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***Evaluation of the role of adenosine in the control of the
inflammatory response and of IL-17A in haemostatic
disorders associated to inflammation***

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CHAPTER I

INFLAMMATION

Inflammation is a defense reaction to an injury of different type (bacterial, physical, chemical etc.), whose ultimate purpose is the elimination of injurious cause and the restoration of tissue integrity. Signs and symptoms of inflammation depend upon the interaction among different cells and mediators into the damaged tissue.

Cells involved are either leukocytes recruited from circulation and those resident into the tissue, such as mast cells, macrophages, but also stromal cells, such as endothelial cells, fibroblasts, smooth muscle. Inflammatory mediators are produced by all cells involved, stromal and recruited.

Depending on their biochemical properties, inflammatory mediators may be classified into seven groups: vasoactive amines, vasoactive peptides, complement components, lipid mediators, cytokines, chemokines and proteolytic enzymes (Medzhitov *et al.*, 2008).

Biogenic amines are preformed mediators, stored into mast cell and platelet granules released following activation. Their main effect is on vasculature, where they cause increased vascular permeability and vasodilatation. Vasoactive peptides can be stored in secretory granules, such as substance P, or generated by proteolysis, such as bradykinin; these peptides also affect vasculature and have pro-algesic effect. Complement fragments, such as C3a, C4a and C5a promote cell

recruitment into the damaged tissue. Lipid mediators, eicosanoids and platelet activating factors (PAF), derive from arachidonic acid as products of cyclooxygenases (prostaglandins and thromboxane) or as products of lipoxygenase (leukotrienes and lipoxins), or from lysophosphatidic acid (PAF). Prostaglandins, leukotrienes and PAF all participate to cause signs of inflammations, vasodilatation or smooth muscle contractions, cell recruitment, pain, fever; on the other hand, lipoxins are involved in resolution of inflammation and tissue repairing processes. Proinflammatory cytokines (IL-1, IL-6, TNF α and many others) have several roles in inflammatory response, they activate cells and induce acute phase response; antiinflammatory cytokines, such as IL-10, can inhibit the production of inflammatory cytokines and down-regulates previously activated cells. Chemokines are responsible of cell recruitment into the damaged tissue. Proteolytic enzymes, metalloproteinases, cathepsins, elastin, have a role in degrading extracellular matrix, in tissue remodeling and leukocyte migration (Pober and Sessa, 2007; Barton *et al.*, 2008).

In other words, all cells, either recruited or stromal participate actively to the inflammatory process through the production of soluble mediators responsible, in turn, for other cell recruitment. In this way the inflammatory process, with its actors, cells and mediators, possess a great “*self-amplifying*” ability (Figure 1).

Over the years the list of soluble mediators involved in the inflammatory response has been growing until nowadays when it's known that close to the early recognized inflammatory mediators, first biogenic amines and then prostaglandins, cytokines, chemokines, growth factors all participates in a coordinated network to orchestrate the inflammatory response. However, the ultimate purpose of inflammation is the restoration of tissue integrity (Medzhitov *et al.*, 2008).

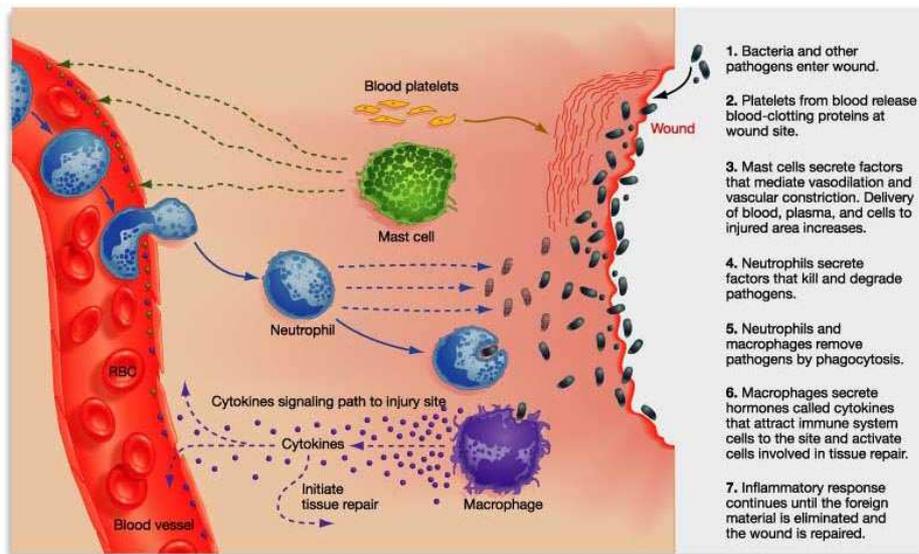


Figure 1. Cellular components of inflammation.

It is known that the natural outcome of the acute inflammatory program is the resolution and repair of tissue damage; failure of this program leads to chronic inflammation and loss of organ function. Thus together with pro-inflammatory mediators there are as many anti-inflammatory whose role is to dampen out inflammation.

Resolution of inflammation may be considered an integral component of the program of acute inflammation. It is an active process regulated by natural immunosuppressive mechanisms, representing a “metabolic switch” to preserve host defense and tissue integrity. During the early stage of an inflammatory response a large number of leukocytes are recruited from circulation; at the end of the inflammatory process, these effectors cells will be cleared due to the loss of survival signals derived from the interactions with stromal cells, leading to apoptosis and subsequent phagocytosis of dead cells by monocytes – derived macrophages. Once phagocytosis is complete, macrophages exit the inflamed site by lymphatic drainage (Serhan and Seville, 2005; Serhan, 2007). Another important aspect of resolution is that stromal cells that hosted the inflammatory event revert back to a no-inflammatory phenotype (Filer *et al.*, 2006). In chronic inflammation, the resolution phase is prolonged and disordered, leading to the persistence of cell infiltrate that become rich in monocytes and T lymphocytes rather than neutrophils.

Thus, inflammation is also a “*self-limiting*” process; its turning into a chronic process can be understood as being the result of either the persistence of a stimulus, or of a deregulation of the endogenous anti-inflammatory mechanisms that normally regulate its resolution (Serhan and Saville, 2005; Lawrence and Gilroy, 2007) (Figure 2). A novel anti-inflammatory therapeutic approach would be to potentiate those mechanisms involved in the resolution phase.

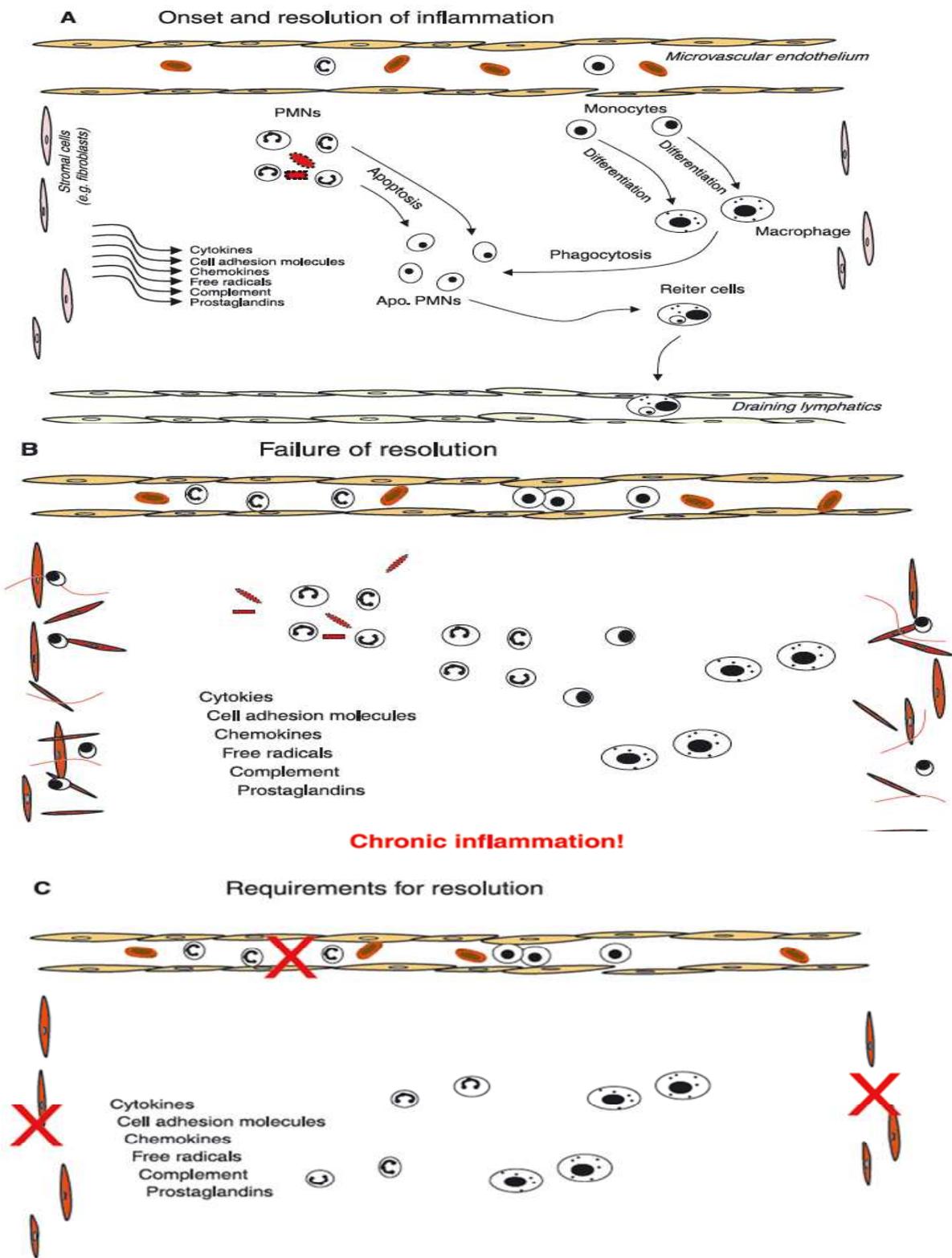


Figure 2. Illustration of the cellular kinetics and sequential release of mediators during the evolution of the inflammatory response from onset to resolution. (A) Inflammation causes the immediate and sequential release of signalling factors to neutralize the injurious agent. (B) Failure of acute inflammation to resolve adequately could result in chronic inflammation (C) Thus, for the effective resolution of acute inflammation we need to curtail further influx of inflammatory leucocytes signal monocytes/macrophages to phagocytose and clear all these cells from the site of injury once the inflammatory stimulus poses no further threat. (Lawrence and Gilroy, 2007).

Linking inflammation and haemostasis

Inflammation and haemostasis cannot be considered as two separate processes, since there are several connecting points making them part of a unique, defensive host response. There is much evidence that inflammation triggers haemostatic imbalance. Experimental and clinical data demonstrate that inflammation is associated to an increased risk of cardiovascular events (Cicala and Cirino, 1998; Jurado and Ribero, 1999; Cicala *et al.*, 2007).

During an inflammatory state, several cytokines by acting on the expression and synthesis of several proteins involved in coagulation and fibrinolysis are responsible for impairing the balance between pro- and anti-coagulant factors toward a pro-thrombotic state (Cicala and Cirino, 1998). Indeed, proinflammatory cytokines (IL-1 and TNF α) increase tissue factor (TF) expression. TF is a 44000 molecular weight membrane-bound glycoprotein binding factor VIIa (Marmur *et al.*, 1996). The TF-VIIa complex activates factor X and factor IX, thereby initiating proteolytic cascades that result in thrombin formation and blood clotting (Fay *et al.*, 2010). At the same time, cytokines, and in particular TNF α , are mainly responsible for the downregulation of thrombomodulin (TM).

TM is a high affinity receptor protein for thrombin and it is expressed on the endothelial surface. The thrombin-TM complex activates Protein C,

stimulating the fibrinolytic pathway. In the case of inflamed tissue, thrombin is not able to bind TM and therefore the fibrinolytic pathway is not activated. This phenomenon is due to the TM down-regulation caused by cytokine release into the inflamed site. In this way, it is clear that there is an alteration of the coagulative cascade that may lead to critical thrombus formation (Esmon, 2003) (Figure 3).

Endothelium might be considered a surface interfacing inflammation and haemostasis. In particular, when the endothelium is under physiological conditions, the balance between its pro-and anti-thrombotic features is preserved; on the contrary, when the endothelium is damaged, it loses these properties and becomes an idoneous surface in which the first contact between inflammation and haemostasis takes place (Cicala and Cirino, 1998; Goran *et al.*, 2006; Esmon *et al.*, 2011).

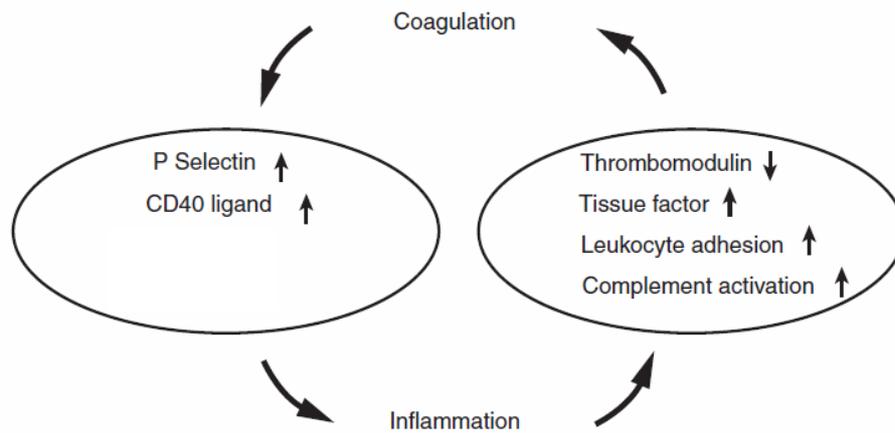


Figure 3. The impact of coagulation on inflammation and the impact of inflammation on coagulation. Coagulation triggers platelet activation and leads to P selectin and CD40 ligand expression platelet surface. Inflammation in turn leads to tissue factor induction, leukocyte adhesion, thrombomodulin down regulation, and complement activation. Thus coagulation increases inflammation that in turn increases coagulation (Esmon *et al.*, 2011).

Another important aspect to be considered when the link between haemostasis and inflammation is examined is the contribution of platelets to both processes (Lindemann *et al.*, 2001; Ruggeri, 2006). Platelets are considered like effective elements of the inflammatory system. Under physiological conditions, platelets circulate freely in the blood. On the contrary, when endothelium is damaged, platelets adhere to collagen fibres of the subendothelium, thereby becoming activated. Activated platelets express on their surface molecules driving platelet-endothelium adhesion and platelet-leukocytes interaction (Figure 4).

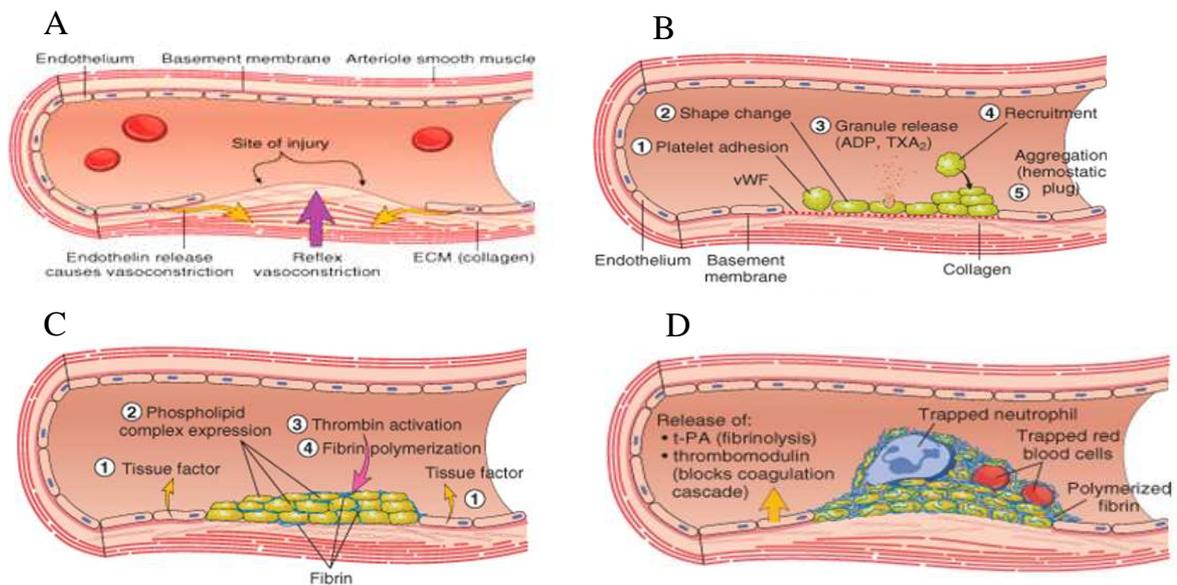


Figure 4. Representation of thrombus development: A) vasoconstriction; B) primary hemostasis; C) secondary haemostasis; D) thrombus and anti-thrombotic events.

For example, P-selectin, translocated on platelet surface following activation, engages its receptor P-selectin glycoprotein -1 (PSGL-1) on polymorphonuclear (PMN) and monocytes; the interaction of platelets with leukocytes may result in local fibrin deposition through an increased TF expression in these cells (Palabrica *et al.*, 1992; Celi *et al.*, 1994; Maugeri *et al.*, 2006).

The platelet receptor GPIIb/IIIa, engaged on platelet surface following activation, binds to P-selectin and von Willebrand factor (vWF) externalized by endothelial cell granules following vascular damage (Nurden, 2011).

Other adhesive proteins that have been described on platelets, important for cell-cell interaction, are CD40 and CD40L. On activated platelet

surface, CD40L by binding CD-40 (its counter-receptor on endothelial cells and monocyte/macrophages), promotes platelets-monocytes and platelets-endothelial cells interactions (Zarbock *et al.*, 2007). The interaction between CD40L-CD40 promotes cell-cell adhesion but also up-regulates several functions in monocytes, such as chemokine and cytokine secretion, expression of tissue factor, upregulation of adhesive receptors and differentiation of monocytes into macrophages (Henn *et al.*, 1998; Li *et al.*, 2008; Cerletti *et al.*, 2011).

Another important molecule promoting platelet adhesion to endothelium and monocytes is C reactive protein (CRP). C reactive protein is an acute phase protein whose synthesis in the liver is under the control of cytokines. CRP has also been found in the walls of damaged vessels and into atherosclerotic plaques (Hirschfield and Pepys, 2003).

CRP consists of five identical subunits of 206 aminoacids each; pentameric CRP can be dissociated in monomers either *in vitro* or *in vivo*. Pentameric and monomeric forms of CRP have been shown to possess different biological activity. Platelets express CRP receptors Fc γ RIII (CD16) and Fc γ RIIa (CD32) (Filep, 2009). It has been shown that pentameric CRP by binding to Fc γ RIIa on platelets inhibits platelets-neutrophils interactions (Khreiss *et al.*, 2004). On the contrary, monomeric CRP, by binding to Fc γ RIII on platelets promotes platelet-neutrophils interaction (Filep, 2009). Furthermore, activated platelets

convert pentameric CRP to monomeric. Thus, it is worth noting that CRP regulates platelet activation and, in turn, platelet activation regulates the conformational status and biological function of CRP. Thus, monomeric CRP, through platelet activation may lead to monocyte activation, thus representing an important mechanism linking platelet/monocyte activation and invasion of the vascular wall (Yaron *et al.*, 2006; Danenberg *et al.*, 2007; Fay, 2010).

Furthermore, CRP inhibits also fibrinolytic pathway by inhibiting the release of tissue plasminogen activator (t-PA) and stimulating the release of plasminogen activator inhibitor-1 (PAI-1) from endothelial cells (Devaraj *et al.*, 2003; Singh *et al.*, 2005). In summary, CRP, that since long time has been considered a marker of cardiovascular risk during inflammation, is now known to play an active role in linking inflammation and thrombosis by affecting the function of blood platelets, coagulation cascade and the fibrinolytic pathway.

Adenine nucleotides (ATP and ADP) are platelet activators; conversely, adenosine, the final product of the nucleotide hydrolysis, is a vasodilator and inhibitor of platelet aggregation (Burnstock, 1990). In an inflammatory environment, ADP released from activated platelets contributes to stimulate other platelets. CD39 is an ecto-nucleoside triphosphate diphosphoydrolase (E-NTPDase), ubiquitously expressed on cell surface, metabolizing ATP to ADP and to AMP, thus reducing

ADP concentration at the site of injury. Hence, CD39 has been considered a key modulator of thrombus formation. In an inflammatory environment the loss of CD39 activity from activated endothelium sustains platelet aggregation and thrombogenesis (Atkinson *et al.*, 2006). On the other hand, within a damaged tissue the increased expression of CD39 on inflammatory cells, working in tandem with CD73 (catalyzing the conversion of AMP to adenosine) might cause inhibition of platelet activation by increasing extracellular adenosine levels (Johnston-Cox *et al.*, 2010).

ADENOSINE

Prolonged and inappropriate inflammation due to the persistence of cytotoxic proinflammatory molecules may cause destruction of normal bystander cells. Thus, prolonged and inappropriate inflammation must be regulated by natural immunosuppressive mechanisms, representing a “*metabolic switch*” to preserve host defense and tissue integrity.

Adenosine and its receptors are possible candidates involved in the natural down-regulation of inflammation (Linden, 2006; Fredholm, 2007)

Adenosine is a nucleoside always present both within and outside cells in nanomolar concentration (10 – 100 nM) under physiological conditions, deriving by the breakdown of intra – or –extra cellular adenine nucleotides. Physiologically, adenosine concentration is constant and finely regulated by an equilibrium between the extracellular release and the cellular re –uptake and its conversion to inosine. Two enzymes regulate this equilibrium: adenosine deaminase (ADA) and adenosine kinase. ADA is mainly a cytosolic enzyme but can also appears on the cell surface of several immune and non immune cells (ectoADA); ADA catalyzes the deamination of adenosine to inosine. Adenosine kinase is an intracellular enzyme catalyzing the adenosine phosphorylation to AMP (Bours *et al.*, 2006).

Following trauma or cellular stress, such as during hypoxia, ischemia or inflammation, extracellular adenosine levels increase rapidly following ATP degradation (Fredholm, 2007).

Two ecto – enzymes working in concert, the nucleoside triphosphate diphosphohydrolases, ecto-apyrase (CD39), and an ecto- 5'- nucleotidase (CD73), are involved in the adenine nucleotides (ATP and AMP respectively) breakdown in adenosine; they are located on cell surface but may be found as soluble forms in the interstitial medium and in body fluids (Schetinger *et al.*, 2007) (Figure 5).

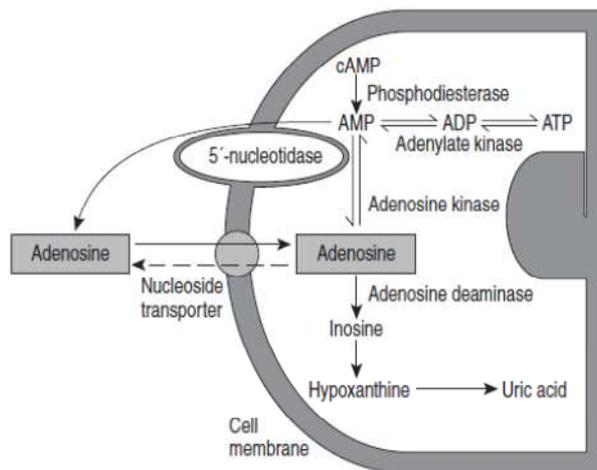


Figure 5. Metabolism of adenosine

Extracellular adenosine accumulation represents an early endogenous signal controlling inflammation and immune responses. Adenosine protective effects fall in four main mechanisms: it is protective against ischemic damage by cell conditioning; it increases the ratio of oxygen

supply to demand; it promotes angiogenesis and it has antiinflammatory effects. (Lankford *et al.*, 2006; Fredholm, 2007) (Figure 6).

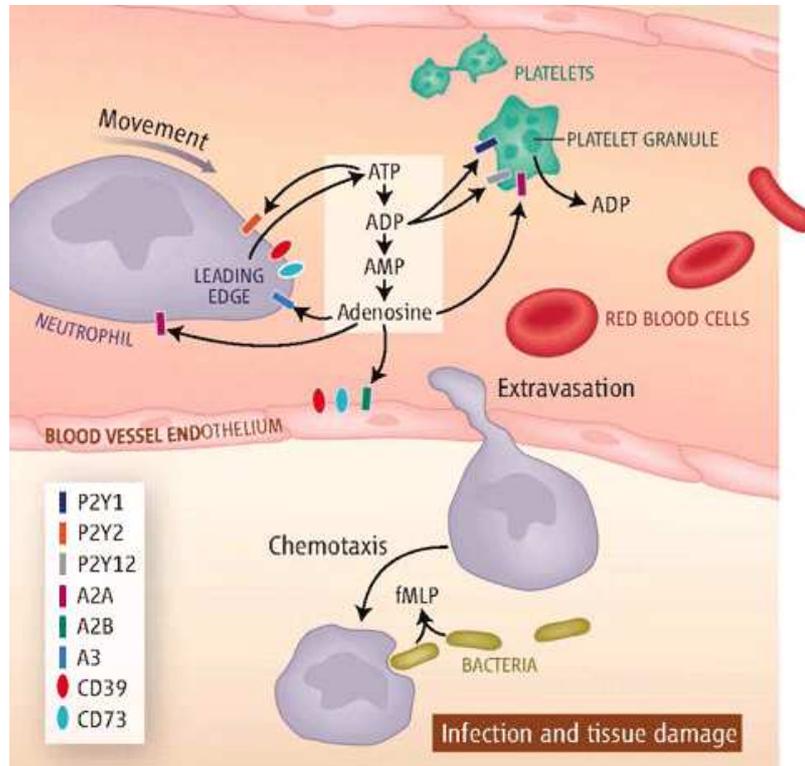


Figure 6. The inflammatory response to infection or tissue damage depends on the coordination of adenine nucleotide metabolism and signaling among many cell types via purinergic receptors that recognize ATP, ADP, or adenosine. A neutrophil migrating toward a chemotactic stimulus (fMLP) releases ATP from its leading edge. ATP is dephosphorylated by ectoenzymes (CD39 and CD73) to ADP and adenosine. Gradients of ATP and adenosine initiate and accelerate directional chemotaxis via P2Y2 and A3 adenosine receptors, respectively, on neutrophils. Other adenosine receptors (A2A and A2B) inhibit neutrophil chemotaxis and adhesion to endothelial cells, as well as platelet aggregation.

Adenosine Receptors

Adenosine effects are mediated through the interaction with four G-protein coupled receptors, indicated as A₁, A_{2A}, A_{2B}, A₃, (Figure 7), belonging to the family of purinergic P₁ receptors. They are widely expressed on a variety of immune and non immune cells (Table I). Although they bind the same agonist, they differ in several aspects, including their affinity binding for the agonist; their expression profile in different cell types; the identity of the G – proteins to which they are coupled and their sensitivity to receptor phosphorylation. All these factors combined determine the extent, the duration and the outcome of cellular exposure to adenosine and, in the end, dictate the nature of the response to adenosine tissue accumulation (Polosa 2002; Hasko and Cronstein, 2004).

Table I: Pharmacological classification and anatomical distribution of adenosine-receptor subtypes.

Receptor subtype	Agonist	Antagonists	Distribution
A1	CHA>NECA>CGS21680	DPCPX>XAC>CGS15943 >SPT	Heart, adipocytes, respiratory smooth muscle, neutrophils, kidney, hippocampus cortex
A2A	CGS21680~NECA>>CHA	ZM241385~SCH58261~C GS15943>XAC>DPCPX	Platelets, neutrophils, vasculature, pancreas, mast cells, striatum
A2B	NECA>CHA>>CGS21680	XAC>CGS15943>DPCPX	Vascular, intestinal and respiratory smooth muscle, chromaffin tissue, mast cells, brain
A3	2-CI-IB- MECA>APNEA>NECA~ CGS21680	MRS1220~IABOPX>L268 605>>XAC>DPCX	Testis, kidney, lung, mast cells, eosinophils, neutrophils, heart, cortex, striatum

A₁ adenosine receptor (A₁R), a high affinity receptor, is coupled to a G_i and G_o proteins, its activation results in adenylyl cyclase activity inhibition and increased activity of phospholipase C (PLC) through G_{βγ} subunits. In the cardiac muscle, A₁R can activate a potassium channel, leading to an increased K⁺ efflux from the cell. This adenosine receptor subtype is found in adipose tissue, heart muscle, central nervous system, airways and inflammatory cells such as neutrophils.

A_{2A} adenosine receptor (A_{2A}R), a high affinity receptor, is coupled to a G_s protein, its activation results in the increase of adenylyl cyclase activity. A_{2A} receptors are expressed in the central nervous system, vascular smooth muscle, endothelium and on neutrophils, platelets, mast cells and T cells.

A_{2B} adenosine receptor (A_{2B}R), a low affinity receptor, is coupled to both G_s and G_q, its activation may lead to increased adenylyl cyclase activity (G_s) or to phospholipase C (PLC) activation (G_q) resulting in calcium mobilization. It has been identified widely including in the brain, human bronchial epithelium, endothelial cells, muscle cells, neurons, glial cells, fibroblasts and mast cells.

A₃ adenosine receptor (A₃R), a low affinity receptor, is coupled to a G_i and its activation results in adenylyl cyclase activity inhibition but it can also stimulate PLC, leading to increased calcium concentration. The A₃

receptor is widely distributed, being found in the kidney, testis, lung, mast cells, eosinophils, neutrophils, the heart and brain cortex.

Adenosine receptors are expressed on all cell types involved in orchestrating an inflammatory/immune response, including monocytes/macrophages, dendritic cells, mast cells, neutrophils, eosinophils, platelets, fibroblasts, epithelial cells, endothelial cells. Through the interactions with its receptors adenosine may be beneficial or detrimental to tissues (Polosa, 2002; Linden, 2001; Burnstock, 2006).

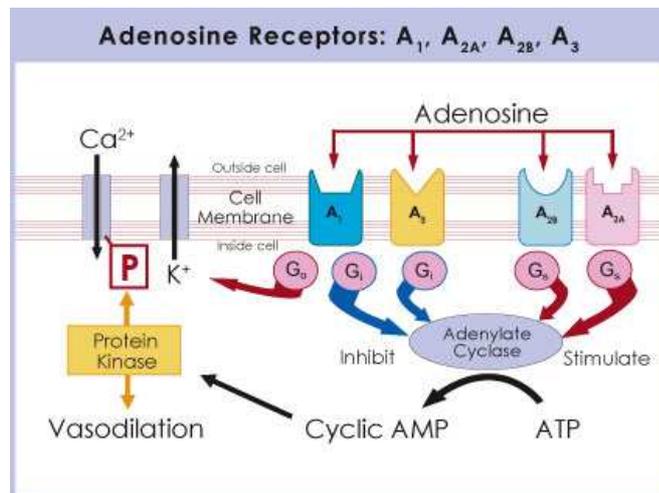


Figure 7. Adenosine receptors

Adenosine and inflammation

As described above, inflammatory tissue injury is accompanied by increased levels of extracellular adenosine; the most important factor causing adenosine accumulation is hypoxia in inflamed tissue.

Recently, considerable evidence has been accumulated on adenosine involvement in inflammation through activation of its receptors. The interest on the role of adenosine in inflammation has been growing also following the finding that some antiinflammatory drugs, nimesulide, salicylates, and methotrexate exert their effects through adenosine signaling (Capecchi *et al.*, 1993; Cronstein,*et al.*, 1993; 1999; Cronstein, 1994; Amann and Peskar, 2002; Bernardi *et al.*, 2007).

On inflammatory cells, adenosine may have opposite effect, being protective or harmful, depending on receptor subtype activation. This discrepancy between pro - and anti- inflammatory adenosine effects might also been attributed to the different receptor distribution in different tissue and/or to an alteration of tissue receptor expression under pathological conditions (Zimmermann, 2000; Bours *et al.*, 2006).

Adenosine A_{2A} receptor and inflammation

There is *in vitro* and *in vivo* evidence that adenosine signalling through A_{2A} receptor plays the major role in controlling inflammation and immune response.

In vitro studies have demonstrated that A_{2A} activation on neutrophils inhibits oxidative burst and chemokine production in response to several stimuli (Cronstein *et al.*, 1986; 1988; Barnes *et al.*, 1995; McColl *et al.*, 2006) and on human monocytes the release of proinflammatory cytokines. Through A_{2A} receptor on lymphocytes, adenosine inhibits activation and expansion. The majority of A_{2A} receptor inhibitory effects on immune and inflammatory processes have been proposed to occur *via* cAMP/PKA activation. (Huang *et al.*, 1997; Haskò and Cronstein, 2004; Haskò *et al.*, 2007).

Studies performed *in vivo* have demonstrated that administration of selective A_{2A} agonists inhibits inflammation in models of ischemia reperfusion injury of various organs; A_{2A} inhibits also lung inflammation caused by pro-inflammatory stimuli (Palmer and Trevethick, 2008).

Furthermore, *in vivo* studies have demonstrated that administration of the A_{2A} agonist, CGS21680 to ovalbumin (OVA) sensitized mice reduces cell influx to the airways (Bonneau *et al.*, 2006). The protective effect of A_{2A} receptor has also been demonstrated in a murine model of LPS-induced acute respiratory distress syndrome (Thiel *et al.*, 2005).

Furthermore, it has been demonstrated that A_{2A} activation also protects from stress – induced gastric lesion in the rat that are dependent upon neutrophil infiltration (Odashima *et al.*, 2005). It has been shown that A_{2A} receptor activation on neutrophils increases COX₂ expression and the following production of PGE₂, a prostanoid with antiinflammatory properties (Cadiaux *et al.*, 2005). More recently it has been demonstrated a beneficial role for A_{2A} agonist, CGS21689, also in a model of chronic inflammation, as collagen induced arthritis in mice (Mazzon *et al.*, 2011).

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 fibrotic disorders and repairing processes (Montesinos *et al.*, 1997; Chan *et al.*, 2006A; Cronstein, 2006) (Figure 8)

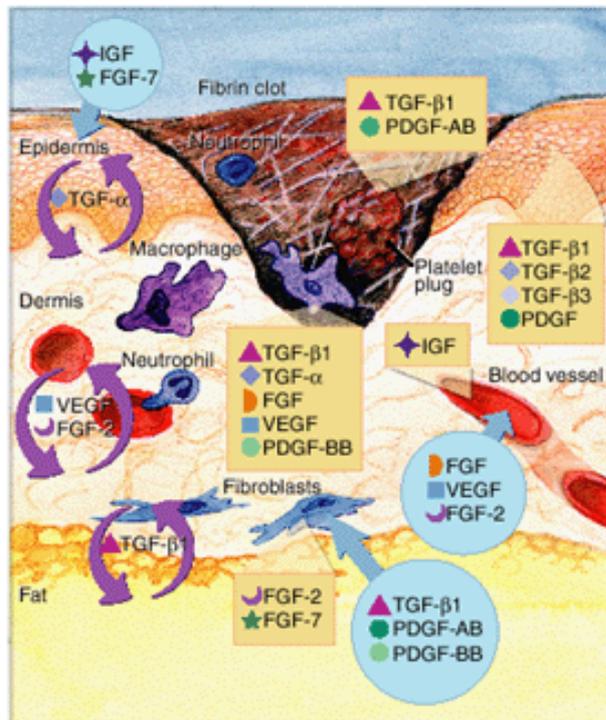


Figure 8. During inflammation, the formation of a blood clot re-establishes hemostasis and provides a provisional matrix for cell migration. Cytokines play an important role in the evolution of granulation tissue through recruitment of inflammatory leukocytes and stimulation of fibroblasts and epithelial cells

By performing experiments in A_{2A} deficient mice it has been demonstrated that A_{2A} receptor signaling represent an endogenous antiinflammatory mechanism; indeed, mice deficient in A_{2A} receptor display an exaggerated inflammatory response in models of hepatitis and sepsis (Ohta *et al.*, 2001; Chan *et al.*, 2006 B). Furthermore, mice lacking A_{2A} receptor have shown exaggerated lung inflammation and cell infiltration following sensitization (Nadeem *et al.*, 2007).

A_{2A} receptor expression on immune and endothelial cells is up-regulated by inflammatory cytokines (IL-1 β , TNF α) (Khoa *et al.*, 2004; Nguyen *et al.*, 2003). All these data support the hypothesis that in an inflammatory environment signaling through A_{2A} receptor functions to switch off the inflammatory process.

Ectonucleotidases

Ectonucleotidases are enzymes that hydrolyze extracellular nucleotides to their respective nucleosides. There are four major families of ectonucleotidases, namely E-NTPDases (ATP to ADP and ADP to AMP), alkaline phosphatases (ATP to ADP to AMP to Adenosine), E-NPPtype pyrophosphatase/phosphodiesterase (ATP to AMP) and Ecto-5'-nucleotidase (AMP to adenosine). The distribution of ectonucleotidases is as ubiquitous as that of nucleotide receptors (Schetinger *et al.*, 2007).

Although there is much work confirming adenosine protective role in inflammation, it is not yet clear the mechanism at the basis of adenosine antiinflammatory effect neither how steps involved in extracellular adenosine accumulation are regulated during inflammation.

Two ectonucleotidases (CD39 and CD73) play a key role in extracellular adenosine accumulation. NTPDase 1 (CD 39), ecto-apyrase, degrades equally well ATP and ADP. Ecto 5'- nucleotidase (CD73) degrades

AMP in adenosine. The central function of these enzymes is to increase extracellular production of adenosine.

To date, it has been shown that stimuli causing adenosine accumulation cause also an increased expression of these enzymes on cells (Robson et al., 2006; Schetinger et al., 2007).

There is evidence that inflammatory stimuli increase CD39 and CD73 expression and/or activity on cells involved in the inflammatory/immune responses, such as neutrophils, monocytes, lymphocytes. It has been demonstrated that following hypoxia activated neutrophils release ATP; at the same time hypoxia induces an increased expression of CD39 and CD73 on vascular endothelium causing a rapid formation of adenosine deriving from ATP breakdown. Adenosine, in turn, via A_{2A} activation inhibits neutrophil function; indeed, knock out mice for these enzymes show increased neutrophil accumulation following hypoxia (Eltzschig *et al.*, 2004). Thus, the increased adenosine levels provide a negative feedback signal that counteract neutrophil activation; high levels of extracellular adenosine, following tissue damage, are conserved by the increased CD39 and CD73 expression (Eltzschig *et al.*, 2004; Bours *et al.*, 2006). *In vivo*, by performing experiments in CD39 null mice it has been shown that CD39 is required for optimal stimulation of hapten reactive T-cell (Mizumoto *et al.*, 2002).

Both CD39, ecto-apyrase, and Cd73 on endothelial cells play a protective role against vascular injury. In a model of hypoxia in vivo, in knock out mice for CD73 it has been shown that this enzyme through the generation of extracellular adenosine plays a crucial role for vascular leakage (Thompson *et al.*, 2004).

In mice airways, CD39 and CD73 have shown to be an innate protective pathway from damage caused by mechanical ventilation (Eckle; 2007). Both enzyme are also over expressed in airways of mice following LPS-induced lung injury and play a role in attenuating polymorphonuclear trafficking (Reutershan *et al.*, 2009).

As described above, CD39 plays a critical role in the control of vascular thrombosis; it has been shown that following endothelial cell activation the ATPDase is lost and this might contribute to vascular damage (Atkinson *et al.*, 2006). Nonetheless, transgenic mice overexpressing CD39 are protected from myocardial ischemia injury (Cai *et al.*, 2011; Deaglio *et al.*, 2011).

Thus, these enzymes might represent a key step of a natural metabolic switch whose final product is represented by adenosine. A better knowledge on the role of these enzymes in inflammation would help to clarify the physiopathology of inflammation and to identify therapeutic target that activate endogenous protective mechanisms.

INTERLEUKIN 17

Interleukin-17: family overview

IL-17A, a 153 amino acid polypeptide, is the oldest described member of IL-17 family. It represents the prototypic member of a family of cytokines that also includes IL-17B, IL-17C, IL-17D and IL-17F (Table II and Figure 9). IL-17E was independently identified; now it has been renamed IL-25 and it is no longer considered as a member of IL-17 family, it has been shown to have anti-inflammatory properties by inducing Th2 responses (Kolls and Linden, 2004). IL-17 (synonymous of IL-17A) is a homodimeric glycoprotein of 155 aminoacids and a molecular weight (MW) of 35 kDa.

All members of this cytokine family, except IL-17B, exert their biological effects as dimers, by binding to IL-17 receptors that are ubiquitously expressed.

IL-17A and IL-17F are the best characterized members of this cytokine family. Both are homodimers, but recent findings show that mouse and human CD4⁺ cells can produce heterodimer forms of IL-17A-IL17F (Chang *et al.*, 2007).

IL-17 is mainly produced by a subset of T helper cells (CD4⁺ cells) termed Th17, phenotypically and functionally distinct from Th1, Th2 and T regulatory cells (T_{reg}).

In mice, the presence of two cytokines, transforming growth factor β (TGF β) and IL-6, is responsible for naïve Th cells polarization towards a Th-17 subtype; both cytokines cause upregulation of IL-23 receptor expression on T cells and, in turn, IL-23, together with IL-12, stimulates IL-17 production in Th17 cells. Humans Th17 display similarities and differences in their differentiation, with mouse Th-17 cells: IL-1 β , IL-23 and IL-6 seems to drive differentiation of naïve Th cells towards Th-17, however is still unclear the role of TGF β . It is worth noting that besides Th-17, other cell types can produce IL-17 such as natural killer T cells and neutrophils (Aggarwal *et al.*, 2002).

Table II: The human IL-17 cytokine family.

IL-17 family member	Molecular weight (kDa)	Receptor	Source	Proposed pathogenic role
IL-17	35	IL-17RA+C	TH-17 cells, CD8 cells, T cells, iNKT cells, granulocytes(?), macrophages (?)	Induction of neutrophil-mobilizing mediators, induction of antimicrobial cytokines, accumulation of neutrophils, stimulation of osteoclastogenesis
IL-17B	41	IL-17 RB	Not determined	Not determined
IL-17C	40	?	Not determined	Not determined
IL-17D	52	?	Not determined	Not determined
IL-17F	44	?	Th-17 cells	Induction of neutrophil-mobilizing mediators, accumulation of neutrophils

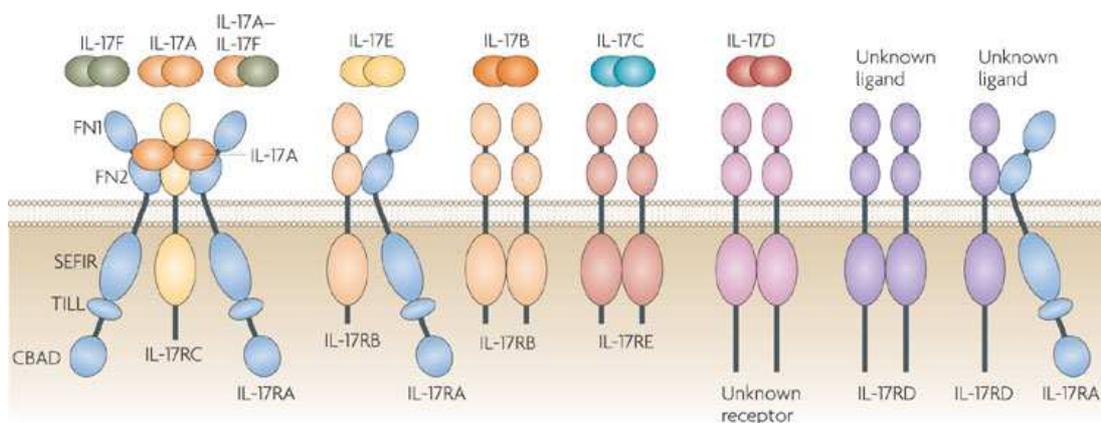


Figure 9. Interleukin-17 family

Interleukin-17: receptor family

The IL-17R family consists of five receptor subunits, from IL-17RA to IL-17RE (Aggarwal, *et al.*, 2002). IL-17RA is a type I transmembrane protein consisting of an extracellular domain (293 AA), a transmembrane domain (21 AA) and a cytoplasmic domain (21AA). IL-17RA is expressed in a variety of cells in humans and mice, such as epithelial cells, fibroblasts, B and T lymphocytes, neutrophils, bone marrow cells (Linden *et al.*, 2005; Dragon *et al.*, 2008). IL-17A stimulates a receptor complex formed of IL-17RA and IL-17RC; the latter shows similarities to IL-17RA while the functional characteristics of the other receptors are still unclear (Ivanov and Lindén, 2008).

Following activation of the IL-17R complex, IL-17A signalling involves at least two downstream pathways; the first involves the adaptor protein

nuclear factor- κ B activator (Act-1) that forms a complex with the SEFIR domain (a cytoplasmic protein segment present in all members of IL-17R family); subsequently, intracellular signalling molecules, such as tumour necrosis factor- receptor associated factor (TRAF 6 and TRAF3) and transforming growth factor activated kinase 1 (TAK1) are activated and, in turn, mediate the activation of transcription factors. The second pathway described is Act-1-independent and involves activation of Janus kinase-1(JAK1) and phosphatidylinositol 3-kinase (PI3K), followed by subsequent inactivation of glycogen synthase kinase (GSK)-3 β and gene activation (Ivanov and Linden 2008) (Figure 10).

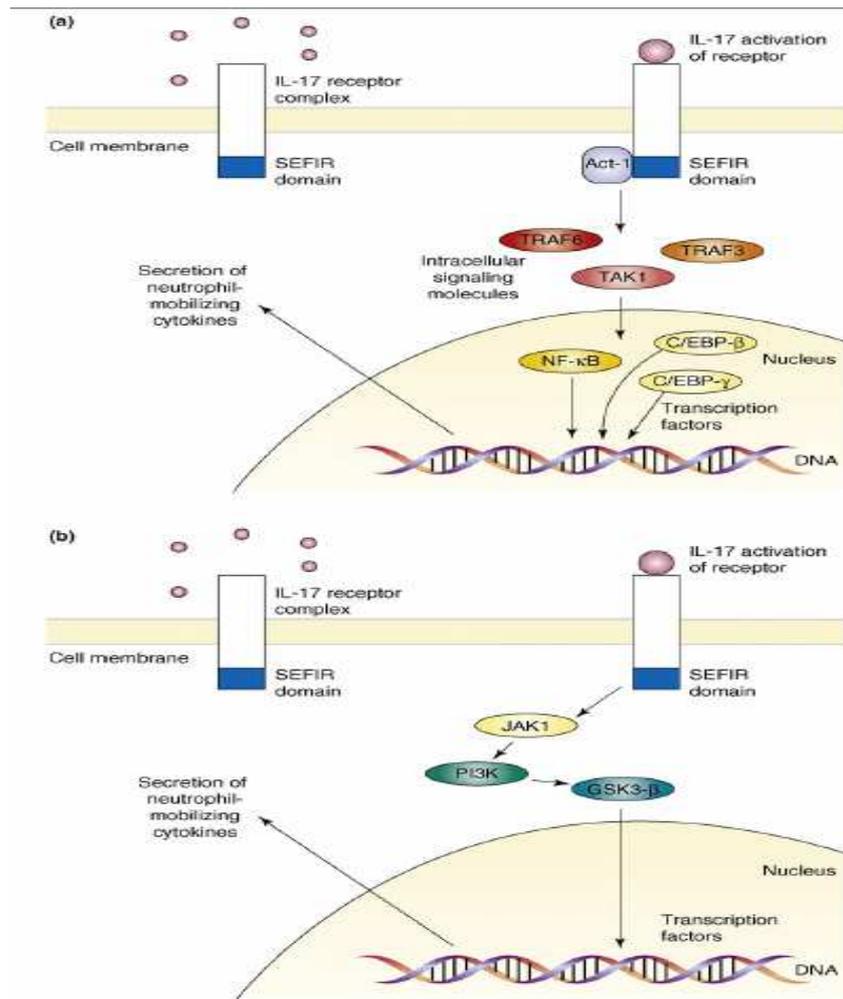


Figure 10. Intracellular IL-17 signaling. (a) Act-1 dependent: after ligand activation of the IL-17 receptor R complex (i.e. IL-17 RA and IL-17 RC), the adaptor protein nuclear factor κ B activator 1 (Act-1) forms a complex with the similar expression to fibroblast growth factor (FGF) genes and IL-17 receptor (SEFIR) domain of IL-17 R complex. Subsequently, intracellular signal molecules (e.g. TRAF3, TRAF6, and TAK1) are activated leading to the involvement of transcription factors such as NF- κ B. As consequence secretion of neutrophil-mobilizing is induced. b) Act-1 independent: it involves Janus kinase (JAK)1 and phosphatidylinositol3-kinase (PI3K) followed by subsequent inactivation of glycogen synthase kinase (GSK)-3 β , gene activation and cytokine secretion. Interleukin-17A and inflammation.

Interleukin 17 and inflammation

IL-17A has a unique role in the context of inflammatory response (Kolls and Linden, 2004). This cytokine is produced mainly by T cells rather than by macrophages or other cells of the innate immune system and thus it is believed to play an important role in the inflammatory events triggered by the adaptive or memory immune system (Dong, 2008). On the basis of these hypotheses, *in vitro* and *in vivo* studies have shown that IL-17A cooperates either additively or synergically with various cytokines or mediators inducing inflammation (Katz *et al.*, 2001). Indeed, recently, by using a murine model of inflammation, it has been shown that IL-17A is not a classical proinflammatory cytokine and it is not able to initiate *per se* an inflammatory reaction. On the contrary, this cytokine is able to further amplify biochemical and cellular events characteristic of the early stages of the inflammatory reaction, when it is injected in pre-inflamed tissue (Maione *et al.*, 2009).

Another important aspect of Th-17 cells is their capacity to produce not only IL-17 but also other cytokines, such as IL-2 and IL-22, IL-26, CCL20. Th17 derived cytokines induce the production of IL-6, TNF α , CXCL1, CXCL2, CXCL8, CCL2 and metalloproteinases, MMP-3, MMP-6 and MMP-13, from various tissues and cell types (Strezpa *et al.*, 2011).

Important differences, as well as many similarities, emerge when the biology of Th17 cells in the mouse is compared with corresponding phenomena in humans (Aggarwal *et al.*, 2002). However, in this context, it is very important to underline that IL -17A has *in vivo* as well as *in vitro* inflammatory properties. One of the main cellular target of this molecule are the neutrophil-cells, in fact it is known that IL-17A stimulates the production of chemokines, such as IL-8 (CXCL8) and granulocyte-chemotactic protein - 2 (GCP-2), growth factors, such as CSF and GM-CSF, the neutrophils activating cytokine, IL-6, from epithelial cells, smooth muscle cells and fibroblasts. Furthermore, IL-17A increases local sign of neutrophil activation, such as the activity of myeloperoxidase (MPO), elastase and matrix metalloproteinase (MMP-9) (Fossiez *et al.*, 1996; Hoshino *et al.*, 2000; Jones *et al.*, 2002).

As reported by Fouser and co-workers, IL-17A sustains, induces and amplifies the inflammatory response on a pre-existing tissue injury (Fouser *et al.*, 2008).

On the basis of the considerations reported here, it is clear that Th-17 cells, through IL-17, drive the inflammatory cascade by stimulating cells to release a large number of inflammatory mediators and growth factors that have important effects on neuroendocrine and metabolic functions and on the maintenance of tissue homeostasis in general.

Interleukin-17 and autoimmune diseases

Growing evidence, gathered over the last few years, indicate that IL-17A might play a key role in the development of autoimmune diseases. IL-17 is an important factor directing disease progression in multiple sclerosis (MS). Multiple sclerosis is a central nervous system (CNS) disease associated with destruction of myelin sheaths, leading to impaired nerve signal transduction. Similar to other proinflammatory cytokines, the concentration of IL-17A is increased in CNS lesions of MS patients and correlates with neutrophil infiltration of the CNS (Lock *et al.*, 2002).

The role of IL-17A in the rheumatoid arthritis (RA) is more complex. The pathological role of IL-17A in arthritic joints involves the stimulation of MMP, vascular endothelial growth factor (VEGF) and proinflammatory cytokine production and increased recruitment of T lymphocytes and innate immune cells (Kotake *et al.*, 1999). However, these processes are not attenuated when arthritis is induced in IL-17 *-/-* mice; this evidence suggests that other factors involved have a substitutive role and demonstrates that the role of IL-17 is still unclear (Doodes *et al.*, 2008). Nonetheless, in experiments performed by using a murine model of collagen-induced arthritis it has been demonstrated a beneficial effect of the animal treatment with an anti IL-17 antibody (Nakae *et al.*, 2003).

In patients affected by psoriasis and chronic skin disease, high levels of IL-17 have been found in skin lesions. Particularly, these types of diseases are characterized by a hyperproliferation of keratinocytes and production of proinflammatory molecules increasing angiogenesis and T cell infiltration. In these cases, it has been shown a possible synergism between IL-17 and other Th17 associated inflammatory cytokines (IL-23 and IL-22) with IFN-gamma in promoting characteristic pathologic changes (Teunissen *et al.*, 1998; Zheng *et al.*, 2007).

Furthermore, IL-17 and IL-23 have also been associated to Crohn's disease and colitis ulcerosa; however, the role of IL-17 in intestinal inflammation is still controversial, indeed this cytokine has been shown to be either protective or proinflammatory in two different models of murine colitis (Zhang *et al.*, 2006; O'Connor *et al.*, 2009).

Allergic asthma represents another disease in which IL-17 plays a crucial role. Allergic asthma is characterized by elevated IgE serum levels, chronic airway inflammation with intense cell accumulation, mucus hyperproduction and airway hyperresponsiveness to a large variety of stimuli (Souwer *et al.*, 2010). Asthma induces many irreversible changes in airway tissues. Accumulating evidence indicates that in bronchoalveolar lavage (BAL) fluid of patients with severe asthma there are elevated levels of IL-17, in comparison to moderate asthmatic patients and to control subjects; a role for IL-17 in inducing neutrophil

accumulation into asthmatic airways have been suggested. (Molet *et al.*, 2001).

In addition to neutrophil recruitment, IL-17 may also stimulate neutrophil activity since it stimulates the release of neutrophil-activating cytokines, IL-6 and IL-8, from bronchial epithelial cells and fibroblasts; furthermore, it may also play a role in causing structural changes in the airways, since causes the production of profibrotic cytokines, IL-6 and IL-11, from fibroblasts (Fossiez *et al.*, 1996; Molet *et al.*, 2001). Recently, by performing experiment on an *in vivo* model of murine asthma, it has been shown that the increased expression of heparin-binding epidermal growth factor (HB-EGF), induced by IL-17, might be responsible of mucus overproduction and airway smooth muscle cell proliferation in chronic asthma (Wang *et al.*, 2010). Targeting IL-17 may be useful for the treatment of asthma. It has been shown that neutralization of IL-17 with monoclonal antibodies reduces neutrophil accumulation in BALF (Helling *et al.*, 2003).

IL-17 plays also a role in chronic obstructive pulmonary disease (COPD) (Figure 11); indeed by releasing chemokines (CXCL1, CXCL6 and CXCL8) and granulocyte survival factors (GM-CSF and G-CSF) from airway epithelial cells it would increase neutrophil chemotaxis and prevent apoptosis (Jones *et al.*, 2002; Vanaudenaerde *et al.*, 2003; Rahman *et al.*, 2005; Traves and Donnelly, 2008).

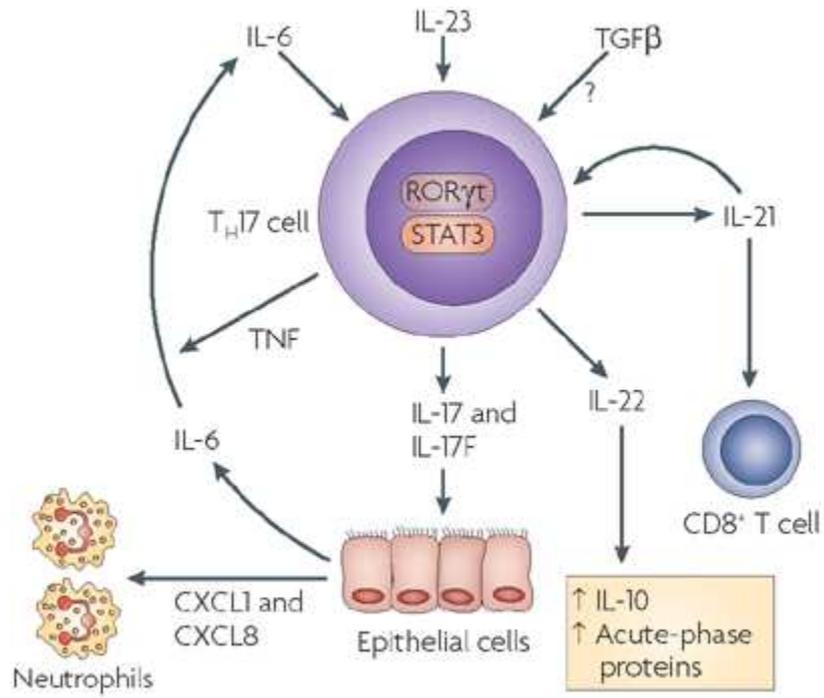


Figure 11. Interleukin-17 and chronic obstructive pulmonary disease (COPD).

IL-17 and cardiovascular risk

As described above, there is much evidence, either experimental or clinical, that thrombosis and atherosclerosis might be closely associated to an inflammatory reaction, (Jurado and Ribeiro; 1999, Esmon; 2003, Strukova; 2006). It is known that inflammation initiates clotting, decreases the activity of natural anticoagulant mechanisms and impairs the fibrinolytic system. Nonetheless, proteases involved in coagulation system contribute to inflammation not only by promoting fibrin formation at site of injury, but also by stimulating several cell functions (Cicala and Cirino,1998; Esmon, 2008). On the other hand, pro-inflammatory molecules are actively involved in the activation and migration of leukocytes to sites of vascular injury and inflammation, and may contribute to the release by activated cells of prothrombotic factors, which in turn may activate platelets and other cell types (Ruggeri *et al.*; 2007, Lambert *et al.*; 2007).

In agreement with this, there is much evidence that patients suffering from autoimmune diseases have an elevated risk of thrombosis (Gisondi et Girolomoni, 2009; Mameli *et al.*, 2009). As described above, multiple factors may be implicated; it is known that circulating cytokines and recruited inflammatory cells cause endothelial dysfunction and haemostatic disorders leading toward a prothrombotic state (Cicala and Cirino, 1997; Nurden, 2011). Furthermore, it has also been hypothesized

that in rheumatic diseases atherosclerotic lesions might be more prone to the rupture leading to acute cardiovascular events (Frostedgard *et al.*, 2011).

Evidence that increased IL-17 levels are associated with coronary artery disease (Eid *et al.*, 2009; Wang *et al.*, 2011) and atherosclerosis (Cheng *et al.*, 2008) has suggested that this cytokine may play a role at the interface between inflammatory immune disorders and cardiovascular risk. *In vitro* experimental data show that IL-17 stimulates C-reactive protein expression in human hepatocyte and coronary artery smooth muscle cells (Patel *et al.*, 2007). It is known that CRP is not only a marker of cardiovascular risk but, as described above, directly participate to endothelial dysfunction and may stimulate platelet aggregation and platelet/leukocytes interaction (Fay, 2010; Hirschfield and Pepys, 2003). Recently, by performing experiments *in vitro*, it has been shown that IL-17A favours the aggregation of murine and human platelets in response to ADP. The effect of IL-17A on platelets, *in vitro*, is paralleled by an increased expression of P-selectin on platelet surface (Maione *et al.*, 2011). It is well-established that platelet adhesion is mediated via glycoprotein GPIb receptors through interaction with the von Willebrand factor and that further physiologic activation of platelets via intracellular signalling pathways leads not only to an increased expression of the GPIIb/IIIa receptor complex, but also to a conformational change and

exposure of the fibrinogen binding site. Subsequent fibrinogen bridging allows firm attachment of adjacent platelets (Clemetson *et al.*, 1995). This process is a prerequisite for platelet aggregation and thrombus formation. Similarly, the increased platelet expression of CD62P is predictive for an elevated risk of circulating platelet-leukocyte aggregates that are typically considered predictive of thrombus formation (Wohner *et al.*, 2008) and observed in patients with acute myocardial infarction (Furman *et al.*, 2001) as well as in patients suffering of autoimmune diseases (Joseph *et al.*, 2001; Hu *et al.*, 2004; Irving *et al.*, 2004).

EXPERIMENTAL SECTION

ADENOSINE AND INFLAMMATION

MATERIALS AND METHODS

Animals

All experiments were performed on male Wistar rats (Charles River; 120-150g). Rats were slightly anaesthetized with enflurane. 100 µl of carrageenan (1 % w/v in saline) were injected in the rat hind paw to obtain an oedema. Then, oedema was measured by the means hydroplethismometer at time zero and each hour for the following 6 hours.

Drug treatments

To investigate on the role of adenosine A_{2A} receptor activation on carrageenan oedema development, animals were divided in 5 groups and treated, just before oedema induction, with the intraperitoneal injection of: A_{2A} agonist, CGS 21680 (0.02, 0.2 and 2 mg / kg); A_{2A} antagonist, ZM 241385 (3 mg/kg); CGS 21680 (2 mg/kg) *plus* ZM 241385 (3 mg /kg) and the respective vehicles. At different times following oedema induction, the paws were excised, cut, frozen in liquid nitrogen or fixed in buffered formalin 10 % (v/v) and stored.

Myeloperoxidase (MPO) assay

Myeloperoxidase activity was measured from animals treated with the selective adenosine A_{2A} receptor agonist, CGS 21680 (0.02, 0.2 and 2 mg/kg ip.) or with the vehicle (DMSO; 0.5 ml/kg ip.), inflamed paws were excised after 3 hours from oedema induction and the soft tissue was removed, frozen in liquid nitrogen and stored. Tissue samples were then defrosted, weighed and homogenized in a solution containing 0.5 % (w/v) hexadecyltrimethylammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged at 12000 rpm for 30 minutes at 4°C; an aliquot of the supernatant was then allowed to react with 0.167 mg/ml o-dianisidine dihydrochloride and 0.001 % H₂O₂. The rate of change in absorbance was measured spectrophotometrically at 650 nm; MPO activity was defined as the quantity of enzyme degrading 1 μmol/min of peroxide at 37°C and was expressed in milliunits per g of wet tissue (mU/g tissue).

Western blot analysis

Tissue samples were defrosted, weighed and homogenized with a Polytron. In order to extrapolate proteins, 1 ml of buffer (β-glycerophosphate 50 mM; sodium ortovanadate 100μM; MgCl₂ 2mM; 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 pellets were then

centrifuged at 12000 rpm. for 30 minutes at 4°C in order to measure the protein content *via* Bradford assay. Proteins were separated by Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE) (12 %); 40 µg of protein were applied on the gel and electrophorezed at 90 mV. for 1.5 hours. The protein samples were then electro-blotted at 250 mA for 1.30 h using nitrocellulose membrane. Afterwards, nitrocellulose membrane was blocked using a blocking solution containing 5% (w/v) non-fat dry milk, 0.1% (w/v) Bovine Serum Albumin (BSA) and 0.1% (v/v) Tween 20 in phosphate buffer solution (PBS), for 2h at room temperature. Then, it was incubated overnight at 4° C on a shaker with anti A_{2A}(R-18, Santa Cruz) goat antibody (dilution 1:500). The nitrocellulose membrane was washed five times for 25 minutes and then incubated with the secondary antibody anti-goat IgG (dilution 1: 5000) conjugated with horseradish peroxidase for 2h at room temperature. After five washes, the proteins bands were detected using the enhanced chemiluminescence (ECL) method and analyze with Image Quant 400 GE Healthcare software (GE Healthcure, Italy) as described by the manufacturer.

Immunohistochemical localization of A_{2A}

Tissue samples removed, as described above, were prepared from paraffin embedded tissues. After deparaffinization and rehydration of tissue section (thickness 7 µm), antigen retrieval was performed for 30

minutes at 100°C with 0.01 M citrate buffer (pH 6.0). To block non-specific binding, slides were incubated for 10 min at room temperature with the protein block serum free solution (Dako). Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin. Then the sections were incubated overnight with primary goat anti-A_{2A} antibody (Santa Cruz) (dilution 1:250) in PBS and BSA 1%, overnight at 4 °C, or with control solutions including buffer alone or non-specific purified rabbit IgG.

After washing, endogenous peroxidase was quenched with 0.3 % (v/v) H₂O₂ in 60 % (v/v) methanol for 10 min. Specific labeling was detected with a biotin-conjugated universal secondary antibody (Universal DakoCytomation LSAB Kit) for 30 min at room temperature followed by incubation with streptavidin-HRP. After washing, slides were incubated with Diaminobenzidine (DAB). The counterstaining was performed with hematoxylin. Negative staining control experiments were performed according to the protocol described above, with omission of the primary antibody.

Picro Sirius red staining for collagen detection

Picro Sirius stain was applied to visualize collagen content. Briefly, the paraffin sections were de-waxed and rehydrate. The sections were stained with Mayer's haematoxylin to visualize the nuclei and then

incubated in the Pico Sirius red solution for one hour. The sections were washed with acidified water, dehydrated and cleared in xylene before mounted in a resinous medium.

Statistical analysis

All results were expressed as mean \pm error standard 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.

RESULTS

Effect of CGS 21680 treatment on carrageenan – induced oedema

Injection of carrageenan in the rat hind paw caused an oedema peaking between 3 and 4 hours. Treatment with CGS 21680 inhibited oedema development in a dose – related manner, and this effect was reverted by co-administration with the A_{2A} antagonist, ZM 241385. On the contrary, ZM 241385 alone did not modify oedema (Figure 12-13)

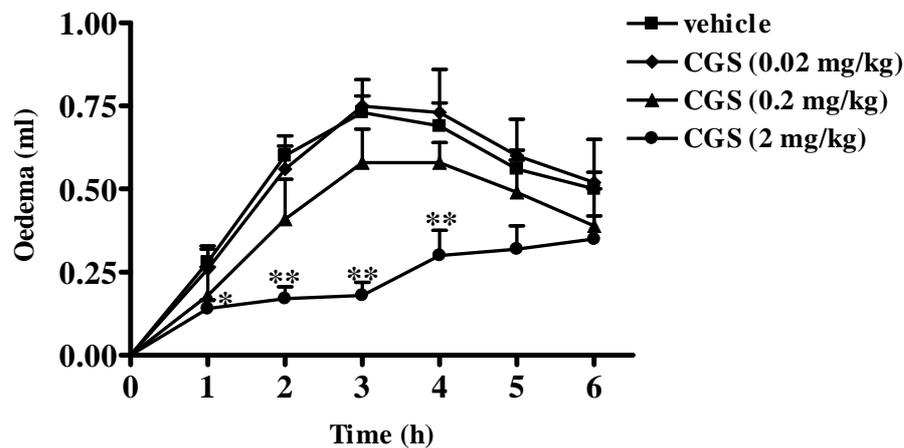


Figure 12. Effect of the selective adenosine A_{2A} receptor agonist CGS 21680 (0.02, 0.2 and 2 mg/Kg ip) on carrageenan oedema; * $P < 0.05$, ** $P < 0.01$ vs vehicle. Dunnett's test ($n = 10$).

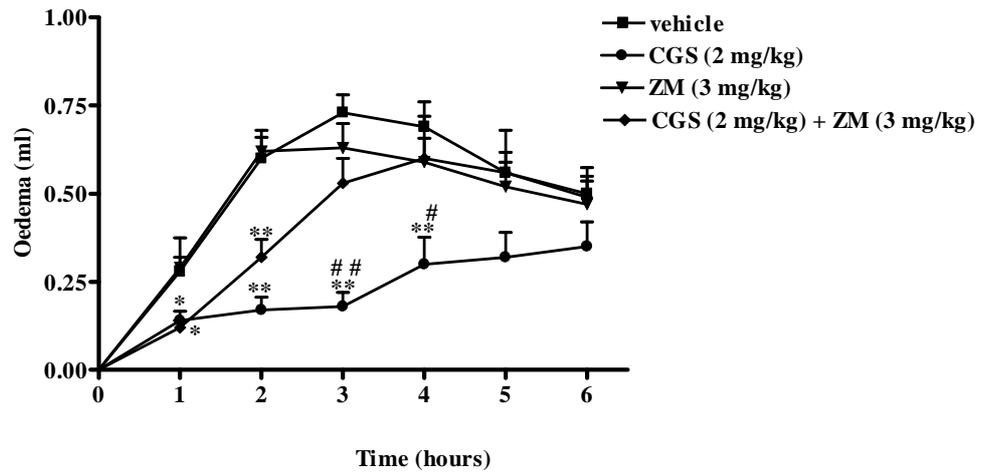


Figure 13. Effects of CGS 21680 (2 mg/kg ip), a selective A_{2A} receptor agonist, ZM 241385 (3 mg/kg ip), a selective A_{2A} receptor antagonist, and CGS 21680 *plus* ZM 241385 on carrageenan oedema; * $P < 0.05$, ** $P < 0.01$ vs vehicle, # $P < 0.05$, ## $P < 0.01$ vs CGS 21680 *plus* ZM 241385. Dunnett's test (n = 10).

MPO assay

MPO activity measured in inflamed paws excised 3 hours after carrageenan injection was reduced after treatment with CGS 21680 at 2 mg/kg ip, compared with control values (Figure14).

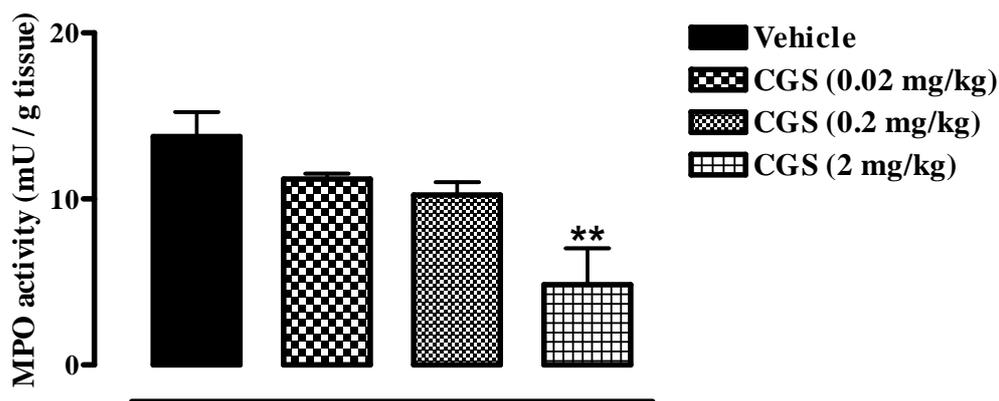


Figure 14: Effect of CGS 21680 (0.02, 0.2 and 2 mg/kg ip.) on myeloperoxidase (MPO) activity of inflamed paws (3 hours after carrageenan injection); ** $P < 0.01$ vs. vehicle. Dunnett's test ($n = 3$).

Western blot

Western blot analysis performed on paw excised at different time following oedema induction, showed an increased A_{2A} protein expression starting 1 hour following oedema induction, and peaking between 3 and 4 hours.(Figure 15). The increased A_{2A} protein expression observed at 3 hours after oedema induction was significantly reduced (like control values) following rat treatment with CGS 21680 (2 mg/Kg ip.) (Figure 16).

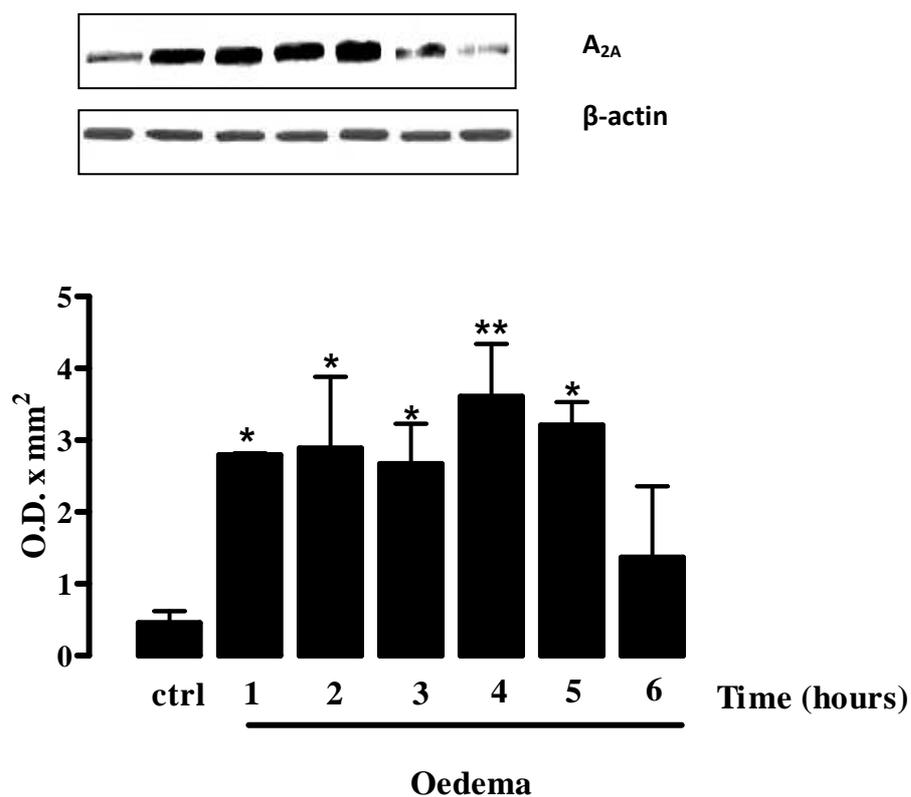


Figure 15. Detection of adenosine A_{2A} receptor and optical density analysis in inflamed paws; * $P < 0.05$, ** $P < 0.01$ vs vehicle. Dunnett's test (n = 3).

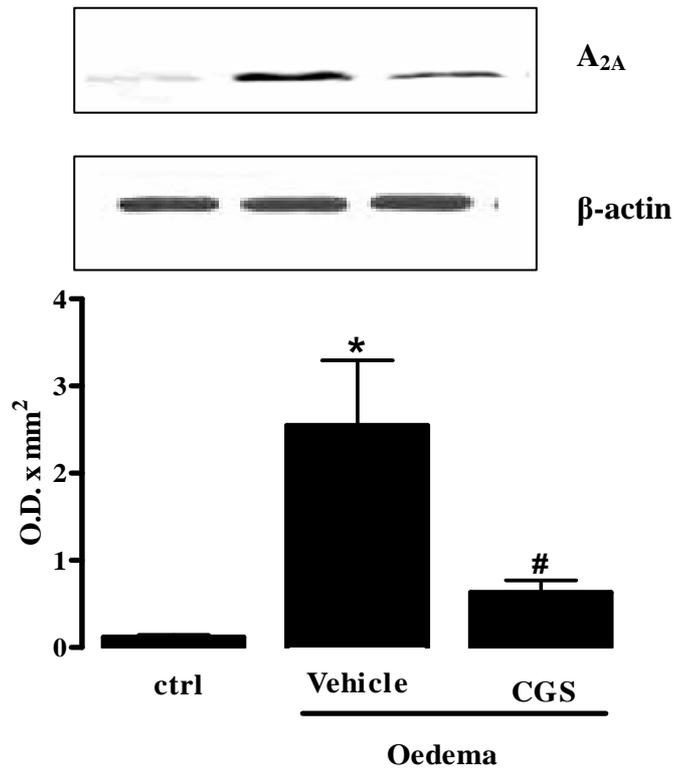


Figure 16. Effect of CGS 21680 (2 mg/kg ip) on the proteic expression of the adenosine A_{2A} in inflamed paws (3 hours after carrageenan injection); * $P < 0.05$, ** $P < 0.01$ vs vehicle. Dunnett's test (n = 3).

Immunohistochemical localization of A_{2A}

Immunohistochemical analysis showed A_{2A} receptor overall staining on vascular endothelium and on dermal fibroblast provided from carrageenan-injected paws, compared to no-injected paws. This staining was greatly reduced in the case of paws obtained from CGS 21680-treated rats (Figure 17).

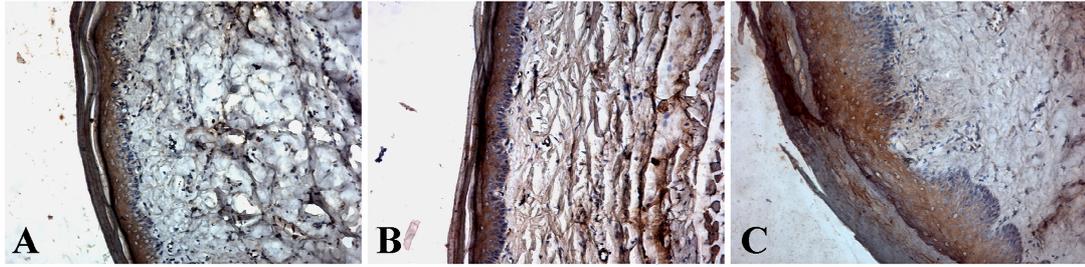


Figure 17: Immunohistochemical localization of A_{2A} receptor paw tissue ($7\mu\text{m}$). The figure represent paw tissue from: A) sham animals; B) vehicle treated animals; C) CGS 21680 treated animals. Original magnification is 10X.

Picro Sirius staining

Picro Sirius red staining for collagen detection showed that in tissue section from inflamed animals, dermal collagen result to be loose compared to control paws (not inflamed) and CGS 21680 treated animals where dermal elastic fibers appear to be well organized. Moreover, the treatment with CGS 21680 enhanced fibroblast infiltration and collagen production (Figure 18).

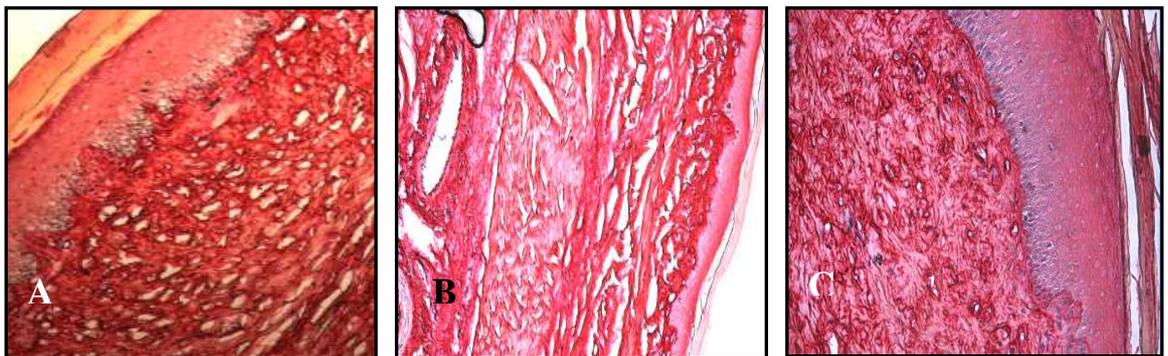


Figure 18. Sections of paw tissue stained for picro sirius red for the detection of collagen. (A) Paw tissue for sham animals, (B) paw tissue from vehicle treated animals, (C) paw tissue from CGS 21680 treated animals. Original magnification is 10X.

INTERLEUKIN 17A AND THROMBOSIS

MATERIALS AND METHODS

Materials

Recombinant mouse IL-17A was purchased from R&D System (Abingdon, UK), reconstituted in 4 mM HCl solution and diluted in phosphate saline buffer (PBS) as reported on the certificate of analysis. Unless otherwise specified, all the other reagents were from Sigma-Aldrich Co. (MI, Italy).

Thrombosis model

Male Wistar rats (300–350 g; Harlan Nossan, Correzzana, MI, Italy) were used for all experiments. Animals were kept under standard conditions, with food *ad libitum* and maintained in a 12 h/12 h light/dark cycle at $22 \pm 1^\circ\text{C}$. All the *in vivo* procedures were in accordance with the Italian legislative decree (D.L.) no. 116 of January 27, 1992 and associates guidelines in the European Communities Council Directive of November 24, 1986 (8676097ECC).

Rats were anaesthetized with urethane (10 % w/v; 10 ml/kg ip.) and placed on a surgical table; an arterial thrombus was induced by FeCl₃ application onto the surface of the right carotid artery, as described by Kurz *et al.* (1990). In brief, following surgery a piece of filter paper (Whatman n°1, 3 x 5 mm) soaked in FeCl₃ (from 5 to 35%), or in IL-17A

(100 µg/ml), was applied onto the external surface of the right carotid artery, for 30 minutes, afterward the paper was removed and the vessel left *in situ* for 60 minutes, to enable thrombus formation. In another set of experiments, an IL-17A (100 µg/ml), or vehicle (saline), soaked paper was applied on the vessel for 30 minutes before applying FeCl₃. On the basis of preliminary experiments we have chosen a percentage of FeCl₃ (5%) that induced a partial carotid occlusion. At the end of 60 minute period, a piece of 2 cm in length of the right carotid artery, and of its controlateral (where only vehicles were applied), was removed and weighed. Thrombus size was evaluated by the difference in weight between the treated vessel and its controlateral. In another group of animals, the experiment was performed as described above and, vessels were removed, rinsed in saline to remove the blood excess, then fixed with formalin (4 % v/v) for 24 hours and successively used for histological analysis, or removed tissues were immediately frozen in liquid nitrogen and successively used for Western blot analysis.

Western blot analysis

Tissue samples were defrosted, weighed and homogenized in liquid nitrogen. In order to extrapolate proteins, 1 ml of buffer (β-glycerophosphate 50 mM; sodium ortovanadate 100µM; MgCl₂ 2mM; 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 pellets were then centrifuged at 12000 rpm. for 30 minutes at 4°C in order to measure the protein content *via* Bradford assay.

Proteins were separated by (SDS-PAGE) (8 %); 35 µg of protein were applied on the gel and electrophoresed at 90 mV. for 1.30 hour. The protein samples were then electroblotted at 250 mA for 1.30 hour onto a nitrocellulose membrane. Afterwards, nitrocellulose membrane was blocked using a blocking solution containing 5% (w/v) non-fat dry milk, 0.1% (w/v) BSA and 0.1% (v/v) Tween 20 in PBS, for 2h at room temperature. It was then incubated overnight at 4° C on a shaker with anti CD39 (A-16, Santa Cruz) goat antibody (dilution 1:200). The nitrocellulose membrane was washed five times for 25 minutes and then incubated with the secondary antibody anti-goat IgG (dilution 1: 2000) conjugated with horseradish peroxidase for 2h at room temperature. After five washes, the proteins bands were detected using the ECL method and analyze with Image Quant 400 GE Healthcare software (GE Healthcare, Italy) as described by the manufacturer.

Morphological analysis

Samples were processed and embedded in paraffin. Sections (thickness 5 µm) were then stained for haematoxylin and eosin (H&E) to be morphologically analyzed. In all cases, a minimum ≥ 5 sections per

animal were analyzed by using a standard light microscope (x 5 and x 10 objective). In each section the thrombus formation was evaluated by calculating the percentage of thrombotic area compared with the total area, by using a computerized program (Leica, MI, Italy). 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 V2.4.0.

ELISA and Proteome Profiler Antibody Arrays

In subsets of experiments, cytokines and chemokines expression from whole IL-17-or vehicle-treated carotids was determined. For this purpose the carotids were excised, the remaining blood was removed by washing with PBS and immediately frozen in liquid nitrogen before being stored at -80°C. Tissues were placed in a mortar, finely chopped and homogenized using liquid nitrogen. The homogenized powder was reconstituted with 300 µl of ice-cold lysis buffer (Aprotinin 3.07 µM, EDTA 100 mM, Leupeptin 2.2 µM, Na-deoxycholate 10%, NaCl 150 mM, NaF 5 mM, NP-40 10%, Ortovanadato 50 µM, PMSF 100 µM, Tris-HCl 65 mM) and collected in 1.5 ml eppendorf tubes. Samples were frozen and thawed three times in liquid nitrogen and then placed under rotation for 30 minutes at 4°C in order to optimize the process of homogenization. After spinning at 10.000 rpm to remove cell debris, the

supernatants were collected and the total protein concentrations were determined by using a Bovine Serum Albumin (BSA) protein assay (Biorad, Italy) following the manufacturer's directions. In order to analyze the expression of a wide range of cytokines and chemokines after the IL-17 or vehicle application a proteome profiler antibody array (R&D System; Abingdon, UK) was used, according to the manufacturer's instructions. For this purpose 1 ml of homogenized tissue from three different rats, treated under the same experimental conditions, was used to incubate each membrane on a rocking platform overnight. Positive dots were then detected by enhanced chemiluminescence (ECL) using Image Quant 400 GE Healthcare software (GE Healthcare, Italy). Aliquots of 50 μ l were diluted (1:1) with assay diluents and analyzed for the levels of MCP-1 by ELISA according to the manufacturer's instructions (eBioscience, UK).

RESULTS

Effect of IL-17A on carotid thrombus model

A dose responsive curve for FeCl₃ was performed to establish the percentage of FeCl₃ that we could use for our *in vivo* model. As showed in figure 19, there was a correlation between the effect of FeCl₃ and the concentration used. In fact, when 5 % and 15 % of FeCl₃ was used a partial occlusion of the carotid artery was observed. On the other hand, an high concentration of FeCl₃ (35%) induced an occlusive thrombus. On the light of these preliminary results, we have chosen FeCl₃ concentration of 5% to perform all the subsequent experiments.

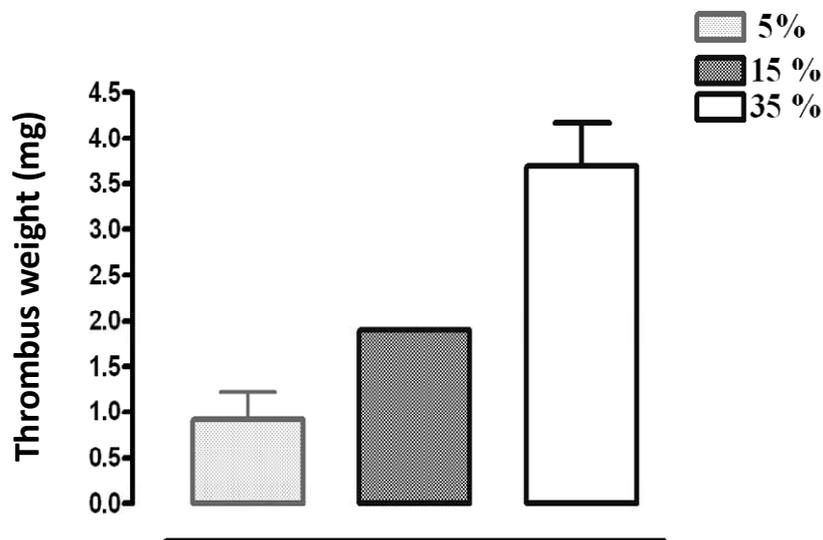


Figure 19. Thrombus weight (mg) after the application of three different concentration of FeCl₃: 5, 15 and 35 % (w/v) respectively.

Results obtained (Figure 20) show that the application of IL-17A on rat carotid artery determined a small intravascular thrombus with a mass of 0.35 ± 0.15 mg (one sample t-test, $P < 0.05$) although not significantly different compared to the thrombus obtained following the application of vehicle. The application of FeCl_3 (5 %) caused an intravascular thrombus of 0.99 ± 0.2 mg ($n=9$); however, thrombus mass increased significantly when the carotid was pre-treated with IL-17A (100 $\mu\text{g}/\text{mL}$) 30 minutes before FeCl_3 application (1.94 ± 0.2 mg; $n=9$. $P < 0.01$).

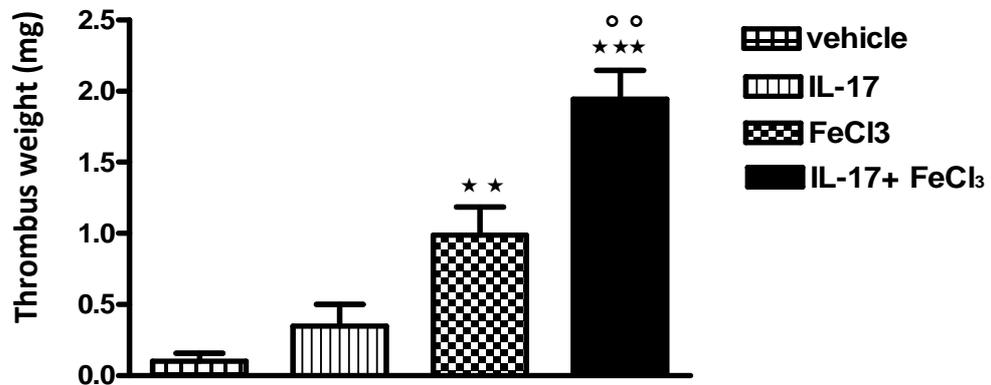


Figure 20. Thrombus weight after different treatments: vehicle, IL-17, FeCl_3 , and IL-17 plus FeCl_3 . ** $P < 0.01$ and *** $P < 0.001$ vs vehicle; °° $P < 0.01$ vs FeCl_3 .

Western blot analysis

The western blot analysis performed on the carotid treated with vehicle and IL-17A showed a reduced expression of CD39 protein in IL-17A treated animals compare to the vehicle (Figure 21).

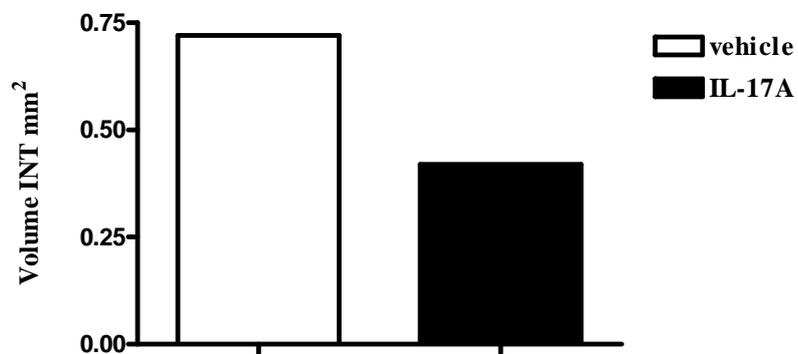
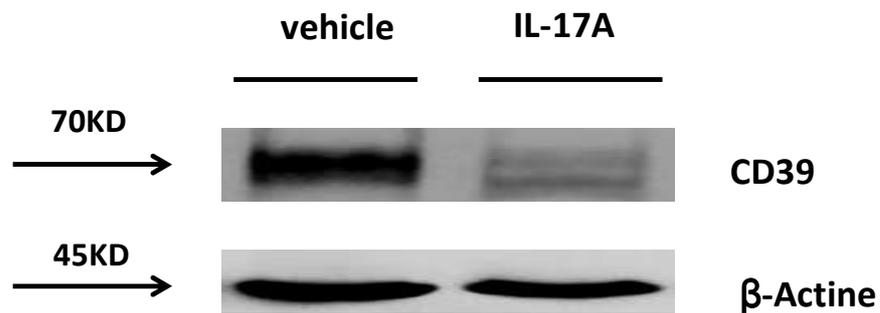


Figure 21. Detection of CD39 receptor and optical density analysis in rats treated with vehicle and IL-17A respectively.

Histological analysis

Morphological analysis of the vessel section occluded, evidenced that the luminal surface of carotid sections from vehicle group was covered by a continuous endothelium. Sections from IL-17A *plus* FeCl₃ treated carotids showed an occluding thrombus; furthermore, the endothelium appeared damaged and vessel wall thickness extremely reduced (Figure 22).

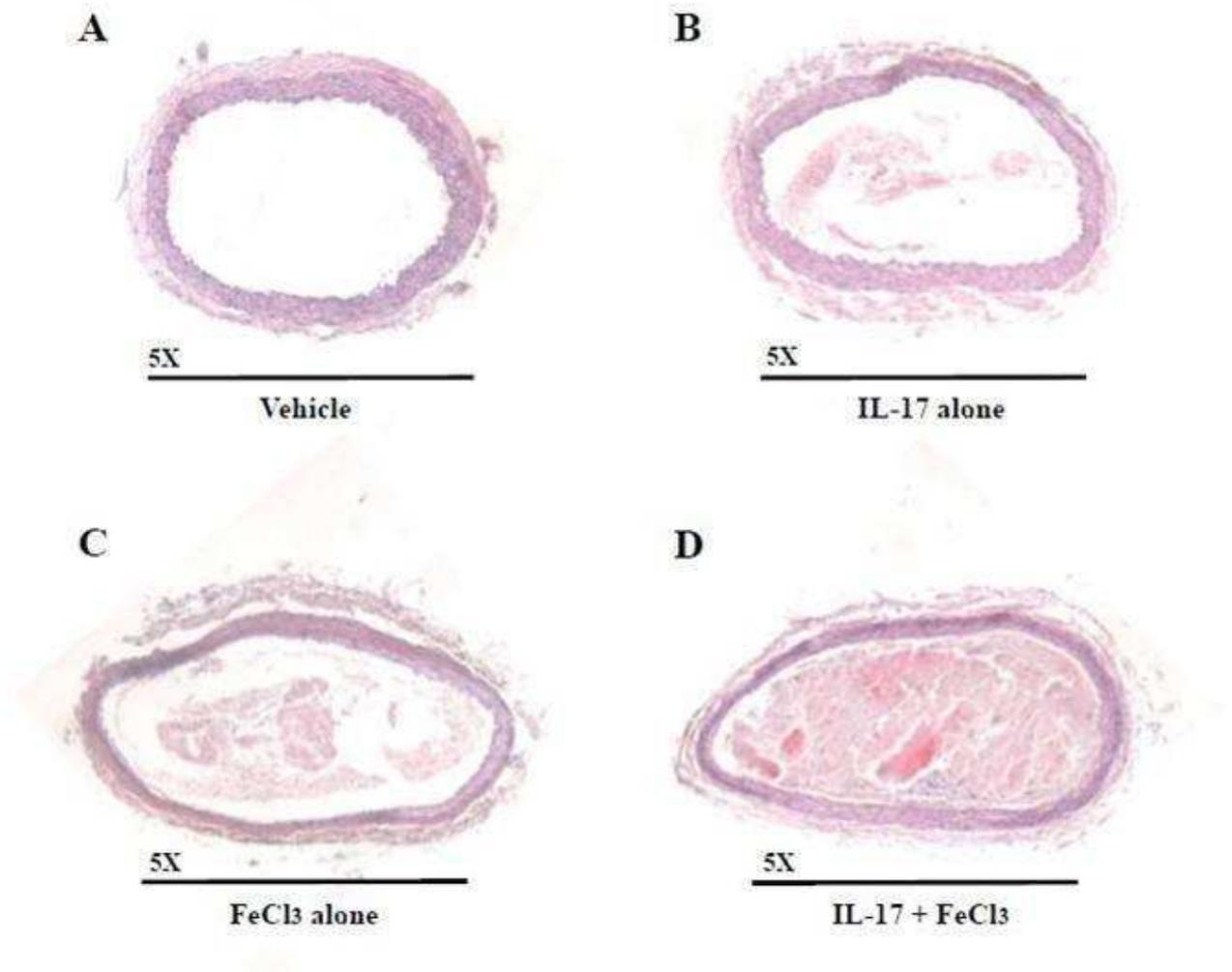


Figure 22. Histological analysis of haematoxylin and eosin (H&E)-stained carotid sections, from : A) vehicle; B) IL-17 alone; C) FeCl₃ alone; D) IL-17+ FeCl₃. Original magnification 5X.

Percentage of carotid area of occlusion

The increased mass of thrombus in the co-administration of FeCl₃ and IL-17A was confirmed by the measurement in terms of percentage of the lumen-vessel occlusion from a computer-assisted planimetry. The percentage of occlusive thrombi resulted not significant in IL-17 sections compare to vehicle, whereas FeCl₃ alone induced an occlusion of 29.9% ± 5.0 that resulted ~ 2 fold- increased in IL-17 *plus* FeCl₃- treated animals (54.5 % ± 7.0).

ELISA and Proteome Profiler Antibody Arrays

The Figure 23 reported the analysis of the inflammatory proteins in the supernatant of vehicle and IL-17A treated carotids. It is possible to observe that the interleukin induced the selective production of a specific set of chemokines such as CCL2 (MCP-1) compared with vehicle.

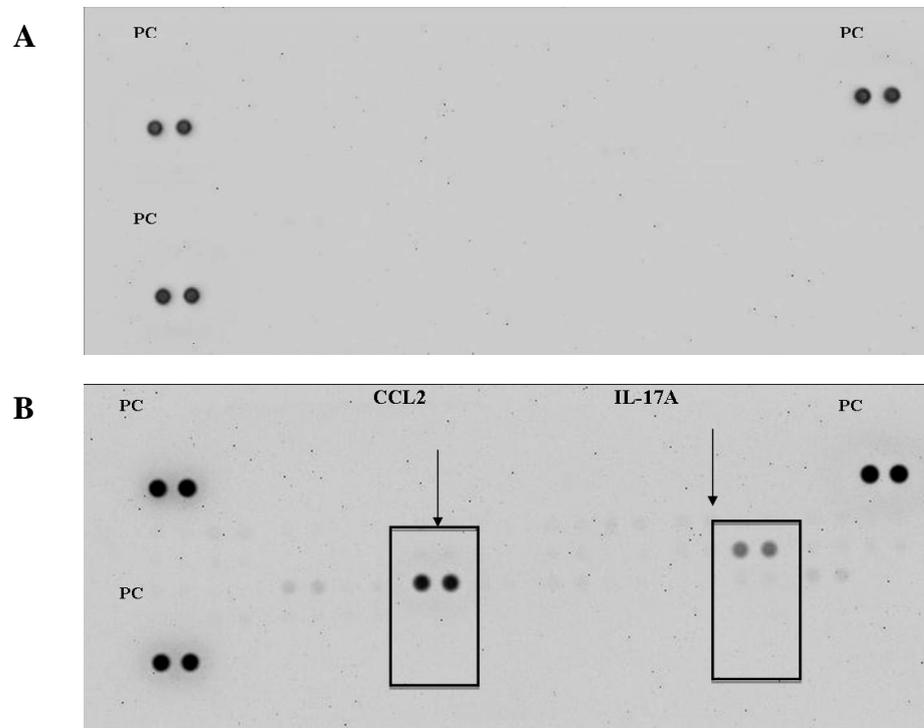


Figure 23. Elisa Spot obtained from the application of IL-17 (A) and vehicle (B) in thrombosis model. (PC: positive control; CCL2: MCP-1; IL-17A).

CONCLUSION

Adenosine and inflammation

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. Aim of the present study was to investigate on the role of A_{2A} adenosine receptor in acute inflammation, in vivo.

The main finding of our research is that following carrageenan edema induction in rats there is a time dependent increase in A_{2A} receptor expression.

Pharmacological stimulation of A_{2A} receptor, by using the selective A_{2A} agonist, CGS21680, prevented edema development in a dose-dependent manner. Furthermore, CGS21680 inhibitory effect was prevented by co-administration with ZM241385.

Conversely, systemic administration of ZM241385 did not have any effect on edema development. These findings confirm that the effect of CGS21680 was specific, through A_{2A} receptor stimulation, nonetheless A_{2A} activation by endogenous adenosine seems not to offer protection against an acute inflammation, since A_{2A} antagonism did not exacerbate oedema. This latter finding is in agreement with previous work showing that systemic administration of ALT-146e, a selective A_{2A} agonist, reduced skin ulceration induced by recurrent ischemia reperfusion in

rats, the effect was reverted by the antagonism ZM241385; however, the antagonist alone did not exacerbate skin ulceration (Peirce *et al.*, 2001).

It is known that, among inflammatory cells, neutrophils express A_{2A} receptor on their membrane surface; many of antinflammatory effect of A_{2A} agonists have been shown to be related to the inhibition of neutrophil sequestration (Linden, 2006).

Moreover, it has been demonstrated that A_{2A} agonist protect from aspirin induced gastric lesions by inhibiting neutrophil sequestration into the gastric mucosal tissue (Odashima *et al.*, 2006). However, the mechanism at the basis of the antinflammatory effect of adenosine through A_{2A} activation has not yet been clarified. Cadiaux and coworkers (2005) have shown that following A_{2A} receptor activation on neutrophils there is an increased COX₂ expression paralleled by increased production of PGE₂, a prostanoid with antinflammatory properties. Thus, the increased PGE₂ production might be the mechanism by which endogenous adenosine, through A_{2A} receptor activation, limits an inflammatory reaction.

Neutrophils play a pivotal role in the development of an acute inflammation, such as carrageenin edema. In the present study, as marker of neutrophil accumulation, we measured MPO activity in the inflamed paw. We found that treatment with CGS21680 significantly inhibited the increase in MPO activity into the inflamed tissue

confirming that the antiinflammatory effect of CGS21680 was associated to a reduced neutrophils infiltration.

Following treatment with CGS21680, edema inhibition was paralleled by downregulation of A_{2A} receptor expression on tissues excised. Immunohistochemical analysis also showed a reduced immunopositivity for A_{2A} receptor on paws obtained from CGS21680 treated rats.

This finding suggests that in an inflammatory environment there is an upregulation of A_{2A} receptor expression, whose function it to keep down inflammation. Further studies are required to better investigate on the mechanism underlying the antiinflammatory effect mediated by A_{2A} activation.

Interleukin 17A and thrombosis

To investigate on the role of IL-17A on thrombus formation we used the model described by Kurz (Kurz *et al.*, 1990) in which thrombosis is induced in rats by topical application of FeCl₃ on the exteriorized carotid artery.

We found that topical pre-application of IL-17A on rat carotid artery had a synergistic effect with FeCl₃ (5 %); while IL-17A alone caused only a small intravascular thrombus. Morphological analysis of the vessel section occluded evidenced that the luminal surface of carotid sections from vehicle group was covered by a continuous endothelium; into the vascular lumen some aggregates of red blood cells were observed but without any fibrin mesh. Sections from IL-17A *plus* FeCl₃ treated carotids showed an occluding thrombus; furthermore, the endothelium appeared damaged and vessel wall thickness extremely reduced.

These results suggest that IL-17A has the ability to facilitate thrombus formation induced by a minimal stimulus, as FeCl₃ at 5 %. There is evidence that IL-17A plays a role in vascular inflammation and atherothrombosis (von Vietinghoff and Ley, 2010). In vitro, it has been shown that IL-17A stimulates C reactive protein expression in human coronary artery smooth muscle cells (Patel *et al.*, 2007). As described above, C-reactive protein is an important marker of vascular diseases playing an active role in atherosclerosis by stimulating chemokine

expression, platelet adhesion at the site of vascular damage, platelet/leukocyte interaction and also inhibit the fibrinolytic pathway (Danenberg *et al.*, 2007). Thus, clinical evidence showing a correlation between IL-17A serum levels and acute coronary syndrome (Cheng *et al.*, 2008; Liang *et al.*, 2009), together with experimental data showing that IL-17A modifies cellular expression of molecules involved in thrombosis (Patel *et al.*, 2007) and also increases ADP-induced platelet aggregation (Maione *et al.*, 2011) has driven the attention of investigator to consider this cytokine has possible link between haemostatic disorders associate to inflammation.

Our data show that IL-17A applied alone on carotid causes only a small not occlusive thrombus, however, the main effect we observed was that IL-17A strongly increased the thrombus induced by a minimal FeCl₃ concentration. The model of FeCl₃-induced thrombosis has been shown to involve platelets and several component of haemostasis (Broersma *et al.*, 1991); thus the effect of IL-17A is consistent with its ability to prime platelets for the effect of ADP (Maione *et al.*, 2011). Our preliminary results aimed to better investigate on the molecules triggering the pro-thrombotic effect of IL-17A observed show a possible involvement of monocyte chemoattractant protein (MCP-1). This is a chemokine that is expressed by several cells, among which are endothelial cells, smooth muscle cells, fibroblasts, monocyte/macrophages. In an inflammatory

environment, activated platelets may induce MCP-1 from endothelial cells, leading to monocyte/macrophages chemotaxis and the consequent interaction among cells (Charo and Taubman, 2004) included platelet/monocyte interactions (Gleissner *et al.*, 2008). We found that following exposure to IL-17A there was an increased carotid expression of MCP-1.

We don't know if MCP-1 is induced by a direct effect IL-17A on endothelial cells, or indirectly, from platelets activated by IL-17A. Since platelet-induced MCP-1 from endothelial cells is secondary to the interaction between CD40L (on activated platelets) and CD40 (on endothelium) (Gleissner *et al.*, 2008), it would be interesting to investigate whether IL-17A causes externalization of CD40L on platelet surface.

We also observed a reduced expression of CD39 on carotid artery following treatment with IL-17A. As described above, CD39 by hydrolyzing ATP and ADP to AMP represents a key modulator of thrombus formation. It has been shown that the loss of CD39 from activated endothelial cells causes platelet sequestration and TF upregulation, key events for thrombogenesis (Atkinson *et al.*, 2006). Platelets from mice lacking CD39 show an increased response to ADP (Enjyoji *et al.*, 1999). Thus, it could be hypothesized that the first event for the prothrombotic effect IL-17A would be a down-regulation of

CD39 on endothelial cells and the following platelet activation; this, in turn, would cause increased MCP-1 expression from endothelial cells.

The mechanistic bases of pro-thrombotic effect of IL-17A needs further investigation; however our findings represent first in vivo evidence for a prothrombotic effect of IL-17A and suggest that this cytokine might be an important molecule at the interface between haemostasis and inflammation.

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CHAPTER II

PROTECTIVE EFFECT OF DIMETHYLSULFOXIDE ON ACUTE MYOCARDIAL INFARCTION IN RATS

Dimethylsulfoxide (DMSO) is an organic compound that has been shown to possess several biological effects, including antioxidant, anti-inflammatory, antinociceptive. Chemically, DMSO is an amphipathic molecule widely used as solvent in biological studies and as vehicle for drug administration (Santos, et al., 2003; Colucci, et al., 2008). Since its anti-inflammatory, antinociceptive and antioxidant effect, DMSO has been proposed to be therapeutic in several disorders, such as gastrointestinal diseases, rheumatologic diseases and for the treatment of several manifestations of amyloidosis (Scherbel, et al., 1965; Rosestein et al., 1999; Swanson et al., 1985; Hsieh et al., 1987) Nowadays, its therapeutic use has been approved for the treatment of interstitial cystitis, by intravesical instillation (Sant, et al., 1987; Parkin, et al., 1997) and in veterinary as analgesic and anti-inflammatory drug (Smith et al., 1998). Nonetheless, DMSO is present in topical dermatological preparations for human use as active vehicle facilitating drug penetration (Turgwell, et al., 2004).

The mechanism at the basis of the therapeutic effects of DMSO has not yet been elucidated; however, among its several biological activities, DMSO has been show to have antioxidant effects (Santos, *et al.*, 2003; Colucci, *et al.*, 2008). In addition, experimental studies have shown that DMSO reduces intracellular calcium accumulation in different biological systems (Zhang, *et al.*, 2004; Choi, *et al.*, 1999; Michel, *et al.*, 1998;

Santos, *et al.*, 2002). However, DMSO - induced relaxation on guinea pig and rabbit papillary muscles has been shown to be dependent upon a reduced sensitivity to calcium of contractile proteins without affecting intracellular calcium concentration (Ogura *et al.*, 1996). Similarly, on rabbit detrusor muscle DMSO causes relaxation by decreasing calcium sensitivity of the contractile apparatus, an effect that has been shown mainly due to the inhibition of myosin light chain phosphorylation (Shiga *et al.*, 2007); this mechanism might be at the basis of its therapeutic activity in interstitial cystitis. There is much evidence that following ischemia reactive oxygen species produced by an abnormal cell metabolism impair myocardial function. Indeed, ischemia is followed by calcium overload and prolonged contractile abnormalities. Both free radical scavengers and calcium channel blocking agents protect myocardial function from ischemic damage (Bolli *et al.*, 1999). Cardiac pharmacology of DMSO has also been investigated in several in vitro studies demonstrating a protective effect on ischemic damage (Shalfer *et al.*, 1983).

In the attempt to investigate on the effect of adenosine A_{2A} receptor agonist, CGS21680, in a model of acute myocardial infarction in rats, we came across to an unexpected effect of the vehicle, DMSO, used to administered CGS21680 to rats.

Thus, on the basis of the literature reported above, showing that DMSO has several biological effects, we have sought to investigate the effect of DMSO pre-treatment in a model of rat acute myocardial infarction.

EXPERIMENTAL SECTION

METHODS

Experimental procedure

All experiments were performed on male Wistar rats (250 – 280 g; Harlan Nossan, Italy). Animals were anaesthetized with an intraperitoneal injection of a solution of ketamine (100 mg/kg) and xylazine (10 mg/kg), placed on a surgical table and artificially ventilated through a tracheal cannula connected to a ventilation pump for small animals (Ugo Basile, Italy).

Myocardial infarction was produced by ligation of left anterior descending coronary artery (LAD), according to a method previously described in Wistar rats (Guerra, *et al.*, 2006). Briefly, the left side of the thorax was opened between the fourth and fifth intercostal space. The heart was gently exteriorized and the pericardium dissected out. The left anterior descending coronary artery was occluded near its anatomical origin by a 5-0 silk suture (Ethicon, *Johnson-Johnson*) for 90 minutes. At the end of the ischemia period, a blood sample was withdrawn from abdominal aorta, and the serum was obtained 24 h thereafter following centrifugation at 3000 rpm for 15 minutes and then kept at -80 °C until the measurement day. Harvested heart following 90 minutes ischemia was placed into a Petri dish containing potassium chloride and cut into 5-6 thick transverse slices from the apex to the basis. Slices were incubated

for 30 minutes at 37 °C in a 1 % solution of 2,3,5-triphenyltetrazolium chloride (TTC) in 1% phosphate buffered solution (PBS) then washed with PBS and stored for three weeks in PBS with 0.01 % sodium azide (PBS-A) at 4 °C , as described by Pitts (Pitts *et al.*, 2007). To differentiate necrotic (pale) from non necrotic (red) area, each section was analyzed by using a computerized image analysis system and infarct size was calculated by calculating the percent (%) of necrotic area compared to the total area, by using a computerized program (Leica, Milan, Italy).

All animal experiments complied with the Italian D.L. no. 116 of 27 January 1992 and associates guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC).

Treatment with DMSO

Ischemic animals were divided in three groups: 1) animals that did not receive any treatment before ligation; 2) animals treated with DMSO 500 µl/kg ip., 15' minutes before the ligation; 3) animals treated with DMSO 500 µl/kg ip. for three consecutive days, the last injection was given 15 minutes before the ligation. Sham animals underwent to the surgical intervention without the LAD ligation.

Determination of biochemical parameters

Quantitative determinations of serum cardiac troponin I (cTpI) and myoglobin (MYO) were performed by immune enzymatic assays, (AxSYM System; Abbott).

Statistical analysis

All data were expressed as mean \pm SEM, of $n = 6 - 8$, and analyzed with the non parametric Kruskal-Wallis test followed by Dunn's post test by using a statistical computer package, GraphPad Prism, v.4.01; a value of $P < 0.05$ was considered statistically significant.

RESULTS

Cardiac damage

Rat treatment with DMSO 500 µg/kg ip. for three consecutive days significantly reduced cardiac damage induced by 90 minutes ischemia. DMSO given acutely, 15 minutes before LAD ligation, also caused a reduction of the damage, but the effect was not statistically significant (Table I and Figure 1).

Biochemical parameters

Serum levels of cTnI and MYO from ischemic rats were significantly reduced by rat treatment with DMSO for three consecutive days. In contrast, DMSO given only fifteen minutes before ligation did not have any effect on biochemical parameters (Table I).

Table I: Effect of rat treatment with two different regimen dosing of DMSO on cardiac damage, serum myoglobin (MYO) and serum cardiac troponin (cTPI).

Treatment	Damage %	MYO ng/ml	cTPI ng/ml
Sham	–	32.25 ± 11.02 (n = 4)	1.25 ± 0.18 (n=4)
Ischemic	18.75 ± 4.88 (n=12)	46.86 ± 10.35 (n=7)	29.35 ± 12.32 †† (n=8)
Ischemic /DMSO 15 min	8.016 ± 3.23 (n=10)	30.44 ± 4.46 (n=9)	15.70 ± 4.65 (n=9)
Ischemic /DMSO 3 days	4.46 ± 2.01 * (n=8)	13.75 ± 0.85 * (n=4)	2.95 ± 1.32 * (n=4)

Data are the mean ± SEM, * $P < 0.05$ vs. ischemic and †† $P < 0.01$ vs. sham, Kruskal-Wallis test followed by Dunn's post test

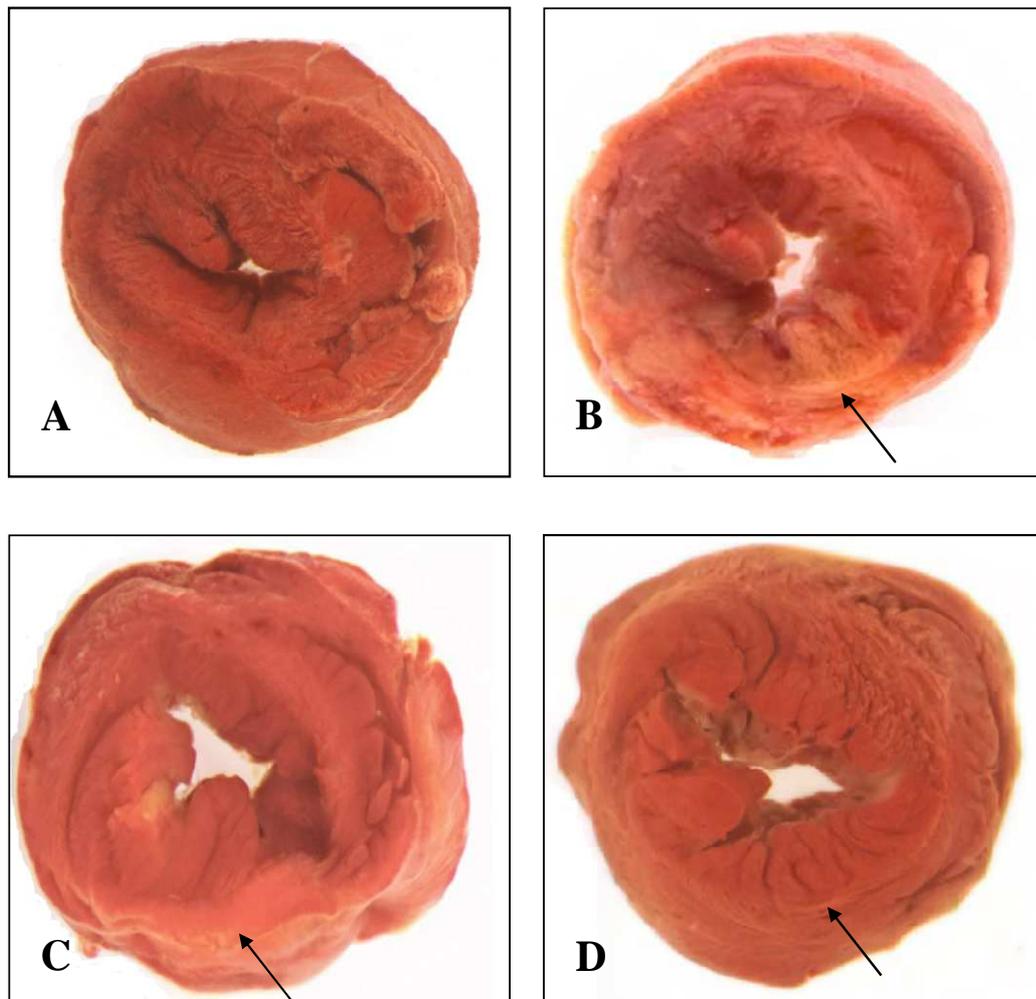


Figure 1: Cross section of the 2,3,5-triphenyltetrazolium chloride stained heart slices. The pale area, indicated by arrows, represents infarction area of the anterior wall of the left ventricle; (A) sham, (B) ischemic, (C) ischemic plus DMSO 500 µl/Kg 15 minutes before, and (D) ischemic plus DMSO 500 µl/Kg for 3 days (x10 photo ocular; x 6.4, magnification charger).

CONCLUSION

Here, we investigated on the effect of DMSO in a model of rat acute myocardial infarction. Our main result is that DMSO reduced myocardial damage following LAD ligation, accordingly to previous results on its protective effect on cerebral ischemia (Shimizu *et al.*, 1997), on hepatic ischemia reperfusion injury (Sahin, *et al.*, 2004) and on in vitro cardiac ischemia (Shalfer, *et al.*, 1983). The mechanism at the basis of this protective effect is not known; it is known that DMSO readily penetrates cell membranes; inside the cells its effect on ischemic damage might be attributed to an antioxidant effect given its properties as free radical scavenger but also to an effect of ion exchange through cell membranes and thus on cell excitation (Santos *et al.*, 2003; Colucci, *et al.*, 2008). An early study performed on perfused rat hearts demonstrates that DMSO protects from oxygen induced cell damage following reperfusion and also reduces contractile force of heart caused by hypoxic contracture (Ganote, *et al.*, 1982), suggesting that this effect could be dependent upon an action of DMSO directly on contractile proteins or, indirectly, on calcium metabolism. However, other works demonstrate that DMSO does not alter ion exchange through cell membranes but has negative inotropic effect probably due to an inhibition of myofilament to calcium responsiveness (Ogura *et al.*, 1996). In our study, the beneficial effect on cardiac function is confirmed by the reduction of serum levels

of myoglobin and cTPI whose increasing levels following ischemia represent contractile protein damage.

In conclusion, here we show for the first time a protective effect for DMSO on an *in vivo* model of rat acute myocardial ischemia, although further studies are required to define the mechanism at the basis of this effect, we think that our work contributes to delineate the pharmacological profile of this neglected compound.

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