**PhD Program in Translational and Molecular Medicine**

# DIMET


**University of Milano-Bicocca**
**School of Medicine and School of Science**

# Study of the role of protective genes against inflammatory stimuli in experimental in vitro and in vivo models

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## *CHAPTER 1*

# Chapter 1

# Introduction

# Inflammation and Transplantation

Transplantation is the preferred treatment for most end-stage solid organ diseases. The first successful transplant is dated 1954, when a surgical team under the direction of Joseph Murray, removed a kidney from a healthy donor and transplanted it into his identical twin [1]. The organ functioned immediately, and the recipient survived for nine years (fig. 1). These clinical breakthroughs were ultimately recognized by awarding of Nobel Prizes to Joseph Murray (and others), for the first clinical transplantation and the first use of immunosuppression.

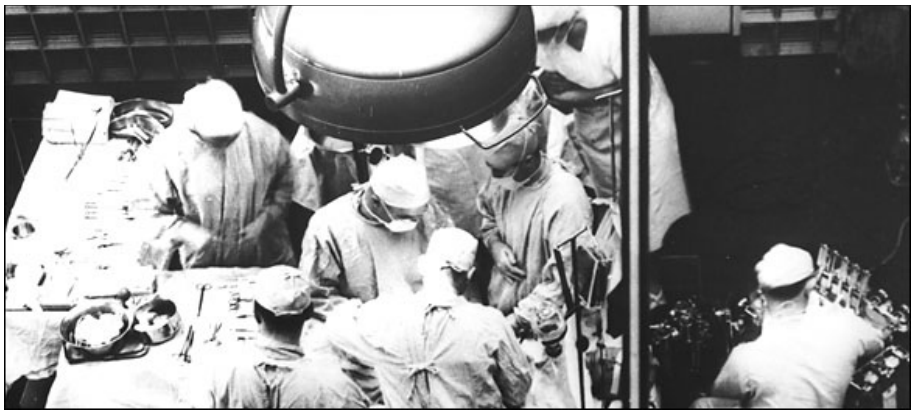

**Figure 1** - The first successful organ transplant took place on Dec. 23, 1954, when Richard Herrick received a kidney from his healthy identical twin brother, Ronald. Richard survived for nine years until the original kidney disease struck again (*Photograph property of Brigham and Women's Hospital*).

Since then the science of organ transplantation has progressed considerably largely because of an improved understanding of the role of the immune system in allograft rejection, the

disentanglement of the molecular mechanisms underlying graft failure, and better management of immunosuppression [2]. But although tremendous progress has contributed to the success of this therapy, nowadays more than 60 years later, immunologic reactions between donor and host still remain one of the major causes of morbidity and mortality after solid organ transplantation.

The innate immune system is one of the most ancient components of immunity. It acts as the first line of defense against pathogen invasion or noxious stimuli [3]. The sensors that are activated by such stimuli are located on the cell surface of sentinel cells such as dendritic cells (DCs) or macrophages [4]. Regardless of how they are activated, signalling via innate immune receptors induces an inflammatory program consisting of the production of pro-inflammatory cytokines, such as IL-6 and TNF-α, and the up-regulation of costimulatory molecules and chemokines [4]. Transplantation research indeed is important not only because of its direct clinical relevance, but also because it can serve as a general model system for a diverse set of pathophysiologic processes that are associated with acute and chronic inflammatory disease. The exact pathophysiologic mechanisms of graft rejection after solid organ transplantation have been extensively studied in the process of development of effective immunosuppressive drugs. Graft rejection can be classified in three groups: hyperacute graft rejection, acute graft rejection, and chronic graft rejection [5].

Hyperacute graft rejection (HAR), also called humoral rejection

or acute antibody-mediated rejection (AMR), is a very rapid antibody-mediated graft destruction which occurs within the first 24 hours, most often minutes to hours after transplantation [6]. It results from preformed donor-specific antibodies and leads to edema of the transplanted organ, platelet aggregation, formation of fibrin thrombi, neutrophil infiltration, and eventually endothelial damage, interstitial edema, haemorrhage, and infarction [7]. In the last years, HAR has been a rare complication due to screening procedures for host antibodies against donor HLA prior to transplantation [8].

Acute graft rejection occurs within the first 4–6 months after solid organ transplantation. It is initiated by T cells activated mostly via their T cell receptor which recognizes nonself MHC molecules on the donor APCs. Activation of $CD4^+$ T helper cells leads to production of pro-inflammatory cytokines which enhance the proliferation and differentiation of $CD8^+$ cytotoxic T cells. After cytotoxic T cells are activated, they can secrete cytotoxic pro-inflammatory cytokines such as IFN-*γ* and TNF-α which can lead to apoptosis [9]. Natural killer (NK) cells also play a supportive role for T- cell activation by secreting IFN-*γ* and TNF-α and amplifying early graft inflammation [10]. Altogether, these mechanisms lead to tissue damage in the transplanted organ and eventually graft dysfunction.

Chronic graft rejection occurs months to years after transplantation and is a main cause for long-term allograft dysfunction. Organs undergoing chronic rejection display many of the features of healing wounds, including fibroblast,

endothelial and epithelial cell proliferation, collagen deposition within the graft parenchyma and blood vessels, increased infiltration of macrophages and elevated concentrations of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1*β* in the extracellular space [11] (fig. 2). Endothelial cells also up regulate the secretion of IFN-*γ* and TNF-α and subsequently show enhanced expression of adhesion molecules like ICAM-1 and VCAM-1 [12]. All of these inflammatory processes result in interstitial fibrosis, ischemia, early atherosclerosis and the loss of graft function [13, 14].

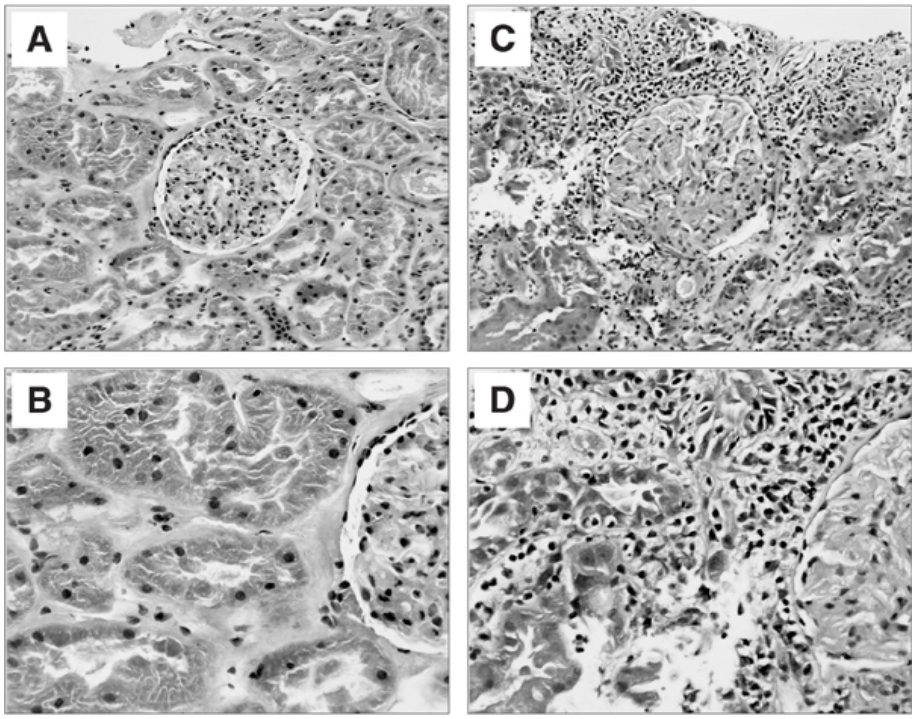


**Figure 2** - Inflammatory infiltrate in acute renal allograft rejection. *A* and *B*, A hematoxylin and eosin-stained renal allograft biopsy specimen obtained from a patient without rejection during a protocol biopsy. *C* and *D*, Intense interstitial inflammatory infiltrate characteristic of acute renal allograft rejection. These inflammatory cells are the source of the inflammatory mediators (cytokines, chemokines, prostanoids) that orchestrate the alloimmune response. Top figures (A and C), 50X magnification; bottom figures (B and D) 100X magnification (*Rocha, P. N., & Carvalho, E. M., 2005*).

We have discussed the intricate relationship between inflammation and the alloimmune response: inflammation is not only central to the initiation of the alloimmune response but also occurs as a result of the effector elements generated by this response. During rejection event inflammatory mediators produced by activated mononuclear cells, such as TNF-*α,* infiltrating the graft can modulate the intensity of the alloimmune response and directly regulate graft function.

Many studies suggest that inducing and maintaining tolerance will require a careful assessment of the inflammatory status of a recipient's immune system and how it responds to the environment [15]. Perhaps, in the future, we will have better drugs to down-regulate innate immunity at the time of transplantation to efficiently shut off the innate immune system. In addition, targeted anti-inflammatory agents that can inhibit only the pro-inflammatory cytokine, might be of great value for the treatment of acute rejection [16].

All the advances in the understanding of the overall transplant process, including ischemia-reperfusion, organ preservation techniques and immunological mechanisms underlying rejection and graft function, together with a more individualized immunosuppressive therapy have been combined to progressively increase the success of the human allo-transplantation. Unfortunately, the supply of human organs is insufficient to treat all the patients who present each year with organ failure, and who could benefit if a compatible graft were available. These considerations has led to the possibility of

addressing the shortage of organs needed for today's aging population, by developing animal organs for human xenotransplantation [17,18].

## Xenotransplantation

Pigs are actually considered the preferred potential source animal for many reasons, among which breeding characteristics, the relatively short gestation time and large litter size, the availability of techniques for oocyte manipulation and artificial insemination and the ability of the genome of these animals to be modified by modern technologies of genetic engineering [19].

Xenotransplantation is not yet world wide a clinical reality because some obstacles still exist. It has now been determined that most of the difficulties inherent within xenotransplantation stem from the independent evolutionary background of pigs and primates. When a pig organ is transplanted into a nonhuman primate (or into a human) can be rejected through several reactions: hyperacute rejection, acute humoral vascular rejection, trombotic microangiopathy, and chronic rejection [20-21]. Hyperacute rejection is mainly mediated by natural preformed anti-pig antibodies, which bind the vascular endothelium of the graft with consequently activation of the complement cascade. The most important target for human anti-pig antibodies is the galactose-α1,3-galactose (Gal) antigen [22], an oligosaccharide very similar in structure to the

B blood group antigen. The immunopathology of hyperacute rejection is very similar to that which occurs when organ allotransplantation is carried out across the ABO blood group barrier [23]. Hyperacute rejection have been significantly reduced via the production of α1,3-galactosyltransferase (GalT) gene knockout (KO) pigs [24].

When hyperacute rejection does not occur, as in closely-related primate species (e.g., chimpanzee to man), or if it is prevented through the use of α -gal KO pigs, xenografts still eventually fail as a consequence of acute vascular rejection (AVR). Several elements have been found to be involved in the pathogenesis of AVR and its pathology is primarily characterised by vascular thrombosis, blood extravasation and oedema. Deposits of fibrin, immunoglobulins and complement in the graft do not differ substantially from those observed in allograft rejection. Cellular infiltrates include neutrophils, macrophages, CD8+ T cells and NK cells secreting TNF-α and amplifying graft inflammation [25] (fig. 3).

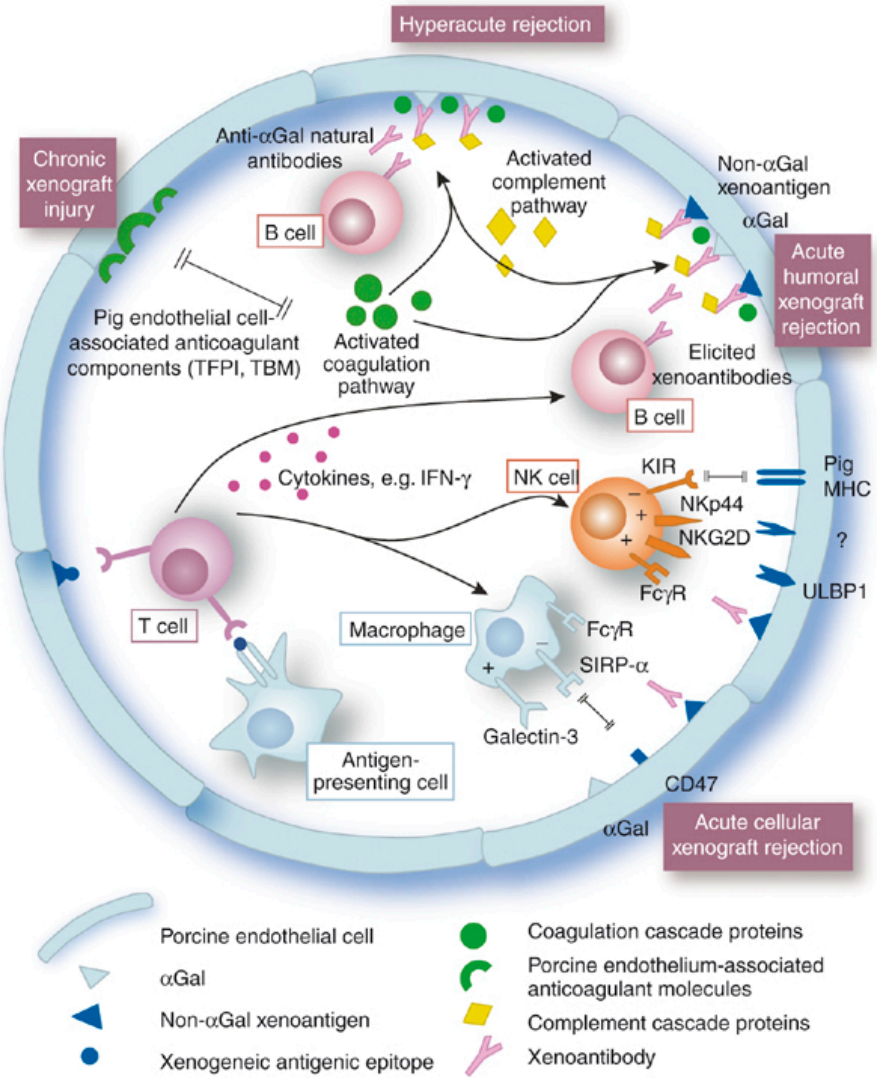


**Figure 3** - Principal mechanisms involved in rejection of porcine xenografts in nonhuman primate. HAR is initiated when preexisting 'natural' xenoantibodies, primarily directed toward αGal, bind to donor endothelium. Endothelial cells are activated, complement and coagulation systems are involved, and endothelial damage and thrombosis occur. In addition to the process described for HAR, binding of xenoreactive antibodies to endothelial cells results (through binding to FcγR) in antibody-dependent cell-mediated cytotoxicity by NK cells and macrophages. T cells are able to recognize and attack porcine aortic endothelial cells directly, and to respond to xenogeneic antigens through the indirect pathway (presented by recipient antigen-presenting cells). Macrophage phagocytosis is triggered when lack of autologous CD47 expression on xenogeneic endothelium fails to sustain inhibitory signaling through SIRP-α. Finally, incompatibilities between the recipient coagulation proteins and porcine endothelial cell-associated anticoagulant components may result in a chronic procoagulant state of the xenograft endothelium, and is thought to play a role in chronic xenograft injury (*B. Sprangers, M. Waer and A.D. Billiau, 2009*).

Recent studies hypothesize that a state of systemic inflammation develops after pig organ xenotransplantation, which is generated by both adaptive and innate immune responses and suggested that inflammation can lead to activation of the coagulation system. Additionally, pro-coagulant proteins, e.g. thrombin, are considered as pro-inflammatory factors. In fact, a considerable crosstalk is deemed to exist between inflammation and coagulation, leading to escalation of each other [26]. Even if T cell-directed immunosuppression can control activation of coagulation induced by adaptive immune responses, pro-inflammatory signals induced by the innate immune system can still promote activation of coagulation [27]. Together, these observations indicate that the immune response to a pig xenograft cannot be considered in isolation and that equal attention needs to be directed to the innate immune, coagulation, and inflammatory responses.

Just as there may be a need for exogenous immunosuppression and/or antithrombotic therapy, there may also be a need for the administration of anti-inflammatory agents. In this respect, in addition to corticosteroids, there is evidence that high-dose statin therapy not only reduces the inflammatory response and platelet activation [28] but also down-regulates the primate cellular response to pig antigens [29]. Anyhow control or reduction of the inflammatory response is also most likely to be controlled by genetic manipulation of the pig. Expression of thrombomodulin, endothelial protein C receptor, and/or CD39 is anticipated to reduce the inflammatory

response in addition to coagulation dysfunction [30]. Furthermore, pigs are now available that express anti-inflammatory hemeoxygenase-1 [31]. Other anti-inflammatory genes that might prove valuable include A20 [32]. Human A20 has a double cytoprotective function by protecting endothelial cells (EC) from TNF-mediated apoptosis and inflammation, via blockade of several caspases, and the transcription factor nuclear factor-κB (NF-κB), respectively [33]. Before xenotransplantation can be introduced successfully into the clinic, the problems of the innate, coagulopathic, and inflammatory responses will have to be overcome, most likely by the transplantation of organs from specifically genetically engineered pigs.

**Genetic engineering and polycistronic vectors**

The application of gene transfer and transgenic technology to xenotransplantation has already led to important breakthroughs. However in order to overcome the inflammatory barrier discussed, additional multiple genetic modifications in donor pigs are necessary and many permutations and combinations of these will need to be tested.

There is no simple process of adding and subtracting genes from the pig genome, and the gestation time and age to sexual maturity cannot be altered. Therefore, even with the success of the GTKO platform, the main limiting factor for assembling multiple genetic modifications from individual pig lines is the

time; and resources are required for breeding, crossing, and screening [34]. Moreover, even when the final multigene pig is generated, the genes are hard to hold together in the offspring as they are random insertions invariably in different chromosomes and so randomly assort on breeding. Fortunately, nuclear transfer technology has superseded the process of traditional microinjection and breeding and screening for transgene integration are bypassed by modification in vitro by transfection in a somatic cell line. Expression is therefore achieved in a single gestation period. However, the problem of bringing together multiple genetic modifications remain.

Co-expression using multigene constructs will resolve this issue. F2A technology allows the expression of multi transgenes at roughly equivalent levels starting from a single open reading frame and is actually considered particularly useful in the production of multi transgenic large animal models in which combining multiple independent genetic modifications by breeding is time consuming and expensive [35]. It fulfills all the functions IRES sequences are currently used for: multicistronic expression in transgenic animals and cell culture, multicistronic expression using viral vectors in entire animals, expression of exogenous coding sequences inserted by targeted recombination into endogenous loci, and so on. In addition, it also provides the advantage of reliable and reportedly almost stoichiometric levels of expression, a particularly useful feature when accurate proportion of the expression levels of two or more proteins is important. 2A

sequence has been used in the construction of efficient multicistronic vectors containing three to four graft-protective genes, that are human CD55, thrombomodulin, CD39 and CTLA4-Ig [36].

For these reasons this technology was chosen for the co-expression of three genes whose over-expression is capable to down-regulate the inflammatory response and limit apoptosis.

# Protective genes against inflammatory stimuli

Over the years several genes, whose over-expression is capable to down-regulate the inflammatory response and limit apoptosis, were identified. A lot of evidences showed among others, three genes candidate to be expressed because of their involvement in the regulation of inflammatory and vascular response: the human heme oxygenase-1 (HO-1), CD73 (Ecto-5'-nucleotidase, E5'N) and CD39 (Ecto nucleoside triphosphate diphosphorylase 1, ENTPDase).

## Heme oxygenase-1

Heme oxygenase-1 (HO-1), which is encoded by the Hmox1 gene, is a stress-responsive enzyme (32kDa) that degrades free heme (iron protoporphyrin IX) to yield equimolar amounts of three products: the gas carbon monoxide (CO), iron (Fe2+) and biliverdin, which is converted to bilirubin by NAD(P)H biliverdin reductase (BVR).

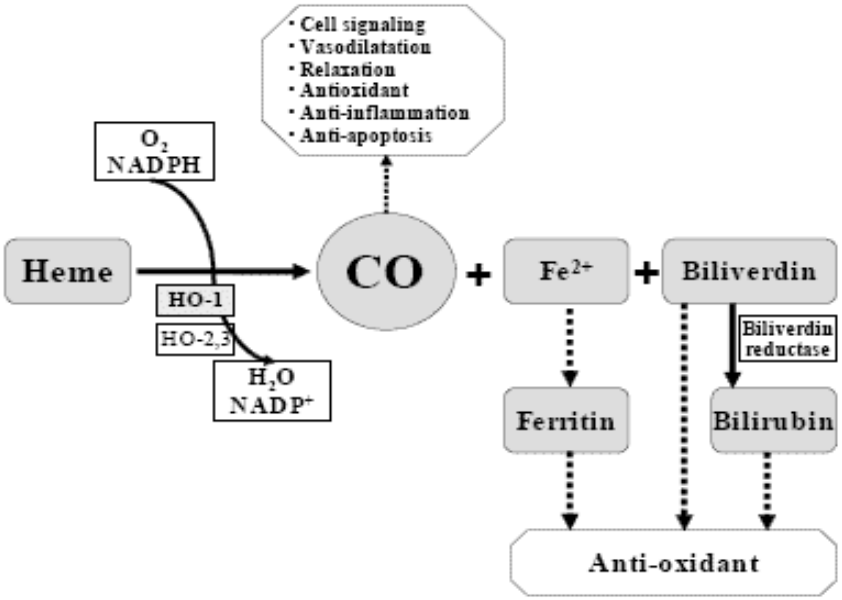


**Figure 4** - The heme/HO-1 system. Free heme is a reactive Fe compound that can catalyze, through the Fenton reaction, the formation of cytotoxic hydroxyl radical (OH*) from hydrogen peroxide ($H_2O_2$). Elimination of the reactive heme-Fe by HO-1 involves cleavage of the protoporphyrin IX ring of heme with production of biliverdin (BV) and CO as well as release of Fe. The Fe released from the protoporphyrin IX ring of heme is then stored by the ferritin H chain (FtH). BV is converted by biliverdin reductase into the antioxidant bilirubin. All three end products of heme catabolism, that is, biliverdin/bilirubin, CO, and Fe/FtH, are cytoprotective. (*Gozzellino R. et al., 2010*. Image from *Babusikova E. et al., 2008*)

Conventionally HO-1 is known to be localized to microsomes and ER, anchored by a single transmembrane spanning region at the carboxy-terminal end. Recent studies have raised the possibility of the functional compartmentalization of HO1 in other subcellular domains beside the ER, including but not limited to the nucleus, plasma membrane and also caveolae [37,38].

Expression of HO1 is regulated essentially at the transcriptional level and is induced by a broad range of chemical and physical stress stimuli, such as ROS generated substances (heme,

hemin, $H_2O_2$), thiol reactive substances, heavy metals, lipid metabolites, nitric oxide and derivatives, LPS, endotoxins, $O_2$ tension, UV light, shear stress, cytokines (IL1, IL6, TNF-α) and growth factors, etc [39]. One common feature of these inducers is their capacity to generate reactive oxygen species.

Presumably the main biologic function of HO-1 is to avoid the accumulation of highly deleterious free heme that can catalyze the production of free radicals through Fenton reaction.

It has been well known that HO-1 plays as a protective gene by preventing cells death because of its anti-apoptotic properties, via inhibition of caspase-3 [40] and attenuating TNF-α induced damage, by suppression of TNF/TNFR1-mediated apoptotic signaling likely via attenuation of DISC adaptor protein expression and their association with TNFR1 [41]. Through the years a wide variety of additional protective effects has been attributed to HO-1 and in particular to its reaction products.

The protective effects of CO were initially demonstrated in a model of acute lung injury [42] and endotoxic shock [43] and subsequently in a mouse cardiac xenotransplantation model [44]. HO-1 expression in the transplanted heart was essential to prevent rejection in this model [45]. If donor and recipient were both treated with CO, a heart that could not express HO1 activity still survived indefinitely [44]. So CO appeared able to substitute for HO1 and suppress the pro-inflammatory response that would otherwise lead to graft rejection.

It was shown that CO suppresses the pro-inflammatory response and promotes the anti-inflammatory response of

macrophages [43]. Three other actions of CO contribute to its anti-inflammatory effects. First, CO prevents platelet activation and aggregation, thereby suppressing thrombosis and the pro-inflammatory response stimulated by activated platelets [46]. Second, CO downmodulates the expression in macrophages of plasminogen activator inhibitor type 1 (PAI-1); this action appears to be crucial for the ability of CO to exert a protective effect in a model of ischemia-reperfusion of the lung [47]. Third, CO prevents apoptosis in several cell types, including endothelial cells [48], fibroblasts, hepatocytes and β- cells of the pancreas [49].

Moreover CO suppresses the proliferative response of smooth muscle cells that contribute to neointimal proliferation associated with inflammatory lesions in vivo [50]. The cytoprotective effect of CO is mediated by the activation of several signal transduction pathways, among which the p38 MAPK signal transduction pathway [51]. CO triggers the proteolytic degradation of the proapoptotic p38α MAPK isoform favoring signaling via the antiapoptotic p38β MAPK isoform [52]. Activation of p38β MAPK by CO is involved in the mechanism by which HO-1 interacts functionally with c-IAP-2 and A1 to suppress TNF-mediated apoptosis, and also induces the expression of Bcl-xl via the phosphatidylinositol-3-kinase (pi3k/Akt) signal transduction pathway [53].

Biliverdin and bilirubin probably exert their protective (including anti-apoptotic) effects largely because of their anti-oxidant properties [54]. Exogenous administration of biliverdin or

bilirubin, mainly tested in rodents, provides beneficial effects in terms of disease occurrence and/or severity, including in ischemia reperfusion injury [55], graft rejection [56], endotoxic shock, neointima formation after balloon injury and the development of autoimmune neuroinflammation [57]. Moreover bilirubin derived from heme degradation suppresses MHC class-II expression in endothelial cells [58].

Labile Fe produced by HO-1 upregulates the expression of H-ferritin, which associates with light-chain (L-) ferritin subunits to form a multimeric protein complex that has a high capacity for storing Fe (4500 mol of Fe per mol of ferritin)). Expression of ferritin is cytoprotective under a variety of conditions, an effect that is in large measure attributable to the ferroxidase activity of the H-chain subunit [59] which catalyzes the oxidation of iron from the ferrous form form ($Fe^{2+}$) to the ferric form ($Fe^{3}$) (fig. 4).

## Ecto-5'-nucleotidase (CD73)

CD73 is a membrane bound 70kDa enzyme that functions downstream of CD39 and catalyzes the hydrolysis of extracellular AMP to adenosine. It belongs to the 5'-nucleotidase family, which also contains other six isozymes, five of which are located in the cytosol and the last one in the mitochondrial matrix [60]. CD73 consists of two glycoprotein subunits that are tethered by non-covalent bonds, binds zinc and other divalent ions at the N-terminal domain and is anchored to the plasma membrane at the C-terminus by a

glycosyl- phosphatidylinositol (GPI) [61,62].

CD73 is expressed to a variable extent in different tissues, with abundant expression in the colon, kidney, brain, liver, heart and lung [61]. In the vasculature, CD73 is predominantly associated with the vascular endothelium of large vessels such as the aorta, carotid and coronary artery [63]. In the case of circulating T- and B-lymphocytes, the enzyme expression is restricted by certain cell types and strongly correlates with cell maturity, while neutrophils, erythrocytes, platelets and other blood cells express little or no CD73 [64]. There are evidences that the expression and function of this enzyme are upregulated under hypoxic conditions [65,66], as well as by the presence of several proinflammatory mediators, such as transforming growth factor (TGF)-b, interferons (IFNs), tumor necrosis factor (TNF)-a, interleukin (IL)-1b, and prostaglandin $E_2$ [67,68]. An increase in CD73 expression has also been reported in several neoplastic tissues [67], suggesting the involvement of this enzyme in the onset and progression of neoplasia. CD73 is essential for the extracellular formation of adenosine, even if nowadays it is known that adenosine is also produced intracellularly and once reached high concentrations, is shunted into the extracellular space through specialized nucleoside transporters [69]. However this pathway is minor than the extracellular catabolism of precursor adenine nucleotides (ATP, ADP, AMP) to adenosine by the action of CD39-CD73 system [70].

## Ectonucleoside triphosphate diphosphohydrolase-1 (CD39)

CD39 is a plasma membrane ectonucleotidase with molecular mass ~58kDa that belongs to the Nucleoside triphosphate diphosphohyrolase (NTPDase) family. It is now known that this family contains other seven NTPDases, three of which being expressed as cell surface- located enzymes: NTPDase1, which is present on Kupffer and vascular endothelial cells, NTPDase2, which is expressed by portal fibroblasts and activated hepatic stellate cells and NTPDase8 that seems to be the major ATPase of the hepatic canaliculus [71]. The vascular isoform of CD39 is abundantly expressed on vascular endothelial and smooth muscle cells [72] dendritic cells [73], neutrophils, monocytes and certain T- and B- cell subsets, but not on platelets and red blood cells [74].

The expression of this gene appears to be induced by several pro-inflammatory stimuli, oxidative stress and hypoxia [75]; in particular, some cloning studies on CD39 promoter region revealed a prominent role for the transcription factor Sp-1 in regulating CD39 transcription in response to hypoxic stimuli. Sp-1 is a member of the family of transcription factors Sp/XFLF, expressed ubiquitously, and it is involved in the transcription of hypoxic genes, such as VEGF and the same CD39 [76].

CD39 is $Ca^{2+}$ and $Mg^{2+}$ dependent and contains two predicted transmembrane domains at the N- and C-terminus with a large extracellular loop containing a more central hydrophobic region

and six potential glycosylation sites, and a palmytoil group that targets the enzyme to caveolae [77].

This enzyme is involved in the modulation of vascular cell and platelets purinergic receptors activities, by the breakdown of extracellular adenine nucleotides, that is ATP being hydrolyzed to ADP and then to AMP. Subsequently, AMP is hydrolyzed to adenosine by CD73 (Ecto-5'-nucleotidase, E5'N) (fig. 5).

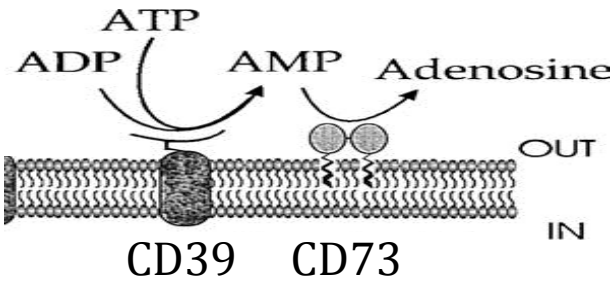


**Figure 5** - The pathway of ATP hydrolysis by the ectonucleosidases, CD39 and CD73 to adenosine.

### Adenosine and protective mechanisms of CD39-CD73 axis

Adenosine is a nucleoside reported to be cytoprotective, anti-thrombotic and immunosuppressive [70]. Adenosine exerts its protective effects binding to the G protein-coupled adenosine receptors (ARs), $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$. By this way, adenosine exerts cardioprotection by vasodilatation and protection against myocardial ischemia, antagonism of the chronotropic and ionotropic effects of catecholamines, promotion of endothelial barrier function, inhibition of platelet aggregation, protection against vascular inflammation and neointima formation,

inhibition of TNF-α, reduction of the complement component C2 level and inhibition of neutrophil adhesion and free radical generation, as well reviewed by Antonioli et al. [75]. NK cells have long been shown to respond to adenosine that inhibits granule exocytosis [78] and attenuates tumour recognition and adhesion [79].

By terminating the prothrombotic and proinflammatory effects of ATP and ADP, CD39 exerts its hemostatic and vascular protective functions against platelets aggregation and vessels occlusion [80]. Thus, ADP is an important mediator of vascular thrombosis in inflammatory states and, consequently, ATP diphosphohydrolase (ATPDase) has a fundamental role in the inhibition of ATP-ADP signal transduction induced in platelets, leukocytes and vascular endothelium mediated by purinergic receptors. The ATPDase-mediated hydrolysis of ATP and ADP reduces these purinergic mediators from the extracellular space and generates adenosine, resulting in protective mechanism against inflammation process [81]. Data from mutant mice deficient in NTPDase1/CD39 or overexpressing human CD39 further confirmed an important role for this ectoenzyme in the control of hemostasis, platelet reactivity, thrombotic reactions and vascular growth in vivo and further support its therapeutic potential in clinical vascular diseases and during transplantation [82,83]. Recently, the protection against myocardial ischemic injury in hCD39 transgenic mice hearts has been reported [84].

The importance of CD73 in producing adenosine for AR signalling has been revealed through studies with CD73-

deficient mice. For example, CD73-generated adenosine reduces inflammation and fibrosis in lungs of bleomycin-treated mice [85] and is tolerogenic for cardiac and airway allografts [86]. CD73-dependent $A_{2B}$ signaling protects mice during renal ischemia inhibits systemic vascular leakage during hypoxia [87], and is also required for cardioprotection as a result of ischemic preconditioning [88].

The purinergic pathway leads to adenosine formation that mediates anti-inflammatory and anti-thrombotic effects mainly by binding to $A_{2B}$ receptor. This molecular binding results in signaling pathways transduction involving Akt and Erk1/2 protein molecules that mediate cell survival.

Therefore, taking in account that CD39 and CD73 degrade ATP, ADP, and AMP to adenosine, they can be viewed as ‘immunological switches’ that shift the ATP pro-inflammatory stimulus toward an anti-inflammatory state mediated by adenosine; thus, the protective effects of the CD39-CD73 axis are mainly related to the final extracellular product formed as a result of their combined action.

## TNF-α-induced inflammation model

Inflammation is a very important part of innate immunity and is regulated in many steps. One such regulating step is the cytokine network, where tumor necrosis factor-α (TNF-α) plays one of the most important roles. Being one of the most important pro-inflammatory cytokines, TNF-α plays an

fundamental role in the immune system during inflammation, cell proliferation, differentiation and apoptosis. It participates in vasodilatation and edema formation, and leukocyte adhesion to epithelium through expression of adhesion molecules; it regulates blood coagulation, contributes to oxidative stress in sites of inflammation, and indirectly induces fever [89]. During last decades, different researchers have investigated about the relationship between TNF-α signaling pathway and inflammation processes, and several evidences were proved. It has been shown that TNF-α regulates immunogenicity of transplanted tissue by amplification of major histocompatibility complex gene expression [90], and provokes rejection by a direct cytotoxic effect or via T cell or macrophage action [91]. Indeed, the expression of TNF-α was observed in heart allograft [92]. Furthermore, evaluation of plasma level of TNF-α could be used as allograft rejection marker [93]. Thus, it has been established that TNF-α plays a central role in the inflammatory response, and it is currently considered that TNF is involved in the development of tumors because of its ability to activate the transcriptional factor NF-κB, which plays a key role in tumor proliferation [94] and also in the induction of angiogenic factors like IL-8 and VEGF [95]. Moreover a recent study demonstrated that human tumor necrosis factor-a levels in human blood increased after contact with pig endothelial cells (PECs) in an antibody/complement-dependent manner and that hTNF-α induced the up-regulation of porcine tissue factor in PECs in vitro. These results suggest that hTNF-α may mediate the

dysregulated coagulation in xenotransplantation.

**TNF-α and HO-1**

A potential mechanism to contrast TNF-α-induced inflammation involved the activity of Heme Oxigenase-1 (HO-1) and of its catabolic products, in particular carbon monoxide (CO). To date, many studies have demonstrated that HO-1 plays important roles in the stress response to various pathological stimuli to serve a cytoprotective function. For example, HO- 1 has been reported to be involved in the atherosclerosis process, and a recent study provides several possible mechanisms underlying the anti-atherosclerosis effects of HO-1 [96]. In particular, the authors assessed the effects of induction/inhibition of HO-1 on two important inflammatory and pro-atherogenic factors, NF-κB and TNF-α, in a rabbit model of atherosclerosis, and show how high fat diet (HFD) induced activation of aortic NF-κB and TNF-α, whereas hemin, which is a potent inductor of HO-1 expression, attenuated the HFD-induced increase of these factors, and consequently, atherosclerosis plaque formation. They also shown that hemin increased CO levels and eNOS activity, and postulated that the influence of hemin on these stress protective molecules may contribute to its beneficial effects [96]. Moreover, Pae and colleagues found that the HO-1 inducer cobalt protoporphyrin can inhibit TNF-α–induced NF-κB activation in human umbilical vein endothelial cells and that the effect could be reversed by

the HO-1 inhibitor tin protoporphyrin [97].

These observations highlight the importance of HO-1 as protective regulator in the genesis and development of inflammation-related diseases, such as atherosclerosis, and may motivate future efforts to explore the possibility of developing new chemical inducers of HO- 1 as a promising drug in the future. Therefore, increased expression of HO-1, with subsequent increased generation of CO is part of a physiological response to injury by which many cells are protected from undergoing apoptosis. It has been demonstrated that exogenous administration of CO dramatically inhibited TNF-α- mediated liver injury in vivo and hepatocyte cell death in vitro, and, the hepatoprotective effect of CO seems to be dependent on NF-kB activation [98]. Moreover, induction of HO-1 or delivery of CO was shown to protect mice from liver injury or lung injury by modulation of caspase or Bcl-2 family via MAPK pathway [99].

**TNF-α and CD39-CD73**

Extracellular ATP release may occur after tissue damage and necrosis, and it represents a danger-associated molecular pattern molecule that can initiate inflammatory responses and activate both innate and adaptive immunity [100]. Understanding mechanisms that modulate ATP effects on target cells may have therapeutic implications. After receptor binding, extracellular ATP may initiate and modulate

inflammation in several ways: activating cells (e.g., endothelium, leukocytes), inducing cytokine and chemokine release, enhancing expression of adhesion molecules, and facilitating chemotaxis. Termination of these responses is mediated by ectonucleotidases that are present in the circulation and on cell surfaces, such as CD39 and CD73.

Moreover studies reveals that extracellular ATP potently stimulated TNF-α release, resulting from TNF-α gene expression in primary cultures of rat brain microglia [101], and that ATP and TNF-α synergized in the activation of dendritic cells [102]. Extracellular ATP has thus been implicated in vascular inflammation, atherosclerosis, and angiogenesis. As support of these observations, the development of shock and organ failure from severe infections or haemorrhage is associated with a decrease in tissue ATP levels [103]. It has been demonstrated that the molecular mechanism by which ATP promotes pro-inflammatory processes involved induction of several genes expression, among which adhesion molecules that play an important role in modulating inflammation and cell trafficking at the endothelial interface such as ICAM-1, VCAM-1 and SELE, but also IL-6, TNF-α and TNFAIP3, and that glucocorticoids enhanced ATP-induced inflammatory response [104].

ATP clearance from the extracellular environment and consequent adenosine generation mediated by the combined action of CD39 and CD73 as described above, could be an important mechanism to contrast the pro-inflammatory effect of

this nucleotide. Together, these observations suggesting that the modulation of these ectonucleotidases activity may have therapeutic implications in several inflammation-related diseases underlying their protective effects against inflammation processes and thrombosis.

# Inflammation and Vascular Injury

Disease of arteries are responsible for more morbidity and mortality than any other type of human disease. Vascular abnormalities cause clinical disease by two principal mechanisms: i) narrowing or completely obstructing the lumen, either progressively (e.g., by atherosclerosis) or precipitously (e.g., by thrombosis or embolism); ii) weakening of the walls, leading to dilation or rupture. To understand the diseases that affect blood vessels, it is essential to consider some of the anatomic and functional characteristics of these highly specialized and dynamic tissues.

The general architecture and cellular composition of blood vessels are the same throughout the cardiovascular system, anyway, certain features of the vasculature vary with and reflect distinct functional requirements at different locations. Arterial walls are generally thicker than the walls of veins to withstand the pulsatile flow and higher blood pressure in arteries. Arterial wall thickness gradually diminishes as the vessels become smaller, but the ratio of wall thickness to lumen diameter becomes greater. The basic constituents of blood vessel walls are endothelial and vascular smooth muscle cells (VSMCs), and extracellular matrix (ECM), including elastin, collagen and glycosoaminoglycans. The three concentric layers, intima, media and adventitia, are most clearly defined in the larger vessels, particularly arteries (fig. 6).

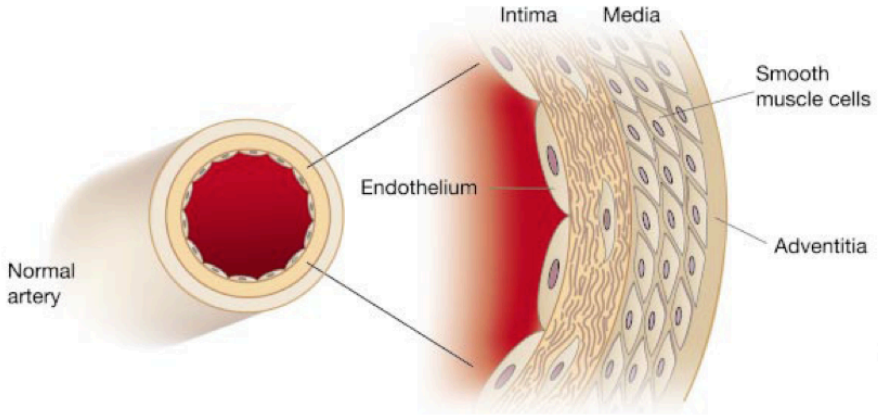


**Figure 6** - General structure of arteries. The normal artery has a typical trilaminar structure. The intima consists of a single layer of ECs in contact with the blood in the arterial lumen. ECs rest upon a basement membrane. The intimal layer can present also some VSMCs scattered within the intimal extracellular matrix. The internal elastic lamina constitutes the barrier between the tunica intima and the underlying tunica media. The media consists of multiple layers of VSMCs, much more tightly packed than in the diffusely thickened intima, and embedded in a matrix rich in elastin as well as collagen. (Image modified from *Libby P. et al., 2002*)

In normal arteries, the intima consists of a single layer of endothelial cells with minimal underlying subendothelial connective tissue. It is separated from the media by a dense elastic membrane called the internal elastic lamina. Basement membranes contain type IV collagen, laminin, and heparan sulphate proteoglycans, such as perlecan and syndecans. The intima may be focally thickened by a hyaluronan-rich matrix containing sparse mesenchymal cells, which are probably modified VSMCs. The underlying media contains mainly VSMCs, surrounded by their own basement membrane. VSMCs are densely packed into an interstitial matrix, which contains types I and III collagen, fibronectin, and chondroitin/dermatan sulphate proteoglycans, such as versican. The media of elastic arteries also contains a honeycomb of

lamellae rich in the hydrophobic protein elastin. The layers of medial VSMCs near the vessel lumen receive oxygen and nutrients by direct diffusion from the vessel lumen, facilitated by holes in the internal elastic membrane. However, diffusion from the lumen is inadequate for the outer portions of the media in large and medium-sized vessels, therefore these areas are nourished by small arterioles arising from outside the vessel (called vasa vasorum) coursing into the outer one half to two thirds of the media. The outer limit of the media of most arteries is a well-defined external elastic lamina.

External to the media is the adventitia, consisting of connective tissue with nerve fibers and the vasa vasorum. The outer adventitia contains fibroblasts in a loose connective tissue, which also contains small blood vessels and fat.

## Histopathology of atherosclerosis

Atherosclerosis is an inflammatory disease characterized by intense immunological activity which increasingly threatens human health worldwide [105]. This is mainly the result of the increasing prevalence of atherosclerosis, owing to the ageing population, the improved survival of patients with atherosclerotic cardiovascular disease and, above all, the widespread under-recognition and undertreatment of individuals with risk factors for atherosclerosis. In addition, the World Health Organization expects atherosclerosis-related diseases to be the major killer worldwide within 15 years owing to both its

rapidly increasing prevalence in developing countries and Eastern Europe and an accumulation of metabolic risk factors, including obesity and diabetes, in the Western world [106].

Atherosclerotic lesions, termed atheromas or atherosclerotic plaques, typically present as asymmetric focal thickenings of the innermost layer of the artery, the intima. Atherosclerotic lesions are characterized by inflammation, lipid accumulation, cell death and fibrosis [107]. Most of the cells found in atheromas are blood-borne inflammatory/immune cells, and the remainder are vascular endothelial and VSMCs. The atheroma is preceded chronologically by so-called fatty streaks, which are sites of accumulation of lipid droplets and immune cells. Fatty streaks are prevalent in young individuals, never cause symptoms, and may either progress into atheromas or disappear with time. Over time, fatty streaks evolve into complicated atheroma through multiplication of VSMCs, which accumulate in the plaque and lay down an abundant extracellular matrix [106,108]. As the lesion becomes more bulky, the arterial lumen narrows until it hampers flow and leads to clinical manifestations (fig. 7).

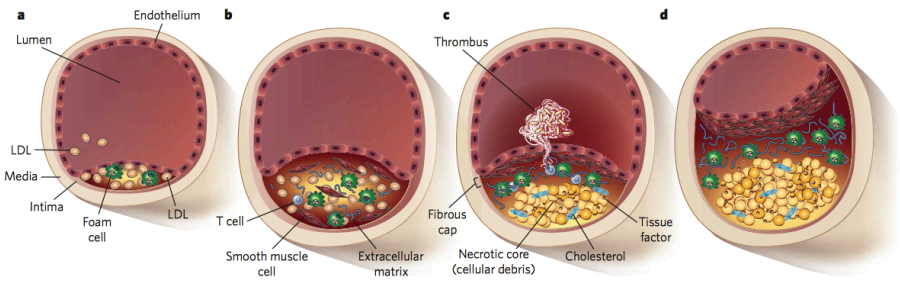

**Figure 7** - Initiation and progression of atherosclerosis. A lesion begins as a fatty streak (a) and can develop into an intermediate lesion (b), and then into a lesion that is vulnerable to rupture (c) and, finally, into an advanced obstructive lesion (d). The atherosclerotic plaque has a core containing lipids (which include esterified cholesterol and cholesterol crystals) and debris from dead cells. Surrounding it, a fibrous cap containing smooth muscle cells and collagen fibres stabilizes the plaque. Immune cells including macrophages and T cells populate the plaque, and are frequently in an activated state. They produce cytokines, proteases, prothrombotic molecules and vasoactive substances, all of which can affect plaque inflammation and vascular function (*Rader D.J., 2008*).

Growth factors elaborated by macrophages in the atherosclerotic intima supposedly stimulated the smooth muscle replication responsible for lesion growth [108]. According to the classical view, this process occurred in an inevitable and progressive fashion gradually during time. The atheroma is structurally more complex than the fatty streak. In the center of the atheroma, macrophage foam cells, dead cells, and extracellular lipid droplets form a core region surrounded by a cap of VSMCs and a collagen-rich matrix. As previously explained for macrophages, many of the immune cells in the atheroma exhibit signs of activation and produce pro-inflammatory cytokines [109].

Symptoms occur late in the course of disease and are usually caused by the narrowing of the lumen of the artery, which can happen gradually (as a result of progressive plaque growth) or suddenly (as a result of plaque rupture and, subsequently, thrombosis). The rupture of a plaque, which exposes the prothrombotic material in the plaque to the blood and causes sudden thrombotic occlusion of the artery at the site of disruption. In the heart, atherosclerosis can lead to myocardial infarction and heart failure; whereas in the arteries that perfuse

the brain, it can cause ischaemic stroke and transient ischaemic attacks. If atherosclerosis affects other arterial branches, it can result in renal impairment, hypertension, abdominal aortic aneurysms and critical limb ischemia [110].

## Hypercholesterolemia and Inflammation

Compelling evidence for the importance of inflammation and atherosclerosis at both the basic and clinical level has evolved in parallel. Accumulating data indicate that insights gained from the link between inflammation and atherosclerosis can yield predictive and prognostic information of considerable clinical utility.

There has been a tendency to consider atherosclerosis as being either a lipid disorder or an inflammatory disorder, but atherosclerosis as an inflammatory disease is initiated by and progresses in the context of hypercholesterolemia. Consequently, much attention has been devoted to defining the changes in morphology and function of arteries that result from chronic elevations in blood cholesterol concentration [111]. Several studies have rather consistently revealed that hypercholesterolemia modifies the structure and function of the arterial wall in a manner that is consistent with an inflammatory response. In a variety of animal models of atherosclerosis, signs of inflammation occur hand-in-hand with incipient lipid accumulation in the artery wall. Total cholesterol can be broken down into a diagnostic lipoprotein profile, including high density

lipoprotein (HDL), low density lipoprotein (LDL), intermediate density lipoproteins (IDL), very low density lipoprotein (VLDL), chylomicron remnants, and triglycerides. Cholesterol has been shown to interrupt and alter vascular structure and function as it builds within the lining of the vascular wall, and can interfere with endothelial function leading to lesions, plaques, occlusion, and emboli; along with a reduction in healing, recovery, and appropriate management of ischemia/reperfusion injury [112,113,114]. The normal endothelium does not in general support binding of white blood cells. However, early after initiation of an hypercholesterolemic diet, patches of arterial endothelial cells begin to express on their surface selective adhesion molecules that bind to various classes of leukocytes. In particular, vascular cell adhesion molecule-1 (VCAM-1) binds precisely the types of leukocytes found in early human and experimental atheroma, the monocyte and T lymphocyte. Not only does VCAM-1 expression increase on endothelial cells overlying nascent atheroma [115], but mice genetically engineered to express defective VCAM-1 show interrupted lesion development [116]. Interestingly, the foci of increased adhesion molecule expression overlap with sites in the arterial tree particularly prone to develop atheroma. The inflammatory phenotype that is seen in microvessels of hypercholesterolemic animals is characterized by increased basal levels of rolling, adherent, and emigrating leukocytes, platelet-endothelial cell adhesion, and enhanced oxygen radical production by endothelial cells [117,118]. Furthermore, the phenotypic

changes in the microcirculation that are associated with hypercholesterolemia can be mimicked by administration of oxidized low-density lipoprotein (oxLDL), suggesting that this putative mediator of fatty streak formation may also contribute to the more rapidly occurring microvascular dysfunction [119].

Once resident in the arterial wall, the blood-derived inflammatory cells participate in and perpetuate a local inflammatory response. As this inflammatory process continues, the activated leukocytes and intrinsic arterial cells can release fibrogenic mediators, including a variety of peptide growth factors that can promote replication of SMCs and contribute to elaboration by these cells of a dense extracellular matrix characteristic of the more advanced atherosclerosis lesion [120].

Additionally, reports have shown a near-complete abrogation in vascular nitric oxide (NO) bioavailability, elevated oxidant stress, and the creation of a strongly pro-inflammatory condition; symptoms which can culminate in profound impairments to vascular reactivity [121,122]. The decreased bioavailability of NO in hypercholesterolemia also diminishes the anti-inflammatory properties of the endothelial cell, permitting the activity of growth factors on the cell surface and platelet activation to act as chemoattractants to a parade of inflammatory events. Leukocytes begin to roll along the lumen and adhere to the cell wall, extravasating due to an increase in vascular permeability, and residing within the intimal space [123]. Macrophages, derived from monocytes, begin to

accumulate LDL and oxidized LDL (oxLDL) which develop into foam cells between the basal lamina of the endothelium and the smooth muscle layer [124]. These foam cells lead to the production of numerous inflammatory and oxidative stress markers, cytokines, chemokines, and growth factors which aggravate the balance of endothelial equilibrium leading to vascular dysfunction [125]. Investigation into vascular consequences of chronic hypercholesterolemia, the mechanisms through which these consequences occur, and the potentially beneficial effects of ameliorative therapies have received considerable attention in recent years [126,127,128].

## Inflammation: genes and molecules

### Inducible Nitric Oxide Synthase

Since its discovery 20 years ago, nitric oxide (NO) has emerged as an important signalling molecule in the nervous, immune, and cardiovascular systems [129]. The mechanism of NO synthesis by NO synthases proceeds through oxidation of one of the amidine nitrogens of L-arginine, yielding NO and L-citrulline [130]. NO is a potent stimulator of guanylate cyclase to form cyclic guanylate monophosphate (cGMP) causing relaxation of VSMC [131]. Three forms of NO synthase (NOS) have been discovered: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). iNOS is typically expressed in response to cellular stress and generates 100-fold to 1000-fold more NO than its constitutive counterparts that are

involved in physiologic regulation [132]. Following arterial injury, NO has been shown to play many vasoprotective roles, including inhibition of platelet aggregation and adherence to the site of injury, inhibition of leukocyte adherence, inhibition of VSMC proliferation and migration, and stimulation of endothelial cell growth. These properties function together to preserve a normal vascular environment following injury [133].

NO production can be impaired by several physical or biochemical injuries to the endothelium. This inhibition results in an intima that is characterized by enhanced thrombus formation, aberrant vessel tone, and dysregulated VSMC growth. One of the hallmarks of a compromised endothelium is represented by diminished levels of NO bioavailability. The endothelial dysfunction is an early marker of atherosclerosis as demonstrated by the observation that fatty streak progression is associated with increasingly impaired vascular relaxation [134]. The reduced presence of NO may result from a decrease in NO synthesis or an increase in NO inactivation owing to locally enhanced production of ROS [135]. This decreased NO bioactivity appears to be a key contributor to vasoconstrictive remodeling and a major determinant of the occurrence of nitrative/oxidative stress. Some of the proatherogenic risk factors identified with endothelial dysfunction, including hypercholesterolemia, tobacco use, diabetes mellitus, and hyperhomocysteinemia, are associated with decreased bioavailable NO [136]. There is also evidence suggesting that atherosclerosis is not only associated with decreased NO

bioavailability, but also with alterations in signal-transduction components downstream of NO, including among others, the NO receptor sGC, particularly in neointima [137].

**Heme Oxygenase-1**

Heme oxygenase was discovered in 1968 when Tenhunen and colleagues described the mechanism for catabolism of heme [138]. Heme oxygenase, as discussed above, catalyzes the oxidative cleavage of heme to yield equimolar amounts of CO, iron (Fe2+), and biliverdin. Biliverdin is subsequently converted to bilirubin through the action of biliverdin reductase, and iron induces increased ferritin synthesis [50].

Free heme can be quite cytotoxic, however, particularly in the presence of oxidants or activated phagocytes. Of all sites in the body, the vasculature may be at greatest risk of exposure to free heme, as erythrocytes contain heme at a concentration of 20 mmol/L and are prone to undergoing unexpected lysis [139]. Heme has been shown to be involved in the pathogenesis of atherosclerosis, which is considered to be an inflammatory disease characterized by the accumulation of lipids and fibrous material in arterial walls [140]. Hemoglobin-derived heme has been demonstrated to act as a catalyst for the oxidation of LDL, which in turn causes atherosclerosis [141]. Beyond heme, the reactive oxygen species released from all types of vascular cells under pathophysiological conditions, including dyslipidemia, hypertension, diabetes, and smoking

regulate various signaling pathways that mediate the development of atherosclerosis [142].

Atherosclerosis is thought to be accelerated when the antioxidative defense is not sufficiently induced in the vascular wall under oxidative and inflammatory stress. Accumulating evidence suggests that HO-1 contributes to the balance of pro-oxidant and antioxidant factors in the vascular wall through multiple mechanisms. HO-1 in vascular endothelial cells, VSMCs and macrophages is markedly upregulated by oxidized LDL, whereas HO-1 is not increased in vascular endothelial cells or VSMCs when exposed to native LDL [143,144]. HO-1 expression has been observed throughout the development of the lesions, from an early fatty streak to an advanced complex atherosclerotic lesion [145]; that is, HO-1 is expressed in vascular endothelial cells and macrophages in the early stages of atherosclerotic lesion formation and in foam cells and VSMCs residing in the necrotic core of advanced lesions. In all these atherosclerotic lesions, HO-1 has been found to be colocalized with oxidized phospholipids, strongly suggesting that HO-1 is induced by oxidized phospholipids in vivo [146]. Numerous findings in animal models have contributed to demonstrate that endogenous HO-1 plays a role in atherosclerotic lesion formation [145]. Mice deficient in both HO-1 and ApoE develop larger and more advanced atherosclerotic lesions, respect to ApoE-/- mice [147].

## Statins

The 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibitors, generically referred to as statins, have emerged as the leading therapeutic regimen for treating hypercholesterolaemia and reducing cardiovascular morbidity and mortality. The clinical use of statins has become so prevalent that they are now prescribed to more than 25 million people worldwide, with the number espected to rise rapidly. These compounds mediate their biological effect by inhibiting HMG-CoA reductase, which is an upstream rate-limiting enzyme in the cholesterol synthesis pathway. The consequent reduction in circulating low-density lipoprotein (LDL) cholesterol, which provided the original rationale for treating cardiovascular disease, was until recently believed to be the main therapeutic effect. The possibility that an agent that inhibited the rate-limiting step in the cholesterol biosynthesis pathway could have useful lipid-lowering properties was quickly appreciated. Clinical benefits originate at least in part from the effects on LDL cholesterol, whose role in atherosclerotic cardiovascular disease (CVD) is well established. Nevertheless, accumulating evidence suggests that these benefits may also be due in part to pleiotropic effects of these drugs.

Evidence from both experimental and clinical studies supports the notion of “pleiotropic” effects of statins. In subjects with cardiovascular risk factors, statins reduce circulating C-reactive protein (CRP) and pro-inflammatory cytokines levels [147]. In human vessels, statins rapidly induce favorable effects on

vascular redox state and reduce vascular reactive oxygen species (ROS) generation [148].

Statins have been shown to reduce tissue factor expression on human EC [149] and macrophages [150]. Inhibition of thrombin formation and its activity by statins has been documented in several in vitro studies [151] and in patients [152]. In addition, statins have been shown to upregulate EC thrombomodulin expression and activity [153], and reverse thrombin-induced downregulation of EC CD39/ ATPDase [154]. Platelet activation is closely linked to vascular inflammation and it is an essential element of atherogenesis. Patients receiving statins have been shown to have reduced platelet activity [155] that decreases with time [156]. The anti-inflammatory properties of statins are also likely to account for their role in primary and secondary prevention of stroke [157], improvement of short-term outcome of acute coronary syndrome patients [158], reduction of the risk for atrial fibrillation post-coronary artery bypass grafting (CABG) [159] and in patients with heart failure [160].

Atorvastatin reduces monocyte chemoattractant protein-1 levels in the intima and media in hypercholesterolemic rabbits [161]. This decrease in monocyte chemoattractant protein-1 is related to a reduction in NF-κB activation, involved in the induction of monocyte chemoattractant protein-1 and other proinflammatory cytokines such as IL-1β and tumor necrosis factor-α.

An important group of intracellular signaling pathways modulated by statins involves the small guanine triphosphate (GTP)-binding proteins, such as Rho, Rac and Ras [162].

These small GTP proteins regulate pro-atherogenic, pro-inflammatory pathways. Rho proteins are involved in the expression of pro-inflammatory cytokines, as well as in the formation and maintenance of actin cytoskeleton. The Ras proteins regulate cell proliferation and hypertrophy, while Rac transduction signaling modulates ROS generation [163].

Nitric oxide is critical for maintaining endothelium homeostasis via its vasodilatory, anti-inflammatory and overall anti-atherogenic effects. It is widely accepted that statins favorably affect important pathways regulating NO bioavailability. With regard to ischemia-reperfusion injury, statin treatment resulted in an improvement in nitric oxide dilator function of the endothelium [164]. In addition, statins have been shown to increase eNOS activity and protect the myocardium in a nitric oxide-dependent manner [165]. Also, EC adhesion molecule expression (P-selectin and intercellular adhesion molecule 1) has been reduced in patients receiving statins [166].

In cultured VSMCs atorvastatin inhibits NF-κB activation by TNF-α or angiotensin II (AngII) by restoring cytoplasmic levels of the NF-κB inhibitor IκB [167]. Thus statins reduce pro-inflammatory cytokines and chemokines release from VSMCs, including down-regulation of MMP-9 activity and expression that is responsible for extracellular matrix remodeling [168].

In the light of ample experimental evidence statins seem an attractive option for treatment of patients with autoimmune or inflammatory diseases. Indeed clinical evidence suggests that statins could be beneficial in a number of pathological disorders

such as in osteoporosis and osteoporotic bone fractures, Alzheimer's disease, Parkinson's disease, multiple sclerosis, organ transplantation, rheumatic diseases, allergic asthma, sepsis and others [169].

## Animal models of atherosclerosis

Animal models have been employed in experiments concerning cardiovascular research since the turn of this century. Direct human research on atherosclerosis is obviously limited by the slowness of lesion development and by the inability to control experiments, therefore animal models represent an alternative, wherein variables can be controlled and statistical data collected in a short period of time. Rabbits have been the first species employed by Ignatowsky, Anitchkov and Chalatov in their fundamental studies concerning the pathogenesis of atherosclerosis [170,171,172].

Animal models have proven invaluable in understanding the pathophysiology of atherosclerosis as well as developing and testing treatment strategies. Genetically modified murine models have led to an understanding of the mechanisms of disease and the role of signaling pathways and genetic factors which play a major role in disease initiation and development. However, mice are limited by their varying lipid profiles, lack of spontaneous coronary artery disease, and development of disease in vascular beds which are in variance with human disease [173,174]. Diabetes has only a small effect on

development of atherosclerosis in mice [175], and their small size limits physiologic evaluation. Rabbits are limited because they do not naturally develop atherosclerosis and require a high cholesterol diet to induce atherosclerosis resulting in cholesterol levels which often exceed 1000 mg/dL, lesions which are largely foam cell rich, and, given size considerations, the need to perform vascular studies in the aortae and iliofemoral arteries rather than coronary arteries [176]. Such differences between rodents and humans have made it incumbent for additional models to be used to assess possible treatments, whether pharmacologic or device related. Porcine models of atherosclerosis have several advantages over small animal models. Individual characteristics that render swine commonly used models for atherosclerosis include: easily inducible disease by high-cholesterol/high-fat diets and a remarkable similarity of lesion distribution, pathogenesis, and morphology to that of humans. Cholesterol levels after dietary induction are similar in range to human levels [177]. Although the arteries of the pig are large in size and are, therefore, easier to work with, the large size of the pig represents a major drawback. The development of the minipig has helped override this disadvantage [178]. Since the original development of the Yucatan miniature pig [179], a number of strains of miniature pigs have been developed including the Gottingen, Sinclair, and Hanford strains [180,181].

# Scope of the thesis

**Chapter 2: The expression of human HO-1, CD39 and CD73 protects NIH3T3 cells from TNF-α injury via the modulation of pro-survival genes.**

Over the years several genes, whose over-expression is capable to down-regulate the inflammatory response and limit apoptosis, were identified. Our aim was to evaluate the effects of the simultaneous expression of human CD39 and CD73 in combination with the overexpression of the human heme oxygenase 1, on the down-regulation of the pro-inflammatory and pro-apoptotic stimuli represented by TNF-α. For this purpose, we have produced a transgenic multicistronic construct by exploiting the features of 2A sequence from Foot and Mouth Disease Virus.

**Chapter 3: Functional analysis of expression of human ecto-nucleoside triphosphate diphosphohydrolase-1 and/or ecto-5'-nucleotidase in pig endothelial cells.**

Adenosine is known to exert immunosuppressive and anti-inflammatory effects. Extracellular pathway that converts ATP and ADP to AMP, and AMP to adenosine mainly mediated by ecto-nucleoside triphosphate diphosphohydrolase 1, (ENTPD1 or CD39) and ecto-5'-nucleotidase (E5NT or CD73) respectively, is considered as important target for xenograft protection and inflammation regulation. To clarify feasibility of

combined expression of human ENTPD1 and E5NT and to study its functional effect we transfected pig endothelial cell line (PIEC) with both genes together.

**Chapter 4: Diet Induced Mild Hypercholesterolemia in Pigs: Local and Systemic Inflammation, Effects on Vascular Injury – Rescue by High-Dose Statin Treatment.**

The aim of the present study was to comprehensively evaluate systemic and local inflammation as well as progression of vascular inflammation in normal and mechanically injured vessels in a large animal model of mild hypercholesterolemia. Our aim was also to test the effect of high-dose statin treatment on these processes.

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# Chapter 2

## The expression of human HO-1, CD39 and CD73 protects NIH3T3 cells from TNF-α injury via the modulation of pro-survival genes

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## ABSTRACT

TNF-α is a pro-inflammatory cytokine and one of the most potent and well studied inducers of apoptosis in mammalian cells. It exhibits its pro-inflammatory activity in part through activation of the transcription factor NF-kB and it has been demonstrated to be a central regulator in ischemia/reperfusion injury, thus limiting the successful rate of organ transplantation. We tested the anti-inflammatory effects of a novel combination of three human genes, namely HO-1, CD73 and CD39, simultaneously expressed in NIH3T3 cells via a transgenic multicistronic construct produced by exploiting the features of F2A sequence. Transgenic cells (TG) were protected against TNF-α-induced cytotoxicity and cell death even in absence of enzymatic substrates as compared to WT cells. Cells were also treated with enzymatic substrates of HO-1 and CD39/CD73, hemin and ATP respectively, demonstrating that the activity of HO-1 and CD39/CD73 systems resulted in a more efficient protection. Moreover, the differences in the activation of TNF- α signalling pathway were evaluated by real-time PCR experiments and we found that the anti-apoptotic *Ikbkg* gene was up-regulated in TG cells as compared to WT cells. In conclusion, this study demonstrated, for the first time, the protection against inflammatory stimuli of a novel combination of three genes expressed in NIH3T3 cells, via a different modulation of key downstream regulators of TNF-α signaling pathway promoting pro-survival phenotype in TNF-α injured cells. These results could provide new insights in the research of protective mechanisms in transplantation settings.

## 1. INTRODUCTION

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine which plays an important role in the immune system during inflammation, cell proliferation, differentiation and apoptosis [1]. Although activated macrophages and T-cells are thought to be the main producers of TNF-α, it can be also produced by other cell types, including macrophages/monocyte lineage, natural killer cells, neutrophils, mast cells, endothelial cells, smooth muscle cells, cardiomyocytes, fibroblasts,

osteoclasts [2]. TNF-α belongs to the TNF superfamily, composed of 19 ligands and 29 receptors, that exhibit, without exception, pro-inflammatory activity in part through activation of Nf-kB pathway [3]. TNF-α is known to interact with 2 different receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2), which are type I transmembrane glycoproteins [1,4]. The ligand-induced activation of TNFR1 receptor, which contains a death domain, leads to two different outcomes for the cells: cell survival and expression of pro-inflammatory genes or apoptosis and cell death [1]. The activation of TNFR2 receptor, which does not contain a death domain, can lead to cell survival outcome via the TRAF2 pathway, except for some cell types and in certain conditions [2,5,6].

TNF-α has been involved in a variety of diseases [1] and in the inflammatory response during ischemia/reperfusion injury in liver [7], heart [8], lung [9] and kidney [10] limiting the successful rate of transplantation, with particular regards to xenotransplantation for which a multiple genetic modification of the donor cells/organs is required [11].

Over the years several genes, whose induction or over-expression is able to down-regulate the inflammatory response and limit apoptosis in the transplantation settings, were identified. Among others, heme oxygenase-1 (HO-1) attenuates TNF-α injury by suppression of TNF/TNFR1-mediated apoptotic signaling [12]. In a model of cardiopulmonary by-pass, we previously demonstrated the protective effects of HO-1/CO against ischemia reperfusion injury [13]. The beneficial effects of heme degradation pathway regulated by HO-1 were well reviewed by Wegiel [14] and Gozzelino [15]. Ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDase 1 or CD39) and ecto-5'-nucleotidase (E5'N or CD73) degrade ATP, ADP, and AMP to adenosine. These enzymatic activities shift the pro-inflammatory stimulus given by ATP toward an anti-inflammatory status mediated by adenosine [16] in different inflammatory settings including ischemia-reperfusion injury.

Our aim was to obtain a multicistronic construct by exploiting the features of Foot and Mouth Disease Virus 2A sequence, which allows multiple genetic modifications in a single transgenesis experiment [17,18], to produce *in vitro* and *in vivo* models for the evaluation of the effects of human CD39, CD73 and HO-1 expression against TNF-α-induced injury. This study

demonstrated, for the first time, the protection against inflammatory stimuli of a novel combination of human genes simultaneously expressed in murine NIH3T3 cells. The three genes have a role in modulating key downstream regulators of TNF-α signaling pathway, as *Tnfaip3* and *Ikbkg*, promoting pro-survival phenotype in TNF-α injured cells.

## 2. MATERIAL AND METHODS

### 2.1. Reagents and antibodies

Recombinant human TNF-α (Sigma Aldrich) was re-constituted at 100 μg/ml in distilled water and stored at −20°C. Cells were treated with 50 ng/ml TNF-α diluted in complete medium, concentration was selected according to the literature and determined in preliminary cytotoxicity experiments (data not shown). Hemin (Sigma Aldrich) was prepared under subdued light by dissolving the powder in 1 mL of 1 M KOH at a stock concentration of 10 mM. Cells were treated with 20 μM hemin diluted in complete medium. ATP powder (Sigma Aldrich) was dissolved in sterile double-distilled water to obtain 10 mM of stock solution. Cells were treated with 200 μM ATP diluted in complete medium. Different time points and different combinations of drug treatments were assessed depending on the type of experiments, as detailed below.

Anti-hCD73 (4G4, Novus Biologicals), anti-hHO-1 (EP1391Y, Epitomics) and anti-hCD39 (BU61, Santa Cruz) primary antibodies, Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 555-conjugated anti-rabbit (Invitrogen) secondary antibodies were used for immunofluorescence analysis. Anti-hHO-1 (EP1391Y, Epitomics), anti-hCD73 (EPR6115, LifeSpan BioSciences), anti-hCD39 (HPA014067, Sigma Aldrich), anti-β-actin (AC-15, Sigma Aldrich) and anti-vinculin (V284, Sigma Aldrich) primary antibodies were used for immunoblotting analysis. Phycoerythrin (PE)-conjugated anti-hCD73 (BD Biosciences) and Alexa Fluor 647-conjugated anti-hCD39 (Invitrogen) primary antibodies were used for FACS analysis and cell sorting.

## 2.2. Triple cistronic vector construction

The triple cistronic vector was prepared following a strategy similar to those previously reported by Ryan *et al*. [18] as detailed in supplementary information. Briefly, two *F2A* sequences were annealed and sequentially ligated into restricted pcDNA3.1+ (Life Technologies) to form plasmid pcDNA3.1-F2A1-F2A2. The coding sequences of each human gene was amplified by PCR and ligated into pcDNA3.1-F2A1-F2A2 plasmid to form pcDNA3.1-hHO1-F2A1-hCD73-F2A2-hCD39 plasmid. The entire coding sequence (hHO1-F2A1-hCD73-F2A2-hCD39) was then excised and ligated into pCX-C1 plasmid (a pCX-EGFP plasmid [19] to which a neomycin resistance cassette has been added) to form the final pCX-hHO1-F2A1-hCD73-F2A2-hCD39-C1, which was called pCX-TRI-2A. Restriction and sequencing analyses were performed on all the intermediate and in the final construct.

## 2.3. Cell culture and electroporation

NIH3T3 cells were grown in Dulbecco's minimum essential medium (DMEM) (EuroClone) supplemented with 10% fetal calf serum (Sigma Aldrich), at 37°C and 5% $CO_2$.

Cells were split and plated to reach 80-90% confluence the following day when they were transfected. pCX TRI-2A plasmid was introduced into NIH3T3 cells by electroporation using Neon Transfection System (Invitrogen) following the manufacturer's instructions. Briefly $2x10^6$ cells were washed with PBS and re-suspended in 100 µl of Buffer R (Invitrogen). Cell suspension were mixed with 20 µg of plasmid DNA and electroporation was carried out at 1350V, 20ms and 2 pulses, according to the conditions suggested for NIH3T3 cells by manufacturer. Cells were immediately re-suspended in growth medium with serum without antibiotics and plated. After 24 hours, cells were transferred in standard medium plus 0.5 mg/ml of G418 (Sigma Aldrich) and maintained in culture for 7 days.

## 2.4. Immunofluorescence and Confocal Microscopy

Transfected cells were seeded at $4x10^4$cells/well in 8-well chamber slides for 24 hours (LabTek Chamber slides, Thermo Fisher Scientific). The next day, cells were washed with PBS and fixed with methanol-acetone 1:1 for 10 min at -20°C. After fixation, cells were blocked with 1% BSA for 30 min. Fixed cells

were co-incubated, for 1 hour, with anti-hCD73 and anti-hHO-1 primary antibodies, or anti-hCD39 and anti-hHO-1 primary antibodies. All primary antibody were diluted in 1% BSA (w/v) in PBS. After three washes in PBS, cells were incubated for 30 minutes with the appropriate secondary antibodies diluted in 1% BSA (w/v) in PBS. Cells were washed twice with PBS and counterstained with DAPI. The stained cells were mounted with mounting medium (Fluoromount; Sigma Aldrich) and analyzed by LSM 710 confocal microscope (Zeiss). Images were acquired by ZEN 2009 software (Zeiss).

**2.5. Immunoblotting**

WT and TG cells were lysed in RIPA buffer and whole protein concentration was quantified by Bradford assay (Sigma Aldrich). 20 µg of total protein extracts were separated in a 10% NuPAGE BT gel (Invitrogen) and then transferred onto nitrocellulose membranes using the iBlot system (Invitrogen). The membranes were probed with anti-hHO-1, anti-hCD73, anti-hCD39, anti-β-actin and anti-vinculin primary antibodies. Immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal West Dura, Thermo Scientific) and digitally acquired using G:BOX (Syngene) instrument.

**2.6. Flow Cytometry analysis and cell sorting**

Cells were detached with trypsin/EDTA and washed once with PFN buffer (serum 3%, $NaN_3$ 0,01% in PBS). Cells were then incubated for 30 min in the dark with primary antibodies fluorophore-conjugated anti-hCD73. The excess and nonspecifically-bound antibodies were removed by washing with PFN buffer. Flow cytometric analysis of stained cells was performed with a FACSAria flow cytometer (Becton Dickinson). Lymphocytes were used as a positive control, whereas wild type NIH3T3 cells were used as a negative control. The not specific cross-reaction of antibody with murine cells was excluded by incubating NIH3T3 cells with isotype-matched immunoglobulins.

**2.7. Cytotoxicity assay**

WT and TG cells were plated in triplicate in 96-well plate at $12x10^3$ cells per well. One day after plating, cells were

incubated in culture medium with of without different combination of drugs (TNF-α 50 ng/ml, hemin 20 μM and ATP 200 μM) for 24h and 48h. Cell death was kinetically quantified by measurement of lactate dehydrogenase (LDH) release into the medium. The released LDH was expressed as a percentage of total cellular LDH, which was determined after complete lysis of the cells. The LDH activity was determined by LDH assay kit (Roche Diagnostics).

### 2.8. Cells death and apoptosis quantification

*2.8.1. Caspase 3/7 activity assay.* The Caspase-Glo 3/7 (Promega) assay was performed on WT and TG cells grown in a 96-well to reach 80% confluence, following manufacturer's instructions. Briefly, after 16h and 24h of treatments with TNF-α 50 ng/ml, hemin 20 μM and ATP 200 μM, lyophilized Caspase-Glo 3/7 substrate was resuspended in its buffer and 100μl of this reagent was added into each well. The contents of the wells were mixed gently and incubated at room temperature for 1 hour. Luminescent signal was measured with a 96 multi-well plate reader (Tecan).

*2.8.2. Propidium Iodide Incorporation.* $1\times10^6$ cells were seeded in 10 ml culture petri and treated with medium containing TNF-α (50 ng/ml) alone or with TNF-α (50 ng/ml), hemin (20 μM) and ATP (200 μM) for 24, 48 and 72 hours. Untreated cells were also cultured, at each condition, as a control of basal level of cell death. Cell death was detected, at each time point, using propidium iodide (PI, Sigma Aldrich) influx evaluation. At the end of treatment, the cells were harvested by centrifugation and suspended in PBS. Subsequently, the cells were incubated with 2 μg/mL of propidium iodide (PI) in the dark for 15 min at room temperature immediately before cytometric evaluation on FACSARIA flow cytometer (Becton Dickinson, San Jose, CA). PI incorporation was detected by red fluorescence on a log scale and cell death percentages were calculated on PI+cells combined with the scatter (FSC) by subtracting the % of untreated cells at each condition. Data were collected (at least 50,000 events) and analyzed using DIVA software (Becton Dickinson) and FlowJo software.

### 2.9. Real time PCR analysis of TNF-α signaling genes

The expression of 84 TNF-α pathway-related genes in mouse were examined using the RT2 Profiler PCR array (PAMM-063C, SuperArray Bioscience). WT and TG cells were treated with TNF-α 50ng/ml alone or in combination with hemin 20 μM and ATP 200 μM for 16h. Untreated WT and TG cells were used as a control. Total RNA was isolated from treated and control cells by using the RNeasy Mini kit (Qiagen) according to manufacturer's instructions. RNA samples were treated with DNase to ensure elimination of genomic DNA, and the extracted RNA was converted to cDNA using the $RT^2$ First Strand Kit from SuperArray Bioscience (Qiagen). Briefly, 1 μg of RNA was combined with 2 μL genomic DNA-elimination buffer and brought up to a final volume of 10 μL using RNAse-free $H_20$. This mixture was incubated at 42°C for 5 minutes, then chilled on ice. Ten microliters of RT Cocktail was then added to this mixture and incubated at 42°C for 15 minutes followed by 5 minutes at 95°C. Ninety-one microliters ddH20 was added to each 20 μl cDNA synthesis reaction, and the diluted cDNA mixture was stored at −20°C until used for gene expression profiling. PCR was performed with the $RT^2$ Profiler PCR array system according to the manufacturer's instructions using Step One Plus instrument (Applied Biosystems). The expression levels of the mRNA of each gene in each cell treatment were normalized using the expression of *B2m, Gapdh, Gusb, Hsp90ab1*, and *Actb,* considered the housekeeping genes. The results were confirmed by quantitative reverse transcriptase-PCR performed using individual RNA samples from the cells in each group by Step One Plus instrument (Applied Biosystems). The primers used for real-time PCR are listed in Table S1.

### 2.10. Statistical Analysis

Statistical analyses were performed using SPSS v.19 for Mac and values of $p \leq 0.05$ were considered to be statistically significant. LDH assay, caspase 3/7 assay and real-time PCR were independently performed 3 times. The results are represented as mean ± standard deviation (SD) Results for WT and TG cells for each treatment at each time point were compared using the Student's t test. Analysis of variance (ANOVA) with Bonferroni *post hoc* test was used for multiple comparisons.

## 3. RESULTS

### 3.1. Transgenic construct design and transfection in NIH3T3 cells.

To express a combination of human genes in eukaryotic cells in a single transfection experiment, we designed and produced a tricistronic plasmid containing the coding sequences of human Heme Oxygenase 1 (hHO1), human Ecto-5'-Nucleotidase (hE5'N or hCD73) and human Ecto-Nucleoside Triphosphate Diphosphohydrolase 1 (hENTPD1 or hCD39). The tricistronic plasmid, named pCX TRI-2A (Fig. 1), was constructed by a series of PCR and cloning steps (see supplementary material and methods). We used the F2A technology to link in frame the three coding sequences [16] obtaining a single open reading frame of 4.3 kb.

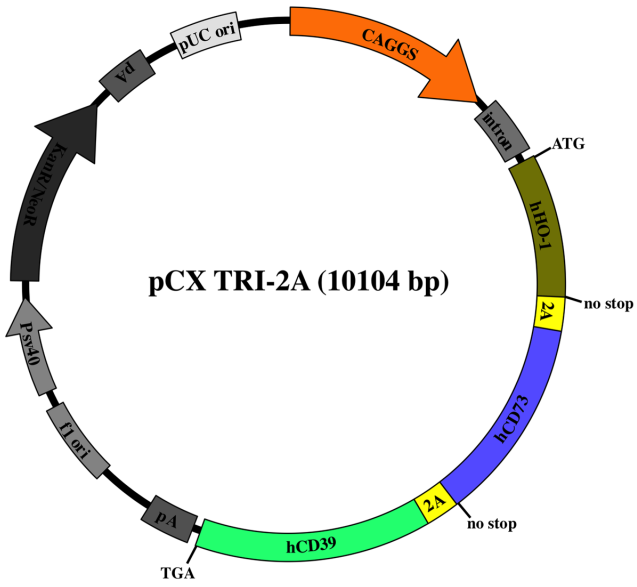



**Figure 1**. Layout of tricistronic pCX TRI-2A construct. The transgenic construct is composed of the CAGGS promoter, followed by the coding sequence of human HO-1 gene without the stop codon, fused in frame to the first F2A sequence (F2A), then the coding sequence of human CD73 gene without the stop codon, the second F2A sequence and the coding sequence of human CD39 gene followed by a polyadenilation signal (pA).

The genes were placed in a specific order designed to maximize the likelihood of correct post-translational processing of each protein: the hHO1 coding sequence was placed upstream the others to avoid the “slipstream translocation” phenomenon [20]; the hCD73, which is known to be cleaved at both its N-terminal and C-terminal ends during the GPI-anchoring processing [21,22], and hCD39 coding sequences were placed downstream hCD73 because they are naturally processed through the secretory pathway [23]. The correct assembly and the absence of mutations at each step of plasmid construction was verified by restriction and sequencing analysis (data not shown). Murine NIH3T3 cells were electroporated with pCX TRI-2A plasmid, and selected for neomycin resistance for one week. In order to verify the presence and the functionality of transgenic vectors, genomic DNA and total RNA were extracted from transfected cells and analyzed for the presence of exogenous molecules. PCR analysis on genomic DNA using transgene-specific oligonucleotides confirmed the genetic modification of the cells (Fig. S1). RT-PCR analyses on total RNA, using oligonucleotides specific for transgenic transcript, also confirmed the correct transcription of the tricistronic cassette (Fig. S2).

### 3.2. The tricistronic transgene encodes for all the three human proteins with a correct subcellular localization

*3.2.1. Immunofluorescence.* To confirm the correct subcellular localization of exogenous hHO1, hCD73 and hCD39 in murine cells, the expression of human proteins was analyzed in NIH3T3 transfected (TG) and wild type cells (WT) by immunofluorescence. Co-staining analysis for hCD73 and hHO1 and for hCD39 and hHO1 indicated that in TG cells both the hCD39 and hCD73 signals had, as expected, a distribution pattern similar to that of plasma membrane proteins, whereas hHO1 signal was detected mainly in the perinuclear area suggesting, for this protein, a cytoplasmic localization related to the ER (Fig. 2).

*3.2.2. Flow cytometry.* In order to enrich the population of transfected cells, the expression of cell surface proteins was firstly assessed by flow cytometry and then the cells expressing the human proteins were sorted and expanded. pCX TRI-2A transfected NIH3T3 cells were analyzed by FACS using

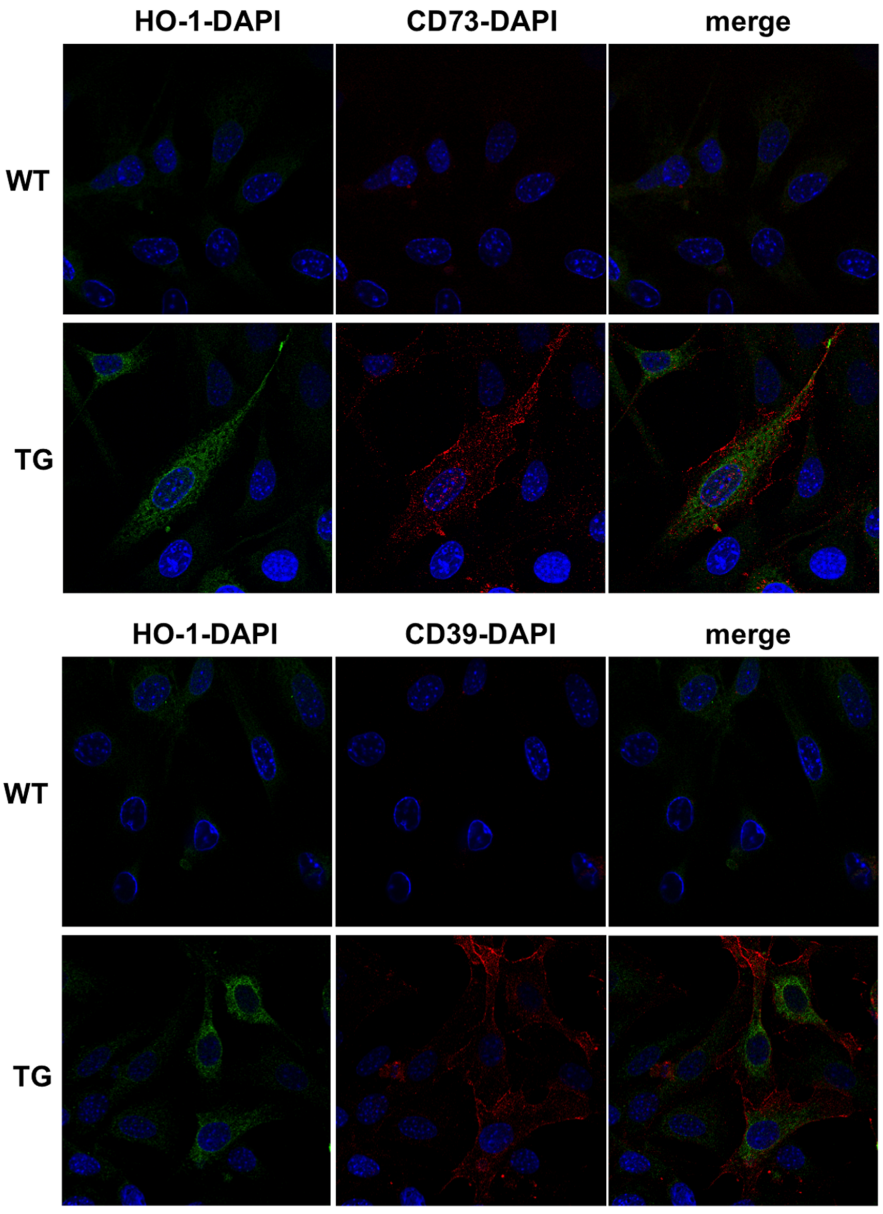



**Figure 2**. Cellular localization of human proteins in NIH3T3 transfected cells. All the three exogenous proteins were correctly localized in TG cell. WT and TG cells were co-stained with anti-hHO1 and anti-hCD73 (upper panels) or with anti-hHO1and anti-hCD39 (lower panels). TG cells positive to hCD73 or hCD39 (red) were also positive to HO-1 (green). hCD73 and hCD39 localized on the cell surface, while hHO-1 had a perinuclear and/or ER membranes cytoplasmic localization.

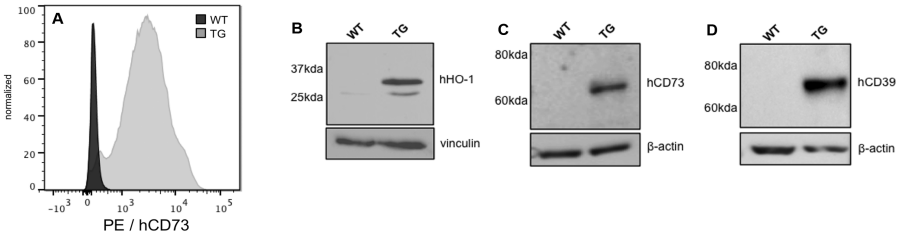



**Figure 3**. TG cells expressed high levels of the three exogenous proteins. NIH3T3 cells transfected with pCX TRI-2A construct were enriched via FACS for the expression of hCD73 (A) and the expression of each human gene was confirmed by immunoblotting analysis (B-D). The three human proteins were found strongly expressed into the transfected cells and expression levels were unaffected by the number of genes in the construct, as there was no evidence of incomplete separation of individual proteins.

antibody anti-hCD73. A fraction of cells between 5% and 7% of cell population analyzed was positive for hCD73 (data not shown). The transfected cells were FACS-sorted based on high hCD73 expression. hCD73-enriched cells were expanded in culture for seven days and then analyzed to evaluate proportion of cell population positive for of hCD73. The analysis of individually marked cells showed that the expression of hCD73 was approximately 98/99% (Fig. 3A).

*3.2.3. Immunoblotting.* To verify if the hCD73-enriched cells expressed all the three human genes, NIH3T3 WT and sorted TG cells were analyzed by immunoblotting. As expected, all the three human HO-1, CD73 and CD39 proteins were found to be strongly expressed in the TG cells and to have the correct molecular weight (Fig. 3B-D).

### 3.3. The expression of hHO1, hCD73 an hCD39 protects cells from TNF-α-mediated cytotoxicity and apoptosis

To evaluate whether the expression of the three human genes protects cells against inflammatory stimuli, WT and TG cells were exposed to 50 ng/ml of TNF-α alone or in combination with appropriate molecules that served as a substrate for the enzymatic activity of exogenous proteins, hemin (20 μM) and/or ATP (200 μM), for up to 48 hours and the cytotoxicity was measured by LDH release.

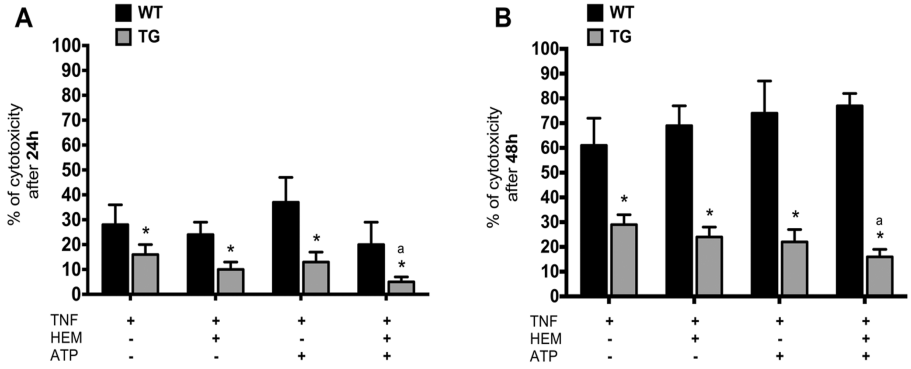



**Figure 4**. TG cells are protected against TNF-α-induced cytotoxicity. WT and TG cells were incubated with 50 ng/ml TNF-α for 24 h (A) and 48 h (B), alone or in combination with 20 μM hemin and/or 200 μM ATP. Cytotoxicity was assessed by lactate dehydrogenase (LDH) release and expressed as follows: relative cytotoxicity (%) = [(*A* e − *A* c) / (*A* b − *A* c)] × 100 (%), where '*A* e' is the experimental absorbance, '*A* b' is the absorbance of lysed controls and '*A* c' is the absorbance of untreated controls. The data are expressed as mean ± SD of three independent experiments. [*] indicates significant difference between TG and WT within the same treatment (t Student, $p < 0.05$); [a] indicates significant difference as compared to TNF-α treatment alone within the same cell type (ANOVA, $p < 0.05$).

As shown in figure 4, the percentage of dead cells was 16% after 24 hours (Fig. 4A) and 29% after 48 hours (Fig. 4B) of treatment with TNF-α alone in TG cells, significantly lower if compared to WT cells treated with TNF-α alone at the same time points (28% after 24 hours and 61% after 48 hours, $p<0.05$). The simultaneous administration of enzymatic substrates, hemin and ATP to TG cells treated with TNF-α, induced a further reduction of cytotoxicity respect to TG cells treated with TNF-α alone ($p<0.05$). In fact, the addition of hemin and ATP to TNF-α treatment induced only 5% of cell death at 24 hours in TG (Fig. 4A), which was significantly lower than cytotoxicity of TG cells treated with TNF-α alone (16%, $p<0.05$). Similarly, the addition of hemin and ATP to TNF-α treatment induced a 16% of cell death in TG cells at 48 hours (Fig. 4B), which was significantly lower than cytotoxicity of TG cells treated with TNF-α (29%, $p<0.05$). On the other hands, the addition of hemin or ATP or both to TNF-α did not have any effect on WT cells as compared to WT cells treated with TNF-α alone.

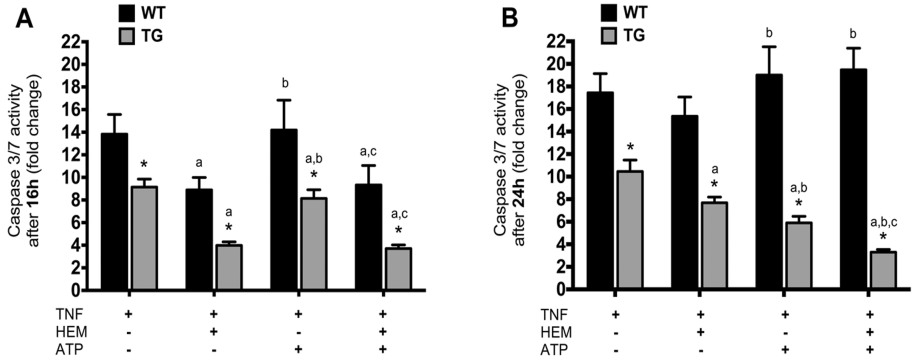


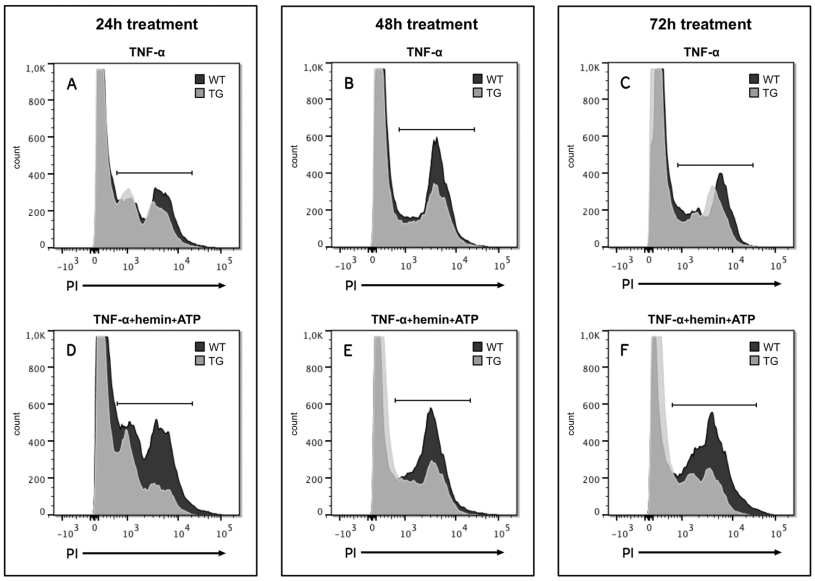

**Figure 5**. TG cells are protected against TNF-α-induced apoptosis. Caspase 3/7 activities were determined in WT and TG cells after 16h (A) or 24h (B) of incubation with 50 ng/ml TNF-α alone or in combination with 20 µM hemin and/or 200 µM ATP. Expression of the three human genes in TG cells significantly reduced the activation of effector caspases 3/7. The data are expressed as mean ± SD of three independent experiments. [∗] indicates a significant difference between TG and WT within the same treatment (t Student, $p < 0.05$); [a] indicates a significant difference as compared to TNF-α treatment alone within the same cell type (ANOVA, $p < 0.05$); [b] indicates a significant difference as compared to TNF-α+hemin treatment within the same cell type (ANOVA, $p < 0.05$); [c] indicates a significant difference as compared to TNF-α+ATP treatment within the same cell type (ANOVA, $p < 0.05$).

Since soluble TNF-α can lead to cell apoptosis [5,25], to verify the protection against apoptotic cell death induced by the three genes, the caspase 3/7 activity assay, which is an early indicator for apoptosis, was performed. As shown in figure 5, TG cells were protected against apoptotic death after challenging with TNF-α (50 ng/ml) in each treatment and at each time-point, as compared to WT cells ($p$<0,05*)*. After 16 hours of treatment, TG cells were protected against TNF-α-induced caspase activation as compared to WT cells (-34%, $p$ <0,05, fig. 5A). After 24 hours TG cells were still protected from TNF-α-induced apoptosis as compared to WT cells (-40%, $p$<0,05, fig. 5B). The addition of hemin (20 µM) to TG cells treated with TNF-α reduced caspase activation at 16 hours (-56% as compared to TG cells treated with TNF-α alone, $p$ <0,05, Fig. 5A) and this anti-apoptotic effect was still observed at 24 hours (-27%, as compared to TG cells treated with TNF-α alone, $p$ <0,05, Fig. 5B). Notably, the addition of hemin, which is known to be an HO-1 inducer [26], was anti-apoptotic also in

WT cells (-35%, as compared to WT cells treated with TNF-α alone, $p$ <0,05, Fig. 5A), but this effect disappeared at 24 hours (Fig. 5B). The combined treatment with TNF-α, hemin and ATP inhibited apoptosis at 16 hours both in WT (-32%, $p$ <0,05) and in TG (-60%, $p$ <0,05) cells as compared to cells treated with TNF-α alone, similarly to the observed anti-apoptotic effect of hemin (Fig. 5A). Interestingly after 24 hours, only in TG cells treated with TNF-α, hemin and ATP it was observed an anti-apoptotic effect, significantly greater than all the other TG cells treatment groups ($p$ <0,05, Fig. 5B).
Taken together, these data suggest that the expression of the three genes is protective against TNF-α-induced cytotoxicity and apoptosis and the protection is more effective when the enzymatic substrates are added.

### 3.4. The simultaneous expression of hHO1, hCD39 and hCD73 protects TG cells from cell death

In order to further evaluate the protective response to TNF-α injury induced by the three exogenous genes, a propidium iodide incorporation assay was performed on WT and TG cells treated with TNF-α alone or with TNF-α, hemin and ATP at 24, 48 and 72 hours post treatment. In agreement with the results of cytotoxicity and caspase assays, the TG cells were protected against TNF-α injury as compared to WT cells at each time point (Fig. 6A, B and C). The percentage of cell death was increasing over the time in both WT and TG cells exposed to TNF-α treatment, although in the latter the injured cells were consistently lower at each time point (Fig. 6G). The simultaneous administration of enzymatic substrates, hemin and ATP, to WT cells treated with TNF-α induced a reduction of cell death at 48 and 72h of treatment as compared to WT cells treated with TNF-α alone. On the other hand, the combined treatment with TNF-α, hemin, and ATP of TG cells exacerbated the reduction of cell death observed in TG cells treated with TNF-α alone at each time point. Furthermore, only in TG cells treated with TNF-α, hemin and ATP the same amount of cell death over the time was found. Taken together these data confirmed the protection against TNF-α injury observed in cytotoxicity assays and suggest that the enzymatic substrates of the exogenous genes confer a persistent resistance of cells to death in TG cells




**G**

| | 24h | 48h | 72h |
|---|---|---|---|
| **WT**+TNF | 45,50% | 68,70% | 80,40% |
| **TG**+TNF | 36,20% | 41,80% | 54,90% |
| **WT**+TNF+HEM+ATP | 48,10% | 54,50% | 61,70% |
| **TG**+TNF+HEM+ATP | 37,20% | 40,70% | 42,60% |

**Figure 6**. TG cells are protected against TNF-α–induced cell death. WT and TG cells were treated for 24, 48 and 72 hours, with 50 ng/ml TNF-α alone or in combination with 20 μM hemin and 200 μM ATP. At the end of treatments cells were stained with propidium iodide and analyzed by flow cytometry. TG cells were protected against TNF-α injury as compared to WT cells at each time point (A, B and C), and the simultaneous treatment with exogenous protein substrates resulted in a further reduction of cell death in TG cells as compared to the same cell line treated with TNF-α alone (panel A and D, B and E, C and F, and G). No relevant presence of dead cells was found in untreated WT and TG at each time point (data not shown).

## 3.5. Molecular characterization of anti-inflammatory response mediated by the combination of the human genes

Trying to unravel the molecular mechanism of the anti-inflammatory response mediated by the combination of the

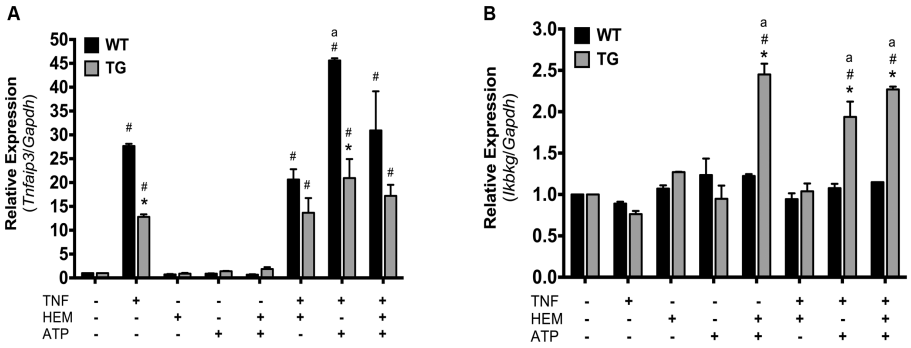



**Figure 7**. Changes in *Tnfaip3* (*A20*) (A) and *Ikgkb* (*Nemo*) (B) mRNA expression in WT and TG cells. Cells were incubated with 50 ng/ml TNF-α for 16h, alone or in combination with 20 μM hemin and/or 200 μM ATP. Murine *Tnfaip3* and *Ikgkb* mRNA were quantified by real-time PCR. The data (mean ± SD of three independent experiments), normalized for *Gapdh* gene, are expressed as fold change respect to the untreated cells. [*] indicates a significant difference between TG and WT within the same treatment (t Student, $p<0.05$); [**#**] indicates a significant difference as compared to untreated cells within the same cell type (ANOVA, $p<0.05$) [a] indicates a significant difference as compared to TNF-α treatment alone within the same cell type (ANOVA, $p<0.05$).

three human genes in transgenic cells, the expression of 84 TNF-α pathway-related genes was analyzed by quantitative $RT^2$–PCR Profiler Array (Qiagen). By this screening, *Tnfaip3* and *Ikbkg* genes were selected for their differential modulation as comparing the WT and TG cells and, within the same cell type, the treatments. The expression of these genes was validated in quantitative reverse transcriptase-PCR independently performed using RNA in each group and the results are summarized in Figure 7. The induction of *Tnfaip3* was observed in both WT and TG cells treated with TNF-α, alone or in combination with hemin and/or ATP (Fig. 7A). WT cells treated with TNF-α showed a strong induction of *Tnfaip3* gene ($p<0.05$, as compared to untreated cells), which markedly increased if TNF-α was combined with ATP ($p<0.05$ as compared to TNF-α treated WT cells). Moreover, in TG cells treated with TNF-α alone or in combination with ATP, *Tnfaip3* expression was induced as compared to untreated TG cells ($p<0.05$) but significantly lower as compared to corresponding treatment in WT cells ($p<0.05$). A significant up-regulation of

*Ikbkg* gene was observed only in TG cells treated with ATP plus TNF-α or Hemin or both (Fig. 7B), as compared to untreated TG cells ($p$<0.05) or to TNF-α treated TG cells ($p$<0.05). Taken together, these data suggest that TG cells exposed to TNF-α injury did not induce *Tnfaip3* gene as compared to WT cells but up-regulated the anti-apoptotic *Ikbkg* gene.

## 4. DISCUSSION

The results shown in the present paper contribute to the understanding of the protective role of a novel combination of human genes in animal cells against inflammatory stimuli. The aim of the work was to obtain a multicistronic construct to produce *in vitro* and *in vivo* models for the evaluation of the effects of human HO-1, CD39 and CD73 expression against TNF-α-induced injury. Each human gene used in this study has been reported to have anti-inflammatory and anti-apoptotic properties when overexpressed or induced in the cells or organisms [12,16,27-30] and we report here the protective effects of the combination of these genes against TNF-α injury.
The coding sequences of the three human genes were included in an expression cassette that allowed the simultaneous translation of three proteins starting from a single mRNA by using the F2A technology [17,18]. The order of genes encoded by the expression cassette was designed to maximize the likelihood of the correct processing and maturation of each protein product and we found the expected subcellular localization for hHO1, hCD73 and hCD39 in transfected (TG) cells. TG cells were sorted and enriched for the expression of hCD73. The western blotting analyses of the exogenous proteins in TG cells confirmed the strong expression of the human proteins and no evidence of incomplete separation of individual proteins was found.
In order to evaluate the protective effects of the combination of human genes in the TG cells against inflammatory stimuli, WT and TG cells were exposed to TNF-α injury. TNF-α was chosen to mimic an inflammatory settings because it plays one of the most important roles in inflammation and in inflammatory conditions [1,2,31]. WT and TG cells were treated with TNF-α alone or in combination with appropriate molecules that served

as a substrate for the enzymatic activity of exogenous proteins. Cytotoxicity and caspase assays were performed on WT and TG cells after exposure to TNF-α injury. TG cells were protected from TNF-α induced cytotoxicity in all treatments and over time. Moreover, TG cells treated with both ATP and hemin added to TNF-α were further protected as compared to TG cells treated with TNF-α alone, suggesting that in presence of substrates of both hHO1 and CD39/CD73, TG cells are able to efficiently counteract TNF-α-induced cytotoxicity.

Taking into account that caspases play an important role in TNF-α-induced apoptotic cell death [5], we determined caspase activity in WT and TG cells at 16 and 24 hours post-incubation with inflammatory stimuli (TNF-α) alone or in combination with enzymatic substrates of human genes (hemin and/or ATP). TG cells resulted protected from apoptosis induced by TNF-α, in each treatment and at each time-point, as compared to WT cells. The hemin treatment significantly decreased the caspase-3/7 activation in WT cells treated with TNF-α as compared to WT cells treated with TNF-α alone, at 16 hours. This is consistent with the protective effect of endogenous HO-1, induced by hemin treatment [32]. This effect disappeared at 24 hours in WT cells. In contrast, in TG cells treated with hemin and TNF-α the anti-apoptotic effect did not decrease over time. The combined treatment with hemin, ATP and TNF-α had an anti-apoptotic effect at 16 hours in WT cells, probably due to the protective effect of endogenous HO-1, which completely disappeared at 24 hours. On the contrary, in TG cells, the combined treatment with hemin, ATP and TNF-α reduced apoptosis at 16 hours and at 24 hours, as compared to all the other treatments. This suggests the anti-apoptotic role of both systems, proposing a beneficial effect of HO-1 and CD39/CD73 co-working. Taken together, these data suggest that the simultaneous activity of the three genes is necessary to further improve the protection against TNF-α injury.

The protective effect mediated by the three human genes against cell death induced by TNF-α was further investigated in longer co-incubation time by a propidium iodide incorporation assay as cell death index. The results demonstrated that the simultaneous administration of enzymatic substrates exacerbated the reduction of cell death observed in TG cells treated with TNF-α alone, thus confirming the protective effects

of the three human genes against TNF-α injury in TG cells. Taken together, these data suggested that the administration of the enzymatic substrates of the exogenous proteins conferred a persistent resistance of TG cells to TNF-α-induced cell death.
In order to better understand how the combined activity of the two systems represented by hHO-1 and hCD39/hCD73 were able to protect TG cells against TNF-α-mediated injury we investigated the molecular mechanisms involved in TNF-α pathway. To this extent, TNF-α pathway-related genes were analyzed by $RT^2$ array and, among the several genes resulted to be differentially modulated between WT and TG cells, we focused our attention on two genes, *A20* (*Tnfaip3*) and *Nemo* (*Ikbkg*). A20 is a zing finger protein known as a NF-κB-induced negative feedback regulator and inhibitor of apoptosis [33,34]. The results of our experiments showed, as expected, that the expression of *Tnfaip3* was rapidly induced in response to TNF-α in both cell types, although the induction was higher in WT cells as compared to each TG cells treated group (Figure 7). Nakajima and colleagues have recently reported that ROS cause induction of *Tnfaip3* and *HO-1* in several cell types, among which murine fibroblasts, leading to inhibition of basal and cytokine-inducible activation of NF-κB. In WT cells, HO-1 is activated by oxidative stress and inhibits NF-kB nuclear translocation, thus limiting the Nf-kB-dependent up-regulation of *Tnfaip3* gene transcription [35]. TNF-α-treated TG cells are not directly exposed to ROS, but they constitutively overexpress human HO-1 that could reduce the TNF-α-induced transcriptional activation of *Tnfaip3* by inhibiting the NF-kB translocation into the nucleus. This observation could explain why *Tnfaip3* gene induction was higher in WT cells as compared to TG cells treated groups, although the TG cells were protected from TNF-α-induced apoptotic cell death (Figure 5). The resistance to TNF-α-induced apoptosis was also reported in presence of the overexpression of a dominant negative C-terminal truncation mutant of p65/ RelA although it suppressed the induction of *Tnfaip3* gene [36], supporting our hypothesis that other anti-apoptotic signals can be activated even in absence of *Tnfaip3* induction.
It has been demonstrated that, upon TNF-α binding, the TNFR1 forms two different and consecutive complexes. The complex I controls the expression of anti-apoptotic proteins and the

complex II triggers cell death process [1,5,25,37]. The complex I is responsible for the downstream activation of the transcriptional activation of NF-kB through the regulatory subunit of the IKK complex, Nemo [5]. In this context, Nf-kb promotes pro-survival signaling within the cells. The gene expression analysis of *Nemo* showed a significant up-regulation of this gene in TG cells treated with ATP plus TNF-α or Hemin or both at 16 hours post-treatment (Figure 7B), as compared to untreated or to TNF-α treated TG cells. This suggested that *Nemo* modulation could be dependent to ATP administration to cells. Moreover, expression of *Nemo* was markedly lower in WT cells compared to TG cells within these three treatments (Figure 7B). On the other hand, TNF-α plus hemin administration to TG cells did not induce *Nemo* expression. This behaviour of the *Nemo* regulation in presence of hemin needs further experiments to be explained, although it can be hypothesized a protective effect of the HO-1 activity that could have abrogated the cell's need of *Nemo*'s up-regulation. In summary, TG cells up-regulated *Nemo*, which promotes pro-survival effects of Nf-kB, in response to TNF-α injury when ATP is added to the medium, resulting in a protection against TNF-α induced cell death.

This study demonstrated, for the first time, the protection against inflammatory stimuli of a combination of three human genes simultaneously expressed in murine cells via a tricistronic vector. The protective effects against TNF-α-induced cytotoxicity and cell death, mediated by hHO1, hCD39 and hCD73 genes were observed in transfected cells and this effect was further improved by administrating enzymatic substrates of the human genes to the cells. Moreover, a gene expression analyses demonstrated that the expression of the three genes has a role in modulating key downstream regulators of TNF-α signalling pathway, as *Tnfaip3* and *Nemo*, that promoted pro-survival phenotype in TNF-α injured cells.

**KEYWORDS**

Tumor Necrosis Factor Alpha; Inflammation; Heme Oxygenase-1; Ectonucleotidases; Cytoprotection; 2A peptide

## ABBREVIATIONS LIST

TNF-α: tumor necrosis factor-alpha; F2A: Foot and Mouth Disease Virus 2A sequence; hHO-1: human heme oxygenase 1; hCD73: human ecto-5'-nucleotidase; hCD39: human ecto-nucleoside triphosphate diphosphohydrolases; NF-κB: nuclear factor κB; TNFR1: TNF receptor 1; TNFR2: TNF receptor 2; WT: wild type; TG: transgenic; PI: propidium iodide.

## ACKNOWLEDGEMENTS

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## SUPPLEMENTARY METHODS.

### Triple cistronic vector construction.

Oligonucleotides F2A1-1 (5'-CTA GCG TGA AAC AGA CTT TGA ATT TTG ACC TTC TCA AGT TGG CGG GAG ACG TGG AGT CCA ACC CAG GGC CCG GCA GCG GCC-3') and F2A1-2 (5'-TTA AGG CCG CTG CCG GGC CCT GGG TTG GAC TCC ACG TCT CCC GCC AAC TTG AGA AGG TCA AAA TTC AAA GTC TGT TTC ACG-3') where annealed form an *Nhe*I-*F2A1-Afl*II fragment that contains the F2A coding sequence. The plasmid pcDNA3.1+ (Life Technologies) was restricted with *Nhe*I and *Afl*II and the *Nhe*I-*F2A1-Afl*II fragment inserted to form plasmid pcDNA3.1-F2A1. Oligonucleotides F2A2-1 (5'-GAT CCG TGA AAC AGA CTT TGA ATT TTG ACC TTC TCA AGT TGG CGG GAG ACG TGG AGT CCA ACC CAG GGC CCG GCA GCG GCC-3') and F2A2-2 (5'-TCG AGG CCG CTG CCG GGC CCT GGG TTG GAC TCC ACG TCT CCC GCC AAC TTG AGA AGG TCA AAA TTC AAA GTC TGT TTC ACG-3') when annealed form a *Bam*HI-*F2A2-Xho*I fragment. The plasmid pcDNA3.1-F2A1 was restricted with *Bam*HI and *Xho*I and the *Bam*HI-*F2A2-Xho*I fragment inserted to form plasmid pcDNA3.1-F2A1-F2A2.

The human Heme Oxygenase 1 (hHO-1) gene was amplified from S/MAR-pcDNA3-hHO1 plasmid (Vargiolu et al., 2010) by the PCR using oligonucleotides *NheI-EcoRI-Kozak-HO1fw* (5'-GCTAGCGAATTCCGGATGGAGCGTCCGCAA-3') which introduced *Nhe*I and *Eco*RI restriction site and a Kozak sequence just upstream the hHO-1 coding sequence and *NheI-HO1NOSTOPrev* (5'-GCTAGCCATGGCATAAAGC-3') which removed the stop codon and introduced *Nhe*I restriction site downstream the hHO-1 coding sequence. PCR product was restricted with *Nhe*I and ligated into pcDNA3.1-F2A1-F2A2 similarly restricted to form pcDNA3.1-hHO1-F2A1-F2A2.

The human Ecto-5'-Nucleotidase (hE5'N or hCD73) gene was amplified from S/MAR-pcDNA3-hCD73 plasmid (Vargiolu et al., 2010) by the PCR using oligonucleotides *AflII-CD73fw* (5'- CTTAAGATGTGTCCCCGAGCCGC-3'), which introduced *Afl*II restriction site just upstream the hCD73 coding sequence and *BamHI-CD73NOSTOPrev* (5'-GGATCCTTGGTATAAAACAAAGATCACTGC-3'), which removed the stop codon and introduced *Bam*HI restriction site

downstream the hCD73 coding sequence. PCR product was restricted with *Bam*HI and ligated into pcDNA3.1-hHO1-F2A1-F2A2 similarly restricted to form pcDNA3.1-hHO1-F2A1-hCD73-F2A2.
The human Ecto-Nucleoside Triphosphate Diphosphohydrolase 1 (hENTPD1 or hCD39) gene was amplified from S/MAR-pcDNA3-hCD39 plasmid (Vargiolu et al., 2010) by the PCR using oligonucleotides *XhoI-CD39fw* (5'-CTCGAGATGGAAGATACAAAGGAGTCTAACG-3') which introduced *Xho*I restriction site just upstream the hCD39 coding sequence and *EcoRI-XhoI-CD39rev* (5'-CTCGAGGAATTCCTATACCATATCTTTCCAGAAATATGAAG-3'), which introduced *Eco*RI and *Xho*I restriction sites downstream the hCD73 coding sequence. PCR product was restricted with *Xho*I and ligated into pcDNA3.1-hHO1-F2A1-hCD73-F2A2 similarly restricted to form pcDNA3.1-hHO1-F2A1-hCD73-F2A2-hCD39. Finally, in order to achieve the expression controlled by the unsilenced promoter CAAGS, the entire coding cassette was moved into the final plasmid pCX-C1 (a pCX-EGFP plasmid(Okabe et al., 1997) to which a neomycin resistance cassette has been added), obtaining the construct pCX-hHO1-F2A1-hCD73-F2A2-hCD39-C1, which was called pCX TRI-2A.

## SUPPLEMENTARY RESULTS

**Fig. S1 - PCR analysis on genomic DNA.**
PCR were performed on 75ng of genomic DNA extracted from WT and TG cells. Two primer pairs were used: 5'HO1 fw (CTGGAGGAGGAGATTGAGCG) / 2A rev (CGCCAACTTGAGAAGGTCAAAA) pair that covers the region from 5' of hHO1 CDS to the first 2A sequence; intern CD73 fw (TGTTGGTGATGAAGTTGTGG) / 2A rev (CGCCAACTTGAGAAGGTCAAAA) pair that covers the region from hCD73 CDS to the second 2A sequence. Results show the presence of amplicons with expected size, respectively 753bp for hHO-1 and 1297bp for hCD73. As positive control 75ng of gDNA from WT cells mixed with $10^2$ copies of pCX TRI-2A plasmid were used.

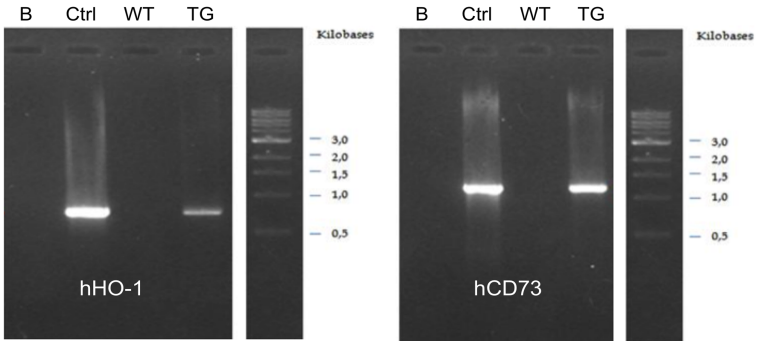


**Fig. S2 - PCR analysis on RNA.**
End-point PCR were performed on 25ng of cDNA from WT and TG cells. Two primer pairs were used: 5'HO1 fw (CTGGAGGAGGAGATTGAGCG) / 2A rev (CGCCAACTTGAGAAGGTCAAAA) pair that covers the region from 5' of hHO1 CDS to the first 2A sequence; intern CD73 fw (TGTTGGTGATGAAGTTGTGG) / 2A rev (CGCCAACTTGAGAAGGTCAAAA) pair that covers the region from hCD73 CDS to the second 2A sequence. Results show the presence of amplicons with expected size, respectively 753bp for hHO-1 and 1297bp for hCD73. $10^3$ copies of plasmids diluted into 25ng of WT cDNA were amplified as positive controls of PCR reaction. Phosphoglycerate kinase (PGK) housekeeping end-point PCR were performed using PGK1-HK-fw (GTATCCCTATGCCTGACAAGT) / PGK1-HK-rev (TTCCCTTCTTCCTCCACAT) primers pair, on 25ng of cDNA from WT and TG cells. Expected size band, 187bp, is visible in RT+ of each type of cells.

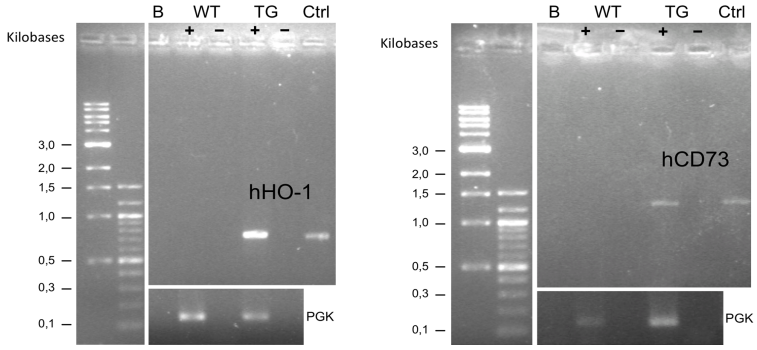

**Table S1 – Oligonucleotides used for real time PCR experiments.** The primers name and sequences are reported. The melting temperature (Tm) is indicated in Celsius grade. Primers for *Tnfaip3* and *Ikbkg* genes were designed by using Primer3 software (Untergasser A, *et al*. Primer3Plus, an enhanced web interface to Primer3. *Nucl. Acids Res.* 2007 35: W71-4). Primer sequences for *Gapdh* gene were recovered from PrimerBank repository (Spandidos A, *et* al. PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. *Nucl. Acids Res*. 2010 38: D792-9).

| Primer Name | Sequence | Tm |
|---|---|---|
| *Tnfaip3 fw5* | 5'-GAA AAC AAG GGC TTT TGC ACT CT-3' | 60 |
| *Tnfaip3 rev5* | 5'-CAG GCA CGG GAC ATT GTT CT-3' | |
| *Ikbkg fw2* | 5'-GAG GCC CTG GTA GCC AAA C-3' | 60 |
| *Ikbkg rev2* | 5'-ATG GCA GCC AAC TTT CAG CTT-3' | |
| *Gapdh PB1 fw* | 5'-AGG TCG GTG TGA ACG GAT TTG-3' | 60 |
| *Gapdh PB1 rev* | 5'-TGT AGA CCA TGT AGT TGA GGT CA-3' | |

# Chapter 3

## Functional analysis of expression of human ecto-nucleoside triphosphate diphosphohydrolase-1 and/or ecto-5'-nucleotidase in pig endothelial cells

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## ABSTRACT

Adenine nucleosides and nucleotides are important signaling molecules involved in control of key mechanisms of xenotransplant rejection. Extracellular pathway that converts ATP and ADP to AMP, and AMP to adenosine mainly mediated by ecto-nucleoside triphosphate diphosphohydrolase 1, (ENTPD1 or CD39) and ecto-5'-nucleotidase (E5NT or CD73) respectively, is considered as important target for xenograft protection. To clarify feasibility of combined expression of human ENTPD1 and E5NT and to study its functional effect we transfected pig endothelial cell line (PIEC) with both genes together. To do this we have produced a dicistronic construct bearing F2A sequence in frame between human E5NT and human ENTPD1 coding sequences. PIEC cells were mock-transfected as transfection control or transfected with plasmids encoding human ENTPD1 or human E5NT. PIEC cells were exposed to 50 μM ATP or 50 μM ADP or 50 μM AMP. Conversion of extracellular substrates into products (ATP/ADP/AMP/adenosine) was measured by HPLC in the media collected at specific time intervals. Following addition of AMP, production of adenosine in the medium of E5NT/ENTPD1- and E5NT- transfected cells increased to 14.2±1.1 and 24.5±3.4 μM respectively while it remained below 1 μM in controls and in ENTPD1-transfected cells. A marked increase of adenosine formation from ADP or ATP was observed only in E5NT/ENTPD1-transfected cells (11.7±0.1 and 5.7±2.2 μM respectively) but not in any other condition studied. This study indicates feasibility and functionality of combined expression of human E5NT and ENTPD1 in pig endothelial cells using F2A sequence bearing construct.

## INTRODUCTION

Extracellular adenine nucleosides and nucleotides are important signaling molecules that mediate different biological effects via cell surface purinergic receptors [1]. Extracellular pathway of catabolism of ATP, ADP and AMP to adenosine is mediated by a cascade of ectonucleotidases such as ecto-nucleoside triphosphate diphosphohydrolase 1, (ENTPD1 or

CD39) and ecto-5' nucleotidase (E5NT or CD73) [2]. It was reported that rejection in xenotransplantation experimental models causes the loss of activity of porcine ENTPD1 and E5NT resulting in a reduced capacity to convert pro-inflammatory and pro-aggregatory nucleotides to adenosine [3]. Overexpression of both human ENTPD1 and E5NT could enhance ATP and ADP breakdown and adenosine production [4]. However, exact role of these enzymes and its impact on levels of nucleotide signaling molecules still needs evaluation. To assess feasibility and to clarify interaction between ENTPD1 and E5NT in regulation of concentrations of adenine nucleotides and adenosine we transfected porcine iliac artery endothelial cell line (PIEC) with human ENTPD1, human E5NT or both genes together.

## METHODS

We have produced a transgenic dicistronic construct bearing the 2A sequence of Foot and Mouth Disease Virus (F2A) in frame between human E5NT and human ENTPD1 coding sequences. F2A technology allows the equimolar expression of discrete protein products starting from one open reading frame [5]. The Porcine iliac artery endothelial cell line (PIEC) was transfected using 10µl of Lipofectamine 2000 (Invitrogen) with 4µg of plasmids encoding for human E5NT or human ENTPD1 or both genes, E5NT/ENTPD1, following manufacturer protocol. As a transfection control, PIEC cells were mock-transfected. Transfected cells were selected by Neomycin (400µg/ml, Sigma) treatment for 2 weeks. Transfection efficiency has been evaluated by FACS analyses and for each transgenic PIEC cell line it was obtained about 20% cells positive for the selected transgenic marker(s) (data not shown). After 15 min of pre-incubation with Adenosine Deaminase (ADA) inhibitor, erythro-9-(2-Hydroxy-3-Nonyl) adenine, EHNA (5 µM), confluent cells were incubated with 50 µM AMP, or ADP, or ATP (Sigma) and supernatant samples were collected after 0, 5, 15 and 30 min. Conversion of extracellular nucleotides into their products was measured by HPLC analysis [6]. Data shown are mean ± S.E.M. (n=5-7). ANOVA with Tukey's post hoc test was used to

compare cell experimental groups. Difference was considered significant with $p<0.05$.

## RESULTS AND DISCUSSION

During incubation with AMP, concentration of adenosine in the medium of E5NT/ENTPD1- and E5NT-transfected cells increased to 14.2±1.1 and 24.5±3.4 μM respectively, while it remained below 1 μM in ENTPD1-transfected cells and in controls (mock-transfected and wild type cells) ($p<0.05$) (Fig.1).

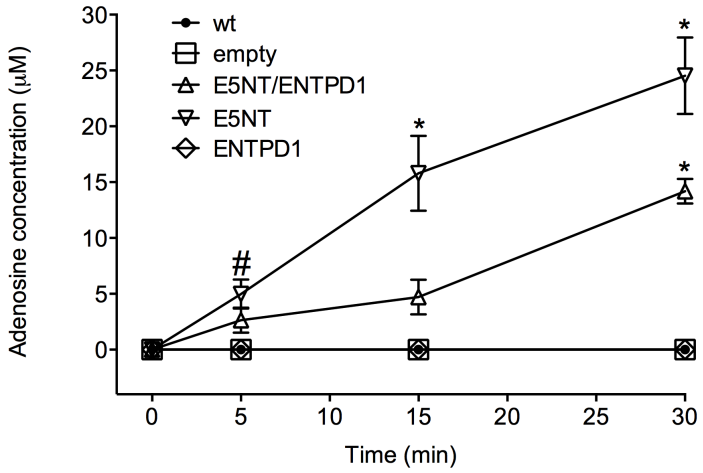



**Fig. 1** - Incubation with 50 μM AMP of transfected or control pig endothelial cells (PIEC). Adenosine concentration values represent mean ±SEM, n=5-7. * $p<0.05$ vs. all other groups; # $p<0.05$ versus other groups except E5NT/ENTPD1-transfected cells.

During incubation with ADP, formation of AMP in ENTPD1-transfected cells increased to 36.4±1.1 μM while it remained below 5 μM in controls and in E5NT-transfected cells ($p<0.05$) (Fig.2A). In E5NT/ENTPD1-transfected cells extracellular AMP reached a maximal concentration at 15 min (19.7±2.1 μM) after which it decreased to 11.4±2.9 μM (Fig.2A). At the same time, a substantial increase in adenosine formation from ADP was observed in E5NT/ENTPD1-transfected cells (11.7±0.1 μM, $p<0.05$), a slight increase in E5NT-transfected cells (0.66±0.17

µM, p<0.05) and not in the other cell groups (Fig.2B) was observed.

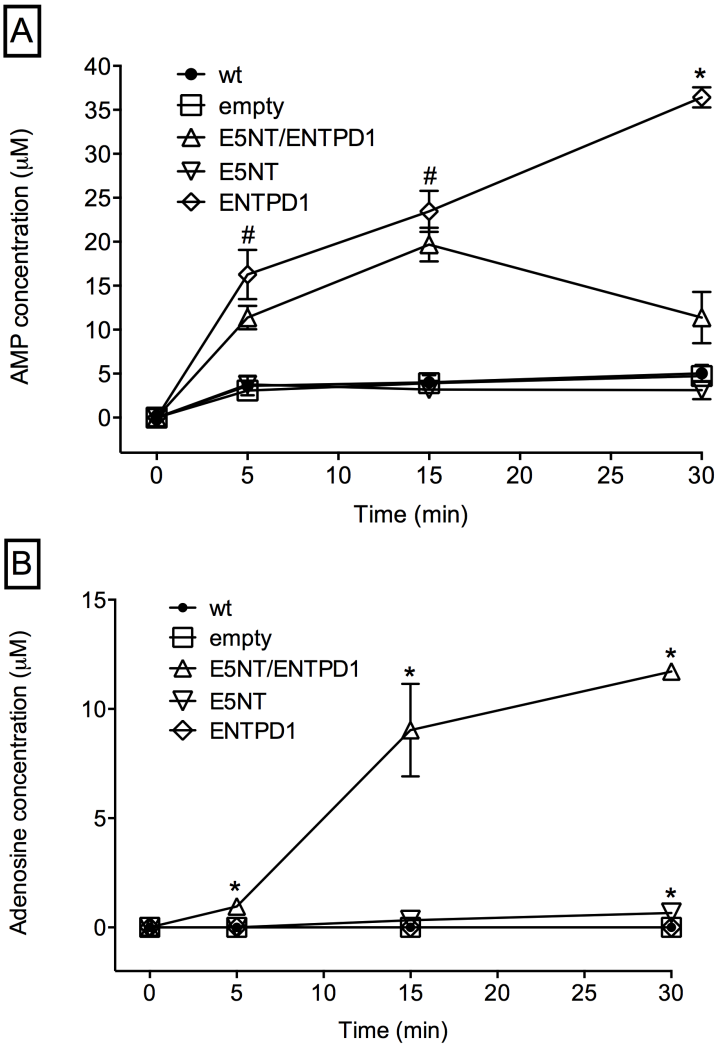
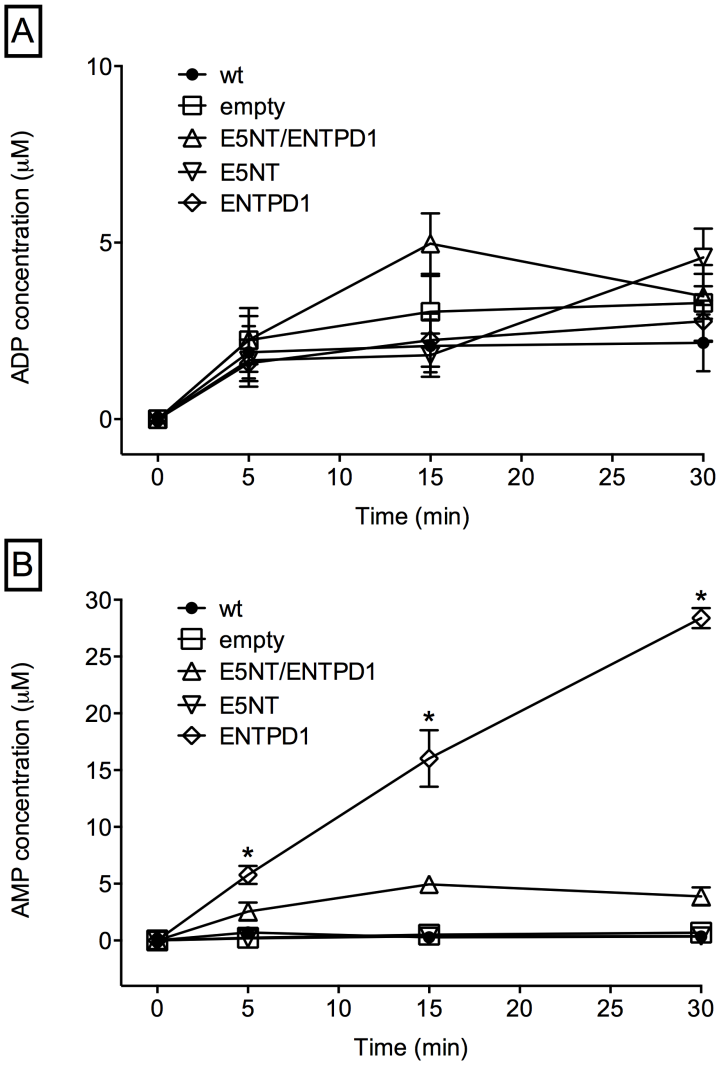



**Fig. 2** - Incubation with 50 µM ADP of transfected or control pig endothelial cells (PIEC). AMP (A) and adenosine (B) concentration values represent mean ±SEM, n=5-7. * p<0.05 vs. all other groups; # p<0.05 versus other groups except E5NT/ENTPD1-transfected cells.

During incubation with ATP, no significant differences were observed in ADP formation between all cell groups (Fig.3A). At

the same time AMP concentration in ENTPD1-transfected cells increased to 28.4±0.9 μM while it remained below 1 μM in controls and in E5NT-transfected cells ($p<0.05$) (Fig.3B). In E5NT/ENTPD1-transfected cells extracellular AMP reached a maximal concentration at 15 min (4.9±0.5 μM) after which it decreased to 3.9±0.6 μM (Fig.3B). A significant increase of adenosine formation from ATP was observed only in E5NT/ENTPD1-transfected cells (5.7±2.2 μM, $p<0.05$) and not in the other cell groups (Fig.3C).



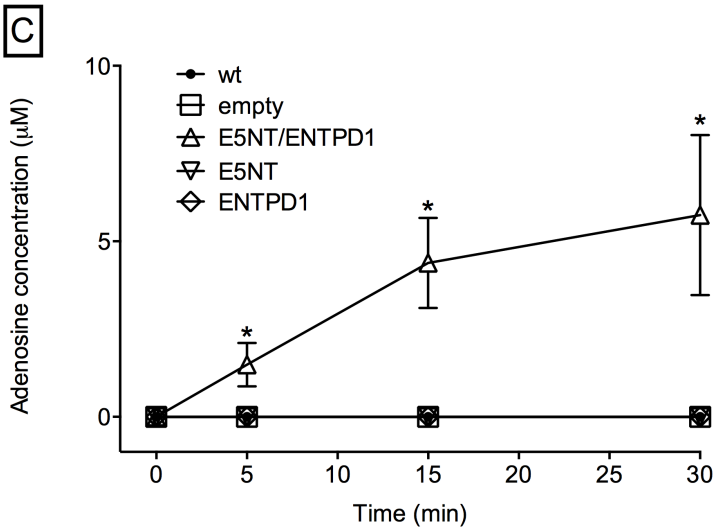



**Fig. 3** - Incubation with 50 µM ATP of transfected or control pig endothelial cells (PIEC). ADP (A), AMP (B) and adenosine (C) concentration values represent mean ±SEM, n=5-7. * $p<0.05$ vs. all other groups.

Our results highlight that the combined expression of E5NT and ENTPD1 modulates the extracellular breakdown of ATP and ADP leading to a marked and significant increase of adenosine production. On the contrary, no significant increase of adenosine formation by ENTPD1-transfected and control cells has been detected with ADP or ATP incubation. Some increase in production of adenosine by E5NT-transfected cells has been detected following 30 min of ADP incubation, suggesting an endogenous catabolic activity of ADP to E5NT substrate, AMP. However, a difference in adenosine formation from AMP between E5NT/ENTPD1- and E5NT-transfected cells has been observed (Fig.1). We hypothesize that this difference could be related to the 2A function. It was shown that F2A sequence allows the equimolar expression of two or more proteins by manipulating the translational apparatus [7]. Although the exact mechanism is not completely understood, it was proposed that during the translation of an F2A-bearing mRNA the ribosome stalls at the end of 2A sequence preventing the formation of peptide bond. At this stage the upstream peptide is released while the ribosome starts the translation of the downstream sequence [7]. Thus in E5NT/ENTPD1- transfected cells, this

particular translation process could slow down the normal processing of the two transgenic proteins causing an early lower amount of active E5NT per cell than the E5NT-transfected cell line. This hypothesis could explain why adenosine formation from AMP in E5NT/ENTPD1- transfected cells is apparently less efficient or delayed as compared to E5NT-transfected cells. Noteworthy, this phenomenon does not appear to affect the combined enzymatic activity of E5NT and ENTPD1 after the incubation with ADP or ATP (Fig. 2 and 3). This study indicates feasibility and functionality of combined expression of human E5NT and ENTPD1 in pig endothelial cells using F2A sequence bearing construct. This vector could be used to produce human E5NT and ENTPD1- double transgenic pigs by a single round of Somatic Cell Nuclear Transfer (SCNT)[8], or by Sperm Mediated Gene Transfer (SMGT)[9]. An increased production of adenosine could protect xenograft's endothelium against pro-inflammatory, pro-thrombotic and immunological effects of ATP and ADP thanks to its anti-inflammatory, anti-thrombotic and immunosuppressive properties [4,5]. This cell model we established could be useful for *in vitro* studies of these mechanisms.

## ACKNOWLEDGMENTS

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# Chapter 4

## Diet Induced Mild Hypercholesterolemia in Pigs: Local and Systemic Inflammation, Effects on Vascular Injury – Rescue by High-Dose Statin Treatment

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## ABSTRACT

**Objective:** The aim of the present study was to comprehensively evaluate systemic and local inflammation as well as progression of vascular inflammation in normal and mechanically injured vessels in a large animal model of mild hypercholesterolemia. Our aim was also to test the effect of high-dose statin treatment on these processes.
**Methods:** Pigs were kept for 120 days on a standard diet (SD, n=7), high-cholesterol diet (HCD, n=7) or high- cholesterol diet with Atorvastatin starting after 50 days (STATIN, n=7). Left carotid artery balloon injury was conducted in all groups after 60 days of diet treatment. Biochemical analysis together with evaluation of blood and tissue markers of vascular injury and inflammation were performed in all groups at the end of experiment.
**Results:** HCD compared to SD induced systemic inflammation demonstrated by increased number of circulating monocytes and lymphocytes. HCD compared to SD induced also local inflammation demonstrated by adipocyte hypertrophy and infiltration of T-lymphocytes in abdominal white adipose tissue, activation of hepatic stellate cells with infiltration of T- and B-lymphocytes and macrophages in the liver and increased macrophage content in lung parenchyma. These changes were accompanied by increased Intima/Media thickness, stenosis, matrix deposition and activated T-cell infiltrates in injured but not in uninjured contralateral carotid artery as we previously reported. The treatment with high-dose statin attenuated all aspects of systemic and local inflammation as well as pathological changes in injured carotid artery.
**Conclusions:** Diet related mild hypercholesterolemia induce systemic and local inflammation in the liver, lung and adipose tissue that coincide with enhanced inflammation of injured vessel but is without deleterious effect on uninjured vessels. High dose statin attenuated systemic and local inflammation and protected injured vessels. However, finding exact role of reduced systemic and remote inflammation in vascular protection requires further studies.

## INTRODUCTION

The long-term consequences of high-fat and/or high-cholesterol diet consumption are associated with an increased risk of cardiovascular disease, fatty liver disease, obesity and type 2 diabetes [1].The fatty streak and atheroma are the most prominent alterations associated with high cholesterol diet.

Most severe consequence of high cholesterol diet is, however, development of vascular changes. Our group and others demonstrated that deleterious effect of hypercholesterolemia is particularly evident in vessels injured by other factors such as mechanical stress during surgery [2,3]. Neointimal hyperplasia after vascular injury mainly consists of a proliferative response of smooth muscle cells, deposition of extracellular matrix, (ECM), systemic and local inflammation. Neointimal hyperplasia plays a decisive role in restenosis, a process actively sustained by the proliferation and migration of vascular smooth muscle cells (VSMCs) in response to various inflammatory stimuli. The change of phenotype of VSMCs results in capability to migrate and synthesize ECM.

Metabolic cells that are exposed to excess of nutrients and energy respond triggering a chronic inflammation not only in vasculature but also in other organs. The architecture of liver, white adipose tissue (WAT) and lung is characterized by a close interaction between metabolic and immune cells. In obese individuals, cells of the metabolic tissues (such as adipose tissue and liver) can eventually initiate the pro- inflammatory signaling cascade causing activation of leukocytes, thus leading to tissue-specific inflammation [4].

Recent evidence in mice suggested that dietary cholesterol exacerbates inflammatory changes due to an increased recruitment of leukocytes in WAT, particularly macrophages and T-lymphocytes [5–7]. Over time, WAT inflammation leads to the induction of cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin 6 (IL-6) with further recruitment of immune cells resulting in a stronger proinflammatory response [8]. High cholesterol loads result in a proinflammatory phenotype with activation of hepatic inflammatory genes and recruitment of several leukocyte subsets [5,9,10]. Cholesterol crystals have been found in early

diet-induced atherosclerotic lesions and it seems that these crystals could play a key role in triggering inflammatory response [11,12]. Interestingly, liver inflammation can develop independently of steatosis upon high-cholesterol feeding. Hence, it has been proven that the removal of cholesterol from the diet prevents hepatic inflammation without affecting steatosis [13]. The lung parenchyma contains alveolar macrophages derived from blood monocytes that patrol the airways to engulf foreign particles. In mice, hypercholesterolemia can increase the number of infiltrating pro-inflammatory macrophages associated with lung remodeling; in addition, hypercholesterolemia is considered a potential risk factor for asthma [14,15].

Heme oxygenase 1 (HO-1) is a stress-induced protein that is expressed in response to a variety of stimuli. HO-1 expression is implicated in protection against atherosclerosis. Previous studies have shown that the increase in HO-1 expression and activity by statins may have an anti-atherosclerotic effect in humans and in animal models of atherosclerosis [16,17].. HO-1 seems to be involved also in the regulation of nitric oxide synthase 2, inducible (iNOS) expression and nitric oxide (NO) production. Recent studies have shown that iNOS may be expressed in the human atherosclerotic plaque. Indeed, one of the hallmarks of a dysfunctional endothelium is diminished levels of bioavailable NO. Oral administration of L-Arginine, the precursor of NO, reduces neointimal hyperplasia in balloon-injured rat carotid arteries [18,19].

Statins, by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase, decrease endogenous cholesterol synthesis. Most data indicate that statins exert pleiotropic effects in addition to the lowering of serum cholesterol [20]. These include a broad range of anti-inflammatory mechanisms that inhibit leukocytes motility and adhesion to the vascular wall reducing the amount of tissue-infiltrating leukocytes [21], associated to a significantly decreased white blood cells (WBCs) count [22,23].

Mouse models have been widely used to evaluate the effects of high-fat feeding, however many of the evidences obtained are biased by differences in anatomy, lipid metabolism and lipoprotein profile. Unlike humans, even an excess of blood cholesterol in mice is carried by high-density lipoprotein (HDL)

and not by low-density lipoprotein (LDL), this could hamper the translation of experimental results obtained in mice to humans [24]. On the contrary, we and others have previously reported that the pig model could help to provide insights into metabolic and cardiovascular diseases because pigs share with humans several aspects of lipoprotein metabolism and the lipoprotein profile. In particular, when pigs were fed with high levels of saturated fat and cholesterol, plasma total cholesterol increased and complex atherosclerotic lesions, with features similar to those seen in humans, appeared [2,25,26]. Clinical studies concerning dietary cholesterol have focused on its effect on plasma lipids and lipoproteins without investigating the effect of hypercholesterolemia on tissue-specific inflammation.
In the present study we evaluated for the first time in a porcine model of mild-hypercholesterolemia whether high- cholesterol diet, besides vascular changes, is sufficient to induce a systemic and tissue-specific inflammation. We addressed the effects of high-dose atorvastatin treatment on entire spectrum of vascular, systemic and tissue specific inflammation to identify key factors involved in pathology.

## METHODS

### Animals

Large White female pigs (n=21) were purchased from a commercial breeder. Experiments involving animals were carried out according to a protocol approved by the Animal Care Committee of the Italian Minister of Public Health. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### Experimental Protocol

Pigs were allocated into three groups: SD (n=7), pigs fed a standard porcine diet (3.6% lipid content); HCD (n=7), pigs fed a high-cholesterol diet (27% lipid content: 5% cholesterol and 22% beef tallow); STATIN (n=7), high-cholesterol fed pigs (the same diet as for HCD group) treated orally with high-dose atorvastatin (Torvast; Pfizer) starting after 50 days of high-

cholesterol diet (80 mg/day/55Kg pig) until the end of the experimental procedure (average weight of pigs, about 70Kg). HCD-fed pigs received nearly 1100 Kcal extra/day compared to SD-fed pigs. The diet had an overall duration of 120 days.
After 60 days, the vascular injury in porcine carotid was induced as previously described [2].

**Laboratory tests**
Blood samples were collected from pigs at the beginning of diet administration and at sacrifice. The plasma obtained was kept at -80°C until the measurements. Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), total, LDL and HDL cholesterol and glucose were determined with colorimetric/enzymatic assays (Olympus system reagent, Olympus, Milan, Italy). All biochemical assays were carried out on an automated analyzer (Olympus AU 400). To determine counts of red blood cells, hemoglobin and hematocrit levels, WBCs, monocytes, lymphocytes, neutrophils, basophils, eosinophils, and platelets, complete blood counts were performed on EDTA anticoagulated blood samples using a Cell-Dyn 3500 system (Abbott Diagnostics). Hemograms were performed using a Cell-Dyn 3500 system (Abbott Diagnostics). Blood smears were May-Grunwald-Giemsa stained and visually evaluated for white blood cell differential counts. Analyses of inflammatory cytokines were performed using the SearchLight Chemiluminescent Array Kit specific for IL-1β, IL-6, interferon-gamma (IFNγ) and TNFα (Pierce Biotechnology). Assays were performed according to the manufacturer's instructions.

**Histological analysis and immunohistochemistry**
Balloon-injured left carotid arteries and contralateral carotid arteries (as negative control) as well as liver, lung and subcutaneous abdominal WAT were harvested at sacrifice, fixed in paraformaldehyde 4% and embedded in paraffin blocks. Serial sections (3 μm thickness) were cut and either stained with hematoxylin&eosin or Masson's Trichrome staining (Bio-Optica).
Immunohistochemical staining was performed on paraffin-embedded tissue sections of carotid arteries, liver, lung and subcutaneous abdominal WAT from each pig. To test antibody specificity, for each primary antibody used, control sections

were incubated a) without the primary antibody or b) with the antibody, which had been previously incubated in presence of the peptide antigen (when commercially available). Antigen retrieval was achieved by boiling in citrate buffer (pH 6.0). Tissue sections were incubated with primary antibodies for staining the following antigens: mouse anti-human CD45RO (M0742) DAKO, dilution 1:200; mouse anti-human MAC387 (sc-66204), Santa Cruz, dilution 1:200; mouse anti-human CD20 (18-0088), Zymed, dilution 1:300; mouse anti-human CD15 (MY1), Histo-Line Laboratories, dilution 1:100; mouse anti-human CD138 (MI15), DAKO, dilution 1:100; mouse anti-human smooth muscle actin (MS-113-P), Neomarkers, dilution 1:3000; mouse anti-human type I collagen (sc-59772), Santa Cruz, dilution 1:400; rabbit anti-porcine TGF-β1 (sc-146), Santa Cruz, dilution 1:250; rabbit anti-human TGFβRII (sc-400), Santa Cruz, dilution 1:50; rabbit anti-human HO-1 (spa-895), Stressgen, dilution 1:1000; rabbit anti-human iNOS (sc-651), Santa Cruz, dilution 1:200. VSMCs in carotid arteries have been detected using an antibody specific for the alpha smooth muscle actin (ab5694, Abcam, dilution 1:200). Proliferation index was evaluated by immunohistochemistry with a mouse monoclonal anti-Ki67 antibody (MM1, Vector Laboratories, dilution 1:100). Apoptosis index was evaluated by immunohistochemistry with a rabbit polyclonal antibody specific for the cleaved form of caspase-3 (Asp175, Cell Signaling Technology, dilution 1:50). A biotinylated secondary antibody was used for streptavidine-biotin-complex peroxidase staining (Vectastain Abc Kit, Vector Laboratories). DAB was used as chromogen (Sigma-Aldrich), and sections were counterstained with hematoxylin (Mayer's Hematoxylin, Lillie's Modification, Dakocytomation). At least three slides per tissue per pig were analyzed for each staining. The Aperio ScanScope GL Slide Scanner (Aperio Technologies, Vista, CA, USA) system was used to capture digital images with a 40x scanning magnification (20x with 2x magnification changer). The ScanScope console was equipped with a Nikon 20x/0.75 Plan Apochromat objective producing a 0.25 μm/pixel scanning resolution. The Aperio ImageScope software (version 8.2.5.1263) was used to acquire and process digital images.

**Morphometric analysis**

The carotid arteries were harvested 60 days after the balloon-injury procedure. The carotid arteries were fixed in paraformaldeyde 4% and embedded in paraffin blocks. Serial sections (3 um thickness) were cut and stained with hematoxylin and eosin. Two independent operators (MB and AF) blindly evaluated all the sections obtained for each carotid artery by microscopic analysis. The ten sections showing the worst damage were selected for a full morphometric analysis, including: Intima-Media Area ratio, Intima-Media Thickness ratio, Intima-Media Thickness, degree of stenosis, intimal thickness, medial thickness, internal elastic lamina perimeter, external elastic lamina perimeter, internal elastic lamina area, intimal area, external elastic lamina area, medial area. The computer assisted analysis has been performed using specific software (Aperio, Imagescope). The degree of stenosis has been evaluated using the following formula: (Area within the internal elastic lamina – Lumen Area)/(Area within the internal elastic lamina)*100. The results reported for each animal for Intima/Media (I/M) Area ratio, I/M Thickness ratio, I/M Thickness and degree of stenosis represent the average value obtained by considering the three sections showing the worst damage.

**Statistical analysis**

Data are presented as the mean ±S.E.M. Datasets from each experiment were tested for normal distribution using SigmaPlot software. For studies involving two groups, comparisons were made using two-tailed t-tests. For multiple group comparisons were made using one-way ANOVA followed by Tuckey post-hoc test. Correlations were determined using Spearman's rank order analysis. Differences were considered significant when $p<0.05$. The statistical analyses were done using SPSS 17.0.

## RESULTS

### High-dose atorvastatin markedly reduces total serum cholesterol and LDL-cholesterol

We already reported that after 120 days the high-cholesterol diet significantly increased serum total cholesterol, LDL-cholesterol [2] (Table 1). The high-cholesterol diet treatment increased also triglycerides as compared to SD (Table 1). The HCD pigs showed a tendency towards an increased glycemia compared with the SD-fed pigs (Table 1). Treatment with high-dose atorvastatin significantly reduced serum total cholesterol and LDL-cholesterol compared to the HCD (Table 1), with a significant increase in HDL-cholesterol compared to the SD. Atorvastatin treatment neither reduced triglyceridemia nor glycemia compared to the HCD (Table 1).

**Table 1.** Serum parameters in SD-fed pigs, HCD-fed pigs and HCD-fed pigs treated with atorvastatin. SD = Standard Diet; HCD = High Cholesterol Diet. Comparisons were made using one-way ANOVA followed by Tuckey post hoc test. * $p < 0.05$ versus HCD; † $p < 0.05$ versus SD.

| | SD (n=7) | HCD (n=7) | HCD + Atorvastatin (n=7) |
|---|---|---|---|
| **Total Cholesterol (mg/dL)** | 65.4 ± 2.7* | 89.9 ± 5.1 | 73.2 ± 3.1* |
| **LDL Cholesterol (mg/dL)** | 37.9 ± 2.1* | 56.5 ± 5.9 | 44.1 ± 4.3* |
| **HDL Cholesterol (mg/dL)** | 24.2 ± 1.6 | 28.1 ± 2.6 | 32.1 ± 1† |
| **Triglycerides (mg/dL)** | 20.1 ± 6.7* | 48.6 ± 25.5 | 38.7 ± 12.3 |
| **Glucose (mg/dL)** | 89.9 ± 19.2 | 123.4 ± 53.8 | 126.7 ± 39.5† |
| **ALT (U/l)** | 30.7 ± 9.3 | 23.1 ± 9.7 | 24.8 ± 2.3 |
| **AST (U/l)** | 22.0 ± 5.6 | 18.7 ± 6.8 | 20.8 ± 2.6 |
| **TNF- (pg/mL)** | < 3 | 4.98 ± 1.1* | < 3 |
| **IL-1 (pg/mL)** | < 3 | 6.15 ± 1.2* | < 3 |
| **IL-6 (pg/mL)** | < 3 | < 3 | < 3 |
| **IFNγ (pg/mL)** | < 3 | < 3 | < 3 |

## Hypercholesterolemia increases the amount of monocytes and lymphocytes

Blood analysis performed after 120 days showed a significantly increased number of total WBCs in HCD-fed pigs compared to SD-fed pigs (Table 2). Blood smear counts demonstrated that this increase depended on monocytes and lymphocytes with a non-significant increase in the number of circulating neutrophils. Compared to the findings in HCD pigs, treatment with atorvastatin significantly reduced the amount of circulating WBCs, monocytes and lymphocytes to levels resembling those found in SD-fed pigs (Table 2).

**Table 2**. Haematological parameters in SD-fed pigs, HCD-fed pigs and HCD-fed pigs treated with atorvastatin. SD = Standard Diet; HCD = High Cholesterol Diet. Comparisons were made using one-way ANOVA followed by Tuckey post hoc-test. *p < 0.05 versus HCD.

| | SD (n=7) | HCD (n=7) | HCD + Atorvastatin (n=7) |
|---|---|---|---|
| **WBCs (x10$^4$ cells/mm$^3$)** | 1.1 ± 0.1* | 2.1 ± 0.4 | 1.3 ± 0.1* |
| **Monocytes (x10$^3$ cells/mm$^3$)** | 0.2 ± 0.05* | 0.6 ± 0.09 | 0.2 ± 0.09* |
| **Lymphocytes (x10$^3$ cells/mm$^3$)** | 7.1 ± 0.7* | 10.2 ± 1.2 | 8.3 ± 0.7 |
| **Neutrophils (x10$^3$ cells/mm$^3$)** | 4.1 ± 0.9 | 6.9 ± 1.7 | 5.5 ± 0.8 |
| **Eosinophils (x10$^3$ cells/mm$^3$)** | 0.1 ± 0.04 | 0.3 ± 0.07 | 0.3 ± 0.02 |
| **Basophils (x10$^3$ cells/mm$^3$)** | 0.05 ± 0.02 | 0.1 ± 0.08 | 0.05 ± 0.04 |
| **Platelets (x10$^4$ Plts/mm$^3$)** | 33.3 ± 3.2 | 40.1 ± 3.8 | 33.3 ± 3.5 |
| **Mean Platelet Volume (fL)** | 4.0 ± 0.1 | 4.3 ± 0.2 | 4.2 ± 0.4 |
| **Red Blood Cells (x10$^6$ cells/mm$^3$)** | 5.5 ± 0.3 | 5.8 ± 0.3 | 5.6 ± 0.5 |
| **Hemoglobin (g/dL)** | 9.7 ± 0.5 | 10.4 ± 0.5 | 10.5 ± 0.3 |
| **Hematocrit (%)** | 30.4 ± 1.5 | 31.2 ± 1.7 | 30.0 ± 1.0 |

**Atorvastatin attenuates hypercholesterolemia-induced adipocyte hypertrophy and T-lymphocyte infiltration in subcutaneous abdominal adipose tissue**

Subcutaneous abdominal WAT from HCD-fed pigs showed adipocyte hypertrophy. The mean cross-sectional area of adipocytes from HCD-fed pigs was significantly increased compared to the adipocytes from SD-fed pigs. An immunohistochemical staining specific for activated T lymphocytes showed that high-cholesterol diet induced a significant increase in the amount of infiltrating T-lymphocytes into the abdominal WAT compared to SD. Treatment with atorvastatin effectively reduced both the adipocyte mean cross-sectional area and the amount of infiltrating T-lymphocytes compared to HCD (Figure 1). The immunohistochemical staining specific for MAC387 in WAT did not reveal a significant increase in the number of macrophages in HCD-fed and SD-fed pigs or after the treatment with atorvastatin (data not shown). In addition, the circulating levels of TNF-α and IFN-γ were significantly higher in HCD-fed compared to SD-fed pigs while no variations in circulating levels of IL-1β and IL-6 were observed (Table 1). Atorvastatin treatment significantly decreased the amount of circulating pro-inflammatory cytokines IFN-γ and TNF-α. (Table 1).

**Atorvastatin prevents the hypercholesterolemia- induced hepatic inflammation by reducing the number of infiltrating leukocytes**

Immunohistochemical liver analysis revealed increased leukocyte infiltration in the hepatic parenchyma of HCD-fed pigs compared with SD-fed pigs. The majority of leukocytes infiltrating the liver were macrophages (Figure 2). A significant increase in the amount of infiltrating T- and B-cells was also detected in livers from HCD-fed pigs compared with SD-fed pigs (Figure 2 B, G, Q and C, H, R). Non significant influxes of neutrophils and plasma cells were also seen in livers from hypercholesterolemic pigs compared to normocholesterolemic pigs (Figure 2 D, I, S and E, J, T). Atorvastatin treatment significantly decreased macrophage and T-lymphocyte infiltrates (Figure 2 F, K, P and G, L, Q), with a borderline reduction of B-lymphocytes (Figure 2 H, M, R).

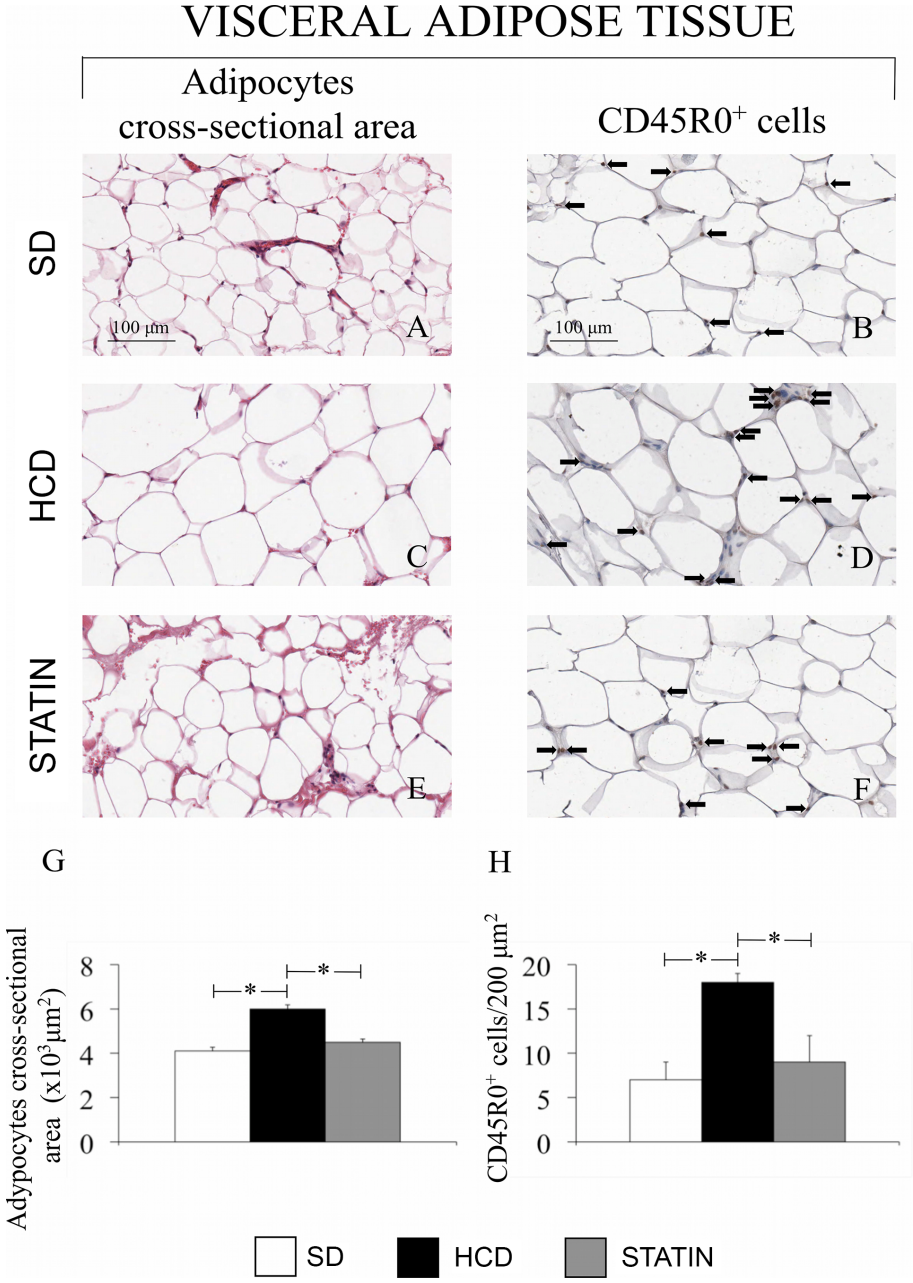


**Figure 1. Adipocyte hypertrophy and T-lymphocyte infiltration in abdominal WAT of HCD-fed pigs.** Hematoxylin and Eosin staining of abdominal subcutaneous WAT (A, C, E, G) and immunohistochemical staining specific for activated T-lymphocytes (CD45RO, B, D, F, H). Adipocyte area and T-lymphocytes are increased in HCD-fed pigs (C, D) compared with SD-fed (A, B) and atorvastatin-treated pigs (E, F). (Magnification 40x). *P<0.05.

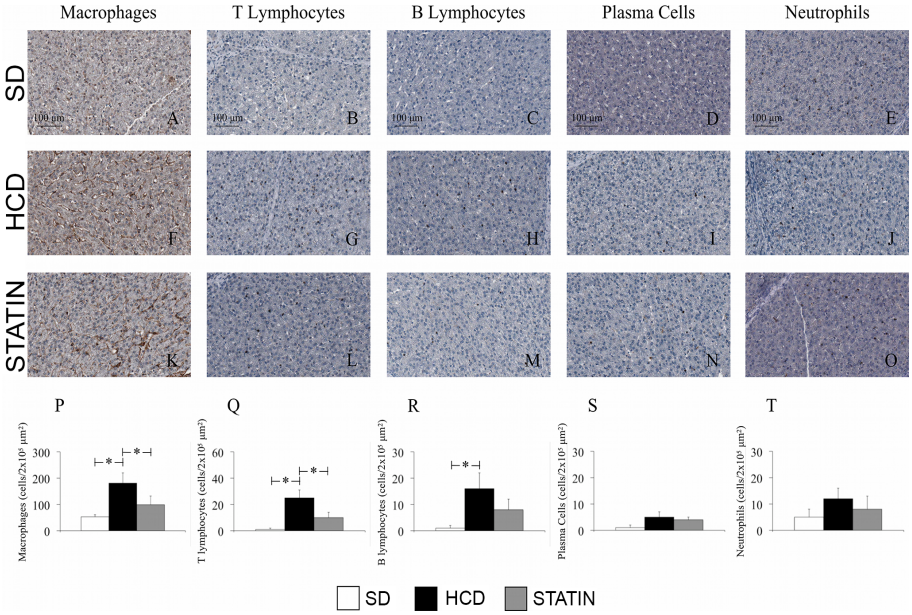


**Figure 2. Increased numbers of macrophages, T- and B-lymphocytes in livers from hypercholesterolemic pigs.** Immunohistochemical liver staining for macrophages (A, F, K, P), T-lymphocytes (B, G, L, Q), B-lymphocytes (C, H, M, R), plasma cells (D, I, N, S) and neutrophils (E, J, O, T). Infiltrating macrophages (A, F, P), T- (B, G, Q) and B-lymphocytes (C, H, R) but not plasma cells (D, I, S) and neutrophils (E, J, T) were significantly increased into the hepatic parenchyma of HCD-fed pigs compared with SD-fed pigs. The treatment of HCD-fed pigs with atorvastatin markedly reduced the amount of infiltrating macrophages (K, P) and T-lymphocytes (L, Q). (40x). *P<0.05.

An immunohistochemical analysis on liver sections specific for alpha-actin (alpha-SMA) showed a significant increase in the number of alpha-SMA-positive cells in HCD-fed pigs compared with SD-fed pigs (Figure 3 A, F, P). There was a significant reduction of α-actin-expressing cells following atorvastatin treatment in comparison to the HCD subset (Figure 3 F, K, P). The histology of liver tissue from HCD-fed pigs did not display steatosis (Figure 4 A, F), notwithstanding the presence of an abundant leukocyte infiltrate with increased activation of hepatic stellate cells (HSCs). Moreover, there were no signs of fibrosis with no differences in ECM deposition (Figure 4 G, L), type I collagen content (Figure 4 M, R), TGF-β1 and TGFβRII expression between HCD-fed, SD-fed and atorvastatin-treated pigs (Figure 3 D, I, N, S and E, J, O, T).

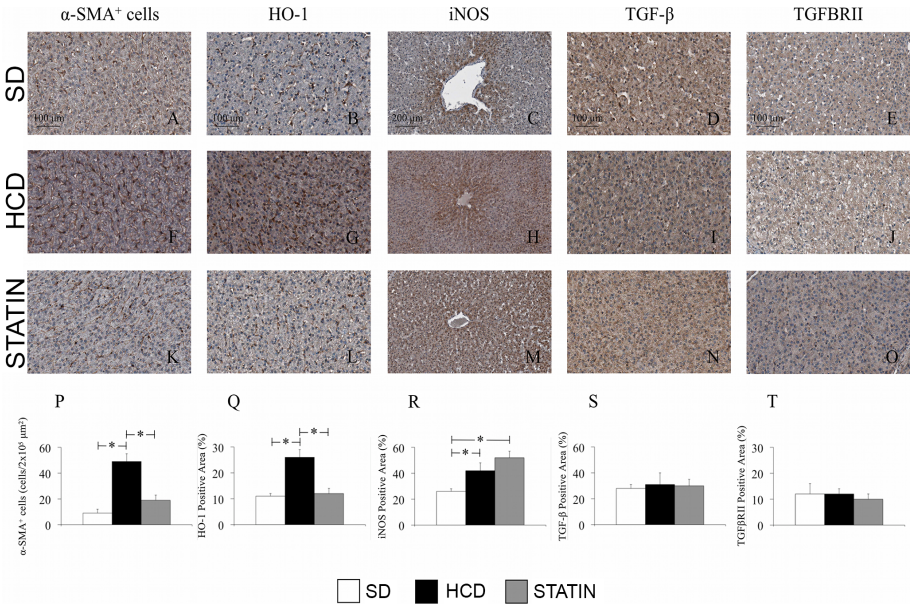


**Figure 3. Atorvastatin reversed hypercholesterolemia-induced liver inflammation by lowering activated-HSCs and stimulating HO-1 and iNOS expression.** Immunohistochemical liver staining for activated-HSCs (α-SMA, A, F, K, P), HO-1 (B, G, L, Q), iNOS (C, H, M, R), TGF-β1 (D, I, N, S) and TGFβRII (E, J, O, T). Hypercholesterolemia significantly increased the amount of activated HSCs (F, P), HO-1 (G, Q) and iNOS (H, R) expression without affecting TGF-β1 (I, S) and TGFβRII (J, T). Atorvastatin significantly reduced HSCs activation (K, P) and HO-1 expression (L, Q) but did not influence iNOS hepatic expression (M, R). (Magnification 40x).

Consistent with the histological findings, ALT and AST levels were not increased in hypercholesterolemic pigs compared to SD-fed pigs (23.1 ± 9.7 vs. 30.7 ± 9.3 U/l, p=ns; 18.7 ± 6.8 vs. 22.0 ± 5.6 U/l, p=ns).

In HCD-fed pigs the expression of the stress-inducible genes HO-1 and iNOS was significantly increased compared to SD-fed pigs (Figure 3 B, G, Q and C, H, R). Atorvastatin had a different effect on HO-1 and iNOS, with a significantly decreased expression of HO-1 compared to the HCD subset (Figure 3 G, L, Q), but a further increase in iNOS expression compared to SD-fed pigs (Figure 3 H, M, R).

**The high-cholesterol diet increases macrophage infiltration in lungs without lung remodeling**

Lung sections from HCD-fed pigs showed a significantly

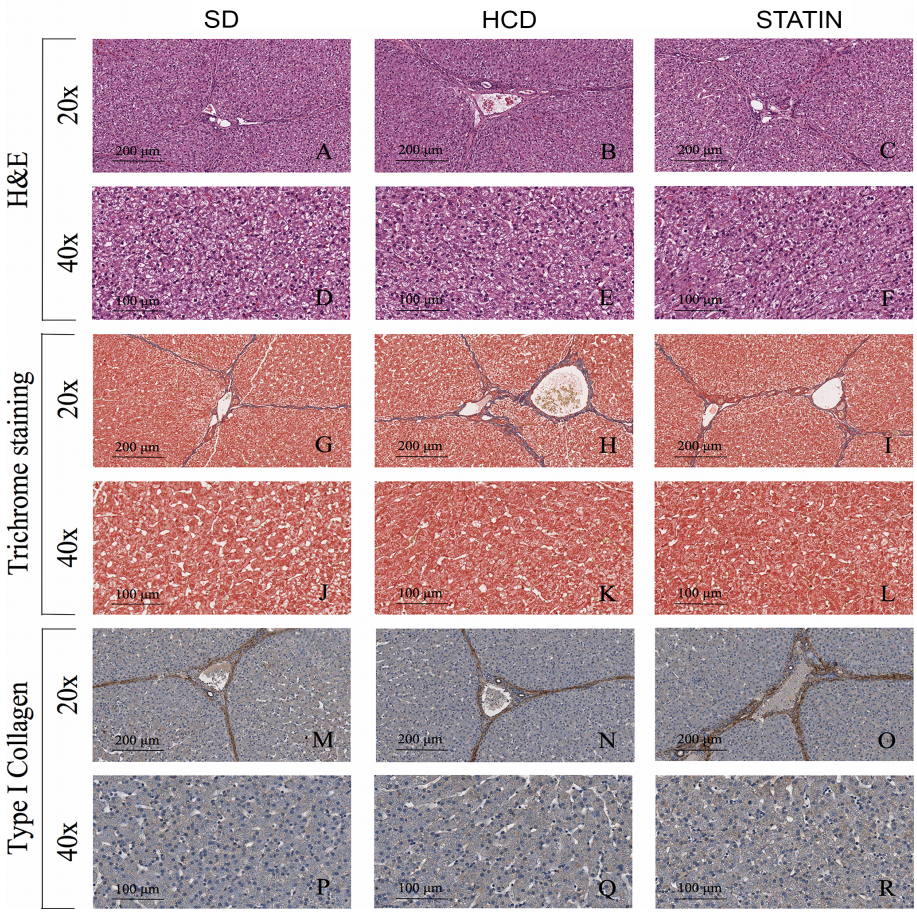


**Figure 4. Hypercholesterolemia did not induce liver steatosis and fibrosis in HCD-fed pigs.** Hematoxylin and Eosin staining (A-F), Masson's trichrome staining (G-L) and immunohistochemical analysis for type I collagen (M-R) of livers from HCD-fed pigs showed neither signs of steatosis (B, E) nor fibrosis (H, K, N, Q) having a parenchymal structure and ECM deposition comparable with those found in SD-fed (A, D, G, J, M, P) and atorvastatin-treated pigs (C, F, I, L, O, R). *P<0.05.

increased recruitment of macrophages compared to the amount of macrophages detected in SD-fed pigs (Figure 5 G, H, J). The treatment with atorvastatin significantly reduced the number of infiltrating mononuclear cells (Figure 5 H, I, J). The amount of lymphocytes was also increased in lungs from HCD- fed pigs even though the increase was not statistically significant (data not shown). No changes in tissue integrity and ECM deposition were detected in lungs from HCD-fed pigs (Figure 5A-C), even

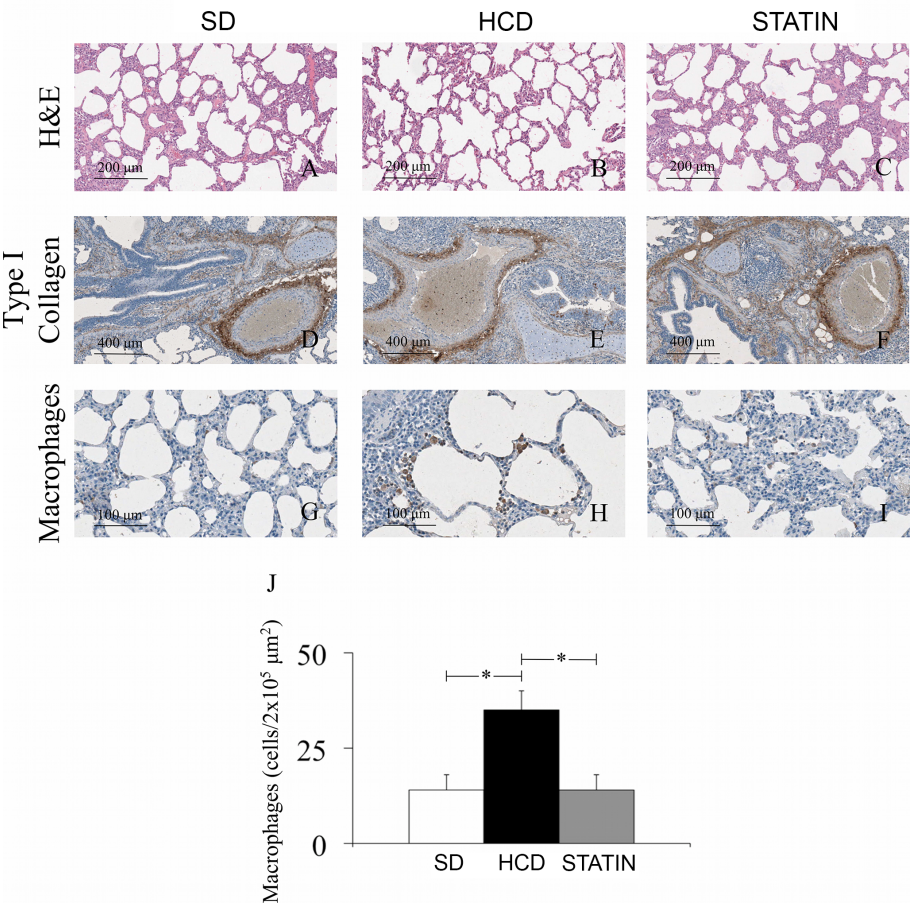


**Figure 5. Increased macrophage infiltration but not tissue remodeling in lungs of hypercholesterolemic pigs.** Hematoxylin and Eosin staining (A-C) and an immunohistochemical analysis specific for type I collagen (D-F) demonstrated that there were no signs of lung remodeling in HCD-fed pigs. The immunohistochemical staining specific for MAC387 showed a markedly increased number of macrophages in lungs from HCD-fed pigs (G, H, J). The treatment with atorvastatin dramatically reduced the number of infiltrating macrophages (I-J).

if a chronic presence of inflammatory cells within the lungs is known to induce tissue remodeling. In accordance with these findings, immunohistochemical analysis on lung sections specific for type I collagen (Figure 5 D-F), TGF-β1 and TGFβRII expression revealed the same degree of ECM deposition independent of diet or treatment with atorvastatin.

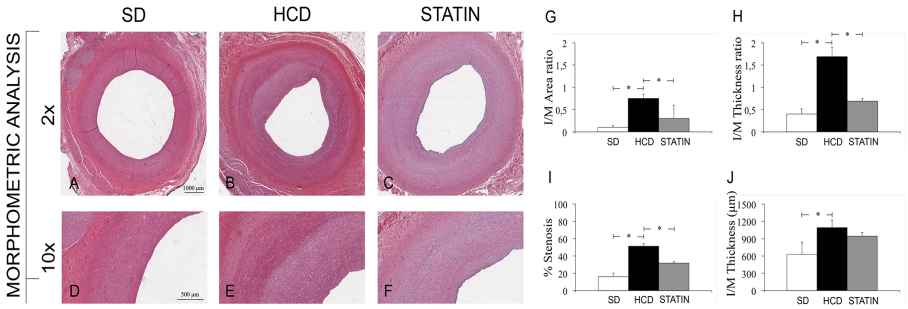


**Figure 6. I/M Area and I/M Thickness ratio in injured and contralateral carotid arteries.** Atorvastatin significantly reduced I/M Area and I/M Thickness ratio compared to HCD (B-C, E-F, G-H). The degree of stenosis was significantly greater in HCD compared to STATIN pigs (I). *P<0.05.

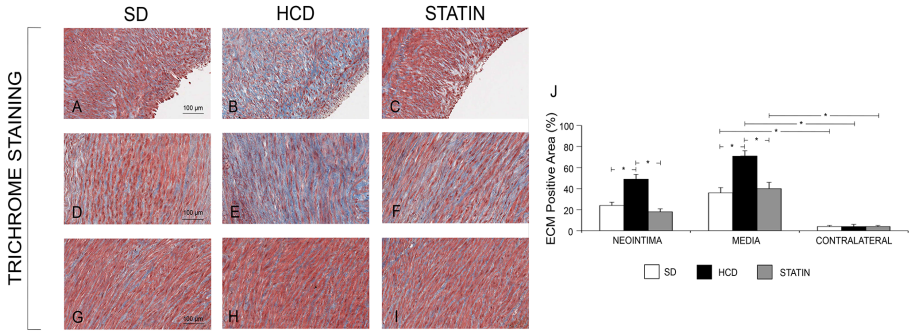


**Figure 7. Hypercholesterolemia induced matrix deposition.** The deposition of ECM was significantly increased in HCD compared to SD pigs, both in the neointima (A, B and J) and in the media (D, E and J). The deposition of ECM was significantly reduced in STATIN compared to HCD pigs, both in the neointima (C, J) and in the media (H, J). *P<0.05.

**Protective effect of atorvastatin treatment in vascular injury**

Atorvastatin significantly reduced I/M Area and I/M Thickness ratio compared to HCD (Figure 6). The degree of stenosis was significantly greater in HCD compared to STATIN pigs. The deposition of ECM was significantly increased in HCD compared to SD pigs, both in the neointima and in the media (Figure 7). The deposition of ECM was significantly reduced in STATIN compared to HCD pigs, both in the neointima and in the media. Atorvastatin restored the expression of the protective genes HO-1 and iNOS at the site of vascular injury,

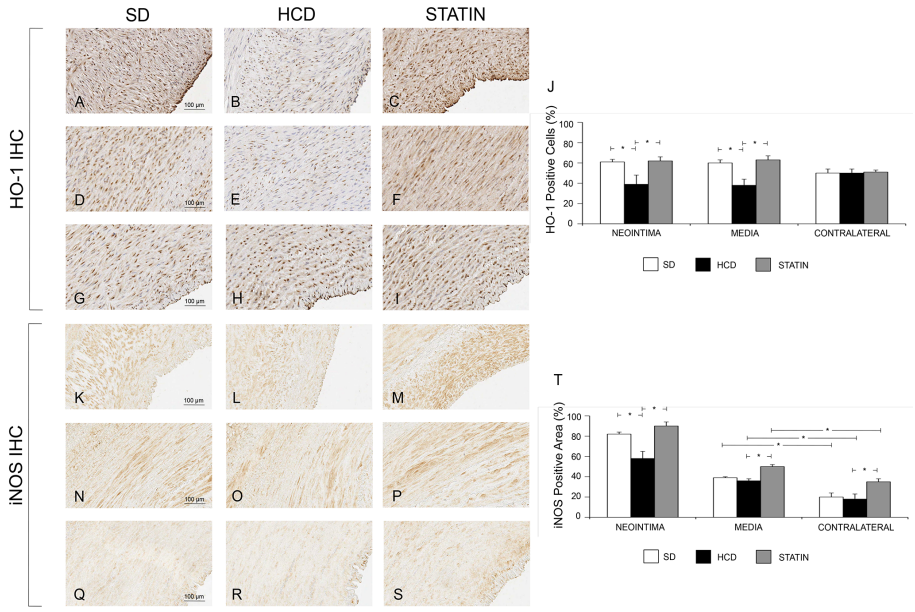


**Figure 8. Atorvastatin restored the expression of the protective genes HO-1 and iNOS at the site of vascular injury, enhancing iNOS expression also in contralateral uninjured arteries.** HO-1 expression was reduced in the neointima and injured media of CHOL compared to SD pigs (39±9% vs. 61±3, p=0.04, panels A, B and J, and 38±6 vs. 60±3, p=0.04,panels D, E and J). Atorvastatin treatment restored the expression of HO-1 to levels similar to SD group (62±4% vs. 39±9%, p=0.04, Figure A-C- J; and 63±4 vs. 38±6%, p=0.04, panels D, F and J). There were no differences in contralateral uninjured arteries (G, H, I and J). There was a significant reduction of iNOS expression following vascular injury in the neointima of HCD compared to SD pigs (58±7% vs. 82±2%, p=0.007, panels K, L and T). Atorvastatin treatment significantly restored the expression of iNOS in neointima compared to HCD (90±4% vs. 58±7%, p=0.005, panels K, M and T) and induced a significant expression in the media of injured and contralateral arteries in comparison to HCD and SD (50±2% vs. 39±1%, p=0.05 panels N, O, P and T; 35±3% vs. 18±5%, p=0.05, panels Q, R, S and T).

enhancing iNOS expression also in contralateral uninjured arteries (Figure 8). HO-1 expression was reduced in the neointima and injured media of HCD compared to SD pigs. Atorvastatin treatment restored the expression of HO-1 to levels similar to SD group. There were no differences in contralateral uninjured arteries. There was a significant reduction of iNOS expression following vascular injury in the neointima of HCD compared to SD pig. Atorvastatin treatment significantly restored the expression of iNOS in neointima compared to HCD

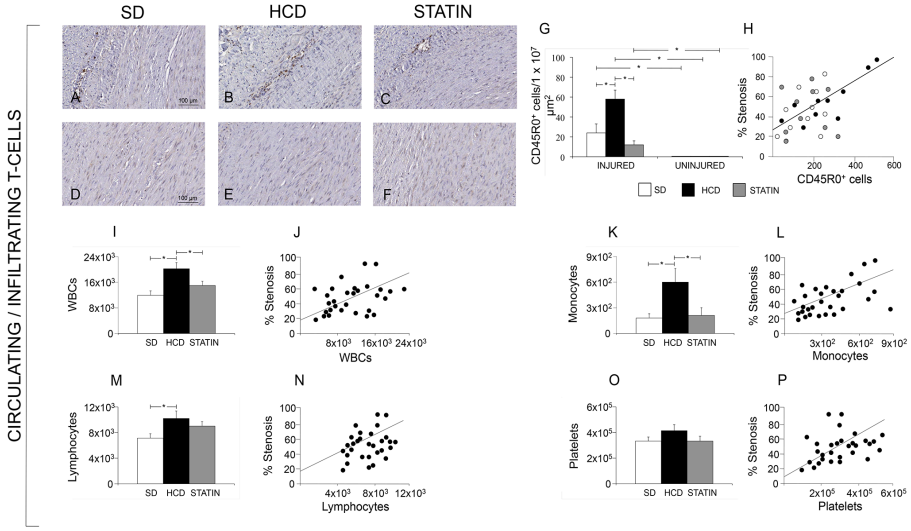


**Figure 9. Atorvastatin significantly decreased circulating leukocytes and activated T-lymphocytes (CD45RO-positive) infiltrates in injured carotids.** The number of circulating WBCs, monocytes and lymphocytes was increased by hypercholesterolemia compared to SD pigs (20215 ±1934/mm$^3$ vs. 11924±1388/mm$^3$, p=0.05, panel I; 600±160/mm$^3$ vs. 180±50/mm$^3$, p=0.04, panel K; and 10200±1160/mm$^3$ vs. 7120±700/mm$^3$, p=0.05, panel M, respectively). This significant difference was abolished by atorvastatin treatment. There was a correlation between circulating WBCs, monocytes, lymphocytes, platelets and the degree of stenosis (r=0.454 p=0.04, J; r=0.710 p=0.01, L; r=0.484 p=0.03, N; r=0.487 p=0.03, P). The CD45RO-positive cell infiltrates in injured carotids was significantly increased in HCD pigs compared to SD ones (58±9 vs. 249± cells/1x10$^7$ µm$^2$, p=0.05, panels A, B and G) while statin treatment reduced T-lymphocytes infiltration (12±4 vs. 58±9 cells/1x107 µm$^2$, p=0.02, panels B, C and G). There were no CD45R0-positive cells in the contralateral uninjured carotid arteries (D-F). CD45R0-positive cells infiltration was positively correlated with the degree of stenosis (r=0.837, p=0.003, H).

and induced a significant expression in the media of injured and contralateral arteries in comparison to HCD and SD. Atorvastatin significantly decreased circulating leukocytes and CD45RO-positive cell infiltrates in injured carotids (Figure 9). The number of circulating WBCs, monocytes and lymphocytes was increased by hypercholesterolemia compared to SD pigs. This significant difference was abolished by atorvastatin treatment. There was a correlation between circulating WBCs, monocytes, lymphocytes, platelets and the degree of stenosis. The activated T-lymphocytes cell infiltrates in injured carotids was significantly increased in HCD pigs compared to SD ones

while statin treatment reduced T-lymphocytes infiltration. There were no activated T-lymphocytes in the contralateral uninjured carotid arteries. Activated T-lymphocytes infiltration was positively correlated with the degree of stenosis.

## DISCUSSION

The present study demonstrates for the first time that statin treatment reduces generalized inflammation in spite of a minimal effect on lipids in a model of vascular injury in pigs fed high cholesterol diet. In fact diet is sufficient to cause a systemic inflammation by increasing the number of circulating WBCs, and to promote tissue-specific inflammation, by raising the amount of infiltrating leukocytes in WAT, liver and lung. These changes could contribute to increased vascular injury in hypercholesterolemic pigs. However statin treatment reversed not only enhanced vascular injury in hypercholesterolemic pigs but also attenuated most of systemic and localized inflammatory responses.

Previous studies have indicated that the pig could represent an ideal preclinical model providing insight into lipoprotein metabolism for several reasons. In particular, pigs have omnivorous habits and a lipoprotein distribution similar to that found in humans; high-cholesterol diets are able to induce human-like changes in the plasma lipoprotein profile of pigs, with ~ 60% of plasma cholesterol distributed in LDL particles [25,26].

In the present study we set up a model of mild-hypercholesterolemic pig, with total and LDL cholesterol concentrations 20% higher than in normocholesterolemic pigs. These supra-physiological concentrations are similar to those of the vast majority of patients, very different from other models showing an increase of cholesterol up to 700%. In fact, nearly 50% of Western Countries adults have total cholesterol concentrations at the level that the National Cholesterol Education Program (NCEP) expert panel considers “borderline-high risk” [2].

Epidemiological studies have reported that leukocyte counts rise in atherosclerotic patients and there is a positive correlation between increased amounts of circulating leukocytes and

coronary artery disease [27,28]. Transgenic and knockout mouse models in particular have proved useful to determine that the number of circulating leukocytes increase profoundly in atherosclerotic animals. Anyway, in these murine models the development of a leukocytosis similar to that seen in humans required the administration of a high-cholesterol diet with plasma cholesterol concentrations approaching 500-700 mg/dL [29–31]. Yet, the extent to which a slight increase of plasma cholesterol levels affects peripheral blood leukocytes remains to be thoroughly defined.

In this regard, blood analysis performed at sacrifice, after 16 weeks of hypercholesterolemic diet, displayed a significant increase in total WBCs, monocytes and lymphocytes in HCD-fed pigs compared to SD-fed pigs. This is in accordance with previous findings from our research group in which pigs fed a high-cholesterol diet showed an increased number of circulating WBCs, particularly monocytes [2]. The structural organization of WAT, liver and lung presents a highly vascularized milieu that allows a close interaction between metabolic and circulating immune cells. Chronic inflammatory reactions occurring in these tissues are characterized by a large infiltrate of lymphocytes that are mobilized to sites of injury where they produce cytokines that further activate macrophages and other inflammatory cells [1].

Previous studies in hypercholesterolemic mice have shown that macrophage and lymphocyte infiltration is of critical importance in WAT inflammation. T-lymphocytes may be enrolled during early adipose tissue inflammation preceding the appearance of macrophages, and the recruitment of these leukocytes to sites of inflammation is usually mediated by chemokines released from preadipocytes and adipocytes [32]. In addition, it has been shown that adipocyte hypertrophy coincides with the accumulation of T-cells and macrophages in mice fed a high fat diet [33,34]. Our results showed a significant increase of the mean cross-sectional area of adipocytes from HCD-fed pigs with a concomitant increase in the number of infiltrating T-lymphocytes, although in the absence of a significant increase in the amount of macrophages.

The occurrence of WAT inflammation in response to the excess of lipids leads to a low-level induction of inflammatory cytokines such as TNF-α, IL-1β and IL-6: this low-grade inflammation

induces the further recruitment and activation of many professional immune cells [4]. In our model, we have observed a significant increase of circulating TNF-α and IFN-γ in mild-hypercholesterolemic pigs compared to normocholesterolemic pigs without significant alterations in IL-6 plasma concentration.
Metabolic disorders related to lipid metabolism such as the increase of serum triglycerides, total cholesterol and LDL-cholesterol, are well known causes of liver injury characterized by inflammation, steatosis and fibrosis [35]. HSCs play a pivotal role in this process. HSCs are vitamin A-storing cells, located in the perisinusoidal space of Disse. Chronic liver injury caused by an excessive intake of lipids can result in a pro- inflammatory hepatic environment with HSCs activation: HSCs start to proliferate in response to cytokines, stimulate the recruitment of inflammatory cells, and finally produce large amounts of ECM [36]. The immunohistochemical analysis of liver in our study displayed a 5-fold increase in the number of activated HSCs (α-SMA-positive cells) in HCD-fed pigs compared with SD-fed pigs. We have also found an increased expression of the stress-inducible genes HO-1 and iNOS in livers from mild-hypercholesterolemic pigs.
The administration of a high-cholesterol diet in our preclinical model did not induce either steatosis or fibrosis, HSCs activation notwithstanding. The evaluation of ECM deposition by trichrome staining and the immunohistochemical analysis specific for type I collagen, TGF-β1 and TGFβRII expression did not show significant differences on liver sections from HCD- and SD-fed pigs. Interestingly, HSCs activation and leukocyte infiltration of hepatic tissue did not alter hepatic function. In particular, HCD-fed pigs did not display increased levels of ALT and AST after 16 weeks on a high-cholesterol diet compared to SD-fed pigs. It is widely demonstrated that the administration of large amounts of dietary cholesterol causes hepatic inflammation in both mice and rabbits and it has been recently showed that high-fat feeding in mice increases the hepatic recruitment of T-lymphocytes and macrophages [5,37,38]. In addition, a previous study showed a correlation between plasma total cholesterol levels and the development of hepatic inflammation rather than steatosis [13]. Our work highlights that the leukocytes infiltrating the liver of HCD-fed pigs were primarily macrophages, but there was also an increased

number of T- and B-lymphocytes. As already hypothesized in the case of WAT, T-lymphocytes may be responsible for the early changes that occur in the liver of hypercholesterolemic animals. The pro-inflammatory crosstalk between T-lymphocytes, hepatocytes and HSCs results in increased macrophage infiltration and consequently in chronic hepatic inflammation.

In our model, the inflammation extended beyond the WAT and the liver to the lung parenchyma, with a significantly increased amount of infiltrating macrophages in HCD-fed pigs compared to SD-fed pigs. This enrichment in lung macrophages was associated with neither parenchyma remodeling nor fibrosis. Moreover, no signs of increased type I collagen deposition as well as significant differences in the expression of TGF-β1 and TGFβRII were are observed. Our results are in contrast with a recent work from Naura et al. in which it has been shown that a high-fat diet induces the persistence of lung inflammation associated with tissue remodeling in apoE-KO mice [15].

To test whether reducing total cholesterolemia could determine a significant decrease of circulating leukocytes, a subset of HCD-fed pigs received high-dose atorvastatin (80mg/ die) for 8 weeks. Statins, the inhibitors of HMG-CoA reductase, are extensively used in medical practice for their cholesterol-lowering effect. Large clinical trials have demonstrated that this class of drugs greatly reduces cardiovascular-related morbidity and mortality in patients with and without coronary disease. Several works have established that statins, in addition to their lipid-lowering effects, exert immuno-modulatory actions being able to decrease the expression of adhesion molecules, chemokines and chemokine receptors on both leukocytes and endothelial cells, ultimately limiting the recruitment of leukocytes across the vessel wall [39,40]. The conventional statin therapy dosage is ranging from 10 to 40mg but there are some studies reporting aggressive lipid-lowering therapy in patients with carotid artery disease demonstrating a reduced echo lucency of the plaques after high-dose stain treatment [41,42].

Consistent with above considerations, we determined that treatment of animals with high-dose atorvastatin resulted in a significant reduction of serum total cholesterol and LDL-cholesterol concentration. However, no significant reduction in

triglycerides was noted.
Compared to the findings in HCD-fed pigs, treatment with atorvastatin markedly lowered the number of circulating WBCs, monocytes and lymphocytes. The immuno-modulatory effects of atorvastatin went beyond the reduction of circulating WBCs. Our findings demonstrated that high-dose atorvastatin treatment leads to a substantial decrease of the hypercholesterolemia-induced inflammatory infiltrates in WAT, liver and lung. In particular, treatment with atorvastatin in HCD-fed pigs prevented adipocyte hypertrophy limiting the number of infiltrating T-lymphocytes. Furthermore, statin-treatment considerably reduced the amount of circulating pro-inflammatory cytokines IFN-γ and TNF-α. In the liver, atorvastatin caused a significant reduction of activated HSCs and of the amount of infiltrating leukocytes, with a concomitant reduction of the stress-inducible gene HO-1 but without affecting the hepatic expression of iNOS. Last, atorvastatin treatment in HCD-fed pigs was effective in reducing the macrophage content in lung parenchyma.
Inflammatory response in liver, lung and adipose tissue correlated with enhanced intimal hyperplasia in carotid artery. Interestingly, no response was observed in contralateral uninjured artery. This highlights the importance of local injury and systemic/remote inflammation in the development of vascular pathology as we previously identified [2]. Atorvastatin treatment attenuated intimal hyperplasia and all its underlying mechanisms such as matrix deposition and activated T-lymphocytes infiltration. Restored expression of HO-1 and iNOS observed following atorvastatin treatment is another molecular event that indicate vascular benefit. Moreover, the atorvastatin-induced increased expression of iNOS in contralateral artery suggests that, besides systemic effects of statins, the local vascular effect could play an important role in vascular protection.
In conclusion, our study demonstrates for the first time in a clinically relevant porcine model of diet induced hypercholesterolemia that even a moderate increase in plasma cholesterol levels can induce a significant increase in the amount of circulating WBCs. This effect extends beyond systemic inflammation to tissue-specific inflammation in WAT, liver and lung. We also establish that a mild-

hypercholesterolemia is sufficient to induce an augmented leukocyte infiltrates in these organs. HCD-fed pigs treated with high-dose atorvastatin results in a reduction of total and LDL cholesterol and in markedly decreased systemic and tissue-specific inflammatory markers by preventing the development of an inflammatory milieu and the accumulation of infiltrating leukocytes.

## ACKNOWLEDGEMENTS

This work is dedicated to the memory of Prof. Fritz H. Bach, a dear friend and colleague whom we miss enormously.

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: ML RG MB SM MGC. Performed the experiments: MB SM AF AV MGC MG AC AZ. Analyzed the data: MB SM AF AV BEL MLB RG ML. Contributed reagents/materials/analysis tools: MF ML. Wrote the manuscript: RG ML RTS GMB MB SM.

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# *Chapter 5*

# Conclusions and Future Perspectives

During my PhD program I addressed my attention on the characterization and possible regulation of the inflammatory response, induced by pro-inflammatory cytokines in vitro, and hypercholesterolemic diet in vivo.

Thanks to the chance I had, to be primary involved in different projects in my PhD program, the study presented here embraces two aspects that at first may appear different, but share the property to act on the tissue as an “insult” to which the cells have to react.

On one side, in our laboratory we performed in vitro studies characterizing the protective effects, against inflammatory stimuli, of the simultaneous over-expression of three human genes well know to be involved in the down-regulation of all the inflammatory events associated with delayed xenograft rejection. On the other, we evaluated for the first time in a porcine model of mild-hypercholesterolemia whether high-cholesterol diet, besides vascular changes, is sufficient to induce a systemic and tissue-specific inflammation.

**Expression of human HO-1, CD39 and CD73 protects murine cells from TNF-α-mediated injury**

Production of genetically modified pigs for xenotransplantation might adequately represent the key step to solve the chronic gap between demand and availability of organs for transplantation. Thanks to the developments in the field of genetic engineering, several advances to “translate”

xenotransplantation to clinical application have been done. Animals transgenic for: α1,3-galactosyltransferase gene (GalT-KO), regulatory proteins of the complement cascade which counteract the humoral response due to non-Gal antigens (DAF, CD46, CD59), anti-coagulant, anti-inflammatory and anti-thrombotic proteins (CD39, CD73, thrombomodulin, TFPI), inhibitory proteins of the apoptotic processes (HO-1) and cellular immune system response modulators (TNFα, HLA-E/beta-2- microglobulin and CTLA-4Ig) has been produced [1].

Recent studies hypothesize that a state of systemic inflammation develops after pig organ xenotransplantation, which is generated by both adaptive and innate immune responses and suggested that inflammation can lead to activation of the coagulation system. Additionally, pro-coagulant proteins, e.g. thrombin, are considered as pro-inflammatory factors. In fact, a considerable crosstalk is deemed to exist between inflammation and coagulation, leading to escalation of each other [2]. Even if T cell-directed immunosuppression can control activation of coagulation induced by adaptive immune responses, pro-inflammatory signals induced by the innate immune system can still promote activation of coagulation [3]. Together, these observations indicate that the immune response to a pig xenograft cannot be considered in isolation and that equal attention needs to be directed to the innate immune, coagulation, and inflammatory responses.

Additional multiple genetic modifications in donor animals are necessary and different combinations of these will need to be

tested in relevant pre-clinical models. The 2A technology is the proposed tool to solve the issue of combining genetic modifications [4].

Numerous reports suggested HO-1, CD73 and CD39 as potentially protective molecules against the still remaining barriers. It is well known that these three genes are involved in the down-regulation of all the inflammatory events associated with delayed rejection such as ROS accumulation, endothelial cells activation with a consequent up-regulated expression of adhesion molecules, platelet adhesion, and ultimately thrombosis and extended intravascular coagulation followed by apoptotic cells death [5,6,7,8]. Although the protective role for each of these genes has been demonstrated in vitro, ex-vivo or in animal models [9,10,11,12], no one has investigated the combination of their beneficial effects in the attempt to reduce the inflammation response.

In our work we presented the anti-inflammatory potential role of these three human genes, through their simultaneous expression at equimolar level in murine cells. To this purpose, a new triple cistronic vector, containing the three genes linked in frame by two 2A sequences in a single ORF was produced, paid particular attention to the order of the genes CDS to maximize the likelihood of the correct processing and maturation of each protein product. To avoid the phenomenon of “slipstream translocation”, in which an intracellular protein encoded downstream of a protein containing a signal sequence can be translocated into the ER [13] and to maximize the

likelihood that each single protein product would be able to correctly reach its functional subcellular compartment we decided to place HO1 as a first gene, followed by CD73 and CD39, respectively as second and third gene of multi-gene coding sequence. We speculated that HO1 should be translated by free polysomes and then released in the cytosol by means of 2A mechanism of action, while ribosomes should proceed through the polycistronic mRNA and translate the N-terminal signal peptide of CD73 allowing its recognition and the formation of traslocon, by which both CD73 and CD39 should be directed to the secretory pathway.

In order to verify the correct enzymatic activity of protein products, as a result of their correct processing, and to test their supposed protective roles against inflammation stimuli, functional assays have been performed on transfected cells. In these experiments, we demonstrated the relevant reduction of cell cytotoxicity and apoptotic signalling and death occurring after exposure to TNF-α inflammatory stimuli in cells simultaneously expressing hHO-1, hCD73 and hCD39.

Among all the known physiological inducers of inflammation processes and apoptosis in mammalian cells, tumor necrosis factor alpha (TNF-α) is perhaps the most potent and well studied [14]. TNF-α elicits its pro-inflammatory signals by initially binding to receptors, TNFR1 and TNFR2, on the cell surface. TNFR1 and TNFR2 stimulate the cellular response to TNF-α via three principal and distinct signalling pathways, leading to the activation of AP-1 and NFκB transcription factors

or the activation of caspases and cell death. This means that by activating the same set of receptors, biological effects as diverse as proliferation, cell survival or cell death can be obtained depending on the quantitative balance between the pro and anti-apoptotic signals. To better evaluate the enzymatic activity of the three human proteins on cell cytotoxicity in response to TNF-α pro-inflammatory stimuli, transfected (TG) and wild type (WT) murine NIH3T3 cells were treated with appropriate molecules that served as substrate for HO-1 or the CD39/CD73 system. Hemin was used as HO-1 enzymatic activity substrate, since it is a porphyrin containing iron which is catabolized by HO-1, as it occurs for the heme in an in vivo context [15]. On the other hand, ATP was administered as a substrate of CD39 and CD73 proteins, to induce their combined enzymatic activity that consists in converting ATP to adenosine as a final product [16]. Cytotoxicity induced by TNF-α has been found relevantly lower in TG cells respect to WT cells in all treatments.

TNF-α-mediated apoptotic signalling depends on caspase 8 activation, a key early regulator of this process. Cleavage of BID (BH3 Interacting Death Domain) by caspase 8 results in the disruption of the mitochondrial membrane and the release of cytochrome c. Cytochrome c finally activates the Apaf-1/caspase-9 apoptosome resulting in effector caspase-3/7 activation and cell death execution [17]. TG cells resulted protected from apoptosis induced by TNF-α, in each treatment and at each time-point, as compared to WT cells.

Most is known about the mechanism by which TNF-α activates NF-kB. NF-kB pathway involves the interaction of TNF-α with its receptor at the cell surface (TNFR), which then recruits a cascade of proteins, resulting in the translocation of NF-kB to the nucleus. In the nucleus it binds to its consensus sequence (5-GGGACTTTC-3) and positively regulates the transcription of genes involved in immune and inflammatory responses, cell growth control and apoptosis [18]. Thus, we decided to investigated the molecular mechanisms involved in TNF-α pathway, in order to better understand how the combined activity of the two systems represented by hHO-1 and hCD39/hCD73 axis was able to protect transgenic cells against TNF-α-mediated injury. To this extent, TNF-α pathway-related genes were analyzed by $RT^2$array. Among the several genes resulted to be differentially modulated between WT and TG cells, we focused our attention on two genes, Tnfaip3/A20 and Ikbkg/Nemo and the modulation of these two genes was then further investigated by single real-time PCR experiments.

The results of our experiments showed, as expected, that the expression of Tnfaip3 was rapidly induced in response to TNF-α in both cell types. Surprisingly, the gene induction was higher in WT cells as compared to TG cells. Nakajima and colleagues have recently reported that ROS cause induction of Tnfaip3 and of HO-1 in several cell types, among which murine fibroblasts, leading to inhibition of basal and cytokine-inducible activation of NF-κB. TNF-treated TG cells are not exposed to ROS, but they constitutively overexpress human HO-1, and this expression

could reduce the TNF-induced transcriptional activation of Tnfaip3 by inhibiting the Nf-kB translocation into the nucleus.

Moreover, taking into account that cytotoxicity and cell death data demonstrated that TG cells were protected from TNF-α injury and apoptosis, we speculated that TG cells were less sensitive and susceptible to damage from TNF-α, and that this resistance may be due to other anti-apoptotic, protective mechanisms, that makes the anti-inflammatory Tnfaip3 induction unnecessary for the survival of the TNF-α injured cells. The gene expression analysis of Nemo suggested that its modulation could be dependent to ATP administration to cells. Upon TNFR1 activation by TNF-α, the so-called complex I is formed and it is responsible for the downstream activation of IKK complex and, thus, of Nf-kb translocation into the nucleus. In this context, Nf-kb promotes pro-survival signaling within the cells. Only if the injurious stimulus becomes persistent, pro-survival signaling pathways switch to pro-death signaling by evolution of complex I into the so-called complex II [17]. This mechanism provides a possible explanation to what it has been observed about Nemo modulation: the early response of the cells to TNF-α stimulus determines an up-regulation of Nemo at 16 hours only in TG cells, promoting a pro-survival signaling in these cells. When damaging stimulus persists for long time, the expression of this gene is switched off and cells can undergo cell death processes.

In summary, TG cells up-regulated Nemo, which promotes pro-survival effects of Nf-kB, in response to TNF-α injury when ATP

is added to the medium, resulting in a protection against TNF-α induced cell death. Taken together, these investigations about the molecular mechanisms involved in cytoprotective effects in response to TNF-α injury, proved that both hHO-1 and hCD39/hCD73 systems have a role in modulating key downstream regulators of TNF-α signalling pathway, as Tnfaip3 and Nemo, promoting important pro-survival and death-resistance effects.

Together with the results of the functional assay, these data support the protective effect against inflammatory stimuli of hHO-1, hCD73 and hCD39 simultaneously expressed in our cells.

**Statin treatment reduces generalized inflammation in a model of vascular injury in pigs fed high cholesterol diet**

Appropriate animal models have been essential in the attempt to understand the atherosclerotic lesion development. A range of experimental models has been developed in the last century, especially including small animal species such as rodents, but also pigeons, dogs, pigs and nonhuman primates.

Nowadays, novel findings from new genetically-modified models continue to enrich our knowledge about atherosclerosis [19,20]. Unfortunately the single ideal animal model for atherosclerosis-related studies has been never conceived. An experimental model should present similarity to the human anatomy and physiology; and above all the lesions should have

pathogenesis, morphology and distribution comparable with the results found in humans. The domestic pig shares many similarities with humans mainly pertaining to the cardiovascular system. Pigs are useful also considering the susceptibility to atherosclerosis, artery dimensions, lesion development, dietary preferences, and similar gastrointestinal metabolism [21,22]. Porcine models have become indispensable for understanding the interaction of the human arteries with medical devices, for understanding intima hyperplasia pathogenesis and providing the knowledge to understand arterial response to injury [21].

In the present study we set up a model of mild-hypercholesterolemic pig, with total and LDL cholesterol concentrations 20% higher than in normocholesterolemic pigs. These cholesterol concentrations are similar to those found in the vast majority of hospitalized patients. In fact, nearly 50% of adults have total cholesterol concentrations at the level that the National Cholesterol Education Program (NCEP) expert panel considers “borderline-high risk” [23].

The idea that atherosclerosis is a chronic inflammatory disease has now gained wide acceptance in the field of cardiovascular research [24]. Sistemically, inflammation has been demonstrated to be an important risk factor for the development of cardiovascular events.

In our experimental model in fact, diet is sufficient to cause a systemic inflammation by increasing the number of circulating WBCs, and to promote tissue-specific inflammation, by raising the amount of infiltrating leukocytes in WAT, liver and lung. The

structural organization of these organs presents a highly vascularized milieu that allows a close interaction between metabolic and circulating immune cells. Chronic inflammatory reactions occurring in these tissues are characterized by a large infiltrate of lymphocytes that are mobilized to sites of injury where they produce cytokines that further activate macrophages and other inflammatory cells [25].

These changes could contribute to increased vascular injury in hypercholesterolemic pigs. Moreover, the systemic inflammation was confirmed by the significant increase in circulating TNF-α and IFN-γ in mild-hypercholesterolemic pigs compared to normocholesterolemic pigs, without significant alterations in IL-6 plasma concentration. In accordance with our results, two experimental works have demonstrated that in several strains of genetically modified mice fed a high-cholesterol diet, there was a significant increase in the number of circulating monocytes [26,27]. Our results are noteworthy also respect to an increasing number of clinical data showing that leukocyte counts rise in atherosclerotic patients and that there is a positive correlation between the number of circulating WBCs and the recurrence of myocardial infarction [28]. These data are of actual interest since it is possible that the measurements of circulating WBCs, WBC sub- populations and cytokine levels should be exploited to gain new careful parameters for risk assessment in patients with atherosclerotic disease.

Oxidative stress and inflammation contribute to

hypercholesterolemia-induced atherosclerosis [29]. Inducible Nitric Oxide Synthase (iNOS) is the enzyme responsible for the production of Nitric Oxide (NO) from arginine. Injury can rapidly upregulate iNOS expression and activity in blood vessels inducing vasodilation, inhibiting platelet adhesion/aggregation and modulating apoptosis and proliferation of VSMCs [30]. At low concentrations NO protects cells against spontaneous or induced apoptosis. Conversely, large quantities of NO are cytotoxic because of the interaction with ROS species [31].

Heme Oxygenase-1 (HO-1) is an inducible enzyme that catalyzes the cleavage of heme into free iron, biliverdin and carbon monoxide (CO). HO-1 plays a pivotal role in vascular protection having antioxidant, anti- inflammatory, antiproliferative and antiapoptotic effects. In particular, HO-1 reduces the inflammatory response following vascular injury [32]. It has been demonstrated that the absence of HO-1 may promote inflammation exacerbating atherosclerotic lesion formation in experimental mouse models. In response to hypercholesterolemia, $apoE^{-/-}$ $ho\text{-}1^{-/-}$ mice fed a high-cholesterol diet developed larger and more advanced lesions than mice deficient in apoE alone [33].

In this work we demonstrated in a preclinical model that HO-1 expression was reduced in the neointima and injured media of mild-hypercholesterolemic pigs compared to normocholesterolemic pigs, while there were no differences in contralateral uninjured arteries. As the same there was a significant reduction of iNOS expression following vascular

injury in the neointima of mild-hypercholesterolemic pigs compared to normocholesterolemic pigs.

Statins potently decrease cholesterol levels and reduce the incidence of cardiovascular and cerebrovascular events. The fundamental prevention of cardiovascular disease and the proved safety of these drugs have led to their widespread use in the clinic [34]. Statins are inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase which is part of the molecular pathway defined as the mevalonate cascade.

Originally designed to decrease the plasma cholesterol concentration, the “traditional” cause of atherosclerosis, statins might also confer cardiovascular benefit by modulating the inflammatory responses in the vascular environment. Several works have established that statins, in addition to their lipid-lowering effects, exert immuno-modulatory actions being able to decrease the expression of adhesion molecules, chemokines and chemokine receptors on both leukocytes and endothelial cells, ultimately limiting the recruitment of leukocytes across the vessel wall [35,36].

Our results showed that the treatment with atorvastatin resulted in a significant reduction of serum total cholesterol and LDL-cholesterol concentration. However, no significant reduction in triglycerides was noted. Compared to the findings in HCD-fed pigs, treatment with atorvastatin markedly lowered the number of circulating WBCs, monocytes and lymphocytes.

Moreover our findings demonstrated that high-dose atorvastatin treatment leads to a substantial decrease of the

hypercholesterolemia-induced inflammatory infiltrates in WAT, liver and lung, and considerably reduce the amount of circulating pro-inflammatory cytokines IFN-γ and TNF-α.

An increasing number of experimental studies demonstrates that statins could mediate their beneficial effects by up-regulating the expression of the inducible protective genes HO-1 and iNOS. The treatment of human umbilical vein and aortic endothelial cells with atorvastatin significantly upregulated HO-1 promoter activity, mRNA expression and protein expression, increasing HO-1 enzymatic activity [37]. Similarly it has been demonstrated that statins activate HO-1 in VSMCs in vitro (human and rat aortic VSMCs) and in vivo (intraperitoneal injection of simvastatin in C57BL/6J), and that the antiinflammatory and antiproliferative effects of statins can occur through the induced HO-1 [38].

Other studies have suggested that statins are able to regulate iNOS in heart and liver diseases. Statins have been reported to enhance hepatic NO production and decrease the vascular tone in patients with cirrhosis [39]. The treatment of cardiac myocytes with lipophilic statins (like atorvastatin) has been shown to induce iNOS mRNA and protein expression via inhibition of the G-protein Rho [40].

In our work we have observed that atorvastatin treatment increased HO-1 protein expression both in the media and in the neointima of injured vessels without modifying the expression in the contralateral healthy artery. In the same way, iNOS expression reached the more prominent staining in the

neointima and in the media of in injured carotid arteries but also in contralateral carotid arteries of pigs treated with atorvastatin. The atorvastatin-induced increased expression of iNOS in contralateral artery suggests that, besides systemic effects of statins, the local vascular effect could play an important role in vascular protection.

In conclusion, our study demonstrates for the first time in a clinically relevant porcine model of diet-induced hypercholesterolemia that even a moderate increase in plasma cholesterol levels can induce a significant increase in the amount of circulating WBCs. This effect extends beyond systemic inflammation to tissue-specific inflammation in WAT, liver and lung. We also establish that mild-hypercholesterolemia is sufficient to induce an augmented leukocyte infiltrates in these organs. HCD-fed pigs treated with high-dose atorvastatin results in a reduction of total and LDL cholesterol and in markedly decreased systemic and tissue- specific inflammatory markers by preventing the development of an inflammatory milieu and the accumulation of infiltrating leukocytes.

**Conclusion and translational application in medicine**

Our study demonstrated, for the first time, the protection against inflammatory stimuli of a combination of three human genes simultaneously expressed in murine cells via a tricistronic vector. To evaluate the potential protective effect of the combination of hHO1, hCD39 and hCD73 in inflammatory-based diseases, such as atherosclerosis and transplantation, in

an in vivo context, three novel transgenic mouse strains were produced using our multicistronic vector, by applying the DNA pronuclear microinjection strategy.

If encouraging results will be achieved in mice, we will use the tricistronic cassette to produce more relevant pre-clinical models, as transgenic pigs. Indeed this vector could be used to produce triple transgenic pigs by a single round of Somatic Cell Nuclear Transfer (SCNT) [41], or by Sperm Mediated Gene Transfer (SMGT) [42]. These animals will allow us to test the potential protective role of this combination of genes against xenograft rejection, and this could bring a further step forward closer to the clinical application of xenotransplantation.

Finally we have clarified the pathogenetic role of hypercholesterolemia in a preclinical pig model of vascular injury resembling clinical settings, and we have demonstrated for the first time that statin treatment reduces generalized inflammation in spite of a minimal effect on lipids in a model of vascular injury in pigs fed high cholesterol diet. In fact diet is sufficient to cause a systemic inflammation that contribute to increase vascular injury in hypercholesterolemic pigs. However statin treatment reversed not only enhanced vascular injury but also attenuated most of systemic and localized inflammatory responses. The use of high-dose atrovastatin attenuating systemic and local inflammation and protecting injured vessels could be proposed as a therapeutic approach to reduce the incidence of restenosis.

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