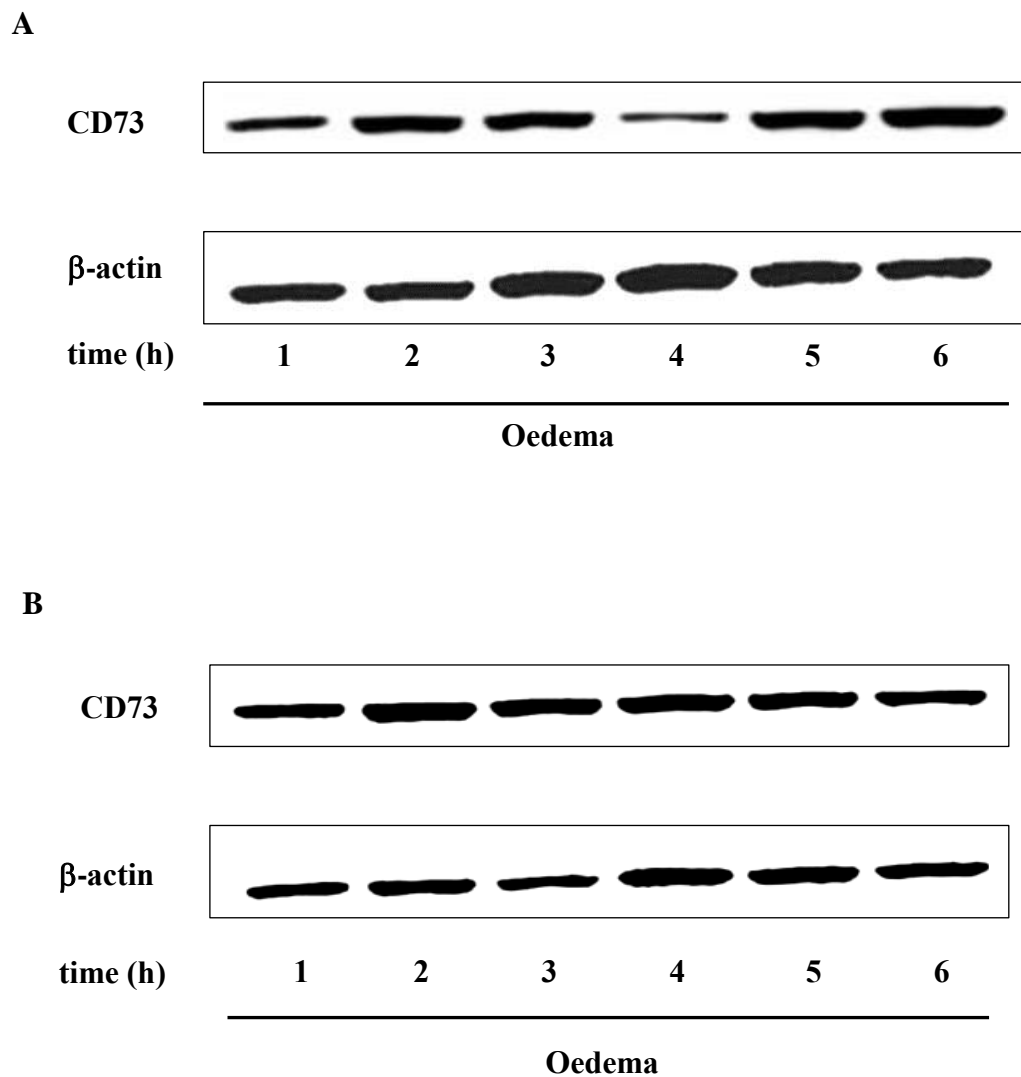
C
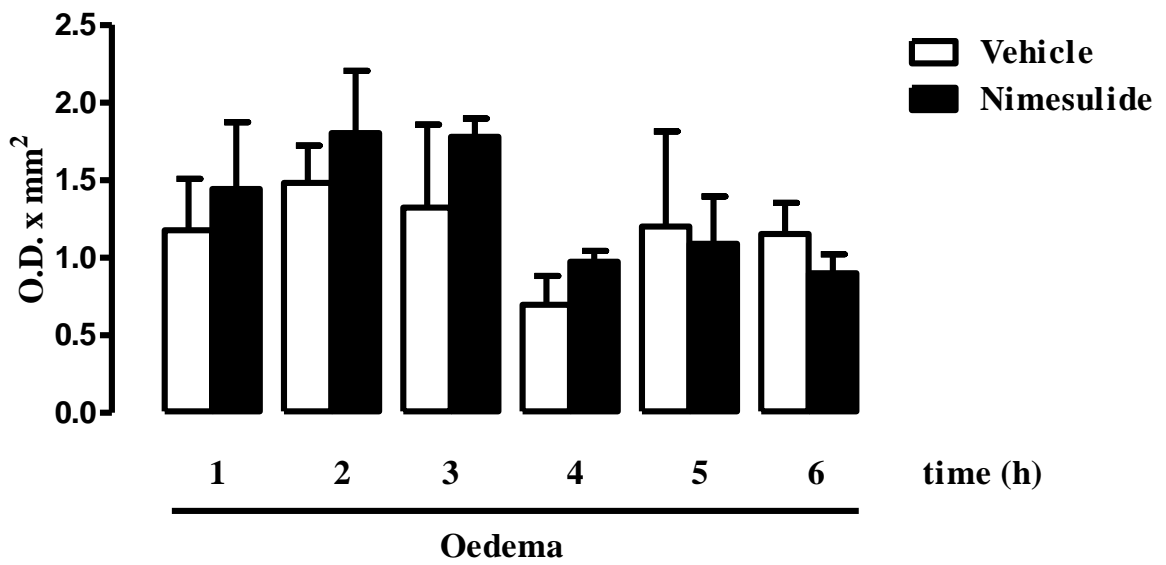
Figure 27. Detection of CD73 by western blotting analysis in inflamed paws from control (A) and nimesulide (B) treated rats each hour following oedema induction and C) densitometric analysis (O.D. optical density). (n = 3).

2.2.2.6 *In vitro* experiments

2.2.2.7 Cell viability assay

Cell viability evaluated 24 hours following incubation with MTT was not modified by treatments described (Figure 28).
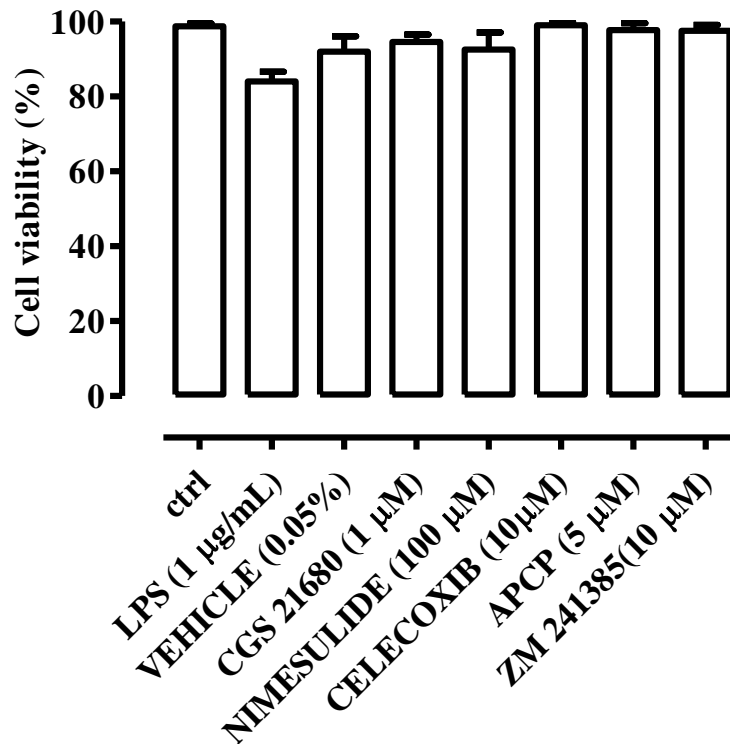
Figure 28. Effect of LPS, vehicle (DMSO), CGS 21680, nimesulide, celecoxib, ZM 241385 and APCP on cell viability evaluated 24 h following incubation with MTT in J774A.1.

2.2.2.8 Nitrite production

Production of nitrite by un-stimulated J774 cells was undetectable (< 0.8 μM). Stimulation with LPS caused a substantial release of nitrite (26.0 ± 0.9 μM, n=5) that was significantly reduced by the adenosine A_{2A} receptor agonist, CGS 21680 (1μM). The inhibitory effect of CGS 21680 (1μM) was reverted by cell co-treatment with the A_{2A} antagonist, ZM 241385 (10 μM; Figure 29 A). Similarly, nimesulide (100 μM) inhibited nitrite production from LPS–activated J774 and its effect was reverted by the A_{2A} antagonist, ZM 241385 (10 μM),

98

6 hours following nimesulide treatment (Figure 29 B). ZM 2141385 did not have any effect when added to J774 together with nimesulide (data not shown). The inhibitory effect of nimesulide on nitrite production from LPS–activated J774 was also fully reverted by the CD73 inhibitor, APCP (5 μM, Figure 29 C). However, the inhibitory effect of celecoxib (10 μM) on nitrite accumulation from LPS-activated J774 was neither affected by ZM 241385 nor by APCP (Figure 29 B and C). Exogenously added PGE_2 inhibited, in a concentration-related manner, nitrite production from LPS–activated J774 cells without affecting nitrite production from not stimulated cells (Figure 29 D).
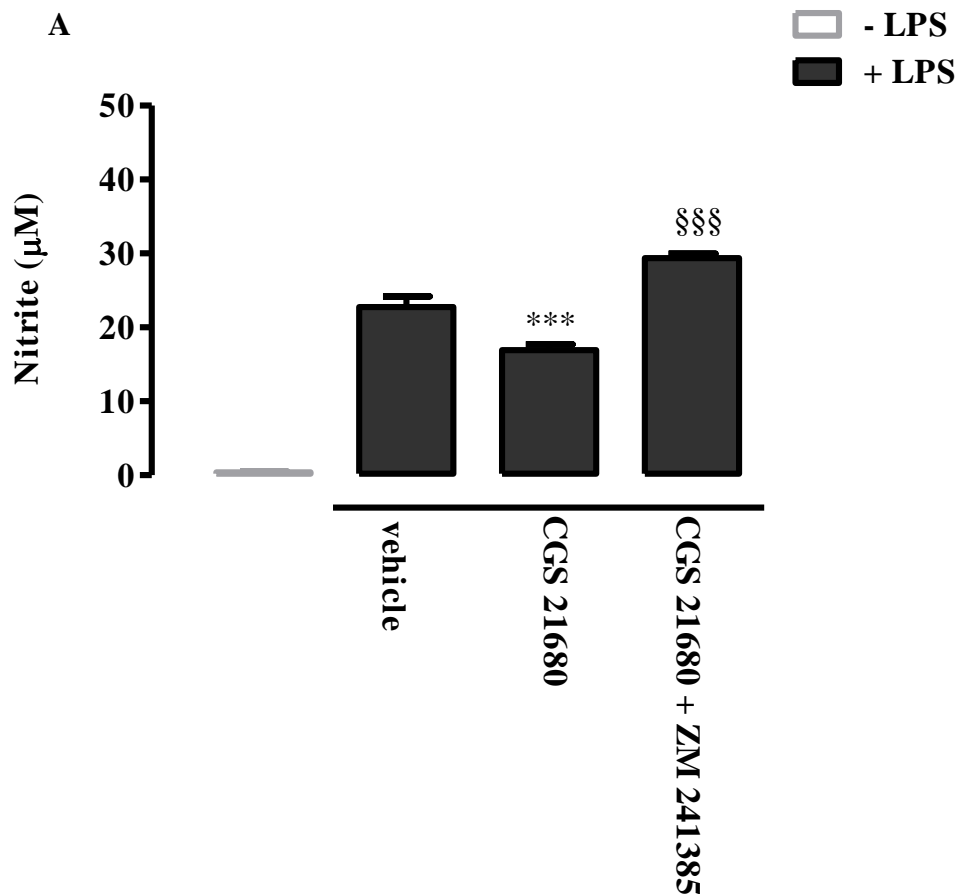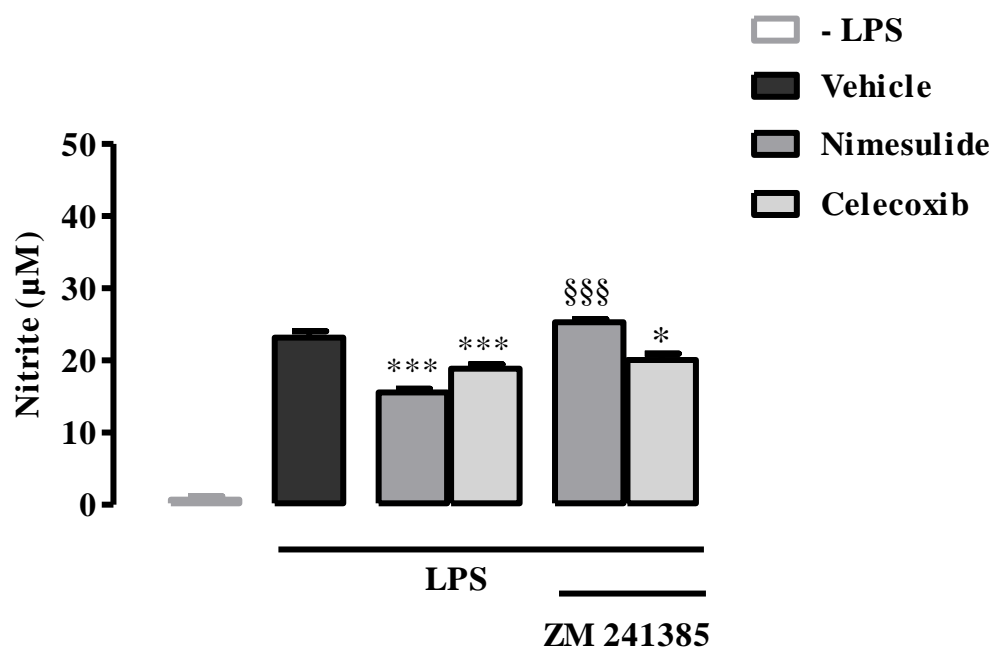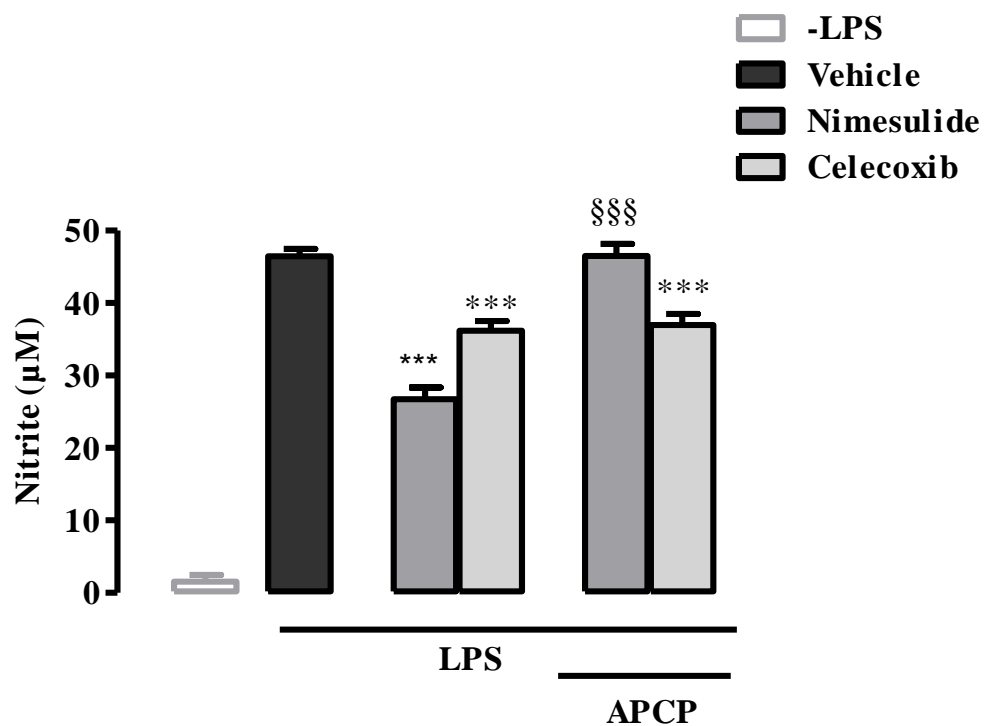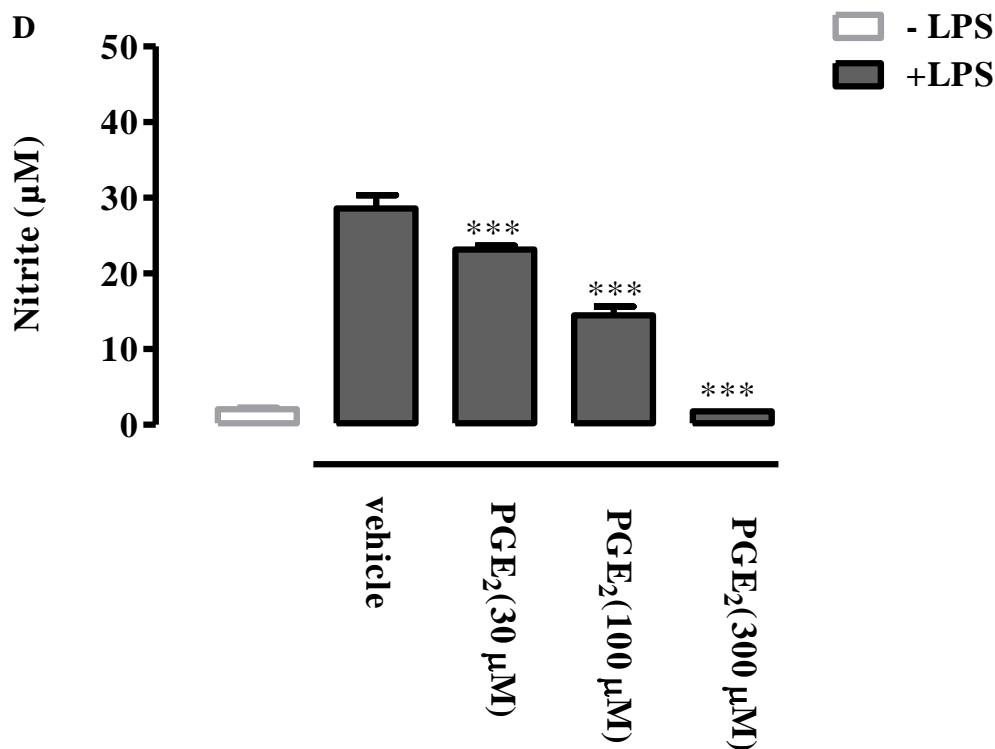
Figure 29. Effect of treatments on nitrite production from LPS-activated J774A.1 macrophages. A) *** p< 0.001 *vs.* vehicle and §§§ p<0.001 *vs.* CGS 21680; **B)** ***p <0.001 *vs.* vehicle and §§§ p <0.001 *vs.* nimesulide; **C)** §§§ p <0.001 *vs.* nimesulide and *** p<0.001 *vs.* vehicle; **D)** ***p <0.001 *vs.* vehicle. Bonferroni's Multiple Comparison Test (n = 5–8).

2.2.2.9 Ecto-5' nucleotidase (e-5'NT)/CD73 activity

LPS–activated J774 (250 x 10^3/well) showed reduced AMPase activity evaluated as Pi (pmol/min) accumulation following incubation with AMP (2 mM). Nimesulide (100 μM) increased Pi accumulation in both non-activated and LPS-activated cells. (Figure 30 A). The specificity of nimesulide effect was proved by evidence that it was blocked by CD73 inhibitor, APCP (5 μM). APCP also significantly reduced Pi accumulation from naïve cells (from 17.67 ± 1.4 μM, n=9 to 3.7 ± 1.8 μM, n= 3; p <0.001). AMP hydrolysis was not affected when J774 were incubated with celecoxib (Figure 30 B). AMPase activity was also evaluated as etheno adenosine-accumulation using ε-AMP as substrate by UPLC analysis. Similar to data described above, nimesulide (100 μM) increased CD73 activity by non-

activated and LPS-activated cells (250×10^3/well) while it was unaffected by celecoxib (10 µM) (Figure 30 C).

A

B

C
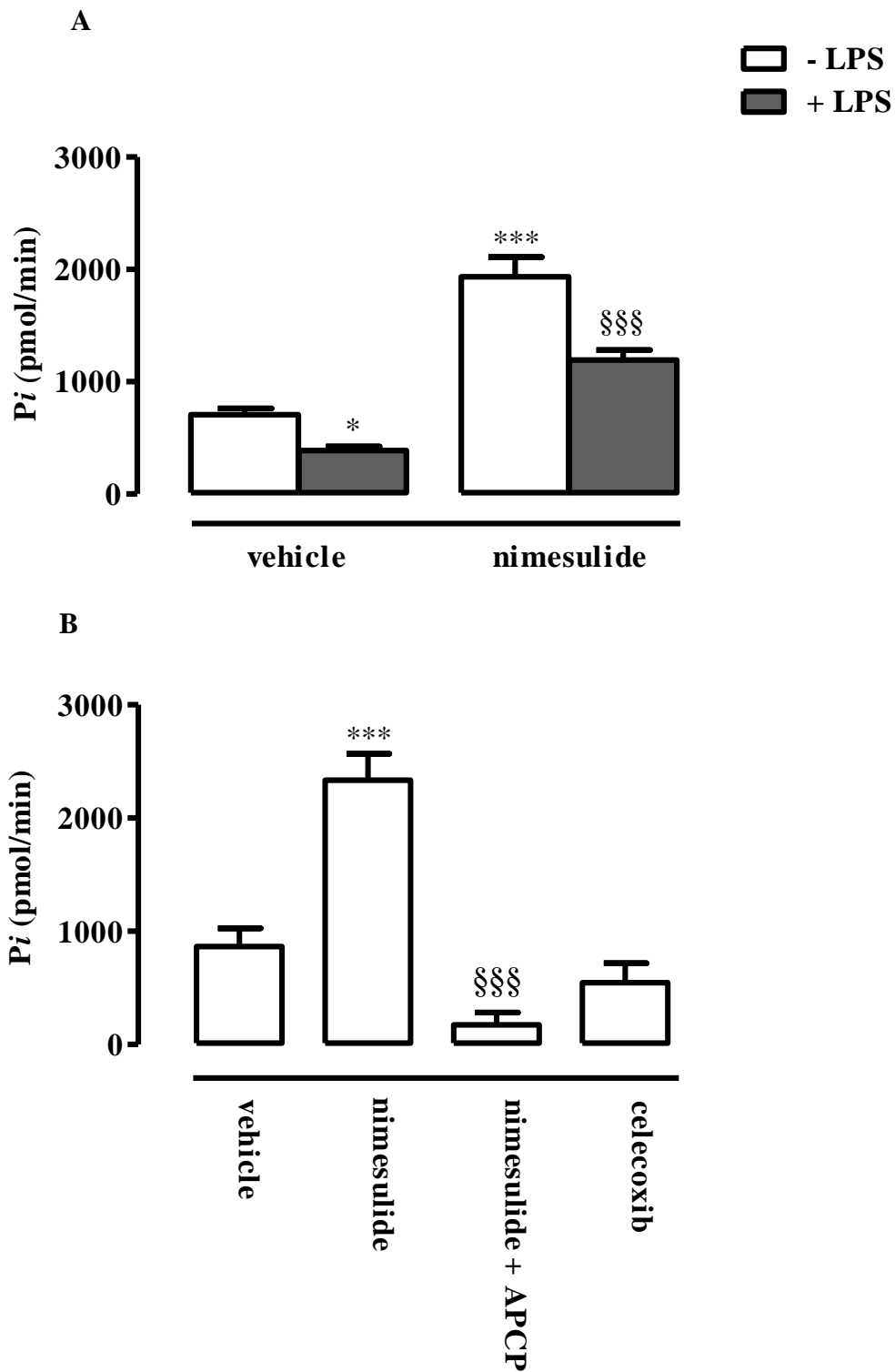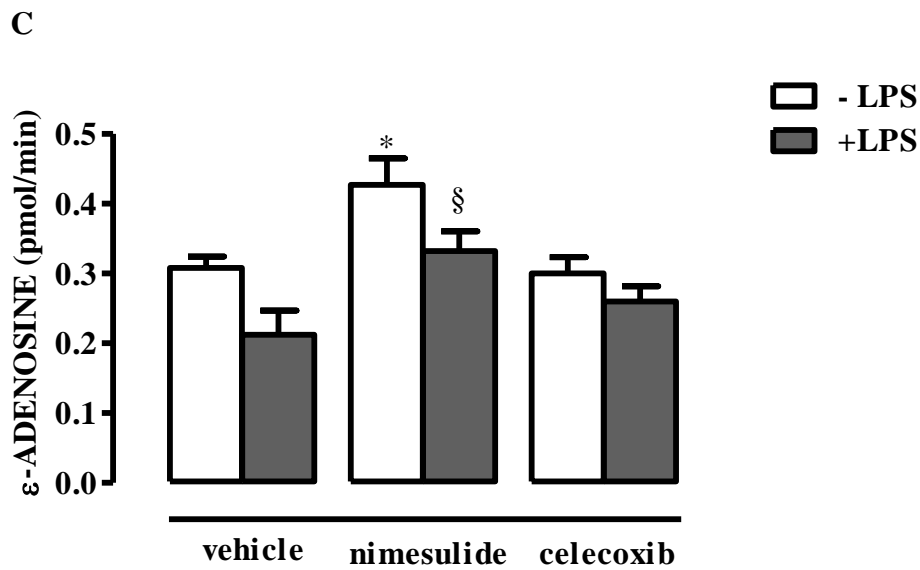
Figure 30. Ecto-5'nucleotidase/CD73 activity in J774 evaluated as P*i* **accumulation (A,B) and as etheno adenosine-accumulation (C). A)**** p <0.05 and *** p <0.001 *vs.*– LPS/vehicle; [§§§] p <0.001 *vs.* + LPS/vehicle; **B**) *** p < 0.001 *vs.* vehicle and [§§§] p <0.001 *vs.* nimesulide; **C**) * p <0.05 *vs.* - LPS/vehicle; [§] p <0.05 *vs.* + LPS/vehicle. Bonferroni's Multiple Comparison Test (n = 6).

2.2.2.10 Nucleotides and nucleosides production by J774A.1

It is well known that nucleotides and nucleosides are involved in the inflammatory process.

UPLC analysis showed that there was no difference in ATP, NAD, ADP, inosine, hypoxanthine production by J774A.1 among different treatments.

Consistent with an increased CD73 activity, following incubation with nimesulide we observed a slight, not significant, reduction in AMP production and a significant increase in adenosine production by both non-stimulated and LPS-stimulated cells.
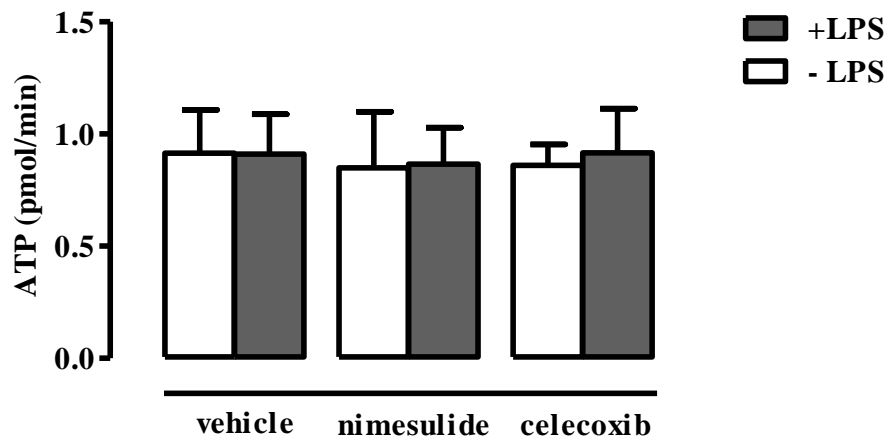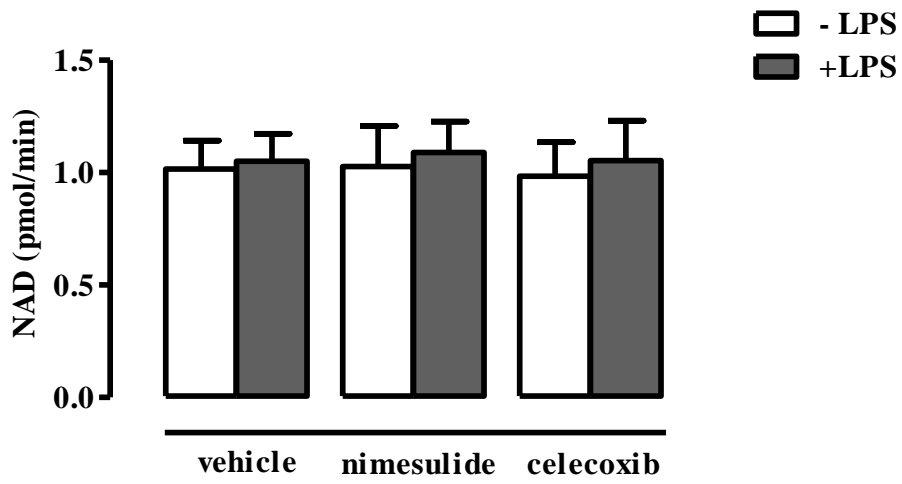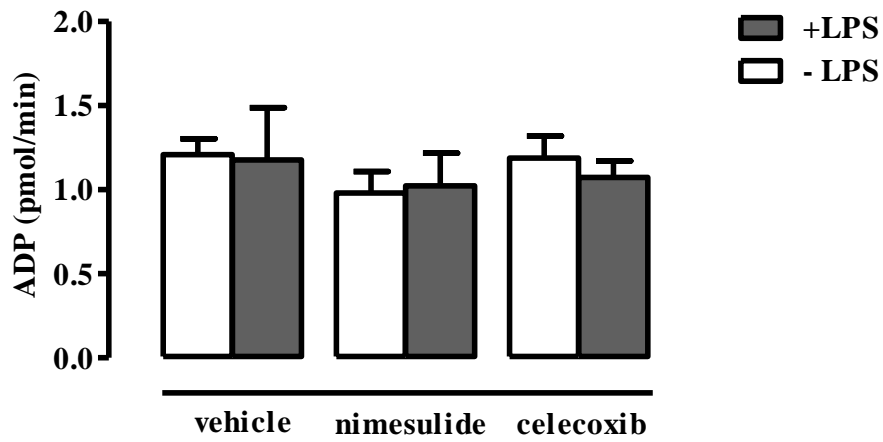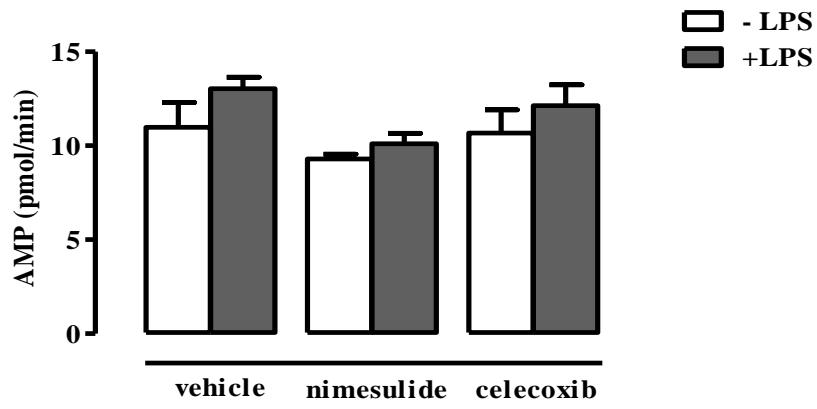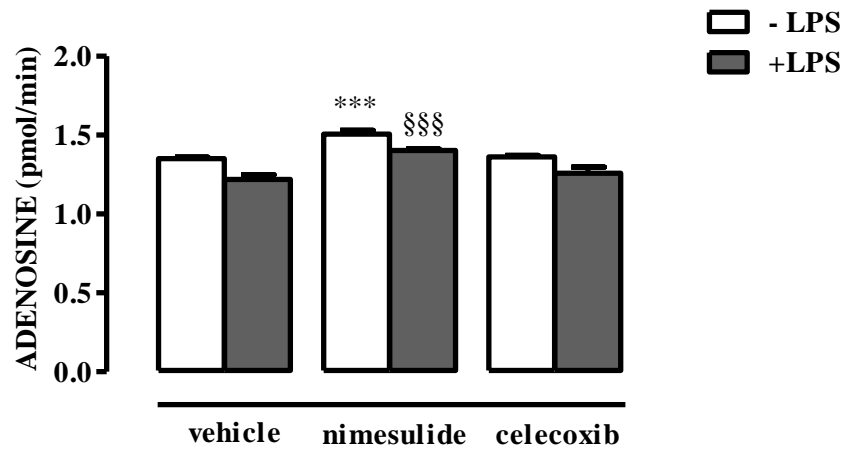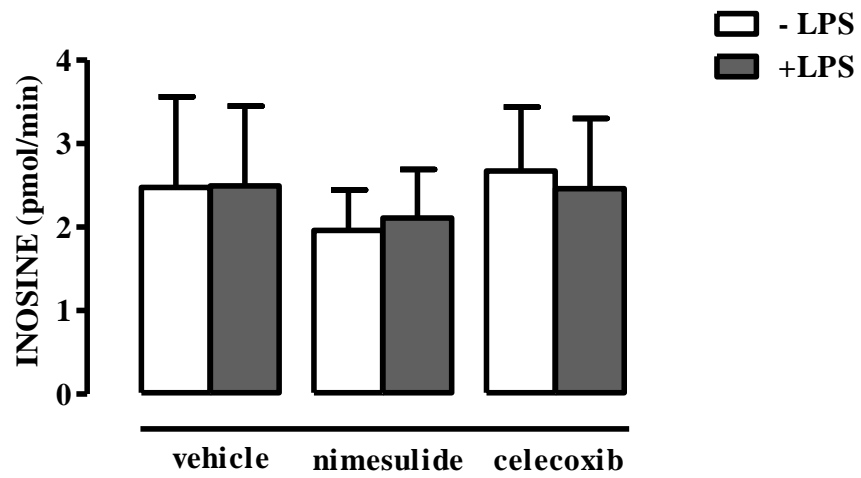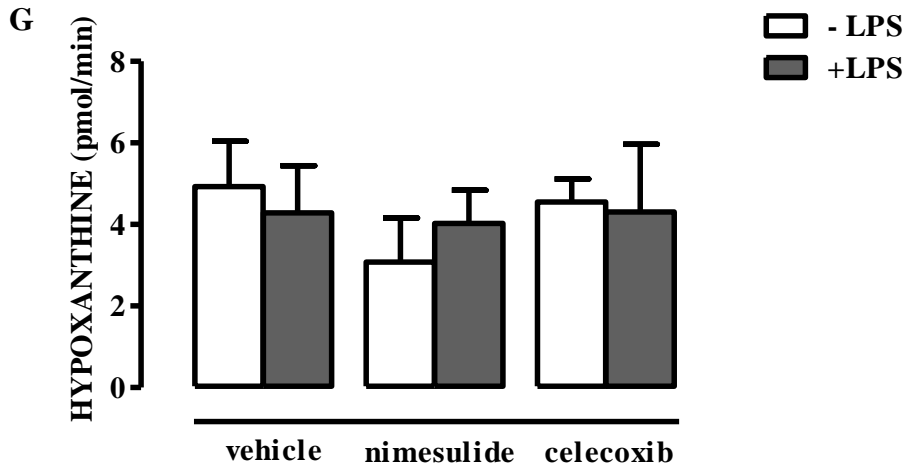
Figure 31. Production of A) ATP; B) NAD; C) ADP; D) AMP; E) Adenosine; F) Inosine; G) Hypoxanthine by J774A.1. *** $p < 0.001$ *vs.* - LPS/vehicle and §§§ $p < 0.001$ *vs.* + LPS/vehicle. Bonferroni's Multiple Comparison Test (n = 6).

2.2.2.11 PGE$_2$ assay

There was a significant increase in PGE$_2$ production following J774 activation with LPS that was almost completely inhibited by nimesulide (100 μM). Either ZM 241385 (10 μM) and APCP (5 μM) increased PGE$_2$ levels of nimesulide-treated cells above control value (Figure 32). Treatment of cells with CGS 21680 (1 μM) did not significantly modify PGE$_2$ production from LPS activated J774 (662.3 ± 53.28 pg/ml *vs.* 770.8 ± 42.56 pg/ml; Bonferroni's Multiple Comparison Test, n= 6).
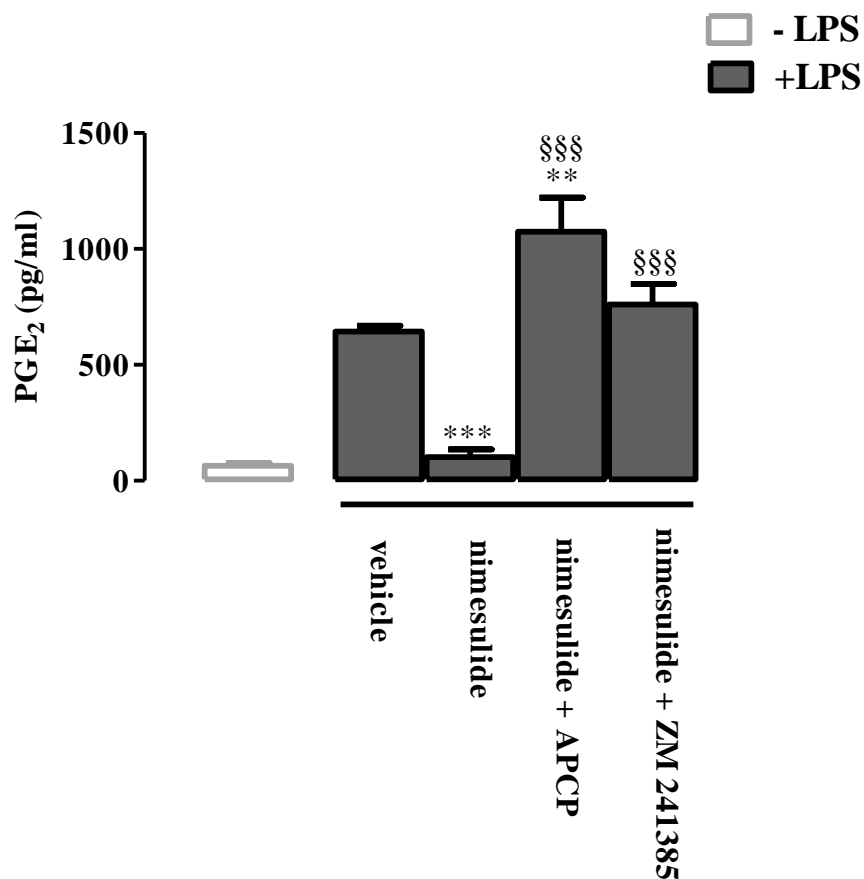
Figure 32. PGE$_2$ accumulation in the culture medium. ** $p<0.01$ and *** $p<0.001$ *vs.* vehicle; §§§ p <0.001 *vs.* nimesulide. Bonferroni's Multiple Comparison Test (n = 6).

2.2.2.12 Western blotting analysis

Western blotting analysis performed on J774A.1 cell line showed that following activation with LPS there was a significant increase of COX-2 expression that was not affected by nimesulide (Figure 33).

A

B
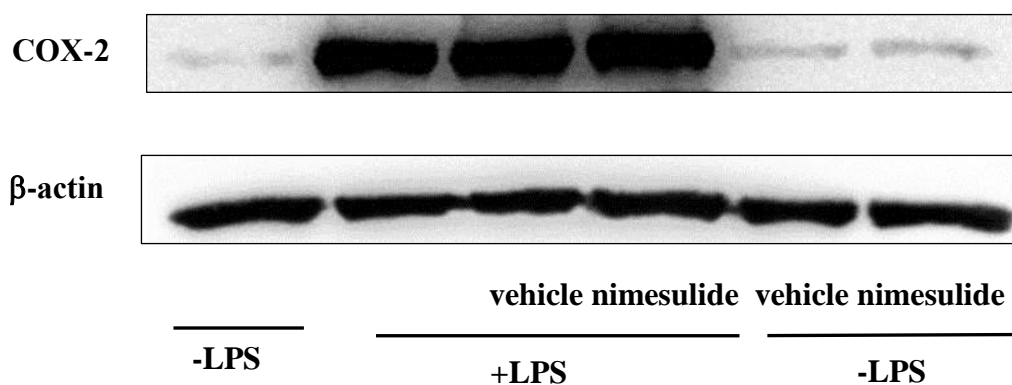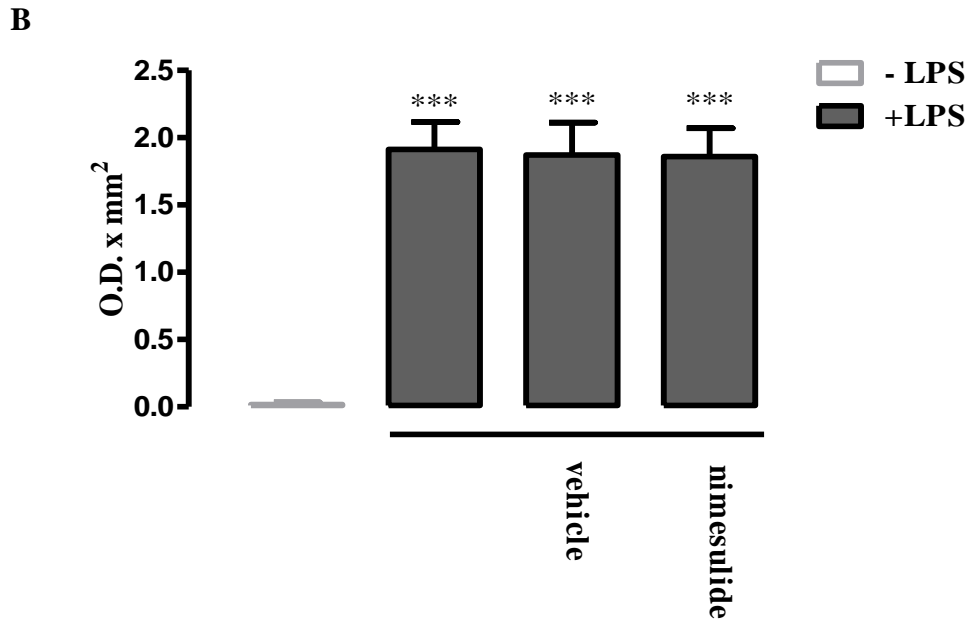
Figure 33. Detection of COX-2 enzyme by western blotting analysis (A) in J774A.1and B) densitometric analysis (O.D. optical density). ***p<0.001 *vs* cells –LPS. Dunnett's Multiple Comparison Test (n = 6).

2.2.2.13 Fluorescence-activated cell sorting (FACS) analysis

LPS significantly increased e-5'NT/CD73 expression on J774A.1 (87.1 ± 3 % CD73^{+} cells; ***p<0.001 *vs.* cells; Dunnett's Multiple Comparison Test (n=5); Figure 34C). Neither nimesulide nor celecoxib modified e-5'NT/CD73 expression on macrophage cell line J774A.1.
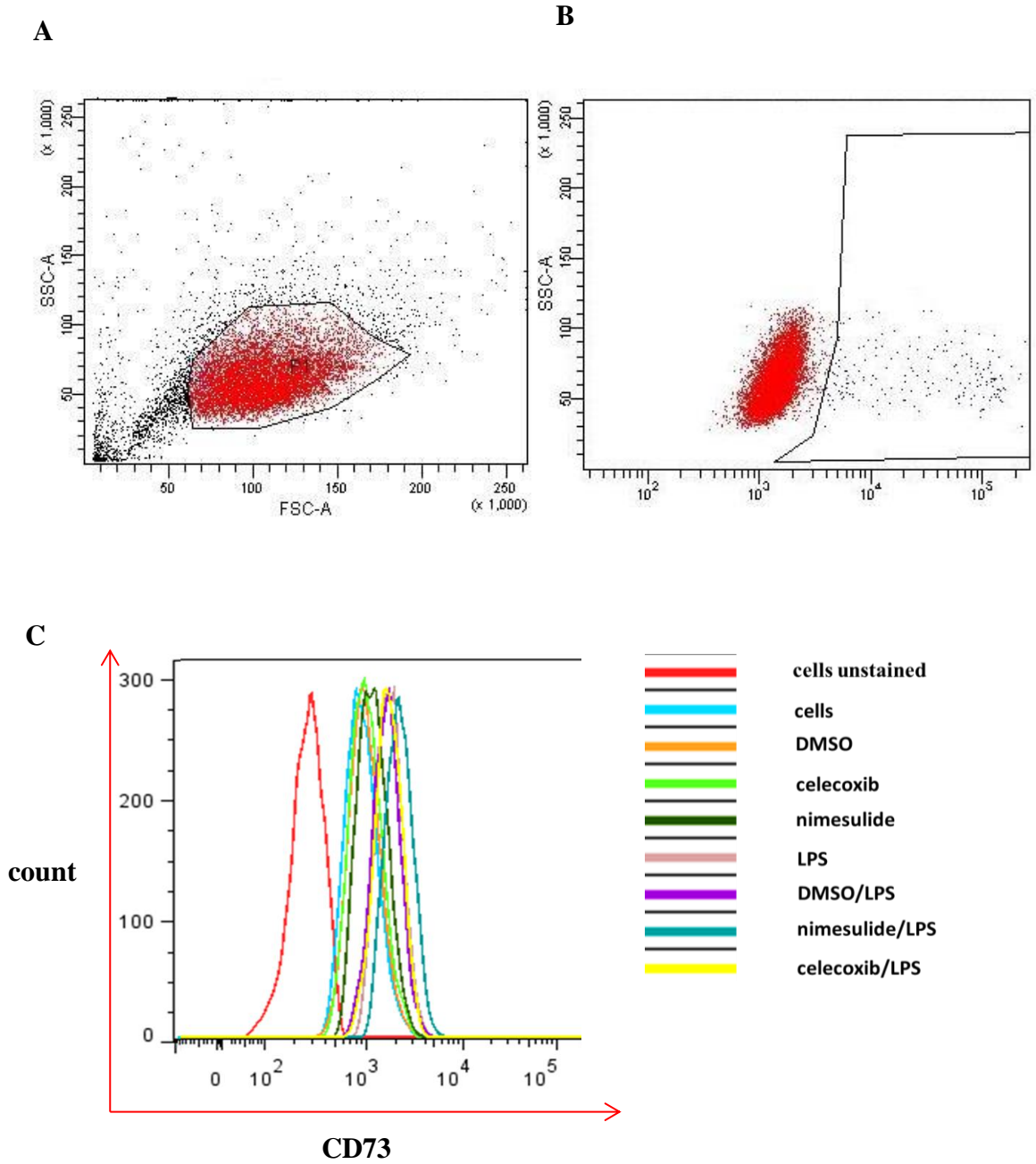
Figure 34. Representative plots illustrating the gating used to identify J774A.1 defined as CD11b+ cells (A) and viable cells defined on the basis of a negative DAPI-staining (B) by flow cytometry. C) histograms representing flow cytometric analysis of the surface expression of CD73 on J774A.1.

2.2.2.14 SiRNA CD73 silencing

After siRNA treatment, CD73 transcripts were reduced by 69 ± 12 % (n=3) as revealed by real-time PCR (not shown). The expression of CD73 protein was confirmed by means of flow cytometry, as shown in Figure 35A and B. The effect of nimesulide (100 μM) on nitrite production was then assessed in siRNA CD73-treated cells. LPS significantly induced nitrite production in siRNA-treated cells (10,0 ± 4,3 μM, n=3), effect that was not significantly different from that of control cells (17,08 ± 2 μM, n=3). Nimesulide (100 μM) significantly inhibited LPS effect in control cells, while it did not impair nitrite production in siRNA-treated cells (Figure 35C).

A
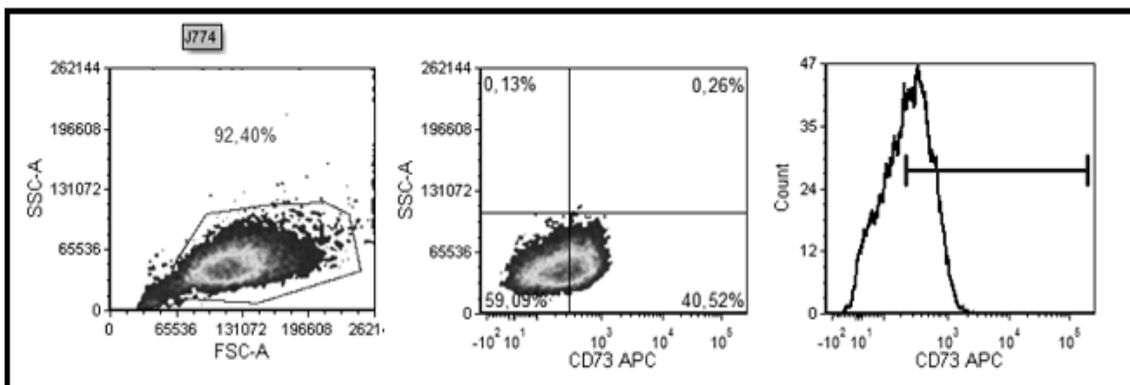
B
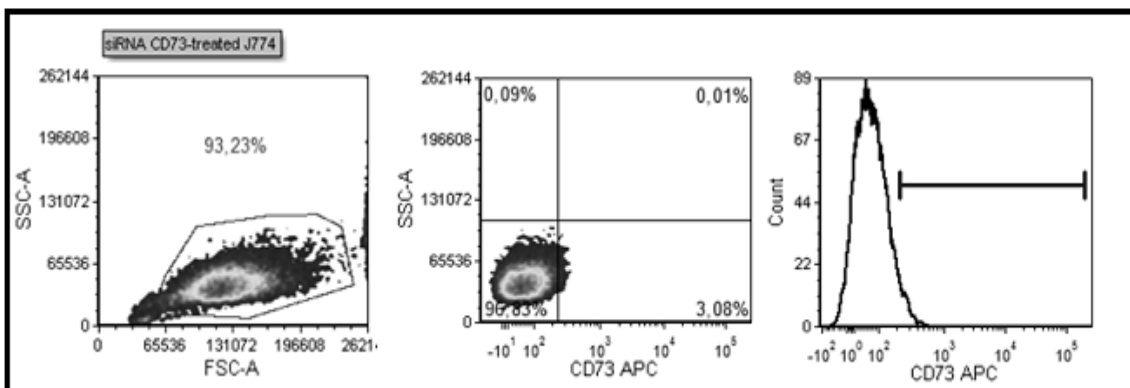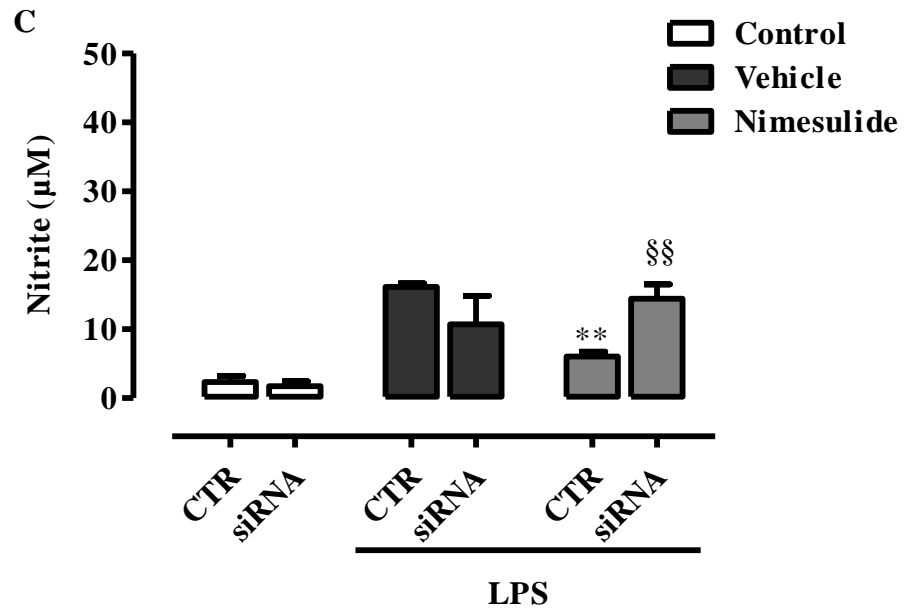
Figure 35. Effect of nimesulide on siRNA CD73 J774. Representative flow cytometry (dot plots and histograms) outlining CD73 expression is shown. A) control J774, **B)** silenced J774 cells (siRNA-CD73-treated J774). **C)** Effect of nimesulide (100 μM) on nitrite production in CTR and siRNA CD73-J774 (siRNA) stimulated with LPS (1 μg/ml). **p<0.01 *vs.* CTR/vehicle, [§§] p<0.01 *vs.* CTR/ nimesulide. Bonferroni's Multiple Comparison Test (n = 3).

2.3 ADENOSINE/A$_{2A}$ SIGNALLING PATHWAY AND FGF-2

2.3.1 MATERIALS AND METHODS

2.3.1.1 Reagents

DMSO and λ-carrageenan, were purchased from Sigma-Aldrich (Milan, Italy); CGS 21680, ZM 241385, were obtained from TOCRIS Bioscience (Bristol, UK); DTT, PMSF and leupeptin were purchased from ICN Pharmaceuticals (Italy). Polyclonal antibody to A$_{2A}$ receptor and polyclonal antibody to FGF-2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-goat IgG conjugated to horseradish peroxidase was purchased from Dako (Denmark); anti-rabbit IgG conjugated to horseradish peroxidase was purchased from Jackson ImmunoResearch (West Grove, PA). Fluorescein isothiocyanate-conjugated anti-rabbit and biotin-conjugated anti-goat were obtained from Jackson ImmunoResearch (West Grove, PA). DAPI and streptavidin-Texas Red were purchased from Vector Laboratories (Burlingame, Ontario, Canada). Paraffin, Hematoxylin of Mayer solution and eosin 0.5% alcoholic solution were obtained from Kaltek (Padova, Italy). All other salts and reagents were purchased from Carlo Erba (Milan, Italy).

2.3.1.2 Animals

Male Wistar rats, body weight 200–250 g, were purchased from Harlan Nossan, (Milan, Italy). Animals were kept under standard conditions with food and water *ad libitum* and maintained in a 12 h/12 h light/dark cycle at $22 \pm 1°$ C. Before the experiments, rats were housed and acclimatized under these conditions for four days. All the *in vivo* procedures were performed according to the Italian and European regulations (DL/2014) on the protection of animals used for experimental and other scientific purposes and were approved by Italian Ministry of Health.

2.3.1.3 Carrageenan-induced paw oedema

Rats, slightly anaesthetized with 4% enflurane mixed with 0.5 L/min O_2, 0.5 L/min N_2O, received in the left hind paw 100 µL of λ-carrageenan suspension (1% w/v). Paw volume was measured at the time zero and each hour for 6 h by a hydropletismometer (Ugo Basile, Comerio, VA, Italy).

2.3.1.4 Drug treatments

Animals were divided in groups of 5 each and treated, just before carrageenan injection, with the A_{2A} agonist, CGS 21680 (2 mg /kg i.p.); with the A_{2A} antagonist, ZM 241385 (3 mg/kg i.p.); with CGS 21680 (2 mg/kg i.p.) plus ZM 241385 (3 mg /kg i.p.) or with an equal volume of vehicle (DMSO). From different groups of animals, 3 hours following oedema induction, paws were excised, cut, frozen in liquid nitrogen or fixed in 10 % (v/v) buffered formalin and stored for further analysis. Controlateral, non-injected paws (sham), were also excised as control tissues.

2.3.1.5 Western blotting analysis

Tissue samples were defrosted, weighed and homogenized with a Polytron. In order to extract proteins, 1 ml of buffer (β-glycerophosphate 50 mM; sodium orthovanadate 100 µM; $MgCl_2$ 2mM; ethylene glycol tetraacetic acid (EGTA) 1mM; DTT 1 mM; PMSF 1mM; Aprotinin 10µg/ml; leupeptin 10µg/ml) was added to 100 mg of tissue samples. The homogenates were centrifuged at 2500 rpm for 10 minutes at 4°C. The supernatants were then centrifuged at 12000 rpm for 30 minutes at 4°C. Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad, Italy). Denaturated samples (50 μg) were subjected to electrophoresis on SDS 12% polyacrylamide gel and transferred onto a nitrocellulose transfer membrane (Protran, Schleicher & Schuell, Germany). The membranes were blocked by incubation with non-fat dry milk (5% w/v) in PBS

supplemented with 0.1% (v/v) Tween 20 for 1 h at room temperature and then incubated with a rabbit polyclonal anti-FGF-2 antibody (1:1000 dilution) overnight at 4° C on a shaker. Successively, membranes were washed and then incubated with the secondary antibody conjugated with horseradish peroxidase, anti-rabbit IgG-HRP (1:5000 dilution) for 2 h at room temperature. Protein bands were detected using the ECL detection kit and Image Quant 400 GE Healthcare software (GE Healthcare, Italy). Protein bands were quantified using Quantity One® software (Biorad, Italy). Successively, to confirm the equal protein loading, membranes were stripped and incubated with anti β-actin monoclonal antibody (1:2000 dilution) and subsequently with anti-mouse IgG conjugated to peroxidase (1:2000 dilution), both for 3 h at room temperature.

2.3.1.6 Morphological analysis

Tissue sample removed as described above were fixed in 10 % (v/v) buffered formalin for 48 h and embedded in paraffin by conventional techniques. For morphological analysis, sections 7 μm thick, were stained with haematoxylin and eosin to demarcate cell type. The sections were analyzed by using a standard light microscope (x 20 objective) and photographed under low power. Images were taken by a Leica DFC320 video camera (Leica, Milan, Italy) connected to a Leica DM RB microscope using the Leica Application Suite software V 4.1.0.

2.3.1.7 Picro Sirius red staining

Picro Sirius red staining and polarization microscopy represent a powerful method for detecting total collagen tissue content (Junqueira *et al*., 1978). Briefly, the paraffin sections were de-waxed and rehydrate. The sections were stained with Mayer's haematoxylin to visualize the nuclei and then incubated with 0.1% (w/v) Sirius red in saturated picric acid solution for one hour at room temperature. After washing with acidified water, slides were

alcohol dehydrated, mounted in a resinous medium, and visualized on a Leica Microscope under 90-degree polarized light.

2.3.1.8 Immunofluorescence

Samples were processed according to standard protocols and a series of sections of the flesh of paw were embedded in paraffin and cut into 7-μm-thick slices. Then, sections were cleared in xylene and hydrated before staining. The sections were incubated with rabbit polyclonal anti-FGF-2 (1:100 dilution) antibody in casein *plus* Triton X-100 0.3% over night at 4°C and then washed with PBS *plus* Tween 0.05% before incubation with goat polyclonal anti-A_{2A} receptor (1:100 dilution) antibody in casein for 3 hours at room temperature. To detect co-localization of FGF-2 and A_{2A} receptor, slides were incubated for 30 min at room temperature with mixture of two secondary antibodies in the dark: fluorescein isothiocyanate-conjugated anti-rabbit antibody (1:250 dilution), and biotin-conjugated anti-goat (1:250 dilution) antibody coupled with streptavidin-Texas Red (1:200 dilution). Slides were then washed in PBS-Tween 0.05% and mounted in Vectashield Mounting Medium with DAPI (1.5 mg/mL). Images were observed using a fluorescence microscope Leica DM RB (Leica Microsystems, Wetzlar, Germany) equipped with appropriate standard filter, acquired using Leica Application Suite V 4.1.0 software and then analysed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA).

2.3.1.9 Statistical analysis

All results were expressed as mean ± standard error (S.E.) and analyzed by one way ANOVA followed by Bonferroni's test for multiple comparisons or Dunnett's test. A value of $p < 0.05$ was taken as statically significant.

2.3.2 RESULTS

2.3.2.1 Carrageenan–induced rat paw oedema

Injection of carrageenan into the rat hind paw caused an oedema peaking between 3 and 4 hours. Treatment with CGS 21680 inhibited, in a dose–related manner, oedema development and this effect was reversed by co-administration with the A_{2A} antagonist, ZM 241385. On the contrary, ZM 241385 alone did not modify oedema (Figure 36).
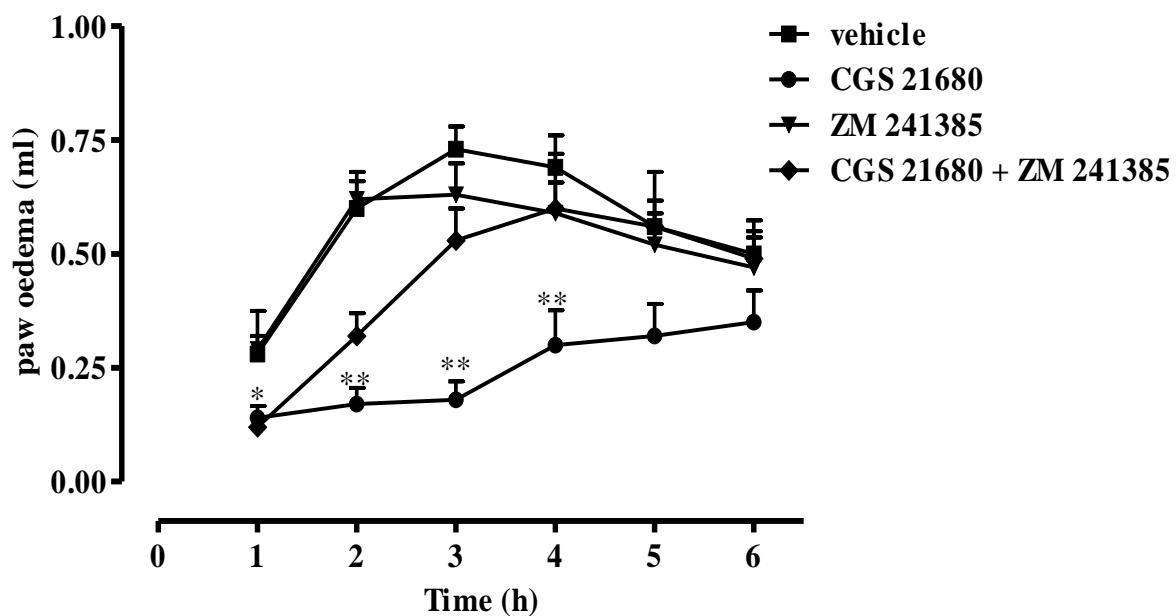
Figure 36. Effect of CGS 21680 treatment on carrageenan–induced oedema. * $p<0.05$ and **$p<0.01$ *vs* control. Bonferroni 's Multiple Comparison Test (n=10)

2.3.2.2 Morphological analysis

Morphological examination of tissue sections showed cell infiltrate in inflamed tissue, characterized by neutrophils and degranulated mast cells (Figure 37B) that was greatly reduced by rat treatment with CGS 21680 (Figure 37C).

A

B

C
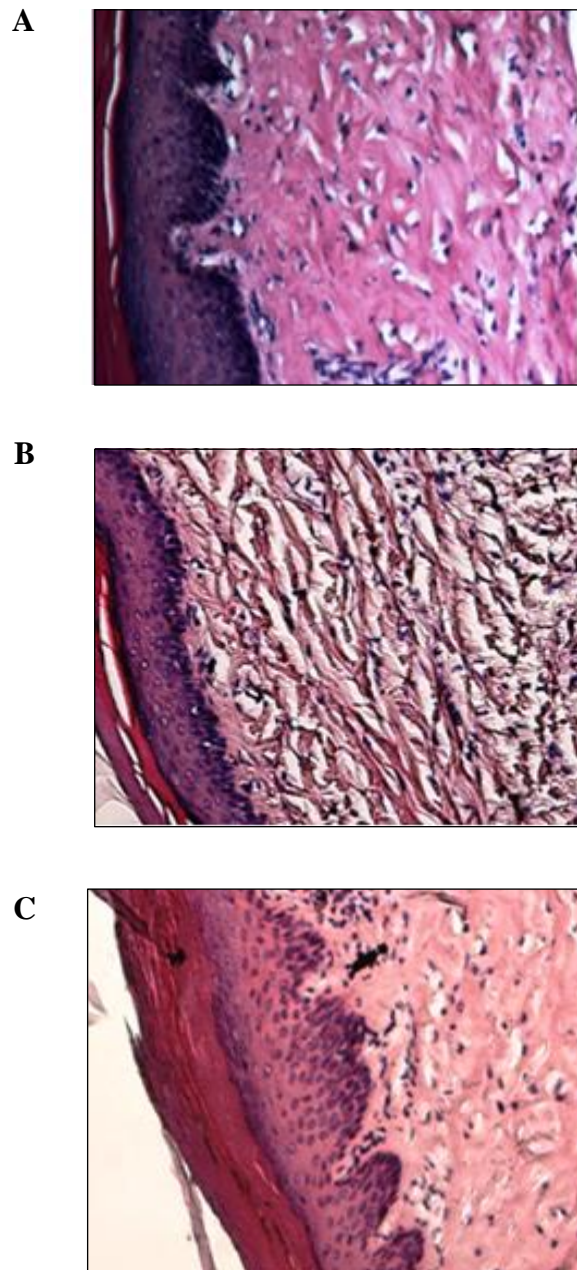
Figure 37. Morphological analysis . Hematoxylin and Eosin (H&E) stained paw tissue from **A**) sham rats, **B**) vehicle and **C**) CGS 21680 treated rats (n=10). Original magnification: x20.

2.3.2.3 Picro Sirius red staining

Picro Sirius red staining for collagen quantification showed that in tissue section from inflamed animals dermal collagen appeared loose compared to paws from non-inflamed and from CGS 21680 treated animals where dermal elastic fibres were well organized (Figure 38 A). Furthermore, when sections were analyzed under polarized light an increased number of yellow/orange collagen fibers were evident in tissue from CGS 21680 treated animals, compared to control tissues were greenish thinner fibers were predominant (Figure 38 B).
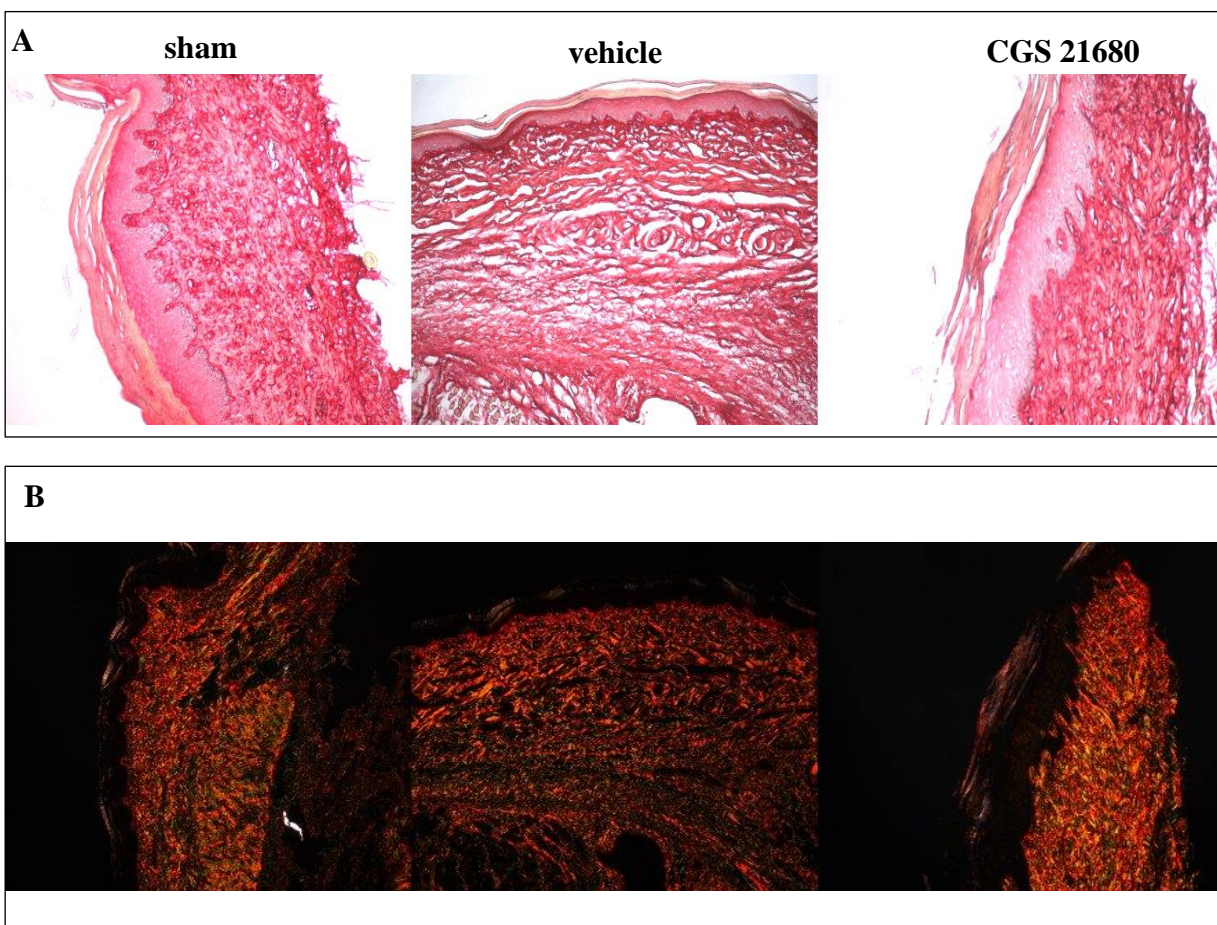
Figure 38. Sections of paw tissue stained with Picrosirius red (A) and polarizing microscopy (B) for the detection of collagen. (n = 5). Original magnification: x10.

2.3.2.4 Western blotting analysis

FGF-2 expression (18 kDa) in paws of CGS 21680 treated rats was increased at each hour following carrageenan injection (Figure 39 B) compared to the FGF-2 expression from inflamed and control paws (Figure 39A).

A

B

C
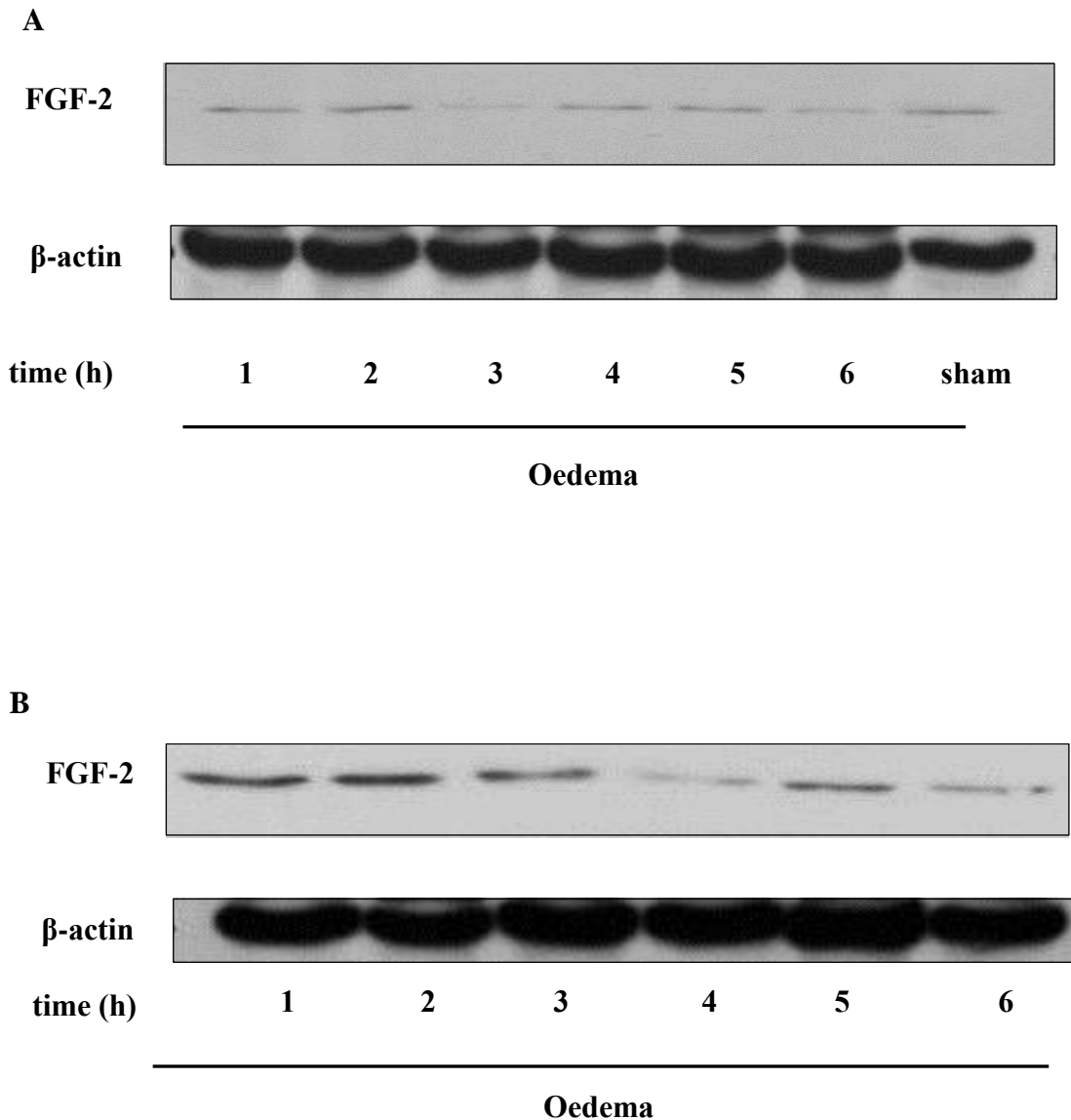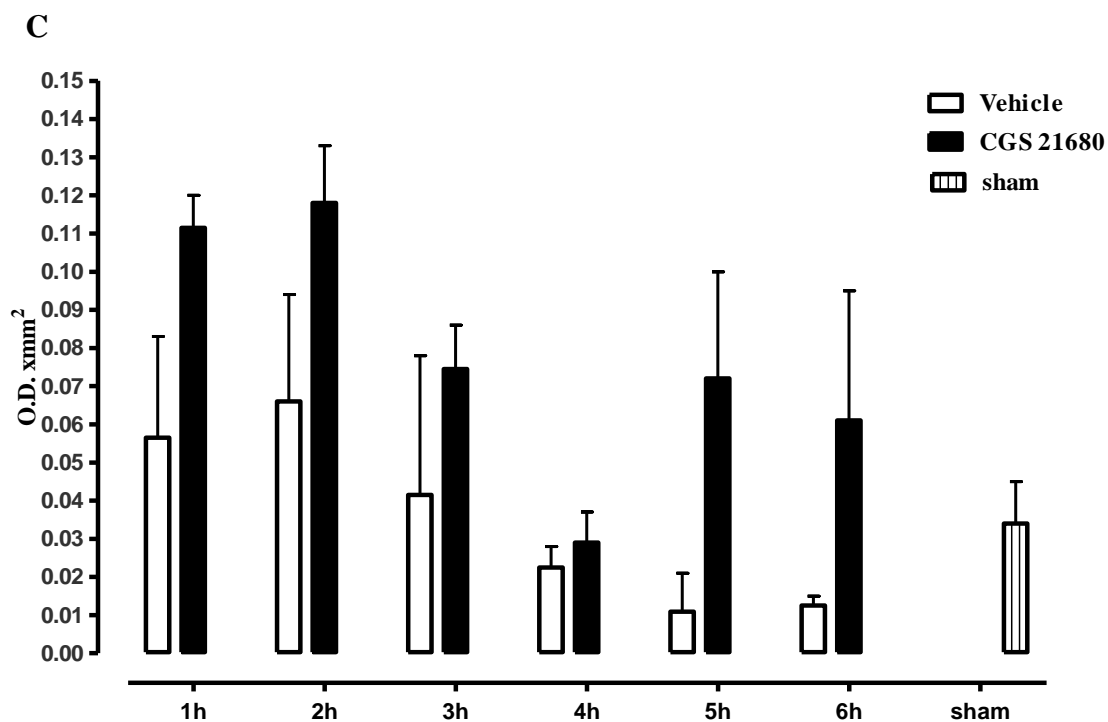
Figure 39. Detection of FGF-2 by western blotting analysis in inflamed paws from vehicle (A) and CGS 21680 (B) treated rats each hour following carrageenan injection and C) densitometric analysis (O.D. optical density). (n=2)

2.3.2.5 Immunofluorescence

On tissue obtained as described above, A_{2A} receptor and FGF-2 (18 kDa) were localized by immunofluorescence analysis (Figure 40). In control paws immunolocalization of $A_{2A}AR$ was increased compared to the immunolocalization observed in paws from CSG 21680 treated rats suggesting that $A_{2A}AR$ was mostly associated with recruited inflammatory cells. Conversely, immunolocalization of FGF-2 was increased in paws from CGS 21680 treated rats compared to immunolocalization obtained in paws from control rats.

Furthermore, spots of co-localization between $A_{2A}AR$ and FGF-2 were also evidenced (arrows).

120
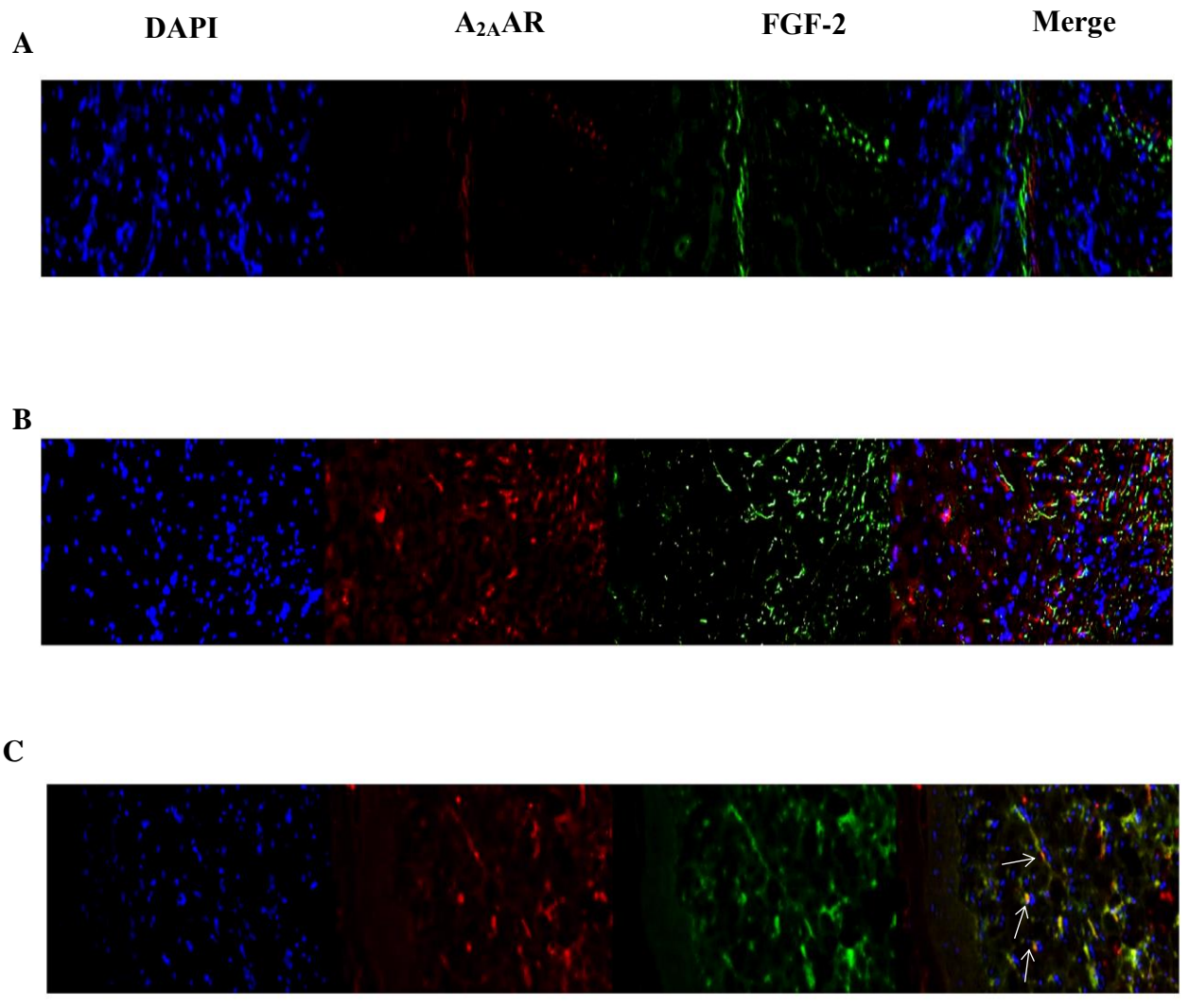
Figure 40. Immunofluorescence detection of A_{2A} receptor and FGF-2 (18 kDa) on rat paws from A) sham, B) vehicle, C) CGS 21680 treated rats 3 h following oedema induction. n=5.

3.1 CD73 AND ACUTE INFLAMMATION

Adenosine has been recognized to be a nucleoside with autocrine/paracrine functions, acting as a signal molecule to preserve host defence and tissue integrity during inflammation and trauma. The ecto-5'nucleotidase/CD73, working in concert with the nucleoside triphosphate phosphohydrolases (NTPDase1/CD39), degrades AMP to adenosine and represents a key enzyme for adenosine accumulation at the site of injury (Antonioli *et al*., 2013). Its function would be to regulate the accumulation of adenosine into the extracellular milieu that, in turn, would limit tissue damage through A_{2A} receptor activation (Palmer and Trevethick, 2008). There is evidence that CD73 on endothelial cells plays an important role in the control of vascular leakage and neutrophil adhesion and migration, and its endothelial expression is increased under hypoxia and correlates to extracellular adenosine accumulation (Thompson *et al*., 2004). A study performed on knockout mice for CD39 and CD73 has shown that these enzymes represent an innate protective pathway from airway damage caused by mechanical ventilation, protecting from increased vascular leakage and cell accumulation into the lung (Eckle *et al*., 2007). Therefore, it has been suggested that CD39 and CD73 contribute to an endogenous anti-inflammatory mechanism. The pharmacological manipulation of endogenous mediators, actively involved in the downregulation of inflammation, might be an innovative anti-inflammatory strategy. Hence, a better understanding of the role of these enzymes in the development of inflammatory responses allow identifying novel molecular targets useful for developing innovative anti-inflammatory agents. The objective of our study was to elucidate the role of CD73 in the development of inflammation, for this purpose we performed experiment on carrageenan-

induced pleurisy model in rats. Carrageenan-induced pleurisy is a well-known model of inflammation characterized by cellular influx, fluid extravasation, and various biochemical parameters involved in the inflammatory response. The carrageenan pleurisy model has been accepted as a reliable method to study acute (4 h) and sub-acute (48-72 h) inflammation, providing information on cells and type of inflammatory mediators involved in the inflammatory process. In our experiments, through pharmacological blockade of CD73 by using the selective inhibitor, APCP, the development of pleural inflammation was assessed 4 and 72 h after the injection of carrageenan. We found that CD73 inhibition significantly increased cell accumulation and exudate formation into the pleural cavity 4 h following carrageenan injection but did not have effect on the sub-acute phase of inflammation. There is evidence that the pro-inflammatory cytokines such as TNF-α,IL-1β, IL-6, MCP-1 help to sustain the extension of a local or systemic inflammatory process. We observed that CD73 inhibition increased pro-inflammatory cytokine content into the pleural cavity in the acute phase of inflammation while only MCP-1 levels were still significantly high 72 h following carrageenan injection thus confirming previous data (Reutershan *et al.*, 2009) that indicate the important role of CD73 in leukocyte migration. To investigate whether the effect of local *in vivo* treatment with CD73 inhibitor was due to a specific chemoattractant ability of APCP or was only secondary to the increase in chemotactic cytokine content that it caused, we also performed *ex vivo* experiments, on cells collected from the inflamed site. Interestingly we found that cells collected from APCP treated rats, 4 hours following carrageenan-induced pleurisy showed increased ability to migrate either spontaneously or under a chemotactic *stimulus*. This finding suggests that the *in vivo* treatment with APCP not only increases the infiltration of the lung with PMNs, but may induce cells (PMNs) to change in a phenotype with increased ability to migrate. In parallel, these cells showed a reduced CD73 expression and activity compared to cells collected from

control group. In contrast, cells from control groups did not migrate in absence of a chemotactic stimulus but their ability to migrate was strongly increased following *in vitro* treatment with APCP. Taken together, our results show that CD73 plays a major role in the early phase of acute inflammation, especially by controlling cell migration. The mechanisms underlying these effects are still to be clarified; they might be due to CD73 enzymatic activity and consequently, to increased extracellular adenosine and/or be independent of adenosine and due to a moonlighting function of CD73. Indeed although biological actions of e-5'NT/CD73 are mainly a consequence its enzymatic phosphohydrolytic activity, CD73 has also been implicated in non-enzymatic functions such as T-cell activation and cell–cell adhesion (Airas *et al*., 1995, Airas and Jalkanen, 1996; Airas, 1998; Loertscher and Lovery, 2002; Andrade *et al*., 2011). Our data, support the view that CD73/adenosine signalling plays an important role as endogenous modulator of inflammation and further highlight the importance of adenosine signalling pathway as a therapeutic target that could be successfully used to treat inflammatory diseases.

3.2 CD73/ADENOSINE/A$_{2A}$ SIGNALLING PATWHAY AND NIMESULIDE ANTI-INFLAMMATORY EFFECT

Accumulated evidence shows that CD39 and CD73 regulate diverse physiological processes that are modulated by adenosine, including inflammation and that adenosine mediates its anti-inflammatory activity primarily through the A$_{2A}$R (Fredholm *et al*., 1996; Antonioli *et al*., 2013). Interestingly, anti-inflammatory drugs that are widely used in clinical practice, such as salicylates and methotrexate, act by enhancing adenosine signalling pathway, at least in part, for their pharmacological activity (Cronstein *et al*., 1994; Montesinos *et al*., 2007). This increase in adenosine is dependent on CD73 activity.

Nimesulide (N-(4-Nitro-2-phenoxyphenyl)-methanesulfonamide) is a selective COX-2 inhibitor that exerts anti-inflammatory activity thanks to unique chemical and pharmacokinetic characteristics, and to a multifactorial mechanism of action.

Indeed, there is evidence that multiple actions of nimesulide on the inflammatory process, such as inhibition of PMNs activity, inhibition of histamine release from mast cells, inhibition of ROS production, are the consequence of regulation of the production and actions of a wide range of inflammatory mediators independently from the sole action on COX-2 activity (Rainsford, 2006).

Various mechanisms have been proposed to explain its particular effects, among wich are a direct interaction with adenosine receptors (Capecchi *et al*., 1993) and the inhibition of the enzyme phosphodiesterase IV (Bevilacqua *et al*., 1994).

More recently, nimesulide has been demonstrated to improve the antirheumatic profile of methotrexate in a murine model of collagen–induced arthritis, with a mechanism that might involve adenosine (Al-Abd *et al*., 2010).

Here we evaluated whether CD73/adenosine signalling was involved in the anti-inflammatory effect of nimesulide, *in vivo* and *in vitro*.

We have first evaluated the effect of adenosine A_{2A} receptor agonist, CGS 21680, in our *in vivo* model of acute inflammation, carrageenan-induced rat paw oedema. It is a well characterised model of inflammation (Moore and Trottier, 1974), useful *in vivo* to analyse the mode of action of anti-inflammatory drugs and also sensitive to COX inhibitors since PGEs are involved (Winter *et al.*, 1962).

In our study CGS 21680 inhibited inflammation, as previously demonstrated in other animal models of inflammation (Fozard *et al.*, 2002; Antonioli *et al.*, 2006; Mantell *et al.*, 2008; Chen *et al.*, 2009; Tang *et al.*, 2010; Vincenzi *et al.*, 2013), and its effect was clearly dependent upon receptor activation, since it was reversed by co-administration with the A_{2A} antagonist, ZM 241385.

Similarly, we found that nimesulide inhibited oedema development and this effect was partially reversed by co-treatment with ZM 241385. This result was thus suggestive of an involvement of adenosine A_{2A} receptor in nimesulide anti-inflammatory effect *in vivo*.

Nimesulide is a COX-2 selective inhibitor; here, we found that its anti-inflammatory effect was paralleled by a reduction of PGE_2 plasma levels, evaluated 4 h following oedema induction, demonstrating that COX-2 was inhibited at the dose used. Conversely, PGE_2 plasma levels were not significantly affected by treatment with CGS 21680. This finding indicates that, under our experimental conditions, the anti-inflammatory pathway mediated by adenosine A_{2A} receptor activation is independent of PGE_2 levels, as also demonstrated *in vitro*, on mouse macrophages (Ezeamuzie and Khan, 2007). Nonetheless, the mechanism linking PGE_2 to adenosine in the control of inflammation remains still poor understood and needs to be investigated more in depth. Since formation of extracellular adenosine by CD73 is crucial to downregulate inflammatory responses either *in vitro* and *vivo* (Haskò *et al.*, 2011; Zimmermann *et al.*, 2012; Antonioli *et al.*, 2013; Chrobak *et al.*, 2015) and CD73 has also been shown to be involved in the molecular mechanism of several well-known anti-

inflammatory drugs, we evaluated the effect of nimesulide on CD73 expression and activity in rat paws.

Western blotting analysis performed on inflamed paws obtained each hour following oedema induction, showed that CD73 was expressed in inflamed tissue and there was no difference in paws obtained from control and nimesulide-treated rats whereas AMP hydrolysis in paws obtained from nimesulide-treated rats was significantly increased. This reflects a functional role of the e-5'NT/CD73 since local treatment with the CD73 inhibitor, APCP, significantly reverted the anti-inflammatory effect of nimesulide, without modifying the oedema development in control animals. These findings indicate that the increased CD73 activity, most likely via the formation of adenosine, contributes to the anti-inflammatory effect of nimesulide in the inflamed tissue. Membrane-bound 5'-nucleotidase can also be shedded into the plasma and can act in concert with a soluble form of NTPDase1/CD39 in the hydrolysis of extracellular ATP to adenosine (Zimmermann *et al*., 2012). Interestingly, we found that systemic administration of nimesulide also significantly increased plasma AMPase activity over basal controls.

On the basis of our *in vivo* findings, we sought to better investigate the involvement of adenosine signalling in mediating the anti-inflammatory effect of nimesulide by performing *in vitro* experiments, on J774A.1 macrophage cell line that represents an *in vitro* model widely used to evaluate inflammatory activation/inhibition and one endpoint to evaluate the extent of this cell line activation is to measure nitrite production following incubation with LPS (D'Acquisto *et al*., 1997; Prestes-Carneiro *et al*., 2007; Shand *et al*., 2011). The anti-inflammatory role of the adenosine A_{2A} receptor was confirmed in the J774 macrophage cell line in that the A_{2A} agonist, CGS 21684, reduced nitrite production following cell activation with LPS and this effect was reversed by the A_{2A} antagonist ZM 241385. Nimesulide inhibited nitrite production from LPS–activated J774 cells and this effect again was reverted

by ZM 241385. Interestingly, the effect of ZM 241385 was lost when added concomitantly to nimesulide (data not shown) but was only evident when it was added 6 hours thereafter, suggesting that adenosine A_{2A} receptor was likely involved but not directly activated by nimesulide. Thus, in the light of these findings, and of results obtained *in vivo*, in the model of carrageenan–induced oedema, we investigated the effect of nimesulide on CD73 activity on J774 cell line. We observed that CD73 activity was reduced following J774 activation with LPS, which is similar to findings in LPS-activated murine peritoneal macrophages (Zanin *et al*., 2012). Similarly, LPS was reported to downregulate CD73 activity in cultured cortical astrocytes (Brisevac *et al*., 2012). We found that pre-treatment with nimesulide strongly increased CD73 activity in naïve J774 cells and this effect was still evident 24 hours following LPS-induced cell activation. Enhanced CD73 activity in the presence of nimesulide was confirmed in independent experiments using ε-AMP as substrate. The increased CD73 activity induced by nimesulide on J774 cell line reflected a functional role as demonstrated by evidence that specific inhibition of CD73 with APCP reverted the nimesulide-induced anti-inflammatory effect as evidenced by the inhibition of nitrite accumulation. Consistent with an increased CD73 activity, following incubation with nimesulide we observed a slight, not significant, reduction in AMP production and a significant increase in adenosine production by both non-stimulated and LPS-stimulated cells. To check if nimesulide could modify CD73 expression on J774 we performed fluorescence-activated cell sorting (FACS) analysis but we observed that nimesulide did not modify CD73 surface expression in both LPS-stimulated and unstimulated J774. While the inhibitory effect of CGS 21680 on nitrite production by LPS-activated J774 was not accompanied by changes in PGE_2 release, cell incubation with nimesulide led to the inhibition of both nitrite and PGE_2 accumulation in the culture medium; both effects were reverted by cell treatment with ZM 241385 or with APCP. Nimesulide did not alter COX-2

expression on LPS-stimulated J774 evaluated by Western blotting analysis. Thus, these findings would correlate the *in vitro* anti-inflammatory effect of nimesulide to COX-2 inhibition; on the other hand, we found that exogenously added PGE_2 inhibited, in a concentration-related manner, nitrite production from LPS–activated J774 cells without affecting nitrite production from non-stimulated cells, in agreement with early studies (Sautebin *et al*., 1996; D'Acquisto *et al*., 1998). These results clearly suggest an important role of the CD73/adenosine/A_{2A} axis to regulate macrophage function and, consequently on the innate immune response, as was already suggested (Alam *et al*., 2014;2015). It is therefore very likely that nimesulide by activating the CD73/adenosine/A_{2A} pathway can inhibit macrophages shifting toward a pro-inflammatory phenotype following activation with LPS. This conclusion is in line with the observation that nimesulide was unable to impair nitrite production when CD73 in J774 cells was downregulated by siRNA. In order to evaluate whether our findings were peculiar of nimesulide or shared with other COX-2 inhibitors, we also performed experiments on J774 macrophage cell line pre-incubated with another selective COX_2 inhibitor, celecoxib. The effect of celecoxib at a concentration that inhibited PGE_2 production and reduced nitrite accumulation from LPS–activated J774 was neither modified by ZM 241385 nor by APCP. Furthermore, consistent with an independent effect of nimesulide on adenosine signalling, celecoxib did not alter CD73 activity in the J774 macrophage cell line. Thus, in addition to the COX-2/PGE_2 pathways, only nimesulide activates the CD73/adenosine/A_{2A} axis. The molecular mechanisms by which nimesulide influences adenosine signalling is presently unclear but might involve a direct or indirect effect on CD73 activation. It is also possible that nimesulide may change CD73 activity by altering membrane fluidity (Horan *et al*., 1994; Sousa *et al.*, 2008). In summary, the *in-vivo* experiments (carrageenan–induced paw oedema) and *in vitro* studies (J744 macrophage cell line) have shown that the anti-inflammatory effect of nimesulide involves the

CD73/adenosine/A_{2A} signalling pathway. These results are in support of a previous hypothesis suggesting that nimesulide may share a common mechanism with methotrexate also involving adenosine acting on immune cells (Al-Abd *et al*., 2010). In conclusion, our findings contribute to delineate the biochemical mechanism underlying the pharmacological effect of nimesulide and the dual mode of anti-inflammatory activity of nimesulide demonstrated in the present study might be exploited in the future by synthesizing COX-2 inhibitors which in addition display a more potent activity on adenosine signalling.

3.3 ADENOSINE/A$_{2A}$ SIGNALLING PATHWAY AND FGF-2

There is broad evidence that endogenous adenosine, generated through the action of two ectoenzymes, CD39 and CD73, exerts anti-inflammatory effects mainly by activation of the A$_{2A}$ receptor subtype localized on several cell types (Sitkovsky, 2003). However, the mechanism of A$_{2A}$ protective effect in inflammation needs still to be clarified. A$_{2A}$ adenosine receptor has also been shown to play a role in matrix deposition and wound healing in a damaged tissue, contributing to either in repairing processes or fibrotic disorders (Montesinos *et al*., 1997; Chan *et al*., 2006). Matrix actively participates to inflammatory process and can act as a reservoir for an increasing number of growth factors including FGF-2 (Burgess, 2009). Given evidence that A$_{2A}$ receptor is involved either in the anti-inflammatory effect and in wound healing and repairing processes mediated by adenosine, and given the importance of extracellular matrix and fibroblasts in either inflammation and repairing processes, we evaluated the effect of a well-known A$_{2A}$AR agonist, CGS 21680, on acute inflammation development and change in extracellular matrix in a model of carrageenan–induced rat paw oedema. As already asserted, carrageenan–induced rat paw oedema is a classical model of acute inflammation, widely used to identify new therapeutic targets and to test the anti-inflammatory potential of new molecules. In its development, the intervention of great arrays of inflammatory mediators has been demonstrated (Vinegar *et al*., 1969; Di Rosa and Willoughby, 1971; Di Rosa *et al*., 1971). Local changes of epidermis and derma following injection of carrageenan have also been described (Vinegar *et al*., 1971). Firstly, to investigate the functional role of A$_{2A}$ in the control of inflammation, we treated animals with the A$_{2A}$ agonist, CGS 21680, or with the A$_{2A}$ antagonist, ZM 241385. We found that systemic treatment with A$_{2A}$ agonist, CGS 21680, prevented oedema development and inflammation, while the A$_{2A}$ antagonist, ZM 241385, did not have any effect. Furthermore, CGS 21680 inhibitory effect was prevented

by co-administration with ZM 241385. This finding confirms that the effect of CGS 21680 was specific, through A_{2A} adenosine receptor stimulation; nonetheless, $A_{2A}AR$ activation by endogenous adenosine seems not offer protection against acute inflammation since $A_{2A}AR$ antagonism did not exacerbate oedema. Moreover, this finding might suggest that CGS 21680 would act through an indirect mechanism, involving stimulation of A_{2A} receptor probably distant from the site of injury and activating anti-inflammatory mechanisms. A similar result was observed by Peirce *et al*., (2001), who demonstrated that systemic administration of ALT-146e, a selective A_{2A} agonist, reduced skin ulceration induced by recurrent ischemia reperfusion in rats, the effect was reversed by the antagonism ZM 241385; however, the antagonist alone did not exacerbate skin ulceration. In our experiments, histological analysis of tissue sections showed cells infiltration in inflamed tissue, characterized by neutrophils and degranulated mast cells, which was significantly reduced in tissue from CGS 21680 treated rats. In addition, tissue sections from CGS 21680 treated rats presented much more packed tissue, without oedema. Morphological analysis was confirmed by performing picro Sirius red staining; indeed, the anti-inflammatory effect of CGS 21680 was paralleled by an increased matrix density, compared to tissue from control animals; furthermore, when sections were analyzed under polarized light an increased number of the larger yellow/orange collagen fibers (collagen type I) was evident in tissue from CGS 21680 treated animals, compared to control tissues were greenish thinner (collagen type III fibers) were predominant. So, we showed that CGS 21680 anti-inflammatory effect was paralleled by an increased collagen deposition and fibroblast proliferation, suggesting that the anti-inflammatory effect mediated by A_{2A} adenosine receptor stimulation was likely due to the acceleration of matrix deposition. It is well known that fibroblasts contribute to the resolution of inflammation (Serhan *et al*., 2007), they can determine the nature and the duration of leukocyte infiltration

(Buckley *et al*., 2001). FGF-2 is a molecule with autocrine, paracrine and intracrine functions; it is a powerful mitogen for fibroblasts. Produced by several cell types and released by shedding vesicles or following cell damage (Taverna *et al*., 2003), FGF-2 plays a role in angiogenesis (Seghezzi *et al*., 1998); wound healing (Ortega *et al*., 1998); gastric ulcer protection (Pohole *et al*., 1999), cell proliferation (Raballo *et al*., 2000; Ahn *et al*., 2009) and inflammation (Hu and Wu, 2001). However, although levels of FGF-2 are increased in inflamed tissues, its role in inflammation is still unclear. An *in vitro* study has shown that A_{2A} activation stimulates FGF-2 production in human retinal endothelial cells (Valls *et al*., 2009). Our hypothesis was that the same mechanism(s) activated to promote wound healing might have been involved in the anti-inflammatory effect of $A_{2A}AR$ activation. This hypothesis was also supported by evidence that, as already mentioned, extracellular matrix is actively involved in resolution of inflammation, A_{2A} stimulation accelerate matrix deposition and that FGF-2, largely involved in wound healing, also shows anti-inflammatory effects (Hu and Wu, 2001; Jeon *et al*., 2007). In our experiments, changes in extracellular matrix morphology, following rat treatment with A_{2A} agonist, were evident by tissue staining with Picrosirius red. Western blotting analysis of inflamed tissues showed increased FGF-2 expression following rat treatment with CGS 21680 compared either to control or to not inflamed paws. To better analyze tissue from control and CGS 21680 treated rats and to further evaluate whether changes observed were peculiar of the rat treatment with the A_{2A} agonist, rather than reflecting a no inflamed paw, we performed immunofluorescent analysis of tissues to localize A_{2A} receptor and FGF-2, 3 h following oedema induction that represented the inflammatory peaking point. Interestingly, we found that A_{2A} receptor was highly localized in derma of inflamed paws (control paws) while scarcely present in the other two groups suggesting that the increased A_{2A} expression in inflamed tissues is mainly representative of receptor expressed by infiltrating cells.

Conversely, FGF-2 was largely localized in derma of tissues obtained from CGS 21680 treated animals; interestingly, there were spots of co-localization between FGF-2 and A_{2A}, leading us to hypothesise about a relationship between adenosine/A_{2A}AR and FGF-2. Our hypothesis would be consistent with the finding of a direct physical interaction between A_{2A}AR and FGF-2 receptor system already reported (Flajolet *et al*., 2008). In conclusion, in our study, by using an *in vivo* model of inflammation, we have demonstrated that the anti-inflammatory effect of A_{2A}AR activation is paralleled by an increased expression of FGF-2 that, in turn, might participate in matrix deposition. Therefore, A_{2A}AR agonists might be valuable anti-inflammatory agents able to target cells as fibroblasts and provide a new range of possibilities in the anti-inflammatory therapy.

4. CONCLUSIONS

Adenosine is a key regulatory molecule, mostly protective but in certain scenarios injurious, in the pathophysiology of inflammatory diseases (Haskó and Pacher, 2008). Activation of adenosine receptors might be advantageous in the treatment of various inflammatory disorders such as rheumatoid arthritis, psoriasis, sepsis (Chen *et al*., 2013); therefore, adenosine receptors represent a promising drug target. At present, due to its widespread and pleiotropic effects and to a short half-life, the clinical uses of adenosine are limited to termination of supraventricular tachycardia (Krenosin®, Adenocard®; 6 mg/2 ml, for iv. use) or in conjunction with thallous (thallium) chloride TI 201 for myocardial perfusion scintigraphy (thallium stress test) in patients unable to undergo adequate stress testing with exercise (Adenoscan® ; adenosine 30 mg/10 ml, for iv. use). For each of the four ARs subtypes, selective agonists and antagonists and positive allosteric modulators have been developed by introducing several modifications in the structure of the lead compounds (adenosine and methylxanthine). CGS 21680 is a well-known, specific A_{2A} agonist. Experiments performed and shown in the present Ph.D. thesis have demonstrated a cross-talk between adenosine/A_{2A} signalling and FGF-2 that may supply a new range of possibilities in the anti-inflammatory therapies. In particular, inflammatory skin diseases, that have a significant impact on the quality of life of patients, could take advantage by A_{2A} receptor agonists since A_{2A} receptor activation shows to inhibit the inflammatory and to promote tissue repair. In agreement with this view, it has been proved that topical application of CGS 21680 effectively protects against phorbol-induced epidermal hyperplasia and inflammation in mice, without the deleterious atrophic effect of topical corticosteroids (Arasa *et al*., 2014).

The development of $A_{2A}AR$ agonists as therapeutic agents in inflammatory disorders is limited by evidence that A_{2A} activation reduces blood pressure (Nekooeian and Tabrizchi, 1996). Therefore, it is necessary to develop A_{2A} agonists able to exert their effect with a tissue selectivity.

Up to now, the only A_{2A} agonists in clinical studies are administered as inhalation formulations in patients with asthma or COPD (Antonioli *et al*., 2014).

Besides A_{2A} agonists, other drugs targeting adenosine receptors are in clinical trials; for instance, based on preclinical pharmacology and encouraging safety data in Phase I studies, the A_3 receptor agonists CF101 and CF102 have been tested in several Phase II trials for rheumatoid arthritis (Silverman *et al*., 2008). Based on anecdotal findings from this trial indicating that CF101 also improves indicators of dry eye syndrome, a follow-up Phase II trial (randomized, double-blind and placebo-controlled) has been carried out, which has determined that CF101 improves the clearance of corneal staining, tear break-up time and tear meniscus height (Avni *et al*., 2010). The efficacy and safety of CF101 has been also tested in a Phase II trial of moderate to severe chronic plaque-type psoriasis; it has been found to be effective and thus advanced to Phase III trials for these indications as an anti-inflammatory agent (Fishman *et al*., 2012).

Despite a large number of selective adenosine receptor agonists and antagonists reported in the literature, the clinical application of adenosine ligands is very limited. Only one adenosine receptor-specific agent, the adenosine A_{2A} receptor agonist regadenoson (Lexiscan; Astellas Pharma), has so far gained approval from the Food and Drug Administration (FDA); it is a coronary vasodilator that is commonly used in pharmacologic stress testing. There are several factors that need to be explored, for example, the function of systemic and local adenosine receptors, in disease models and in humans; the careful examination of adenosine signalling and the effects of the drug over the *continuum* of

specific disease courses (for instance, acute and chronic stages of a disease); clinical studies in which individual differences are carefully monitored and related to background factors, such as caffeine use (the daily consumption of three to four regular cups of coffee results in approximately 50% A_1 and A_{2A} receptor occupancy for several hours; Fredholm *et al*., 1999) and the examination of the possibility of combining direct adenosine receptor actions with drugs targeting other pathways and/or targets (Chen *et al*., 2013).

Moreover, adenosine receptors are found on many cells in the body, so that agonists of these receptors might have negative side effects which make them difficult to use. Another potential complication is the differential effects of adenosine receptor activation (or inactivation) at different stages in development; for instance in newborn mice, A_1 receptor agonism appears to have a harmful effect in response to hypoxia-induced brain injury, whereas in adult mice it has a protective effect (Turner *et al*., 2003).

A possible approach for achieving tissue selectivity could be the use of partial agonists that would predominantly act where there is a high number of "spare" receptors or the use of allosteric modulators. CF602, an A_3 adenosine receptor allosteric modulators, developed by the biotechnology company Can-Fite BioPharma, could be useful in a variety of autoimmune and inflammatory disorders (Antonioli *et al*., 2014). Alternatively, strategies that are capable of interfering with the function of enzymes and transporters that are responsible for the accumulation of extracellular adenosine at inflammatory sites may have more general applicability. For example, beneficial effects have been observed with the pharmacological blockade of ADA in experimental model of colitis (Antonioli *et al*., 2007; Brown *et al*., 2008) and sepsis (Adanin *et al*., 2002; Cohen *et al*., 2002; Kayhan *et al*., 2008) and using AK inhibitor in experimental model of arthritis, colitis and in diabetic retinopathy (Suzuki *et al*., 2001; Siegmund *et al*., 2001; Elsherbiny *et al*., 2013) or nucleoside transporter inhibitors in models of inflammatory pain and inflammatory lung injury (Maes

et al., 2012; Morote-Garcia *et al*., 2013). To date, the adenosine uptake inhibitor dipyridamole is used in patients for pharmacological stress echocardiography (as a coronary vasodilator) or as an inhibitor of platelet aggregation. An FDA-approved adenosine deaminase inhibitor, deoxycoformycin (Nipent; Astex Pharmaceuticals), can increase extracellular adenosine levels and is currently in clinical use for the treatment of haematological malignancies. At the same time, the development of adenosine-regulating compounds as prodrugs, based on our understanding of the ATP-dependent adenosine signalling cascade, that are able to increase adenosine concentration in a site and event specific manner, seems to be very promising (El-Tayeb *et al*., 2009). Flögel and co-workers (2012) have synthesised a phosphorylated prodrug $A_{2A}R$ agonist, which is specifically activated by CD73 overexpressed on immune cells. Once it is locally administered into the joint of arthritic mice, this prodrug reduces inflammation, without inducing vasodilatory side effects, due to profound up-regulation of CD73 and adenosine $A_{2A}R$ expression in neutrophils and inflammatory monocytes in the synovial fluid of arthritic mice. The modulation of CD73 enzymatic activity, in order to enhance the extracellular adenosine formation, may thus represent a therapeutic strategy in the treatment of inflammation. At present, clinical trials are being carried out to increase extracellular adenosine levels in humans (Chen *et al*., 2013). Our data obtained in carrageenan-induced pleurisy in rat support the view that CD73/adenosine signalling plays an important role as an endogenous modulator of inflammation and further highlight the importance of adenosine signalling pathway as a therapeutic target that could be successfully used to treat inflammatory diseases. CD73 directed therapies have not yet been developed. Alternatively, administration of soluble 5'-nucleotidase/CD73 has been shown to be advantageous in a number of experimental inflammatory models, especially by promoting vascular barrier function and by decreasing neutrophil accumulation, according to our results collected in

carrageenan-induced pleurisy model. With regard to this, a big limitation could be the identification of a reliable source of purified protein.

As already asserted, anti-inflammatory action of some drugs is explained by the increase of extracellular adenosine concentrations (Amann and Peskar, 2002; Montesinos *et al*., 2007). For instance, on the basis of the finding that the beneficial effect of IFN-β in multiple sclerosis has been associated to CD73 upregulation, an open-label study has been initiated, where IFN-β-induced up-regulation of human lung CD73 expression and soluble 5'-nucleotidase activity has shown to be associated with reduced mortality in patients with acute respiratory distress syndrome (Bellingan *et al*., 2014). On this basis, a section of the research work described in this thesis demonstrates that the anti-inflammatory effect of the COX-2 inhibitor nimesulide is, in part, mediated by CD73-derived adenosine acting on A_{2A} receptors. Thus, pharmacological agents able to raise the extracellular levels of endogenous adenosine, including nimesulide, might be exploited in the future to project drugs that might have an anti-inflammatory effect by targeting an endogenous anti-inflammatory pathway, representing, in this way, an innovative anti-inflammatory strategy.

In conclusion, it has become increasingly clear the need for identification of novel molecular targets for the development of new anti-inflammatory agents. Adenosine signalling represents a powerful modulator of the inflammatory response, as well confirmed by our findings described in this thesis. An innovative strategy can be represented by the pharmacological manipulation of endogenous anti-inflammatory pathways. Targeting multiple steps and pathways involved in adenosine signalling, such as adenosine generation and metabolism as well as adenosine receptors themselves, may be synergistic and more efficacious than targeting an individual step or pathway (Chen *et al*., 2013) and may represents a valuable strategy to project innovative anti-inflammatory drugs with a more favourable pharmacodynamics/pharmacokinetic and safety profile.

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