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ARGININE TRANSPORT AND NITRIC OXIDE PRODUCTION: ROLE IN THE INFLAMMATORY RESPONSE

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CHAPTER I - INTRODUCTION AND AIM OF THE STUDY

1. L-ARGININE METABOLISM

The occurrence of L-Arginine (2-amino-5-guanidinovaleric acid) in mammalian protein was discovered by Hedin in 1895, although its existence as a naturally occurring molecule had already been known since 1886[1].

Since then, several studies have been performed to characterize the metabolism of this basic amino acid and its biological function in cellular homeostasis. As a result, it is now demonstrated that, although arginine is, strictly speaking, not an essential amino acid, its de novo synthesis does not always seem able to sustain an adequate supply. Particularly under conditions of high demand, such as growth, sepsis, or wound healing, the endogenous arginine supply may become limiting, suggesting that the homeostasis of the plasma concentration of this amino acid can be regulated by a variety of anabolic and catabolic pathways, more than by dietary intake[2]. Thus, arginine is now considered a semiessential amino acid in humans and most mammals; its recommended dietary intake is 1–3 g/d. The complexity of its metabolism arises not only from the diversity of the enzymes involved (Fig.1), but also from their cell-specific patterns of expression.

For what concerns L-Arg catabolism, four sets of enzymes are known to be mainly involved in mammalian cells: nitric oxide synthases (NOS), arginases (ARG), arginine:glycine amidinotransferase (AGAT), and arginine decarboxylase (ADC) (Fig.1)[3].

In 1932, Krebs & Henseleit reported that arginine is an essential component in the

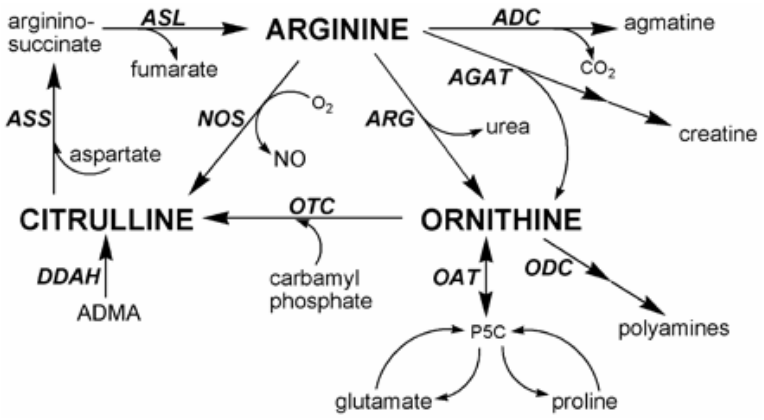


Fig.1 Schematic representation of L-Arginine metabolism.

ADC, arginine decarboxylase; AGAT, arginine:glycine amidino-transferase; ARG, arginase; ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; OTC, ornithine transcarbamylase; P5C, L-Δ1-pyrroline-5-carboxy-late.
(from Morris, *The American Society for Nutritional Sciences, J. Nutr.* (2004), 134:2743S-2747S).

urea cycle, an ubiquitous, cyclic metabolic pathway which allows the elimination of

continuously generated toxic ammonia; it consists of the synthesis of L-arginine and of its subsequent disintegration into L-ornithine and urea catalyzed by the activity of arginase[4]. The "byproduct" of this reaction, L-ornithine, is also a precursor for the synthesis of polyamines, molecules essential for cell proliferation and differentiation; a quantitatively minor pathway for their synthesis in mammals is constituted by direct the Arginine Decarboxylase (ADC)-dependent convertion of L-arginine to CO_2 and agmatine, which can be further catabolized by agmatinase to produce putrescine and urea. Although in the 1990s evidence of arginine decarboxylase activity has been presented for mammalian cells and a putative human arginine decarboxylase clone has been reported, the existence of arginine decarboxylase in mammals is still somewhat controversial. The physiologic roles of agmatine in mammals have not been conclusively defined; however, various studies have indicated that it may act as a neurotransmitter, an inhibitor of cell proliferation, or an inhibitor of NOS suggesting a possible role for it as an endogenous modulator of NO production if local concentrations are sufficiently high[3].

In 1939, Foster et al. discovered that L-arginine is also required for the synthesis of creatine[5]. Arginine:glycine amidinotransferase catalyzes the first and rate-controlling step in the synthesis of this molecule, which, in its phosphorylated form (creatine phosphate), is an essential energy source for muscle contraction. Its degradation product, creatinine, is eliminated by glomerular filtration in the kidney and is used as a surrogate measure of glomerular filtration rate[1]. Flux of L-arginine through arginine:glycine amidinotransferase is determined in part by dietary levels of creatine, which acts as a feedback repressor of this enzyme.

Finally, in the 1980s it was discovered that L-arginine is one of the main precursors of nitric oxide (NO), the chemical entity previously known as endothelium-derived relaxing factor (EDRF)[6].

2. NITRIC OXIDE

2.1. NO BIOSYNTHESIS

NO is a gas produced by many cells of the immune system, such as dendritic cells, NK, mast cells and phagocytic cells, including monocytes, macrophages, microglia, Kupffer cells, eosinophils, and neutrophils, as well as other cells involved in immune

reactions (endothelial cells, epithelial cells, vascular smooth muscle cells, fibroblasts, keratinocytes, chondrocytes, hepatocytes, mesangial cells and Schwann cells)[7].

The enzyme that catalyzes the oxidation of the terminal guanidino nitrogen group of L-Arg to produce NO and L-Citrulline, with NADPH and O_2 serving as cosubstrates (Fig.2), is the NO synthase (NOS) isolated, cloned, and characterized in 1991 from macrophages[8], endothelial cells[9], and neuronal cells[10].

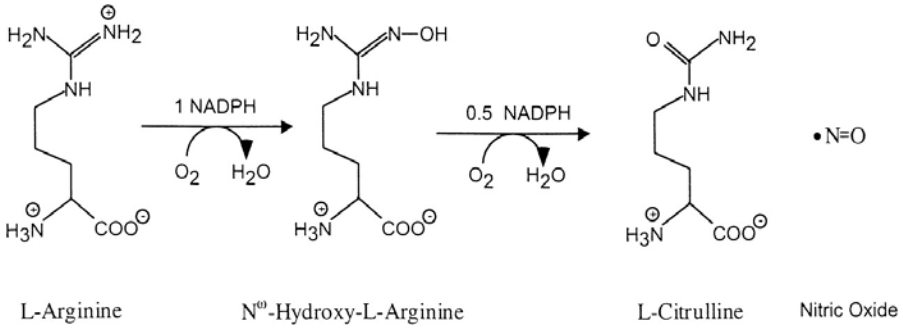


Fig.2 The two reactions of NO synthesis as catalyzed by NOS.

Three related NOSs are expressed in mammal cells, encoded by distinct genes: inducible NOS (iNOS, Type II NOS or NOS2), neuronal NOS (nNOS, Type I NOS or NOS1) and endothelial NOS (eNOS, Type III NOS or NOS3). For the most part, nNOS and eNOS are constitutively expressed at low levels in a variety of cell types, whereas iNOS, which normally is not expressed in most cell models, is highly inducible by bacterial endotoxin and inflammatory cytokines in several cell models, such as stimulated murine macrophages, hepatocytes, pancreatic islet cells, vascular smooth muscle cells, glial cells, retinal epithelial cells and keratinocytes[11]; human hepatocytes have also been shown to produce iNOS-derived NO in the presence of the same combination of endotoxin and cytokines as rodent hepatocytes[12], whereas, as far as its expression in human endothelium is concerned, conflicting evidences report either its constitutive absence under both basal and inflammatory conditions, or, rather, its induction following stimulation with inflammatory compounds[13,14]; in our hands, however, the mRNA of iNOS remains completely undetectable in HUVECs, even in the presence of TNFα[15].

Actually, also the activity of eNOS and nNOS can be modulated in several ways, suggesting them to be inducible enzymes too. In particular, the shift to a more active eNOS is known to be triggered by several stimuli, such as shear stress, PKC inhibitors, insulin, TGFβ1, H_2O_2, VEGF and autacoids that are generated locally by tissue injury, while other inflammatory stimuli such as TNFα, hypoxia, lipopolysaccaride, thrombin, and oxidized

LDL can decrease eNOS mRNA levels[16]. Besides the observation that steady-state levels of eNOS mRNA may be influenced by changes in transcription, many studies have also underlined the importance of posttranscriptional regulation mechanisms in response to numerous stimuli, particularly those that appear to have the greatest effect on mRNA expression[17]. The reason for the importance of such posttranscriptional regulation seems to relate to the finding that eNOS mRNA has a long half-life at baseline (10–35 h): since stable mRNA species are able to pool in the cytosol, synthesis of the encoded proteins is likely to persist long after gene transcription has been repressed; thus, altering the half-life of stable transcripts in this pool may be the most rapid and efficient means of modulating steady-state mRNA levels and gene expression. This level of regulation would provide endothelial cells with flexibility to perform rapid phenotypic changes in response to different stimuli[17].

Among the best characterized posttranslational modification of NOS enzymes, it is widely accepted that activities of the constitutive eNOS isoenzymes are dynamically regulated by Ca^{2+}/calmodulin or by the availability of essential cofactors such as tetrahydrobiopterin[18]. Moreover, also the subcellular localization of NOS isoenzymes seems to be involved in the regulation of their activity, particularly in the cases of eNOS and nNOS; such regulation probably involves dynamic changes in direct protein-protein interactions or placement near ion channels and transporters. For example, eNOS is associated with caveolae at localized regions of the plasma membrane; this may allow more efficient modulation of eNOS activity via local changes in flow-induced shear stress and in calcium fluxes, and may also affect the rate of NO production by placing eNOS in close apposition to arginine transporters at the plasma membrane[19]. Similarly, nNOS is primarily associated with the rough endoplasmic reticulum and postsynaptic membranes in brain and with the sarcolemma of skeletal muscle; as in the case of eNOS, it is thought that the subcellular localization of nNOS near calcium channels allows highly precise regulation of its activity[18].

Finally, protein phosphorylation is known to regulate eNOS activity, too. In particular, eNOS can be phosphorylated primarily on serine residues and, to a lesser extent, on tyrosine and threonine residues. Among the several regulatory cascades likely involved in the stimulation of eNOS activity, one of the best characterized pathways is that of the serine/threonine kinase Akt (protein kinase B), which increases eNOS activity several-fold by directly phosphorylating eNOS at Ser1177 (human eNOS) and Ser1179 (bovine eNOS)[20].

2.2. NO FUNCTION IN HEALTH AND DISEASE

Although only a minor portion of L-arginine is metabolized via NOS-dependent pathways in vivo, they have attracted a lot of interest in recent years because of the prominent role that NO plays in vascular physiology and pathophysiology. Indeed, nitric oxide physiologically produced by constitutive endothelial NOS in the vasculature in vivo is known to play a key role in many cellular events, acting as a paracrine-signaling molecule mediating vasodilation[21], inhibiting platelet activation[22] and monocyte-leukocyte adhesion[23], impairing smooth muscle cell proliferation[24], and controlling vascular oxidative stress and the expression of redox-regulated genes[25]; consistently, in certain animal models and in human diseases where the biological functions of endothelium-derived NO are impaired, the deregulation of endothelial control of vascular tone and blood flow appears evident[1].

Besides the well known beneficial effects of nitric oxide under physiological conditions, NO is known to play a beneficial role even in chronic inflammatory disease, and the increasing evidence of the protection afforded against inflammation and immunity by NO represents a new particular interest in the immunology world. At the same time, however, clinical evidence also supports the assumption of a key role for harmful NO-dependent mechanisms in a variety of autoimmune or chronic inflammatory diseases[26,27].

The resulting conflicting literature attributing to nitric oxide either pro-inflammatory or anti-inflammatory effects has been solved by the assumption that the local concentration of the molecule is a crucial determinant of NO-triggered cytotoxicity: picomolar amounts of the gas are sufficient for intracellular signalling, whereas higher concentrations can be at the same time microbicidal as well as pro-inflammatory and damaging to the surrounding cells and tissues[26]. Consistently, nitric oxide produced in the millimolar range during an inflammatory insult, besides exerting protective actions in mammalian tissues due to cytostatic and cytotoxic antimicrobial activities towards pathogens[28], may also lead, because of its high concentrations, to lipid peroxidation, DNA damage, oxidation of thiols and nitration of tyrosine residues[18,29], acting indirectly via reactive nitrogen oxide species (RNOS), generated through interaction of NO with O_2 or O_2^-.

A paradigm has been thus far accepted, concerning the specific contribution of the different NOS isoforms to the production of nitric oxide under basal and inflammatory conditions: the amounts of NO responsible for basal intracellular signalling are generally

ascribable to the activity of eNOS isoform, while both the beneficial and detrimental effects of nitric oxide produced under pathological conditions are usually referred to the sole iNOS. However, in light of the recent demonstration of eNOS being an inducible enzyme too, this model can no more be accepted[30]. Actually, the characterization of the events following an inflammatory insult has shown that the early phase of cellular response is characterized by the conversion of L-Arg by iNOS to trigger an high-output generation of NO; however, under these conditions, also eNOS is present and the stimulus can increase also its activity and production of NO[17].

Apart from the specific contribution of the different NOS isoforms to the production of nitric oxide under inflammatory conditions, what appears of peculiar interest is the experimental evidence demonstrating the key role of L-arginine in such event[1]. Indeed, a plethora of reports from animal studies have shown that acute or chronic administration of L-arginine in vivo improves vascular responsiveness, probably via enhanced NO elaboration: acute administration of L-arginine augments endothelium-dependent vasodilation in cholesterol-fed rabbits, while long-term oral administration of L-arginine has been associated with a significant improvement in NO-dependent vasodilation in cholesterol-fed rabbits[31,32] and in low-density-lipoprotein receptor knockout mice[33]. In the same animal models, other NO-dependent vascular functions are also modulated by chronic supplementation with L-arginine: endothelial leukocyte adhesion is reduced[34], platelet aggregation is inhibited[35], and vascular smooth muscle cell proliferation in vivo is attenuated[36]. Moreover, very soon after the first animal experiments had proven a beneficial effect of L-arginine on endothelial function, it was shown that local intracoronary infusion of L-arginine normalized coronary vasomotor responses to acetylcholine in hypercholesterolemic humans. A similar observation was also made upon systemic (intravenous) infusion of L-arginine in hypercholesterolemic subjects, in whom endothelium-dependent forearm vasodilation was improved[1].

Although the mechanisms behind these phenomena have not yet been fully elucidated, experimental evidences suggest that, under certain conditions, L-arginine availability regulates endothelial cell NOS activity. For example, reduced activity of endothelial NOS has been shown to occur in the presence of low-density-lipoprotein cholesterol, and this effect can be overcome by excess L-arginine. Consistently, NOS is known to be inhibited by L-arginine analogs that are substituted at the guanidino nitrogen atom, but, again, the inhibitory action of these molecules is overcome by excess L-

arginine, indicating that there is competition for NOS binding between L-arginine and its inhibitory analogs[1].

In light of these findings, particular attention has been devoted in the last decades to better define the role of L-Arg availability, as nitric oxide supplier, in the maintenance of cellular homeostasis, under both physiological and pathological conditions.

3. L-ARGININE SYNTHESIS AND TRANSPORT

As far as the anabolic pathways responsible for L-Arginine synthesis are concerned, it is now evident that the main tissue in which endogenous synthesis occurs is the kidney, where the amino acid is formed from L-citrulline mainly released by the small intestine[1]. The liver is also capable of synthesizing considerable amounts of L-arginine; however, this is completely reutilized in the urea cycle so that the liver contributes little or not at all to total plasma L-arginine concentration. Cell types containing nitric oxide synthase (NOS) have been demonstrated to be able to reutilize L-citrulline, the byproduct of nitric oxide (NO) synthesis, to produce L-arginine via the so called arginine-citrulline cycle[37]. This pathway is mediated by enzymes also involved in the hepatic urea cycle; however, the fact that L-citrulline accumulates in the medium of NO-producing cells indicates that the arginine-citrulline cycle is far less efficient than the urea cycle[18].

Anyway, irrespective of whether nutrition or biosynthesis is the primary source, arginine needs to be exchanged between different tissues and, therefore, every cell needs to transport arginine across the plasma membrane. Thus, since the lipid bilayer of biological membranes is impermeable for hydrophobic solutes such as amino acids, specialized carrier proteins are necessary to provide adequate import and export routes.

Pioneering work by Halvor Christensen concerning the study of L-Arg transporters demonstrated that arginine shares the same transport system with other cationic amino acids (CAAs) such as lysine and ornithine, and that, in most cells, arginine transport through the plasma membrane is not energized by coupling to the Na^+ gradient: in particular, a single Na^+-independent transport system termed y^+ was postulated to be the major entry route for CAAs in most cells[38,39]; this transport system has been shown to be pH insensitive[40] and strongly stimulated by hyperpolarization and by substrate at the opposite membrane side (trans-stimulation)[41].

More recently, several additional Na^+-independent transport systems, further distinguished by their interaction with neutral amino acids (NAAs), and one Na^+-dependent

system for CAAs have been discovered[2]: system y^+L, first defined by Deves et al.[42], transports both CAAs and large NAAs, the latter in a Na^+-dependent manner; system $b^{0,+}$ transports both CAA and NAA in a Na^+-independent manner and prefers large NAAs that are not branched at the α and β carbon[43]; finally, system $B^{0,+}$ mediates the Na^+-coupled transport of CAAs and NAAs and accepts a wide range of substrates, including branched and small NAAs[43] (Table 1).

At molecular level, system y^+ activity is mediated by the CAT (for ***c***ationic ***a***mino acid ***t***ransporter) family of single carrier transporter proteins, CAT1, -2, and -3. Conversely, system y^+L comprises the heteromeric transporters named HAT, formed by a glycoprotein (also called *heavy chains*) combined with a carrier protein. Finally, $ATB^{0,+}$, the protein that mediates system $B^{0,+}$ activity, belongs to the SLC6 family of Na^+- and Cl^--dependent transporters[2].

			Arginine transport		**Leucine transport**		
Protein	Gene	Transport system	Na^+-dependent	Apparent K_m	Na^+-dependent	Apparent K_m	Trans-stimulation
				mmol/L		*mmol/L*	
CAT-1	SLC7A1	y^+	No	0.10–0.16	—	—	Yes
CAT-2A	SLC7A2	ND	No	3.40–3.90	—	—	No
CAT-2B	SLC7A2	y^+	No	0.25–0.70	—	—	Moderate
CAT-3	SLC7A3	y^+	No	0.20–0.50	—	—	Moderate
CAT-4	SLC7A4	—	—	—	—	—	—
y^+LAT1 + 4F2hc	SLC7A7 + SLC3A2	y^+L	No	0.34	Yes	0.02	Yes
y^+LAT2 + 4F2hc	SLC7A6 + SLC3A2	y^+L	No	0.12–0.14	Yes	0.20–0.30	Yes
$b^{0,+}$AT + rBAT	SLC7A9 + SLC3A1	$b^{0,+}$	No	0.08–0.20	No	0.30	Yes
$ATB^{0,+}$	SLC6A14	$B^{0,+}$	Yes + Cl^-	0.10–0.15	Yes + Cl^-	0.01	No

Table 1. Plasma membrane transporters that accept arginine as subbstrate

(modified from Closs, *The American Society for Nutritional Sciences, J. Nutr.* (2004), 134: 2752S-2759S).

3.1. CAT TRANSPORTERS

CATs are integral membrane glycoproteins with 14 putative transmembrane domains and intracellular N- and C-termini[44]. Among the members of this SLC7 subfamily, four carrier proteins are recognized: CAT1, -2A, -2B, and -3, with CAT2A and -

2B being splice variants that differ only in a stretch of 42 amino acids. The product of another related gene (SLC7A4), expressed in brain, testis, and placenta, is only 40% identical to CAT1 to -3[45].

As evidenced by transport studies in *Xenopus laevis* oocytes and mammalian cells, CAT1 to -3 all mediate Na^+-independent transport of CAAs, but differ in their substrate affinities and sensitivities to trans-stimulation. In good agreement with system y^+ activity, human CAT1 exhibits an apparent K_m of 0.10 to 0.16 mmol/L for cationic amino acid, is strongly trans-stimulated and is mainly pH independent. Human CAT2B and -3 both exhibit apparent K_m values of 0.25 to 0.70 mmol/L for arginine and 0.2 to 0.5 mmol/L for lysine, and their affinities to ornithine are slightly lower; both carriers are only moderately trans-stimulated. Like CAT1, CAT3 is pH independent in a range of 5.5 to 8. In contrast, CAT2B shows only 50% activity at pH 5.5, compared with pH 7.5. CAT2A is clearly distinguishable from system y^+, because of its low affinity for CAA (apparent K_m 2–5 mmol/L), insensitivity to trans-stimulation, and moderate pH dependence[2].

CATs are widely expressed. In spite of its almost ubiquitous presence, CAT1 expression is highly regulated at the transcriptional and post-transcriptional level; therefore, modulation of CAT1 mRNA, dependent, for example, upon bacterial LPS, TGFβ, interleukin-1, insulin or IFNγ, might not be reflected in corresponding changes in CAT1 protein, or these changes might not involve the surface expressed CAT1. Furthermore, expression-independent factors such as membrane potential, trans-stimulation, and subcellular distribution of CAT1 may cause pronounced changes in CAT1 activity [2].

For what concerns the two CAT2 splice variants, they exhibit quite distinct expression patterns: CAT2B is significantly expressed only upon treatment of many cell types with cytokines or LPS, whereas the low-affinity splice variant, CAT2A, is expressed in skeletal muscle, pancreas, cardiomyocytes, cardiac microvascular endothelial cells and vascular smooth muscle cells, although its highest expression occurs in the liver, where it most likely serves to clear plasma of excess arginine[46]; substantial CAT2A expression can be induced by surgical trauma (hepatectomy and spleenectomy) as well as by food deprivation[47], when CAT2A probably serves as an export route for arginine derived from the breakdown of muscle proteins.

CAT3 is widely expressed during embryonic development, but seems to be confined to central neurons in adult mice and rats. In humans, it is strongly expressed in the thymus, moderately expressed in the uterus, testis, mammary gland, and brain and only weakly expressed in the ovary and stomach. The specific function of CAT3 in these tissues

is not understood, although a role for CAT3 in the substrate supply of neuronal NOS has been postulated[48].

3.2. HAT TRANSPORTERS

Heterodimeric HAT transporters are formed by a glycosylated type II membrane protein (heavy chain), with a single transmembrane helix and a large extracellular domain, and an associated protein with 12 putative transmembrane helices (light chain). This latter protein is distantly related to CAT and, together, they comprise a subfamily of SLC7. Other than the CATs, members of this subfamily are not glycosylated and localize to the plasma membrane only when co-expressed with the respective glycoprotein. Eight different HATs have been identified to date; in all but one, the heavy chain 4F2hc associates with different light chains to form amino acid transporters with diverse substrate selectivity; two of these accept arginine as a substrate: association of 4F2hc with the light chains y^+LAT1 and y^+LAT2 gives rise to y^+L-like transporters. In the only HAT transporter which does not comprise 4F2hc heavy chain, the glycoprotein rBAT interacts with the light chain $b^{0,+}$AT to mediate system $b^{0,+}$-dependent L-Arginine transport[49].

All arginine-accepting HATs function as obligatory exchangers (i.e., uptake or efflux of arginine by these transporters can occur only in exchange with another CAA or NAA). Their physiological function is best understood in the absorbing epithelia of the small intestine and renal tubule, where the combined action of rBAT/$b^{0,+}$AT and 4F2hc/y^+LAT1 causes trans-epithelial CAA flux. In addition, rBAT/$b^{0,+}$AT in the apical membrane is essential for the (re)absorption of cystine[2].

The vectorial transport of substrate across the epithelium is brought about by an asymmetry of substrate binding at extra- and intracellular binding sites of the apical (rBAT/$b^{0,+}$AT) and basolateral (4F2hc/y^+LAT1) transporters. Uptake of CAAs by apical rBAT/$b^{0,+}$AT is favoured because of its extracellular high affinity for these amino acids and the negative membrane potential; conversely, NAAs are preferentially exported owing to their high intracellular concentration. In return, the basolateral 4F2hc/y^+LAT1 has a higher extracellular affinity for NAAs, which are therefore the preferred uptake substrates; because their transport by 4F2hc/y^+LAT1 is coupled to Na^+, they cannot exit cells by this route (against the Na^+ gradient) and CAAs are thus the exclusive export substrates for 4F2hc/y^+LAT1, although the resulting low extracellular affinity combined with substantial competition by NAAs make this an inefficient entry pathway for these amino acids. Taken

together, rBAT/$b^{0,+}$AT and 4F2hc/y^+LAT1 serve as the import and export routes, respectively, for arginine in epithelial cells of the kidney and small intestine.

The arginine-accepting HATs are expressed in several cell types. 4F2hc/y^+LAT expression is strong in lung (y^+LAT1) and stomach and colon (y^+LAT2) cells, where it may serve as an export route for amino acids accumulated due to the action of apical Na^+-dependent carriers, such as $ATB^{0,+}$ [2,50]; moreover, in organs with blood–tissue barriers (e.g., brain, placenta, and testis), these transporters may contribute to arginine transport across the barrier[2]. However, HAT expression is also found in a number of nonpolarized cells, where their function is less well understood. It can be generally assumed that in these cells, rBAT/$b^{0,+}$AT and 4F2hc/y^+LAT1 or 4F2hc/y^+LAT2 also serve as influx and efflux routes, respectively, for arginine. It is therefore highly unlikely that 4F2hc/y^+LAT1 provides arginine to intracellular enzymes such as NOS[46].

3.3. $ATB^{0,+}$

$ATB^{0,+}$ is the only known Na^+-dependent transporter for arginine, whose transport activity is coupled to Na^+ and Cl^- [51]. It is a glycoprotein with 12 putative transmembrane helices and recognizes a wide range of CAAs and NAAs with high affinity. Its expression is most abundant in lung and salivary gland tissue but can also be found in mammary gland, pituitary gland, stomach, and colon tissue. In mice, $ATB^{0,+}$ is expressed on the apical membrane of epithelial cells of the trachea and bronchi (predominantly in ciliated cells), but also in the bronchioles and alveoli[52]. In human airway epithelial cells $B^{0,+}$ is responsible for the apical influxes, involved in transepithelial arginine transport[50]. It has therefore been suggested that the transporter contributes to protein clearance by removing amino acids from the airway lumen, thus playing a role in lung defense through the maintenance of a low-nutrient environment. In the gastrointestinal tract, the expression pattern of ATB^0 is similar to that of the basolateral 4F2hc/y^+LAT2[53]. In the pituitary gland, human ATB^0 has been proposed to play a role in hormone secretion induced by amino acids (e.g., arginine and leucine).

4. AIM OF THE STUDY

The research performed during my PhD course has concerned the characterization of the pathways responsible for arginine recruitment in different human cellular models, in

light of the importance of this amino acid as the obliged substrate for nitric oxide biosynthesis. In particular, human endothelial cells and human monocytes/macrophages have been employed, since they are among the cell types more directly linked to NO pathway: nitric oxide acts in the endothelium as a versatile mediator, involved in a variety of endothelial functions, such as the regulation of vascular contraction, leukocyte adhesion, vascular smooth muscle cell growth and platelet aggregation; cells of the monocyte/macrophagic lineage, instead, specifically produce it in the context of an inflammatory response, because of its cytotoxic, antimicrobial activity towards pathogens.

A preliminary characterization of L-arginine transport and nitric oxide production has been performed in monocytes and macrophages, since most of the contributions available in literature about these cell types concern animal, rather than human, models; as for arginine transport in human endothelium, experimental evidences, reported in the last years and now widely accepted, have been initially confirmed in our models of foetal (HUVECs) and adult (HSVECs) endothelial cells, so as to verify possible tissutal- or species-specificities.

Afterwards, once defined the molecular pathways involved in the maintenance of physiological concentration of L-Arg under resting conditions, the studies have been extended to activated cells: pro-inflammatory cytokines, known to be effective in stimulating the transport of cationic amino acids in other models, have been tested on our cells, to define whether amino acid transport has a role in the inflammatory response of to exogenous stimuli and which are the molecular pathways responsible for such cytokine-dependent events. In this context, particular attention have been paid to the production of nitric oxide and, specifically, to the study of the relation likely connecting the availability of L-arginine and the synthesis of nitric oxide under inflammatory conditions; indeed, the existence of such link could give a rationale to the clinically evidenced protective effects of arginine subministration under specific pathological, inflammatory conditions.

CHAPTER II – EXPERIMENTAL PROCEDURES

1. CELL CULTURES AND EXPERIMENTAL TREATMENTS

1.1. ENDOTHELIAL CELLS

Human saphenous vein endothelial cells (HSVECs) were obtained from vessel remnants of patients undergoing coronary artery bypass grafting; human umbilical vein endothelial cells (HUVECs) were isolated from normal, full-term deliveries umbilical cords. The cultures of both cell models were established according to the method of Jaffe and colleagues[54], with minor modifications. Briefly, vein lumen was cannulated at both ends and 50-100 ml of phosphate-buffered saline (PBS) were flushed through the preparation to remove any blood; after isolation with 0.48% dispase solution (0.5 U/mg) (Dispase II; Boehringer-Mannheim Italia) in a sterile water bath (37°C) for 15-20 min, the vein lumen was flushed with M199. The cell suspensions were then collected and centrifuged at 1000 rpm for 5 min; pellets were resuspended in complete growth medium consisting of M199 supplemented with 20% fetal bovine serum, heparin (90 U/mL), endothelial cell growth supplement (50 μg/mL), glutamine (2 mmol/L) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

Human aortic endothelial cells (HAECs) were purchased by Cambrex Bio Science. Cultured cell monolayers were screened for typical endothelial cobblestone morphology by phase-contrast microscopy and periodically tested for staining for von Willebrand's factor and CD31/PECAM-1 antigens with indirect immunofluorescence.

All endothelial cells were routinely grown in the same complete growth medium described above, on collagen-coated (2 μg/cm^2 Collagen Solution, Sigma), 10-cm diameter dishes, and kept at 37°C, pH 7.4, in an atmosphere of 5% CO_2. Culture medium was always renewed 24h before each experiment. For the experiments, all the cytokines and LPS (from E. coli, serotype O55:B5) were added from stock solutions in sterile water to complete growth medium for the times and at the concentrations indicated for each experiment; the inhibitors required by the experimental protocols were added 1h before the cytokines.

1.2. MONOCYTES AND ALVEOLAR MACROPHAGES

For the isolation of human monocytes, mononuclear cells were separated from buffy coats, obtained from normal healthy volunteers, supplied by the Unità di Immunoematologia e trasfusione of Azienda Ospedaliera di Parma. The buffy coats, diluted 1:4 with PBS, were layered on 10 ml of Fycoll Hypaque and centrifuged at 750 g for 30 min at 20 °C. Peripheral blood mononuclear cells (PBMC) at the interface were removed, washed three times in PBS, and centrifuged at 150g for 10 min at 20°C. After the final wash, PMBC were suspended in RPMI 1640 (Cambrex Bio Science) containing 2% endotoxin-free fetal bovine serum (FBS; Euroclone) and seeded on plasticware appropriate for the various determinations. After a 30 min incubation at 37°C in an atmosphere at 5% CO_2, non-adherent cells were removed with vigorous washes with prewarmed sterile Earle's Balanced Salt Solution (EBSS). Adherent monocytes were employed immediately (for characterization of arginine transport and expression of CAA transporters in freshly isolated cells) or collected, resuspended in RPMI supplemented with 10% FBS in the absence or in the presence of IFNγ (10 ng/ml), and maintained at 37°C under gentle agitation for the indicated times. After the incubation, monocytes were let to sediment for 1h before the experimental determinations. To assess the purity of the preparation, cells were stained with monocyte-specific anti-CD14 mAb; more than 75% of the isolated cells expressed CD14.

To obtain alveolar macrophages (AM), subjects were enrolled by Clinica Pneumologica of University of Parma and put through the lavage procedure for routine diagnostic purpose. After written informed consent and local anaesthesia of the patient, fiber-optic bronchoscopy and broncho-alveolar lavage (BAL) were performed as described by D'Ippolito et al.[55]. Briefly, the bronchoscope was wedged into a segment of the right middle lobe and three 50 ml aliquots of sterile saline solution, warmed at 37°C, were instilled into the subsegmental bronchus. Fluid was gently aspirated immediately after each aliquot was introduced and collected into a sterile container. AM isolation started within 2h of bronchoalveolar lavage. The fluid obtained was filtered through two layers of sterile surgical gauze and centrifuged (200g x 10 min). The whole BAL pellet was washed once in RPMI 1640, centrifuged at 200g for 10 min, re-suspended in RPMI 1640 supplemented with 10% endotoxin-free FBS, 2 mM glutamine, 10U/mL penicilline and 10 μg/ml streptomycin. Cells were plated in 12-well or 96-well dishes and allowed to adhere (37°C, 5% CO_2). After 2h, the medium was renewed to remove non-adherent cells and AM were

used immediately or cultured in RPMI supplemented as above described for the times indicated in each experiment in the absence or in the presence of LPS (1 μg/ml) and cytokines (20 ng/ml IFNγ or 10 ng/ml GMCSF). Differential cell counts were carried out on DIFF-Quick (Don Baxter)-stained cytospin smears, counting at least 400 cells. The adherent cell population consisted of more than 95% alveolar macrophages. Cell viability exceeded 95%, as assessed by trypan blue exclusion.

2. L-ARGININE INFLUX

For transport studies, endothelial cells were seeded on 2-cm^2 wells of collagen-coated disposable 24-well trays, while monocytes and AM were cultured on 96-well dishes. All the experiments were performed using the cluster-tray method for the measurement of solute fluxes in adherent cells[56] with appropriate modifications.

Cell monolayers were washed twice in Earle's balanced salt solution (EBSS), containing 117 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5.5 mM glucose, buffered at pH 7.4 with 20 mM Tris/HCl. In the experiments in which Na^+-independent transport was to be measured, a modified EBSS named NMG-EBSS was employed, with N-methyl-D-glucamine replacing NaCl. To further discriminate the different components of CAA uptake in cultured cells, L-arginine uptake was measured in the presence of increasing concentrations of L-leucine or after a 5 min pretreatement with 0.5 mM N-ethylmaleimide (NEM): in the presence of sodium, 2 mM leucine completely abolishes system y^+L activity, while NEM specifically inhibits system y^+ but not system y^+L[57]. In all cell models, L-Arginine influx was assayed with a 30 s incubation of the cells in the same solution used for the washes, supplemented with L-[^{3}H]arginine (4-6 μCi/ml, 100 μM); in this interval of time arginine uptake approached linearity. The experiments were terminated by two rapid washes in ice cold 0.3 M urea. Cell monolayers were extracted in 0.2 ml ethanol and the radioactivity of extracts was determined with a Wallac Microbeta Trilux (Perkin Elmer). Extracted cell monolayers were then dissolved with 0.5% sodium deoxycholate in 1 M NaOH and protein content was determined directly in each well using a modified Lowry procedure, described by Gazzola et al.[56].

Amino acid influx is expressed as nmoles • mg of $protein^{-1}$ • min^{-1}.

Kinetic parameters of arginine influx were determined by non-linear regression analysis using GraphPad Prism3™ software.

The equations used for fitting the experimental data were:

$$v = \frac{V_{max} \cdot [S]}{Km + [S]} + K_D \cdot [S] \qquad (1)$$

for a transport process resulting from the additive operations of a saturable system and a non-saturable component, where v is the initial influx, V_{max} the maximal influx and Km the Michaelis constant, or

$$v = \frac{V_{max}1 \cdot [S]}{Km1 + [S]} + \frac{V_{max}2 \cdot [S]}{Km2 + [S]} + K_D \cdot [S] \qquad (2)$$

for the additive effects of two saturable systems and a non-saturable component.

3. RNA ISOLATION AND ANALYSIS

3.1. RNA EXTRACTION AND REVERSE TRASNCRIPTION

For expression studies, cells were seeded onto 6-well trays and total RNA was isolated with RNeasy Mini Kit® (QIAGEN S.p.a.), according to manufacturer's instructions. RNA (2 µg), along with 150 ng of random primers and dNTP (0.5 mM), was heated at 70°C for 10 min, placed on ice for 1 min, then incubated with a mixture containing 5 mM dithiothreitol, 1X first-strand buffer, 40 U of RNAse inhibitor (Invitrogen s.r.l.), 200 U of SuperScript™ III RT (Invitrogen s.r.l.) and water to a final volume of 20 µl for 1h at 42°C; the reaction was stopped by heating at 70°C for 15 min.

3.2. SEMI-QUANTITATIVE PCR

100 ng of single-strand cDNA from each sample were amplified with 1.0 x PCR buffer, 0.2 mM each dNTPs, 2.5 mM $MgCl_2$, along with proband primers (see Table 2 for sequences) and 1.25 U of Hot Master Taq DNA polymerase. The reaction program consisted of an initial denaturation step at 92 °C for 2 min, followed by 32 cycles with a 2-min denaturation step at 94°C, the annealing at 59°C for 30 s, and the extension step at 72°C for 1 min. Images of the electrophoresed cDNAs were recorded with a digital DC 120 Kodak camera and quantified by ID Image Analysis Software (Kodak Digital Science). In semi-quantitative experiments GAPDH primers were also added in the

amplification mixture and the results expressed as the densitrometric ratio of proband vs. GAPDH product.

3.3. REAL TIME QUANTITATIVE PCR (qPCR)

For real time PCR (40 cycles), cDNA (25 ng) was amplified with 2X Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen s.r.l.), along with the forward and reverse primers (5 pmol each), designed according to the known sequences reported in GenBank with the help of Primer 3 program (Table 3). Quantitative PCR was performed in a 36 well Rotor Gene 3000 (Corbett Research). For all probands each cycle consisted of a denaturation step at 95°C for 15 s, followed by separate annealing (30 s, 57 °C) and extension (30 s, 72°C) steps. Fluorescence was monitored at the end of each extension step. A no-template, no-reverse transcriptase control was included in each experiment. At the end of the amplification cycles a melting curve analysis was added. The analysis of the data was made according to the Relative Standard Curve Method[58].

4. PROTEIN ANALYSIS

4.1. WESTERN BLOT

Cells were washed twice with ice-cold phosphate-buffered saline, scraped in the same solution and collected by low speed centrifugation.

For the analysis of E-selectin, cells were lysed in 0.5 ml of RIPA buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA 630, 1% sodium deoxycholate, 0.1% SDS) containing a cocktail of protease inhibitors (Complete Mini EDTA-free, Roche S.P.A, Monza, Italy), sonicated for 30 s and centrifugated for 20 min at 16000g. For CAT2 and NOS3, cells were suspended in 0.3 ml of Laemmli buffer (62.5 M Tris–HCl, pH 6.8, 2% SDS, 20% glycerol, 0.2 M DTT), sonicated for 15 s and centrifugated for 5 min at 12000g. After total protein quantification by a modified micro Lowry protein assay, 30 µg of protein samples were separated on 8% acrylamide gels by SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Bio Rad). Membranes were blocked at 4°C overnight with an incubation in Tris-buffer saline (TBS; 50 mM Tris-HCl pH 7.5, 150 mM NaCl), containing 3% BSA, 1% Casein, 0.33% gelatin and 10% goat serum and exposed for 2h at room temperature to primary antibodies (NOS3 1:200; E-selectin 1:200) or to a filtered anti-hCAT2 polyclonal

antiserum (diluted 1:2000). This antiserum was generated by Neosystem Groupe SNPE (Strasbourg) against the C-terminus peptide Y-17-F (Y-R-N-L-S-S-P-F-I-F-H-E-K-T-S-E-F) of human CAT2 proteins.

The expression of total and phospho-p70S6 kinase was determined with the PhosphoPlus® p70S6 Kinase Antibody kit from Cell Signaling (Celbio) following manufacturer's instructions. The same cell lysates were analyzed also for total and phospho-Akt expression with antibodies anti-Akt and anti-phospho-Akt (Ser 473) (Cell Signaling).

Immunoreactivity was visualized with enhanced chemiluminescence (Millipore). GAPDH, detected with a monoclonal antibody (1:500) was employed for standardization.

4.2. IMMUNOCYTOCHEMISTRY

Immunostaining was performed on cell monolayers grown on two-well chamber slides (Falcon). Cells, after the experimental treatments, were washed twice with PBS and fixed with 3.7% paraformaldehyde in PBS (pH 7.4).

After additional washing, cells undergoing immunostaining for actin were permeabilized with a 10 min incubation in 0.1% Triton X-100 in PBS, then incubated for 20 min at 37 °C with Alexa Fluor 488-phalloidin (15 U/ml) in PBS.

At the same time, cells which had to be stained for CAT2 were instead incubated for 1h in PBS containing 2% bovine serum albumin, to block non-specific absorption of antibodies; they were then incubated for 1h at 37 °C with anti-CAT2 polyclonal antiserum (see Western analysis) diluted 1:500 in blocking solution, and finally incubated for 1h at room temperature with Alexa Fluor 488–conjugated secondary antibodies (Molecular Probes, Invitrogen) (1:400 dilution in blocking solution).

After immunostaining with the different antibodies, the cells were finally washed, the slides were mounted with Pro-Long® antifade kit (Molecular Probes, Invitrogen), and examined with a confocal microscope ZEISS, LSM 510 META equipped with inverted microscope Axiovert 200M (Carl Zeiss Meditec GmbH). Alexa Fluor 488 signal was acquired at a λ_{ex} of 488 nm and the emission recorded through a 510 nm primary beamsplitter and a 530 nm dichroic filter. For each condition, a series of confocal images of representative fields were taken and the section yielding the maximal signal was selected.

4.3. ELISA

Cells seeded on 96-well plates were washed with PBS and fixed with 2% paraformaldehyde for 15 min at room temperature. After an overnight incubation in PBS with 3% BSA at 4°C, the monolayers were exposed for 1h at 37°C to anti-E-selectin polyclonal antibody (1:50). Cells were then washed twice in PBS and incubated for 30 min in biotin-conjugated mouse anti-rabbit secondary antibody (1:300); after additional washes with PBS, they were finally exposed to the Streptavidin-HRP conjugate (1:100). Optical density was measured at 490 nm with a Victor^2 Multilabel Counter (Perkin Elmer); the values obtained were then referred to the correspondent protein content in each well.

5. AMINO ACID CONTENT

The intracellular content of amino acid was determined on cell monolayers washed twice with ice-cold $MgCl_2$ and extracted in a 5% solution of acetic acid in ethanol. The intracellular content of the single amino acid species was determined by HPLC analysis with a Biochrom 20 amino acid analyzer (Biochrom) employing a high-resolution column (Bio 20 Peek Lithium) and the physiological fluid chemical kit (Biochrom) for elution. The column effluent was mixed with ninhydrin reagent, passed through the high-temperature reaction coil, and read by the photometer unit. Cell contents of the single amino acid species are expressed as nanomoles per milligram of protein.

For the determination of the intracellular concentration of amino acids, cell volumes were determined in parallel cultures under the same experimental conditions by urea distribution space[59]. Briefly, cells were incubated for 10 min in [^{14}C]urea (1.5 μCi/ml, 0.5 mM), then rapidly washed with ice-cold urea (300 mM) to terminate the incubation; cell monolayers were extracted as described in section 2. Values of cell volume are expressed as microliters per milligram of protein.

6. NITRIC OXIDE PRODUCTION

6.1. NITRITE PRODUCTION

The accumulation of nitrites, stable derivatives of NO, in the culture media of cell monolayers was assessed through a fluorimetric approach, based upon the production of

the fluorescent molecule 1-(H)-naphtotriazole from DAN in the presence of nitirites in an acid environment[60]. Briefly, 100 µl of medium were put in wells of a black 96-well plate with a clear bottom, along with 20 µl of DAN (0.025 mg/ml in 0.31 M HCl); after 10 min at room temperature, the reaction was stopped with 20 µl of 0.7 M NaOH. Standards were performed in the same medium from a solution of 1 mM sodium nitrite. Fluorescence was determined with a Victor^2 1420 Multilabel Counter (Perkin Elmer). Nitrite production was expressed in nmoles per ml of extracellular medium (µM).

6.2. BIOACTIVE NO PRODUCTION (RFL-6 REPORTER CELLS ASSAY)

The RFL-6 reporter cell assay was performed as already described by Simon and colleagues[61].

Cells were grown to confluence in 6-well plates and treated as indicated. After removing the culture medium, monolayers were washed twice with Locke's solution (LS) (NaCl 154.0mM; KCl 5.6mM; $CaCl_2$, 2.0; $MgCl_2$ 1.0 mM; $NaHCO_3$ 3.6 mM; glucose 5.6 mM; N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) 10.0 mM, pH 7.4) and equilibrated in the same solution for 20–30 min; SOD (20 U/ml) was added to prevent the oxidation of NO.

Since RFL-6 fibroblasts express significant amounts of soluble guanylyl cyclase, but no nitric oxide synthase, they can be used as reporter cells for exogenous NO. RFL-6 cells cultured to confluence in 6-well plates were washed twice with 2 ml LS containing 3-isobutyl-1-methylxanthine (IBMX, 300 µM), to inhibit phosphodiesterases, preventing the formation of cGMP from degradation, and equilibrated for 20 min in the same solution at 37 °C. The medium was removed and conditioned medium from treated cells (1 ml) was transferred onto the reporter cells. After a 2 min incubation, the medium was removed, ice-cold sodium acetate buffer (50 mM, pH. 4.0) was added to each well, and the cells were frozen and lysed with liquid N_2. cGMP levels in the RFL-6 cells were determined by radioimmunoassay as described[62].

7. CELL VIABILITY AND DEATH

7.1. CELL NUMBER AND VIABILITY

Cell number was assessed with Cell Counter ZM (Coulter Electronics Ltd), after detachment of adherent cells by trypsinization.

Cell viability was tested with the fluorescent molecule resazurin, a widely used viability indicator[63]. For the assay, the culture medium was replaced with a solution of resazurin (44 μM) in complete growth medium. After 2h of incubation at 37°C, fluorescence was measured at 572 nm with a Wallac 1420 Victor^2 Multilabel Counter fluorimeter (Perkin Elmer).

7.2. PROPIDIUM IODIDE STAINING

Cells were seeded on 10 cm diameter dishes and treated as indicated for each experiment. After culture trypsinization and centrifugation of the medium (to collect both adherent and floating cells), cells were fixed in ice-cold methanol for 30 min at 4°C. After washing with PBS, cells were resuspended and incubated for 1h at 37°C in the dark in 1 ml of Propidium Iodide solution, containing Propidium Iodide (PI, 20 μg/ml), Triton 0.1% and RNAse A (10μg/ml) in PBS. Propidium staining of at least 10^5 cells was acquired by FACSCalibur Flow Cytometer (Becton Dickinson); the analysis was performed using Cell Quest software. The number of cells undergoing late apoptosis or non apoptotic death is expressed as the percentage of the sub G0 population on the whole cell content.

7.3. ANNEXIN V STAINING

Cells were grown onto 10 cm diameter, collagen coated Petri dishes and treated according to the experimental settings. After incubation, the floating cells in the different conditions were collected in 50 ml-tubes, while the monolayers were detached with trypsin and collected in the same tube, together with the correspondent media. The solutions containing adherent and floating cells were then centrifuged at 1800 rpm for 10 min and apoptotic cells were estimated according to the kit protocol of Annexin V/FITC (Bender MedSystems). Briefly, cells were incubated with 10 μl Annexin V-FITC conjugate in binding buffer (Bender MedSystems) for 30 min at room temperature in the dark, then washed with PBS and resuspended in 190 μl of binding buffer added with 10 μl of Propidium Iodide solution (PI, 1 μg/ml). Cell staining was acquired in at least 10^5 cells with a FACSCalibur Flow Cytometer (Becton Dickinson); analyses were performed using Cell Quest software. The number of apoptotic cell is expressed as percentage of total cells binding Annexin V but negative for PI on total cells.

7.4. *CASPASE 3 ACTIVITY*

The activity of caspase 3 was determined by Caspase 3 Colorimetric Assay Kit (Sigma Aldrich); this method is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3, which results in the release of the chromophore p-nitroaniline (p-NA). Briefly, cells were grown on 10 cm diameter dishes and treated as indicated. After the experimental treatments, culture monolayers were washed with ice-cold PBS and lysed in 1X lysis buffer. Cell lysates were then tested for protease activity using Ac-DEVD-pNA as caspase substrate (1h incubation at 37°C); the concentration of the p-NA released in each condition was calculated from the absorbance values at 405 nm, using a standard curve of p-nitroaniline. The caspase 3 activity is expressed as nmol pNA/min/mg of protein.

7.5. *LDH MEASUREMENT*

Lactate Dehydrogenase (LDH) released from dead cells into the medium was assessed with a CytoTox 96® Non-Radioactive Cytotoxicity assay (Promega), based on an enzymatic reaction, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. Briefly, 50 µl of culture medium were incubated with 50 µl of Substrate Mix provided by the manufacturer; after 30 min at room temperature in the dark, absorbance at 490 nm was measured for each well with a Wallac 1420 Victor^2 Multilabel Counter fluorimeter (Perkin Elmer). The amount of LDH released in each experimental condition is expressed as a percent of the maximum LDH released in lysed untreated cells (% of cytotoxicity).

8. ENDOTHELIAL MIGRATION IN VITRO

To assess endothelial migration in vitro, endothelial wound healing assay described by Weber et al. [64] was used, with minor modifications. Cells were cultured onto 6-well collagen coated Petri plates; once grown to confluence, fresh complete growth medium was added to the wells, along with the compounds required by the experiments. After 12h, the endothelial monolayers were wounded by two parallel strokes across the diameter of the well with a 0.4 mm-wide 200 µl Gilson-style extension length tip and incubated at 37°C. After 0 (control), 12 and 24h, images of the same field were acquired with a Nikon Eclipse Inverted Microscope equipped with a Nikon DS Cooled Camera Head DS-5Mc

employing a Proximo 32 Image Analysis software. The number of cells migrated into 1 mm^2 of wound area was quantified.

9. MATERIALS

Endotoxin-free fetal bovine serum (FBS, Euroclone) and culture mediums (Medium 199 for endothelial cells and RPMI 1640 for human monocytes and macrophages) were purchased from Celbio (Pero, Italy). [L-2,3,4-^{3}H]Arginine (58.0 Ci/mmol) was obtained from Perkin-Elmer Italia, while [^{14}C]urea (58.0 Ci/mmol) was purchased from GE Healthcare Italia, as well as Fycoll Hypaque. GM-CSF, INFγ, TNFα, anthra [1,9-cd] pyrazol-6 [2H]-one (SP600125), 2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1 (CFPD) and CD14 mAb were from Vinci Biochem. NF-kB inhibitors, peptide aldehyde Cbz-Leu-Leu-Leucinal (MG132), 2'-amino-3'-methoxiflavone (PD98059), the PKCα pseudosubstrate inhibitor peptide, and 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580) were from Calbiochem. Hot Master *Taq* polymerase for semi-quantitative PCR was from Eppendorf s.r.l., while all reagents for Reverse Transcription and qPCR were purchased from Invitrogen s.r.l., together with 2,3-diaminonaphthalene (DAN), Alexa Fluor 488-phalloidin and Alexa Fluor 488-conjugated secondary antibodies were from Molecular Probes. All the primary antibodies (Santa Cruz Biotechnology) were purchased from DBA Italia. Biotin conjugated mouse anti-rabbit and Streptavidin-HRP were from DAKO Italia. Sigma (Milano, Italy) was the source of LPS, rapamycin, FK506, AICAR as well as of all the other chemicals, unless otherwise indicated.

Table 1. Primers employed for semi-quantitative PCR

GenBank Accession No.	cDNA	Sense	Antisense	Product Size, bp
NM_003045	SLC7A1	5'-CGTCCCTCTTGATCTGCTTC-3'	5'-GGCTGGTACCGTAAGACCAA-3'	427
	SLC7A1	5'-ACTTGCTTCTATGCCTTCGTG-3'	5'-TGTGGCGATTATTGGTGTTT-3'	387
NM_003046	SLC7A2 (CAT2A)	5'-GTTGACTGCAGGGGTCATTT-3'	5'-ACATTTGGGCTGGTCGTAAG-3'	163
NM_001008539	SLC7A2 (CAT2B)	5'-CCCAATGCCTCGTGTAATCT-3'	5'-ACATTTGGGCTGGTCGTAAG-3'	262
NM_001012661	SLC3A2	5'-GTTTGTCTCAGGCAAGGCTC-3'	5'-GGAACAAGGAAAGGAGGGAG-3'	270
NM_003983	SLC7A6	5'-CTTTCTACTTCATGGGTGTTTACC-3'	5'-ATCCTGAGTCTCCTATAGCTTACCAA-3'	332
NM_003982	SLC7A7	5'-AGACATCTTCCAGCTCATTAACTACTACAG-3'	5'-CTTTTCAACTTCCTTAGCTCTAGCCAGTA-3'	481
NM_000625	NOS2	5'-TCTGTTCAAGACCAAATTCCACC-3'	5'-CGGGGGACTCATTCTGCTGC-3'	151
NM_002046	GAPDH	5'-CTCTGACTTCAACAGCGACACCCACTCCT-3'	5'-GTCTCTCTTCTTCCTCTTGTGCTCTTGCT-3'	209

Sense primers for CAT2A and CAT2B have been formulated on the basis of the different sequences resulting from the alternative splicing of SLC7A2 transcript.

Table 2. Primers employed for Real Time quantitative PCR

GenBank Accession No.	cDNA	Sense	Antisense	Product Size, bp
NM_003045	SLC7A1	5'-CTTCATCACCGGCTGGAACT-3'	5'-GGGTCTGCCTATCAGCTCGT-3'	100
NM_003046	SLC7A2 (CAT2A)	5'-TTCTCTCTGCGCCTTGTCAA-3'	5'-TCTAAACAGTAAGCCATCCCGG-3'	95
NM_001008539	SLC7A2 (CAT2B)	5'-TTCTCTCTGCGCCTTGTCAA -3'	5'-CCATCCTCCGCCATAGCATA-3'	82
NM_003983	SLC7A6	5'-CTTTCTACTTCATGGGTGTTTACC-3'	5'-ATCCTGAGTCTCCTATAGCTTACCAA-3'	332
NM_003982	SLC7A7	5'-GAAGGAGGAGCATCAGACCA-3'	5'-CCCAGTTCCGCATAACAAAG-3'	481
NM_000625	NOS2	5'-TCTGTTCAAGACCAAATTCCACC-3'	5'-CGGGGACTCATTCTGCTGC -3'	151
NM_000603	NOS3	5'-TGGTACATGAGCACTGAGATCG-3'	5'-CCACGTTGATTTCCACTGCTG-3'	148
NM_000576	IL1β	5'-ACAGACCTTCCAGGAGAATG-3'	5'-GCAGTTCAGTGATCGTACAG-3'	127
NM_000660	TGFβ1	5'-TAGACCCTTTCTCCTCCAGGAGACG -3'	5'-GCTGGGGGTCTCCCGGCAAAAGGT-3'	226
NM_000450	ELAM	5'-ACCTCCACGGAAGCTATGACT-3'	5'-CAGACCCACACATTGTTGACTT-'	173
NM_002046	GAPDH	5'-AGCCTCAAGATCATCAGCAATG-3'	5'-CACGATACCAAAGTTGTCATGGA-3'	87

CHAPTER III – ARGININE AND HUMAN ENDOTHELIUM

1. INTRODUCTION AND AIM OF THE STUDY

Since L-arginine–nitric oxide signalling pathway has emerged as one of the key second messenger systems involved in the regulation of vascular tone and permeability, the research of the last decades has been devoted to define the role of circulating and intracellular concentrations of the amino acid and the regulatory mechanisms involved in their maintenance in endothelial cells, in health and disease[65].

An extensive characterization of cationic amino acid transport has been performed in human umbilical vein endothelial cells (HUVEC) by different groups. The results of these studies have demonstrated that, under physiological conditions, L-arginine transport in these endothelial cells is mediated predominantly by systems y^+ and y^+L[15,65]. The same contributions also demonstrate that, in HUVECs, system y^+ activity is referable predominantly to CAT1 transporter, while CAT2A and CAT2B are detectable only at very low levels; system y^+L is also present, as demonstrated by the expression of y^+LAT1, y^+LAT2 and 4F2hc transcripts, but its contribution to the overall L-Arg transport under basal conditions is considered minimal[15].

While a regulatory mechanism for system y^+L is scarcely documented, a lot of evidences demonstrate that the expression of CAT mRNAs and proteins can be modulated by a variety of hormonal and inflammatory stimuli[66]; in particular, it is demonstrated that bacterial LPS and pro-inflammatory cytokines are effective in stimulating arginine transport in different cell models[15,67-69]. In light of these findings, several studies have been performed to verify whether the cytokine-dependent stimulation of arginine transport observed in different animal and human models is somehow ascribable to the cellular inflammatory response, possibly through the increased production of nitric oxide, a well known signalling molecule produced during inflammation.

Among the pro-inflammatory stimuli able to modulate arginine transport, Tumor Necrosis Factor alpha (TNFα) appears of peculiar interest in vascular context, since it plays a pivotal role in the induction of the endothelial response to inflammation; in particular, this cytokine is known to promote the activation of endothelial cells, profoundly affecting the expression of many genes and proteins involved in the regulation of endothelial function, such

as COX2, the adhesion molecules ICAM, VCAM and E-selectin, growth factors and secondary messengers, as nitric oxide.

As far as a possible connection between the stimulation of L-arginine transport and nitric oxide production under pathological conditions is concerned, conflicting results has lead to the idea that CAT proteins and NO synthesis can not be considered always induced in parallel[70-72]. In further support of this hypothesis, experiments performed in our laboratory have shown that the TNFα- and LPS-dependent modulation of arginine transport in HUVECs is dissociated from the stimulation of nitric oxide production: Sala and colleagues have indeed demonstrated that, although TNFα and LPS induce a transient stimulation of arginine influx, mediated specifically by system y^+, nitrite accumulation upon incubation with TNFα is not significantly different, although lower in cytokine-treated cells with respect to control ones; moreover, the mRNA of iNOS is not detectable either in the absence or in the presence of the inflammatory stimuli, while eNOS expression is even decreased by the treatment with the cytokine[15].

In the same paper, it is also shown that the stimulation of L-Arg transport upon treatment of endothelial cells with TNFα or LPS can be ascribed to the increased expression of the sole SLC7A2 mRNA, while SLC7A1 levels remain substantially unaffected, as well as system y^+L expression and activity. Thus far, however, little is known about the signal transduction pathways responsible for such induction. Nuclear factor-[kappa]B (NF-kB) and p38 mitogen-activated protein kinase have been identified as essential mediators of CAT2B induction in rat alveolar macrophages and RASMCs, respectively[73,74]; furthermore, the activation of PKC is known to be required for TNFα stimulation of L-Arg transport in HUVEC[75]. However, given the network of signalling pathways acting downstream the cytokine, it is possible that several transduction routes may overlap to modulate arginine transport.

Therefore, the aim of the first part of this research project has been the definition and the characterization of all the molecular events leading to the induction of L-Arg influx and system y^+ activity in human endothelial cells upon treatment with pro-inflammatory stimuli (i.e. with TNFα). Since differences between the metabolic features of endothelial cells obtained from different vascular districts are well established, we have analyzed human cells derived from both foetal (umbilical vein) and adult (saphenous vein) endothelium, so as to verify whether the results obtained were specific for an endothelial model or more generally referable to human endothelium.

In light of the many evidences describing the dependence of endothelial cells response to TNFα on new protein synthesis (for review see[76]), particular interest has been initially devoted to the study of NF-kB activity.

NF-kB is a transcription factor, involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, and bacterial or viral antigens[77]. Its activity is regulated primarily by the phosphorylation of specific inhibitory proteins, the IkBs, which withhold the factor in the cytoplasm of non-stimulated cells; in response to proper stimuli, IkB kinase (IKK) phosphorylates the IkBs, rendering them suitable of ubiquitination and consequent degradation by proteasome, thus permitting the nuclear translocation of the NF-kB to the nucleus. In endothelial cells, TNFα specifically causes degradation of IkBα, IkBβ and IkBε, producing sustained activation of p50 (NF-kB1), p65 (RelA) and c-Rel subunits[78,79]; furthermore, in the same model, cytokine-dependent NF-kB activation is known to require the intracellular complex TRADD/RIP/TRAF2. Although the precise molecular events underlying this effect remain undefined, several TNFα responsive protein kinases, such as MAPK, PI3K/Akt and PKC, are supposed to be involved in the connection between RIP/TRAF2 and NF-kB, since all of them can phosphorylate and activate IKK.

Mitogen activated protein kinases (MAPKs) could be good candidates for such a role, since they are known to be activated by TNFα in endothelial cells[80]. These proteins are serine/threonine specific kinases that transduce a large variety of external signals, leading to a wide range of cellular responses, including growth, differentiation, inflammation and apoptosis. In mammals, three major MAPK pathways have been identified: MAPK/ERK (Extracellular-signal regulated kinase), SAPK/JNK (stress activated protein kinase/Jun N-terminal kinase), and $p38^{MAPK}$. All these pathways are characterized by the following general signalling cascade, highly conserved from yeasts to mammals:

Stimulus > MAPKKK > MAPKK > MAPK > Response

where MAPKK is the kinase of MAPK and MAPKKK is the kinase of MAPKK.

Another molecular pathway linked to TNFα signalling is that of protein kinases C (PKCs). This family comprises three subfamilies of kinases, based on their second messenger requirements:

- ✓ classical or conventional PKCs, (c)PKCs, includes the isoforms α, βI, βII, and γ, and requires Ca^{2+}, diacylglycerol (DAG) and a phospholipid such as phosphatidylcholine for activation (Fig.3);
- ✓ novel PKCs, (n)PKCs, composed by the δ, ε, η, and θ isoforms, require DAG, but not Ca^{2+};

- ✓ atypical PKCs, (a)PKCs, includes protein kinase Mζ and ι / λ isoforms and requires neither Ca^{2+} nor DAG for activation.

In endothelial cells from human saphenous vein three known isozymes have been described: the classical PKCα, the novel PKCε and the atypical PKCζ[81]; moreover, in the same model, PKCα and PKCε, but not PKCζ, are known to be stimulated by TNFα[81].

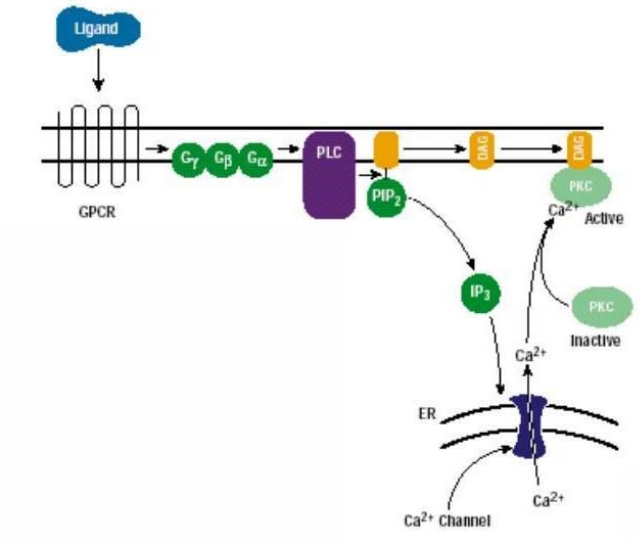


Fig.3 Activation of a conventional PKC.

An external stimulus activates a G protein-coupled receptor (GPCR), which, in turn, activates phospholipase C (PLC), to cleave phosphoinositol-4,5-bisphos-phate (PIP2) into DAG and IP3. The IP3 interacts with a calcium channel in the endoplasmic reticulum (ER), releasing $Ca2^{+}$ into the cytoplasm. The increase in $Ca2^{+}$ levels activates PKC, which translocates to the membrane, anchoring to DAG and phosphatidylserine.
(modified from Signal Transduction Resource, Promega Corporation)

Finally, among the different signalling pathways leading to NF-kB activation, also Akt is of great interest, since it is reported to phosphorylate IKK and mediate activation of NF-kB in response to specific agonists[82]. Akt is a serine/threonine kinase which requires a double phosphorylation to become fully activated: phosphorylation at Thr308, catalyzed by PI3K/PDK1 and essential for Akt activation, and phosphoSer473 due to the activity of the mTOR/rictor complex, TORC2. In particular, in endothelial cells TNFα is known to activate Akt in a PI3K-dependent way[79]. PI3Ks are ubiquitous, heterodimeric enzymes that play a central role in the regulation of many cellular processes, including cell growth, motility, proliferation, and survival. Activated PI3-kinases phosphorylates phosphoinositol (PI) substrates to produce PI(3)P, PI(3,4)P2, and PI(3,4,5)P3, which act as second messengers and recruit the PI3K dependent serine/threonine kinases (PDK1) and Akt from the cytoplasm to the plasma membrane; lipid binding and membrane translocation lead to conformational changes in Akt, which let it be phosphorylated in a residue of Thr in the activation loop by PDK1.

For what concerns mTOR, there are evidence demonstrating that it can be activated by TNFα, through different intermediates, such as Akt[83,84]. mTOR (mammalian Target Of

Rapamycin) is a serine/threonine kinase, which plays a pivotal role in the regulation of cell size and proliferation, in response to different extracellular signals, such as nutrients, energy and growth factors availability or stress associated conditions[85]. Following proper stimuli, mTOR complexes with the adaptor proteins raptor or rictor, forming respectively TORC1 and TORC2; the first is involved in the regulation of protein translation through the control of the phosphorylation status of ribosomal S6 kinase and 4EBP1[86], while TORC2 seems to be mainly involved in the regulation of actin cytoskeleton[87]. What is noteworthy, the activities of the two complexes have different effects on Akt (Fig.4): once the kinase has activated TORC1, this acts as an inhibitor for it, eliciting a negative feedback loop to stop Akt-dependent activation; on the contrary, the other complex, TORC2, is known to promote Akt activation, by phosphorylating it in Ser473[88].

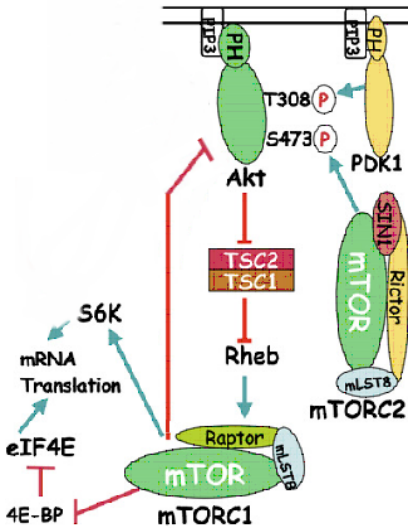


Fig.4 Schematic representation of the interplay between Akt and TORCs, TORC1 and TORC2.

(modified from Bhaskar and Hay, *Developmental Cell* (2007), 12(4), 487)

Studies about mTOR pathway are greatly favoured by the availability of a specific inhibitor, rapamycin (sirolimus), a macrolide antibiotic that, after binding to the protein FKBP12[89], inhibits the signalling cascade dependent upon mTOR and leads to the arrest of both protein synthesis and cell cycle progression. In light of the effects of the drug on cell proliferation, rapamycin has been proposed in clinical practice as both an immunosuppressant and an anticancer agent[90,91]; for the same reason, more recently, the Food and Drug Administration has also approved its use in drug-eluting stents (DES) for the prevention of restenosis after percutaneous coronary revascularization[92]. This is another reason why studies about mTOR involvement in the maintenance of endothelial function appear of great interest: the employ of the drug in clinical practice, and particularly in DES, is currently the object of an open discussion turned to evaluate the safety of the compound for human health. Indeed,

the results of recent clinical investigations have shown that, beyond reducing the incidence of in-stent restenosis, thus preventing the need for repeating percutaneous intervention[92], the use of DES is associated to an increased rate of late stent thrombosis (LST)[93,94]. Although the molecular basis of this effect have not been fully elucidated thus far, recent evidences report a connection between the implantation of rapamycin-eluting stent and a long-term endothelial dysfunction, suggesting that the observed increased risk of LST could be due to a deleterious effect of the drug on the endothelium[95].

In this context, once clarified the involvement of the kinase in the regulation of L-Arg transport in human endothelium, the second part of the research has been intended to define more generally the role of mTOR in the maintenance of endothelial viability and function, that is not only the regulation of vascular tone and permeability under physiological conditions, but also endothelial ability to counteract an exogenous insult by inducing an activated phenotype. More in detail, the consequences of mTOR inhibition at molecular vascular level have been assessed under basal conditions, but also in cells incubated simultaneously with rapamycin and pro-inflammatory cytokines; in this way, the role of the kinase in the modulation of nitric oxide production, as well as of the expression of adhesion molecules, stress-associated proteins and other known markers of endothelial activation has been defined both in normal cells and during the endothelial response to an inflammatory insult.

2. RESULTS AND DISCUSSION

2.1. CHARACTERIZATION OF L-ARG TRANSPORT IN HUMAN ENDOTHELIAL CELLS

Preliminary characterization experiments indicate that HSVECs, like HUVECs[15], transport L-Arg through two Na^+-independent components: one, insensitive to N-ethylmaleimide (NEM) and suppressed by excess of L-leucine (2mM) in the presence of sodium, corresponds to system y^+L; the other, inhibited by NEM and relatively insensitive to leucine, is identifiable with system y^+ (Fig.5).

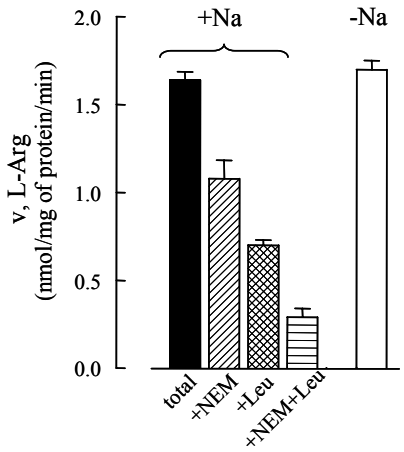


Fig.5 Characterization of L-Arginine influx in HSVECs.

In endothelial cells from saphenous vein arginine transport occurs through both systems y^+ and y^+L.

The effects of pro-inflammatory stimuli, such as cytokines and bacterial LPS, in HSVECs have been therefore studied on arginine influx, discriminated for its different components.

As shown in Fig.6, both TNFα and LPS, but not IL-1β and INFγ, produce an increase of L-Arg transport, which appears referable to the sole NEM-sensitive component (Panel A),

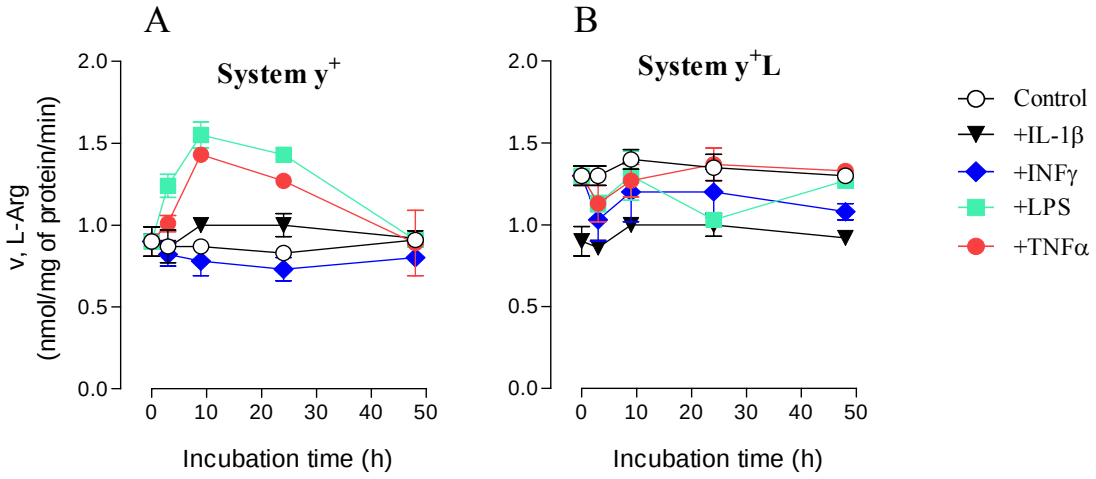


Fig.6 Effects of inflammatory cytokines and LPS on L-Arg transport in HSVECs.

Panel A. System y^+. Data represent the NEM-sensitive arginine influx calculated as the difference between total and NEM-resistant influx; only TNFα and LPS stimulate system y^+ activity. Panel B. System y^+L. NEM-resistant arginine influx is unaltered under any of the experimental conditions tested.

since no significant change of NEM-resistant component, i.e. system y^+L, is detected under any of the experimental conditions tested (Panel B). Conversely, the significant induction of system y^+ activity is evident throughout the 24h incubation, with a maximal stimulation after 9h of treatment, when the influx of the amino acid is almost doubled in treated cells with respect to control, untreated cells.

We can thus conclude that, as already demonstrated in HUVECs, also in HSVECs the specific target of TNFα and LPS, as far as arginine transporters are concerned, is system y^+.

The molecular mechanisms underlying this stimulatory effect of the cytokine on endothelial amino acid transport have been then investigated.

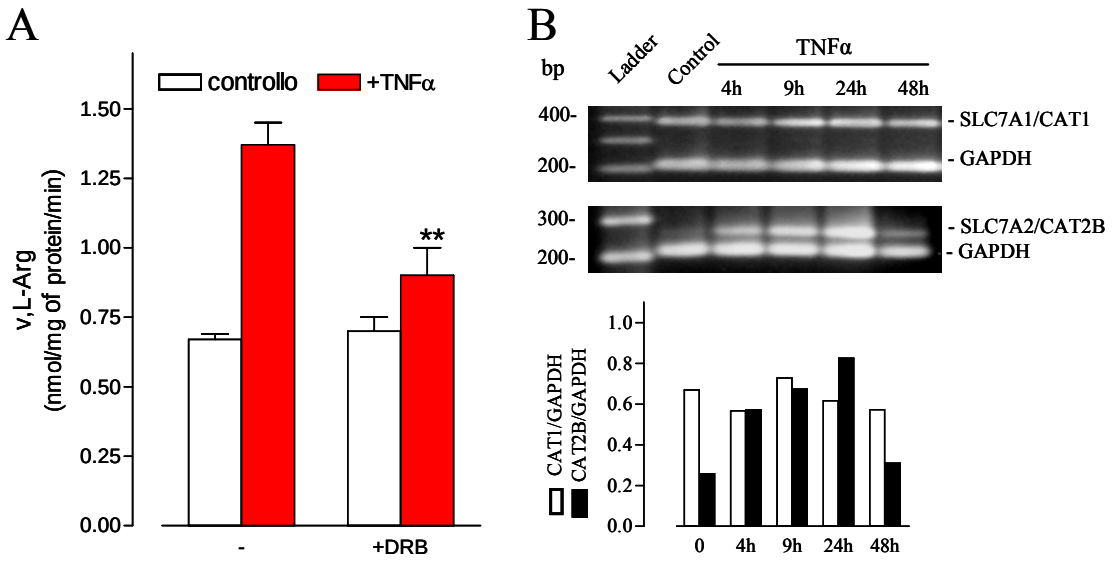


Fig.7 Effects of TNFα on gene transcription.

Panel A. DRB (50 μM), added 1h before TNFα, inhibits cytokine-induced increase of system y^+ transport activity, without affecting the basal L-Arg uptake. $^{**}P<0.01$inhibitor present *vs.* inhibitor absent in TNFα treated cells. Panel B. TNFα-dependent stimulation of L-Arg transport is referable to the induction of SLC7A2/CAT2B transcript, as assessed through RT-PCR and the relative densitometric analysis.

As shown in Fig.7, the treatment with the reversible transcription inhibitor 5,6-dichloro-1-β-D-ribobenzimidazole (DRB) substantially hinders TNFα-induced increase of system y^+ transport activity without altering the basal uptake of arginine (Panel A); consistently, the analysis of the expression of genes involved in L-Arg transport in human endothelial cells shows that only the mRNA of SLC7A2/CAT2B undergoes a significant induction upon treatment with the cytokine (Panel B), whereas SLC7A1/CAT1 (Panel B), SLC7A6/y^+LAT2, SLC7A7/y^+LAT1 and SLC3A2/4F2hc (data not shown) do not show any significant variation with either TNFα or LPS. Therefore, it looks evident that the stimulatory

effects of inflammatory cytokines on arginine transport in human endothelium likely depend on transcription mechanisms, particularly concerning SLC7A2/CAT2B mRNA.

Most of the best characterized endothelial responses to TNFα are known to involve, among the possible transcription factors, NF-kB; we have thus investigated its role also in cytokine-induced stimulation of system y^+ activity. For this purpose, we have tested the effect of several inhibitors of the transcription factor, with different mechanisms of action: CAPE and PDTC, that inhibit transcription activation suppressing the interaction of NF-kB proteins with the DNA[96]; PPM-18, that blocks the removal of IkBα from NF-kB/IkBα complex[97]; and MG132 that inhibits the proteasome-dependent degradation of IkB[98].

As shown in Fig.8, all the inhibitors have no significant effect on basal arginine transport, while, in the presence of TNFα (9h), PDTC, CAPE, and MG132 completely suppress the stimulatory effect of the cytokine, and PPM-18 too, although only partially (Panel A). Consistently, RT-PCR analysis indicates that PPM-18 markedly reduces, but does not abolish, the induction of CAT2B caused by TNFα (Panel B).

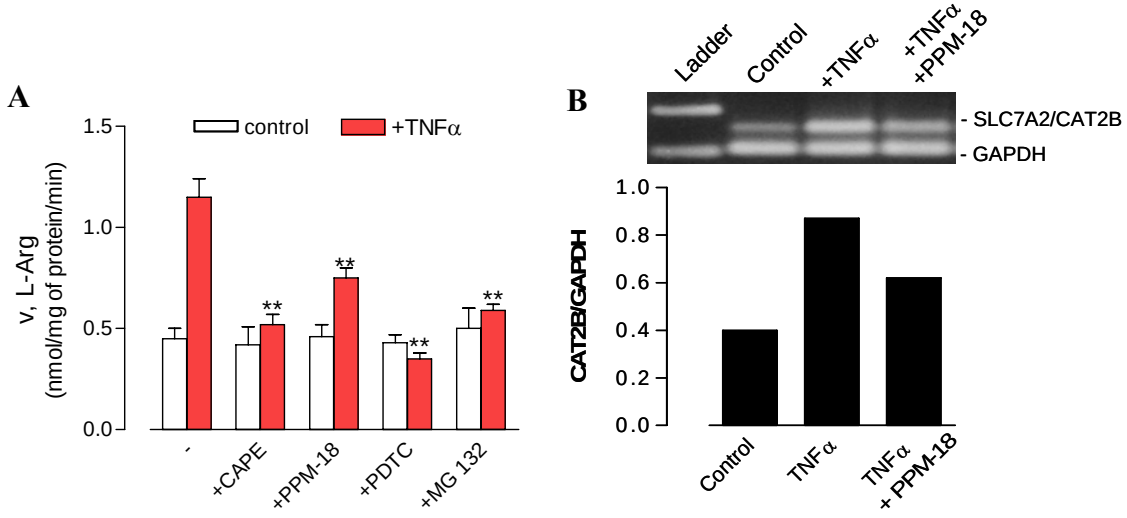


Fig.8 Role of NF-kB on TNFα-induced stimulation of system y^+ activity.

Panel A. CAPE (25 μg/ml), PPM-18 (1 μM), PDTC (100 μM), MG132 (2 μM) inhibit cytokine-induced increase of system y^+ transport activity, without affecting the basal L-Arg uptake. $^{**}P<0.01$(inhibitor present *vs.* inhibitor absent in TNFα treated cells). Panel B. PPM-18 reduces TNFα-dependent stimulation of SLC7A2/CAT2B transcript, as assessed through RT-PCR and the relative densitometric analysis.

In light of these results, the involvement of NF-kB in the molecular mechanism responsible for inflammation-dependent stimulation of L-Arg transport, previously described only in non endothelial models[73], appears now evident also in human endothelial cells; in this model, SLC7/CAT2 gene can be added to the enlarging list of targets of the transcription factor.

Several transduction pathways have been then studied, to ascertain their role in the NF-kB-mediated stimulation of arginine transport by TNFα.

Initially, MAPK cascades have been considered, since evidences demonstrate that in HUVECs short term, adenosine-[99] or D-glucose-dependent[100] stimulation of arginine influx via system y^+ are blocked by the inhibition of p44/42 MAPK; moreover, the stimulation of arginine transport by LPS in smooth muscle cells also appears to involve MAPK, and in particular p38[74]. Nevertheless, in our hands, inhibitors of the main MAPK groups, ERK1/2 (PD98059), $p38^{MAPK}$ (SB203580 and CFPD), and JNK (SP600125), do not interfere with TNFα stimulation (9h) of endothelial arginine transport (Fig.9), excluding the involvement of these transduction pathways in the stimulatory effect of the cytokine on human endothelium.

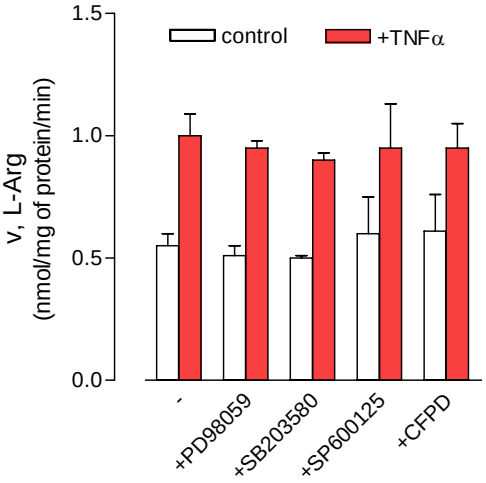


Fig.9 Effects of MAPK inhibitors.

Cells have been incubated with PD98059 (50 μM), SB203580 (20 μM), SP600125 (5 μM), or CFPD (15 μM) for 9h. Both basal and TNFα-stimulated activities of system y^+ are unaffected by any of the inhibitors tested.

Afterwards, also PKC pathway has been studied, since in HUVECs its inhibition is known to counteract the effects of TNFα treatment on the expression of adhesion molecules [101]. Three distinct approaches have been employed to evaluate the role of PKC in TNFα-induced stimulation of system y^+ transport activity:

- ✓ chronic exposure to 100 nM PDBu to downregulate (c) and (n)PKCs activity[81,102];
- ✓ use of chelerythrine (inhibitor of the catalytic domain of PKCs) at the maximal concentration non toxic for HSVECs (Visigalli, unpublished results);
- ✓ use of the inhibitor peptide for PKCζ.

Results in Fig.10 show that TNFα-dependent stimulation of system y^+ transport activity is still clearly detectable under each of the three conditions adopted. This is in contrast with the paper by Pan et al., where cytokine-mediated stimulation of arginine transport in HUVECs is shown to be blocked by chelerythrine[75]; moreover, results published by Krotova and colleagues indicate that the downregulation of (c)PKC, through a 18h exposure to phorbols, produces an increase of arginine transport[102]. Whatever the discrepancy with literature may be due to differences in the endothelial model employed and/or in the

isozyme pattern, PKC involvement in the regulation of arginine transport in endothelial cells deserves further investigations; however, in our hands any of the PKC isozymes appears to be requested for TNFα effect on L-Arg transport in HSVECs.

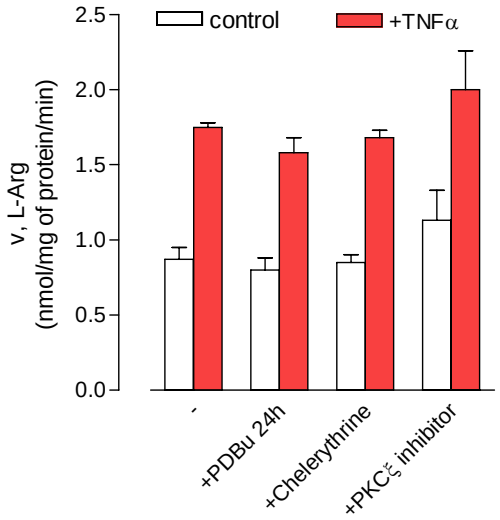


Fig.10 Effects of PKCs inhibitors.
Cells, pre-incubated with chelerytrine (5 μM) for 1h, PKCζ inhibitor peptide (10 μM) for 1h, or PDBu (100 nM) for 24h, have been further treated with TNFα for 9h. L-Arg transport looks stimulated under all the experimental conditions, even in the presence of the inhibitors.

Finally, also mTOR involvement in TNFα-induced stimulation of system y^+ transport activity has been investigated. L-Arg transport has been measured in human endothelial cells upon treatment with rapamycin, a known inhibitor of the kinase (Fig.11).

Surprisingly, mTOR inhibition not only doesn't inhibit the TNFα-dependent stimulation of arginine transport, but, rather, it significantly enhances the effects of the cytokine. Indeed, when combined with TNFα, L-Arg transport is significantly higher than in cells incubated with the only cytokine; moreover, also in the absence of TNFα, rapamycin stimulates arginine transport, with values ranging from +10% to +50%, depending on the cell strain employed. Consistently with recently published evidences[103,104], these results suggest that, in spite of the antagonism described for rapamycin and TNFα in vascular smooth muscle cells[105], the effects of the two compounds may actually synergize in endothelial cells.

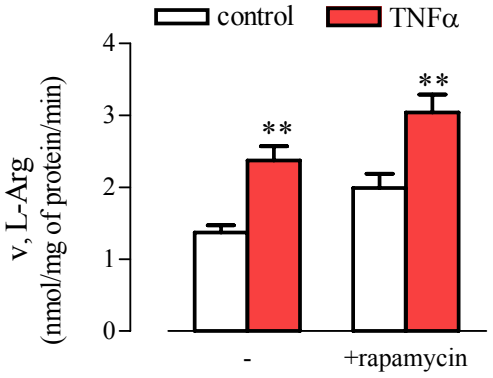


Fig.11 Effects of mTOR inhibition.

Cells have been incubated with rapamycin for 9h, with or without TNFα. The activity of system y^+ under both basal and cytokine-stimulated conditions appears induced by the macrolide in HSVECS.
**P<0.01 vs. control, untreated cells

In conclusion, the first part of the research thus far described demonstrates that

- ✓ human endothelial cells from foetal (HUVEC) and adult (HSVEC) veins respond similarly to pro-inflammatory cytokines or bacterial LPS by stimulating the sole transport system y^+ activity for L-Arg influx, through the induction of SLC7A2 transcript;
- ✓ the enhancement of arginine transport induced by the cytokine requires mRNA transcription and, more precisely, the activation of NF-kB transcription factor;
- ✓ neither MAPK or PKC pathways appear to be involved in the regulatory mechanism responsible for transport stimulation;
- ✓ mTOR inhibition is ineffective in lowering TNFα-dependent stimulation of L-Arg transport, and even strengthens it, suggesting that rapamycin relieves a downregulatory effect of the PI3-kinase/Akt/mTOR transduction pathway on system y^+-mediated amino acid transport .

The second part of this study has then concerned the further characterization of the role of mTOR in TNFα-dependent stimulation of arginine transport in human endothelium, so as to define the mechanism responsible for the strengthening of cytokine-dependent stimulation of L-Arg transport upon incubation of endothelial cells with rapamycin.

2.2. DEFINITION OF THE ROLE OF mTOR IN CYTOKINE-DEPENDENT STIMULATION OF ARGININE TRANSPORT IN HUMAN ENDOTHELIAL CELLS

The consequences of mTOR inhibition on human endothelium have been assessed also on foetal endothelial cells derived from umbilical vein (HUVECs), to verify whether the

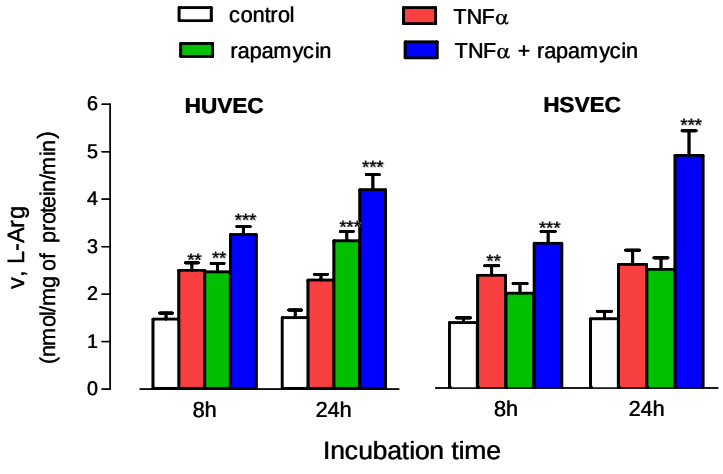


Fig.12 Effects of rapamycin on Arg transport.

In HUVEC and HSVEC rapamycin stimulates the activity of system y^+, both in the absence and in the presence of TNFα.
**P<0.01, ***P<0.001 (vs. control, untreated cells)

effects observed in HSVECs are tissue specific or not. The results obtained are comparable in the two models: also in HUVECs, rapamycin stimulates L-Arg transport, with effects even more marked than in HSVECs. Such stimulation, already observable after 8h treatment, becomes even more evident after 24h: at this time, in cells incubated with both compounds, system y^+-mediated arginine transport is 2- or 3-fold increased, compared with untreated, control cells (Fig.12).

To better characterize this effect, a kinetic analysis of system y^+ transport activity (i.e. arginine transport in the presence of excess extracellular leucine) has been performed with untreated HSVECs or with cells treated for 24h in the presence of TNFα and rapamycin. The

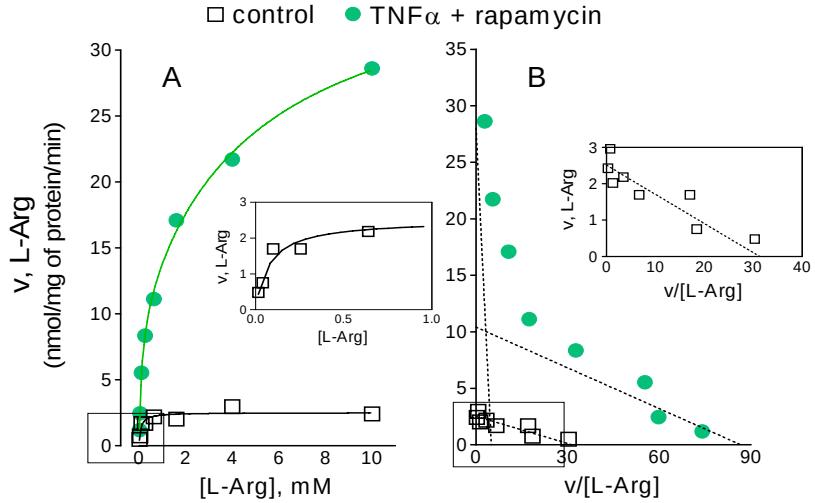


Fig.13 Kinetic analysis of L-arginine transport in HSVEC.

Panel A. Curves represent the best fit of the data to Eqn. 1 for control cells or to Eqn. 2 for TNFα+rapamycin-treated cells (see Experimental Procedures). In the *inset*, data of arginine influx obtained with arginine concentrations up to 1 mM (control) are represented in an amplified scale. Panel B. Eadie-Hofstee graphical representation of data in Panel A. In the *inset*, data of arginine influx obtained in control are represented in an amplified scale.

	High affinity component	***Low affinity component***
control	V_{max} 1.67±0.114 nmol/mg of prot/min Km 0.08±0.027 mM	-
+ TNFα + rapamycin	V_{max} 10.4 ±2.35114 nmol/mg of prot/min Km 0.12±0.035 mM	V_{max} 27.3±2.48114 nmol/mg of prot/min Km 5.0±1.48 mM

Table 4. Kinetic constants of L-Arg transport

diffusive component (Kd of 0.95±0.02 min^{-1}), derived from the linear regression of influx values at high arginine concentrations (from 2 to 10 mM) in control cells, has been subtracted to influx data to yield the saturable arginine influx. The results are presented both in *v* vs. *[S]* representation (Panel A) and in the Eadie-Hofstee graphical transformation (Panel B) so as to allow a better discrimination among the transport components. As shown in Fig.13, saturable arginine transport in untreated cells is satisfactorily fitted with a single system (insets in Panels A and B), while, in cells treated with TNFα and rapamycin, transport data are best fitted by the operation of two saturable transport systems with very divergent affinities (Panel B): the high affinity component, whose V_{max} is markedly increased while its Km is not significantly modified, and a low affinity component, not detectable in control cells (Table 4). Similar analyses indicates that incubation with TNFα or rapamycin alone raises the V_{max} of the high affinity component and causes the appearance of the low affinity component, although at a lesser extent than the two compounds together (results not shown). While the Km of the high affinity component is in the range attributed to the high affinity system y^+-related CAT isoforms (CAT1 and CAT2B), the Km of the low affinity component is compatible with the operation of CAT2A transporter[106].

Consistently with this hypothesis, molecular analyses at protein level reveal that rapamycin-treated cells express higher amounts of CAT2 with respect to control, untreated cells. The cell content of CAT2 has been determined in HUVECs incubated with TNFα, rapamycin, or both, by using, in both Western Blot and immunocytochemistry analyses, a polyclonal antiserum that does not discriminate between CAT2A and CAT2B isoforms. The results of the Western Blot analysis (Fig.14) indicate that, while carrier proteins are barely

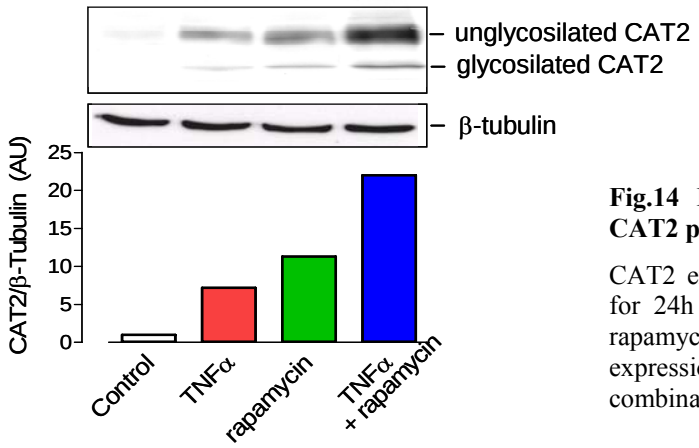


Fig.14 Effect of rapamycin on the expression of CAT2 protein.

CAT2 expression has been assessed in cells incubated for 24h in the absence or in the presence of TNFα, rapamycin, or both. Rapamycin stimulates CAT2 expression, both when employed alone or in combination with TNFα.

expressed in control, untreated HUVECs, a 8-fold increase in CAT2 expression is detected in response to a 24h treatment with either rapamycin or TNFα, confirming that either

compounds stimulate L-Arg transport through the induction of the amino acid transporter; however, the largest induction (20-fold) is detectable in cells treated with both compounds together.

The same results are confirmed by the immunocytochemical analysis of CAT2 expression in HUVECs (Fig.15): while the carrier proteins are almost undetectable in control cells, they become evident in cells treated for 24h with TNFα or rapamycin. More interestingly, cells treated with both rapamycin and TNFα uniformly present a much higher CAT2 signal, with an evident intracellular distribution, although a clear cut positivity of plasma membrane is also evident.

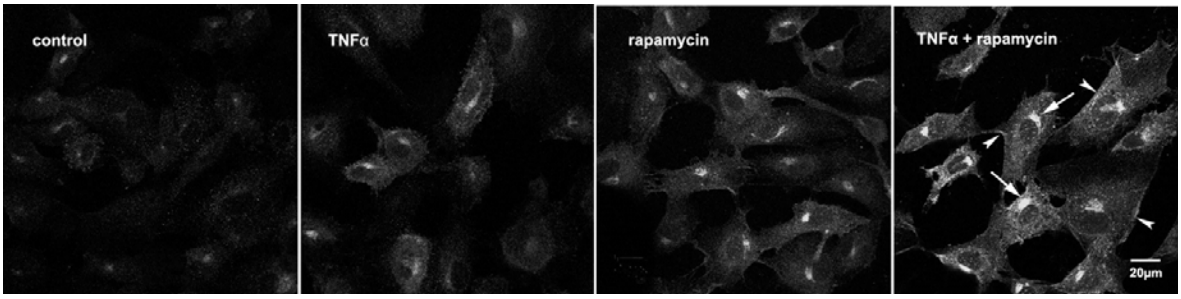


Fig. 15 Expression of CAT2 proteins in HUVECs.

CAT2 proteins signal, undetectable in control, untreated cells, becomes evident upon treatment with rapamycin or TNFα, and, even more, when the two compounds are employed together. Arrows indicate perinuclear areas of high CAT2 expression, arrowheads the membrane expression of CAT2 proteins.

To characterize the mechanism responsible for the induction of CAT2 protein, a qPCR analysis of genes related to system y^+ activity has been also performed (Fig.16). While the levels of SLC7A1/CAT1 are unaffected by any of the experimental conditions adopted (data not shown), transcripts for both SLC7A2/CAT2A (Panel A) and SLC7A2/CAT2B (Panel B) appear induced by the treatment with both TNFα or rapamycin, in the absence and in the presence of the cytokine; in this latter case, a massive (>5-10 fold) increase in both CAT2A and CAT2B mRNA levels is consistently observed after 8h and, even more clearly, after 24h of incubation. The relative increase in mRNA abundance is of the same order of magnitude as the increase in protein levels, thus suggesting that no gross regulations at post transcriptional level are active; moreover, intermediate regulatory steps at the translational level are unlikely, since the increase observed for the immature, non glycosylated form of CAT2 is proportional to that of the mature, fully glycosylated form (Fig.14). Thus, rapamycin-dependent stimulation of L-Arg transport in human endothelial cells appears as quite a straightforward process, with increased SLC7A2 transcription followed by increased abundance of CAT2

proteins and the stimulation of transport V_{max}; the stimulatory effects of the drug on system y^+ activity seem therefore to contrast with other regulatory mechanisms of arginine transport, in which the expression of CAT transporters has been described to be finely regulated at post-transcriptional[107,108] or protein level[109].

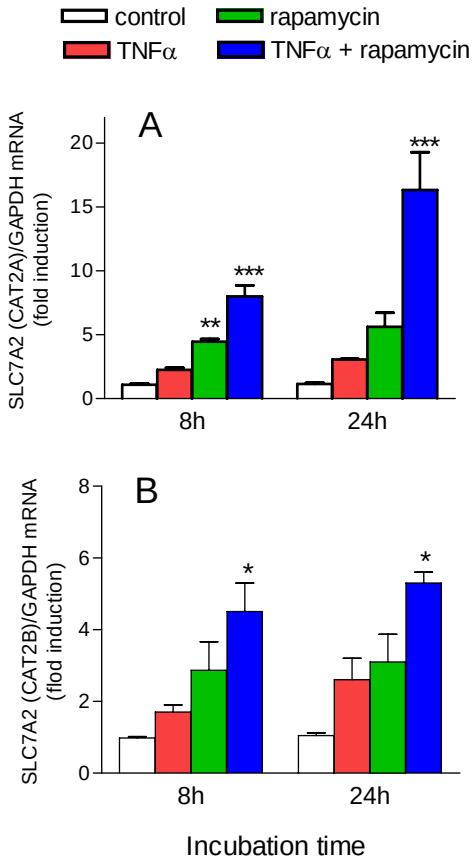


Fig.16 Effect of rapamycin on the expression of genes related to system y^+.

In cells treated for 8h or 24h, rapamycin induces the levels of SLC7A2/CAT2A (Panel A) and SLC7A2/CAT2B (Panel B), particularly when combined with TNFα.
*P<0.05, **P<0.01, ***P<0.001 vs. control cells.

For what concerns L-Arg transport and the induction of the system y^+-related genes, what also appears of peculiar interest is the induction of the low affinity (Km = 5 mM) CAT2A isoform. In human endothelium, SLC7A2/CAT2A expression is almost undetectable in control cells, suggesting that the expression of the low affinity isoform is, at best, marginal under basal conditions. High constitutive expression of CAT2A has been described only in hepatocytes, while the induction of the transporter by cytokines has been reported in other animal cell models, such as rat cardiac myocytes[109] or rat vascular smooth muscle cells [47]; moreover, CAT2A expression is also known to be induced in rat skeletal muscle under stress conditions, such as surgical trauma or food deprivation [2]. Our results demonstrate that in human endothelial cells, while CAT2A contribution appears negligible under basal conditions, it becomes clearly detectable in cells treated with both TNFα and rapamycin, when arginine transport can be satisfactorily explained by two mechanisms, one with an high and one with a low affinity for the substrate. The physiologic significance of this induction remains however unclear since, even at the supraphysiological arginine concentrations of the culture medium (0.3 mM vs. normal plasma concentrations ranging around 0.1 mM), the kinetic parameters reported in Table 1 indicate that no more than 20% of arginine influx in cells treated with both rapamycin

and TNFα occurrs through CAT2A, with the remaining 80% attributable to CAT2B and CAT1 activity.

A possible explanation of CAT2A function derives from the analysis of cationic amino acids cell content under the different experimental conditions (Fig.17): treatment with rapamycin, either alone or combined with TNFα, induces a significant increase of the intracellular concentration of both lysine and arginine, consistent with the observed

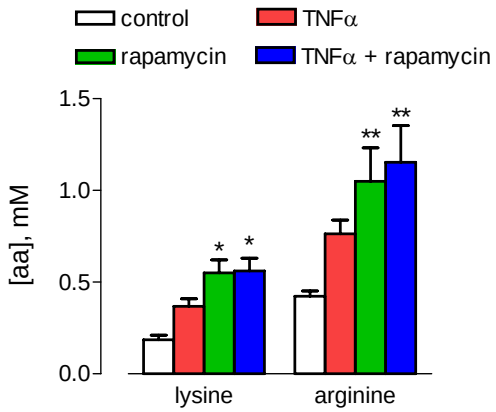


Fig.17 Intracellular concentration of cationic amino acids.

Rapamycin, both alone and together with TNFα, causes an increase in intracellular concentration of arginine and lysine.
*P<0.05, **P<0.01 vs. control cells.

stimulation of system y^+ activity; since rapamycin is known to induce autophagy in cultured cells[110,111], this increase in intracellular arginine levels could be the result of both the induction of arginine influx, referable to CAT2B activity, and the stimulation of cell proteolysis. If this is the case, the stimulation of CAT2A could be explained as a device to prevent the excessive accumulation of cationic amino acids in the intracellular compartment, since this low affinity transporter has been proposed as an export route for arginine derived from the breakdown of proteins[2].

In summary, the second part of this research has led to the following observations:

- ✓ mTOR inhibition is not associated to the suppression of TNFα-dependent stimulation of endothelial L-Arg transport, but, rather, to its further strengthening;
- ✓ even when employed alone, rapamycin stimulates arginine transport across the cell membrane through the induction of the transcription of both SLC7A2/CAT2A and CAT2B messengers;
- ✓ rapamycin-dependent stimulation of SLC7A2 is followed by the straightforward increase in expression of CAT2B isoform and, more interestingly, by the induction of the low affinity, stress-associated CAT2A protein;

- ✓ consistently with the stimulation of system y^+ activity, rapamycin causes a significant increase of both arginine and lysine intracellular concentrations, probably referable to an induced amino acid transport (CAT2B) and to a higher rate of cell proteolysis, likely involving CAT2A.

These results suggest that, under basal conditions and, even more, in the presence of pro-inflammatory stimuli, mTOR is responsible of the repression of CAT2 transcription, although the functional consequences of the observed changes in CAT expression and arginine transport remain undefined. However, given the association of CAT2A transporters to stress conditions, its induction by rapamycin could insinuate that mTOR inhibition is responsible of a severe cellular stress in human endothelium and, as a consequence, that the kinase plays a central role in the preservation of endothelial function and viability.

2.3. EFFECT OF mTOR INHIBITION ON NITRIC OXIDE PRODUCTION

Given the central role of L-arginine in the biosynthesis of nitric oxide and the relevance of this signalling molecule in the maintenance of vascular integrity, a possible link between nitric oxide and the processes involved in the intracellular concentration of its precursor have been investigated in a variety of endothelial cell models, with controversial results: for example, Durante and coworkers found that inflammatory cytokines induce the expression of both CAT transporters and iNOS in vascular smooth muscle cells, to direct the metabolism of L-arginine to the synthesis of the anti-proliferative gas[112]; conversely, protein tyrosine kinase inhibition seems to attenuate iNOS, but not CAT2B induction in RAMSCs[74], indicating differences in the stimulation of the two genes.

In light of these contributions, we have assessed whether the high concentration of L-arginine detected, upon inhibition of mTOR, in HUVECs might be somehow related to changes in NO production, thus providing a rationale for the massive stimulation of cationic amino acid transport in rapamycin-treated human endothelial cells.

As expected from endothelial cells[17,113-115], incubation with TNFα lowers nitric oxide output and NOS expression in HUVECs (Fig14-15); on the contrary, what stands out as particularly interesting is that cells treated with rapamycin, in spite of their higher levels of CAT2 and their higher intracellular concentration of arginine, rather synthesizes less NO than untreated controls or cytokine-treated cells (Fig.18).

These results clearly demonstrate that the transport of cationic amino acid, as well as the expression of the related transporters, can be uncoupled from the production of nitric

oxide; even in endothelial cells, where this molecule plays a key role under both physiological and pathological conditions, being the endothelium one of the first-line defences to inflammatory insults, neither rapamycin nor TNFα are able to stimulate NO production, although they both highly induce the transport of L-arginine.

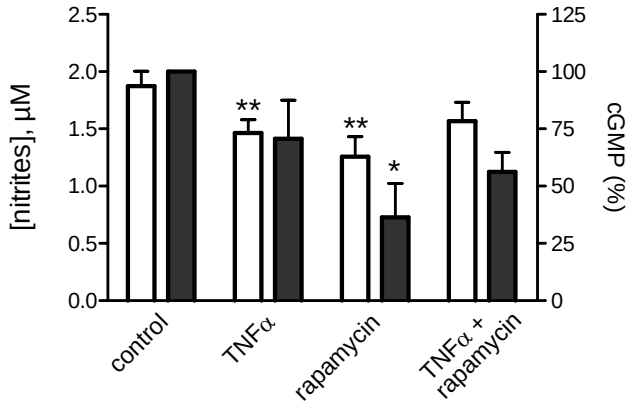


Fig.18 Rapamycin lowers NO production.

The amount of both nitrite and bioactive NO produced by HUVECs is decreased by 24h-treatment with rapamycin or TNFα.
*P < 0.05, **P < 0.01, treated *vs.* control, untreated cells.

At molecular level, consistently with reports demonstrating HUVEC's lack of iNOS activity even under inflammatory conditions[13,15], our results show that the enzyme is completely undetectable neither in the presence of TNFα nor of rapamycin (data not shown). Conversely, the observed decrease of nitric oxide production correlates with the inhibition of the endothelial isoform of nitric oxide synthase, eNOS (NOS3), at both mRNA and protein levels (Fig.19): indeed, together with a diminished synthesis of nitric oxide, the treatment with rapamycin also produces a significant decrease of NOS3 mRNA (Panel A), particularly

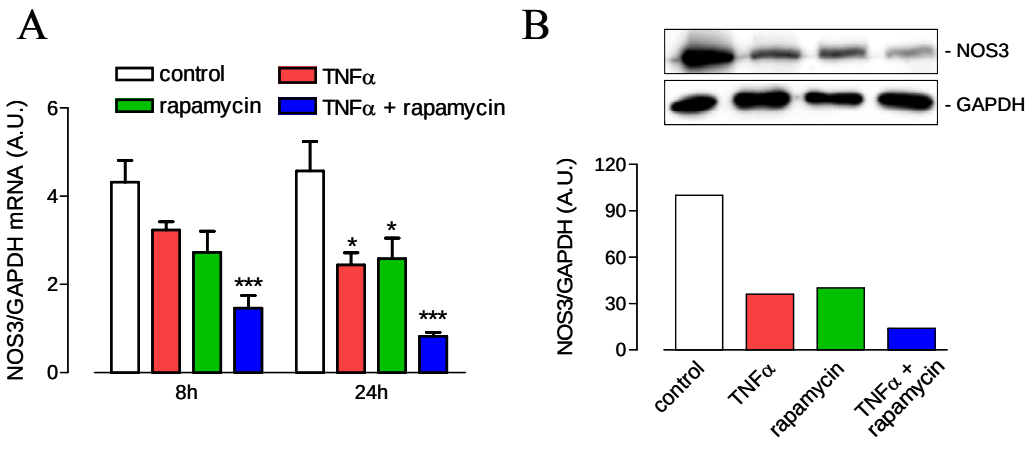


Fig.19 Effects of rapamycin on NOS3 expression.

Panel A. NOS3 transcript levels are lowered after 8h and 24h treatment by both TNFα and rapamycin, especially when employed together. Panel B. Consistently with mRNA results, also NOS3 protein levels are significantly lowered after 24h-treatment with rapamycin, TNFα or TNFα+rapamycin, with respect to control cells.
*P < 0.05, ***P < 0.001, treated *vs.* control, untreated cells.

evident after 24h-treatment, and a likewise reduced expression of the correspondent protein (Panel B). In the same experiment, an even more marked decrease of NOS3 mRNA and protein is caused by the combined treatment with rapamycin and TNFα, both at 8h and at 24h of incubation (Panels A and B), although under this condition, the production of NO is reduced to a lesser extent than in cells treated with rapamycin alone. This latter result may be however explained by the finding recently described by Yang and Rizzo[116] that TNFα exert a stimulatory effect on NOS3 activity, thus explaining why, in cells treated with TNFα and rapamycin, there is no significant decrease in NO output even in the presence of the lowest NOS3 expression.that the inhibition of NO output may derive also from the inhibition of Akt that has been described to stimulate NOS3 activity by direct phosphorylation[20]; indeed, it has

In light of the fundamental role of eNOS-derived nitric oxide in the maintenance of physiological vascular tone and permeability, this rapamycin-induced hindrance of endothelial NO output suggests that the drug may be deleterious for endothelial function, either *per se* or, even more, when combined with pro-inflammatory cytokines. Indeed, in our experiments, the results obtained through the measurement of the stable derivatives of nitric oxide, nitrites, are confirmed by the assessment of the amount of actual bioactive NO produced under the different experimental conditions (Fig.18), thus confirming the real lowering of the production of endothelial relaxing factor upon inhibition of mTOR in HUVECs. Since impaired endothelial-dependent NO production in blood vessels is responsible for attenuated endothelial-dependent vasodilation, characteristic of many different cardiovascular disease processes and usually referred to as endothelial dysfunction[117,118], our results clearly suggest detrimental effects of rapamycin on human endothelium. According to our *in vitro* findings, clinical evidences reported by Fuke et al[119] suggest that the abnormal vasoconstrictive response to acetylcholine, observed in the peri-stent area after implantation of rapamycin eluting stents, are likely related to an impaired NO production and to a more general endothelial dysfunction of the vasculature; this effect could also be responsible of the increased incidence of Late Stent Thrombosis (LST) observed in patients with rapamycin-medicated DES[120]. The molecular basis for the rapamycin-dependent endothelial defect has not been fully elucidated yet; the possibility exists that rapamycin is toxic *per se* or, rather, exerts detrimental effects on endothelium due to peculiarities of stent microenvironment, such as, for example, the pro-inflammatory cytokines, whose levels are particularly increased in sites of arterial injury[121] and after coronary stenting[122].

As a consequence, the last part of the research has been intended to define the role of mTOR in the maintenance of endothelial welfare, under both physiological and pathological conditions. In particular, the experiments of this last part focused on the consequences of the inhibition of the kinase in both unstimulated or TNFα-treated endothelial cultures, in order to verify whether the detrimental effects reported for rapamycin eluting stents are due to the generation of an inflammatory response in the vascular district by the drug itself or, rather, by its hampering the vascular response triggered by exogenous pro-inflammatory stimuli.

2.4. EFFECT OF mTOR INHIBITION ON ENDOTHELIAL FUNCTION AND VIABILITY

In accordance with the assumption of toxic effects of rapamycin, other known markers of endothelial activation appear induced by the drug, both in the absence and in the presence of TNFα. Indeed, besides our findings demonstrating rapamycin-dependent induction of SLC7A2/CAT2A, an indicator of cellular stress, also TNFα-induced expression of Tissue Factor is known to be reinforced by rapamycin in human endothelial cells[104].

Moreover, as a further support, the expression of other markers of endothelial function, i.e. the expression of the adhesion molecules and endothelial cell mobility, have been considered upon incubation with the mTOR inhibitor, both in the absence and in the

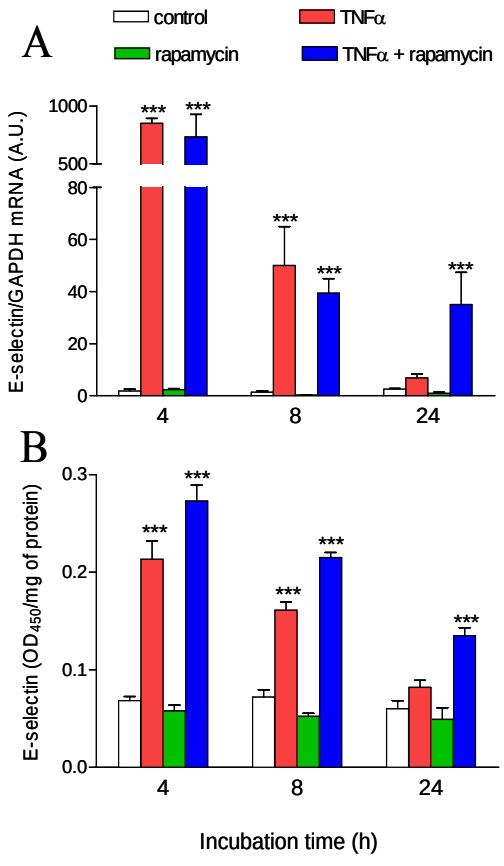
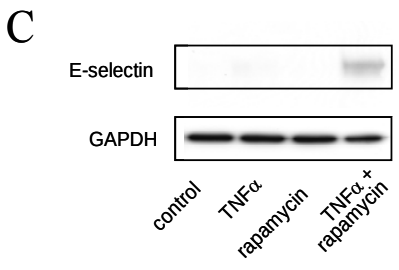


Fig.20 Rapamycin stabilizes TNFα-induced expression of E-selectin

Panel A. E-selectin transcript levels are increased by TNFα after 4 and 8h incubation, but not by rapamycin; this latter, however, strengthens the cytokine's effect, extending it till 24h. Panel B. Consistently with mRNA, also protein levels, detected through ELISA, are significantly higher in TNFα+rapamycin-treated cells with respect to controls and still detectable in TNFα+rapamycin treated cells after 24h, as confirmed by Western Blot (Panel C). ***P < 0.001, treated *vs.* control, untreated cells.



presence of pro-inflammatory stimuli. As expected[123], TNFα induces in HUVECs a strong and transient stimulation of E-selectin at both mRNA and protein level (Fig.20, Panels A, B and C), with maximal effects at 4h and values substantially comparable to basal levels within 24h. Conversely, rapamycin alone does not stimulate E-selectin expression, neither in the absence nor in the presence of TNFα, at any incubation time. Interestingly, the combined treatment with the two compounds exerts the same effect as the cytokine, up to 8h; then, after 24h, E-selectin mRNA levels appear much higher in cells incubated with both compounds than in cells treated with each compound alone. Consistently, under this condition, protein levels are still significantly higher than in control cells (Panel B) and, consistently, still detectable with Western Blot (Panel C). These findings suggest that rapamycin toxicity may be reinforced by the simultaneous presence of exogenous inflammatory stimuli, thus explaining why clinical endothelial dysfunction is particularly evident in peri-stent areas, where inflammatory conditions are present.

As another indicator of endothelial function, cells mobility in vitro has also been

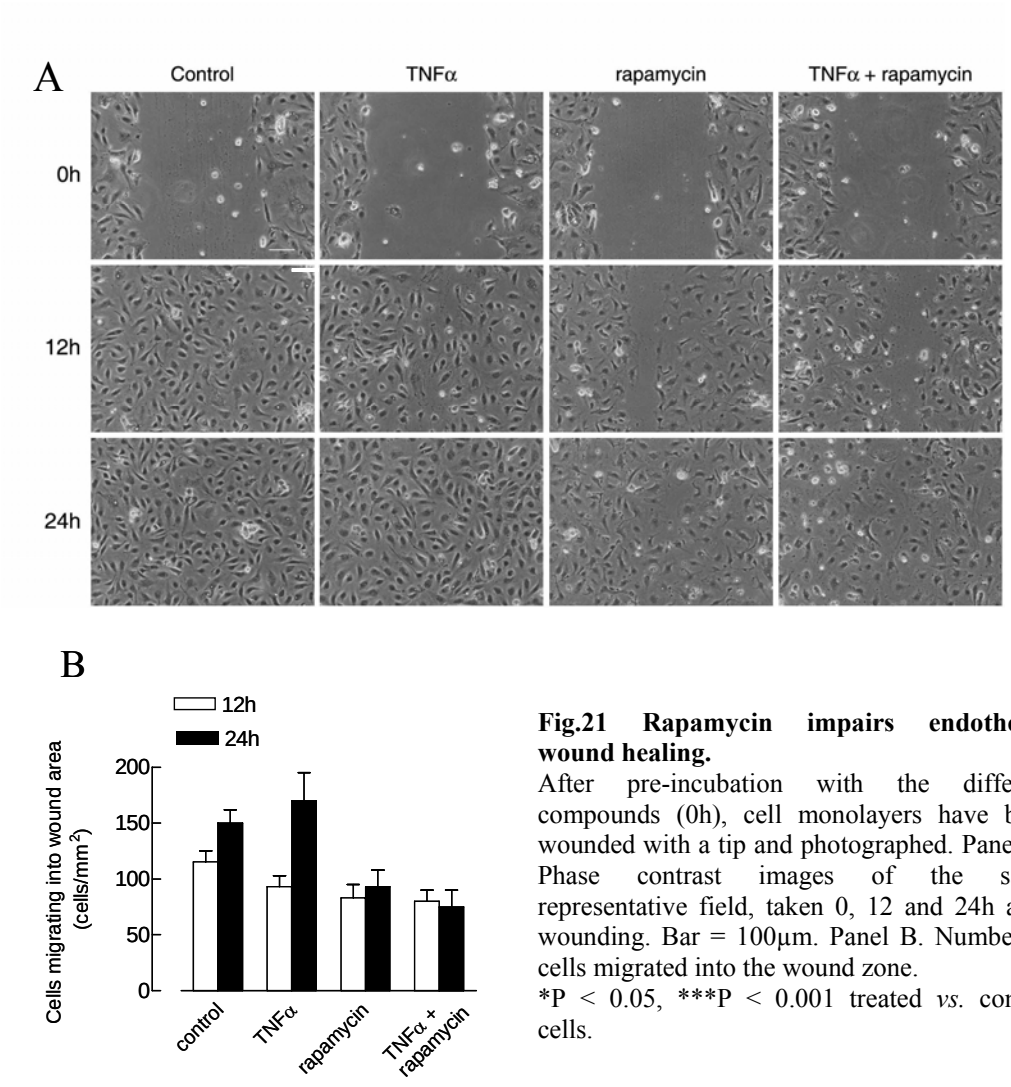


Fig.21 Rapamycin impairs endothelial wound healing.
After pre-incubation with the different compounds (0h), cell monolayers have been wounded with a tip and photographed. Panel A. Phase contrast images of the same representative field, taken 0, 12 and 24h after wounding. Bar = 100μm. Panel B. Number of cells migrated into the wound zone.
*P < 0.05, ***P < 0.001 treated *vs.* control cells.

assessed with a wound healing assay, an in vitro test that evaluates the capability of an endothelial culture to react against a mechanical damage[64].

The results obtained (Fig.21) show that this peculiar endothelial function is hampered by rapamycin, independently of TNFα: indeed, while the wound is almost completely repaired after 12h in both control and TNFα-pretreated HUVECs, wound borders are still clearly detectable in cells preincubated either with rapamycin or TNFα and rapamycin, where they remain evident till 24h. Under the same conditions, the estimation of the endothelial re-colonization indicates that, both at 12h and at 24h, fewer cells have migrated into the wounded area, compared to either control, untreated cultures or cultures treated with the cytokine alone (Fig.21, Panel B). In agreement with this observation, a recent autopsy study by Schaffer and colleagues describes evident defects of wound healing in rats undergone dorsal skin incision, after treatment of the animals with rapamycin; in light of this observation, the authors suggest that one of the major mechanisms responsible for stent thrombosis with DES may reside in rapamycin-dependent defects or delays of stent reendothelization[124].

Looking at the phase contrast images of the different cultures, what appears also particularly relevant is that a 24h treatment with rapamycin, either alone or together with TNFα, leaves endothelial cultures still apparently disorganized, although the wound is almost repaired, with evident morphological changes, such as loss of cobblestone organization and enhanced cell pleomorphism; in the same fields, frankly apoptotic cells are evident too, indicating that the antibiotic may exert cytotoxic effects on endothelial cultures. In order to verify this latter hypothesis, dose-dependent effects of rapamycin on HUVEC cell number have been assessed, both in the absence and in the presence of inflammatory stimuli (Fig.22, Panel A); in parallel cultures, cell viability has been quantified with the fluorescent indicator resazurin, under the same experimental conditions (Fig.22, Panel B). Identical measurement have been also repeated in cells obtained from human aorta (HAECs), so as to determine whether the effects of the drug on cell viability are specific of human foetal cells or also reproducible in adult endothelial cells (Fig.22, Panels C and D).

As shown in the plot, rapamycin causes a significant decrease in HUVEC cell number at concentrations higher than 1 nM when employed alone while, in the presence of TNFα, determines a significant cell loss even at this concentration (Panel A); consistently, cell viability is significantly affected at rapamycin concentrations higher than 1 nM both in the absence and in the presence of TNFα (Panel B). Similar effects on cell number (Panel C) and viability (Panel D) upon treatment with rapamycin 100nM are detectable also in endothelial

cells from human aorta (HAEC), thus demonstrating that the cytotoxic effects of the drug are independent of the endothelial cell model employed.

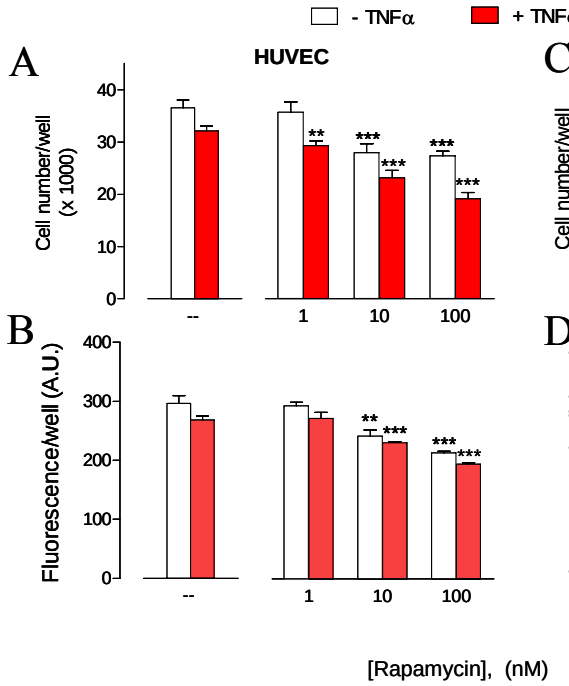


Fig.22 Dose-dependent effects of rapamycin on HUVEC and HAEC viability.
Rapamycin causes a significant cell loss, in both HUVEC (Panel A and B) and HAEC cultures (Panels C and D), particularly when combined with TNFα. Detrimental effects of the drug have been assessed as decrease of both cell number (Panels A and C) and viability (Panels B and D).
*P < 0.05, **P < 0.01, ***P < 0.001 treated *vs.* control, untreated cells.

Following these first observations, a further characterization of rapamycin-dependent cell loss has been performed, studying indicators of apoptosis and necrosis in both HUVECs and HAECs treated with the drug (Fig.23). Rapamycin and TNFα, either alone or used together, increase the percentage of the PI stained sub-G1 population, a marker of late apoptotis or necrosis, although only the combined treatment with the two compounds

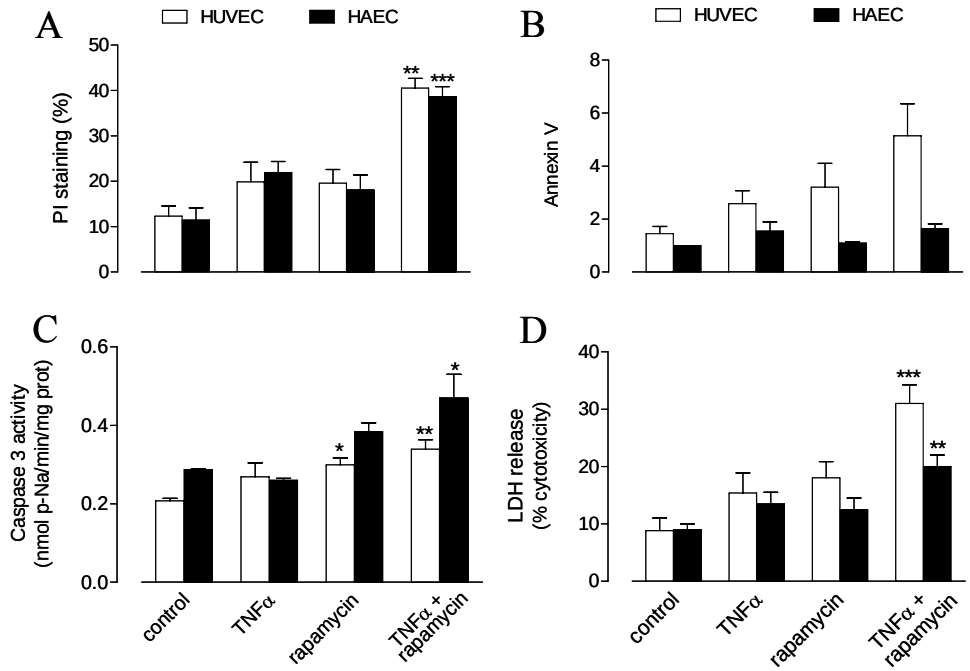


Fig.23 Effects of rapamycin on HUVEC viability.

Combined treatment with rapamycin and TNFα for 24h causes an increased number of cells in the sub-G1, propidium iodide-stained population (Panel A), a more evident staining by Annexin V (Panel B), a higher Caspase3 activity (Panel C) and a more evident release of LDH (Panel D).
*P < 0.05, **P <0.01, ***P < 0.001, treated *vs.* control cells.

produces a significant increment of this index in both cell models (Panel A); a similar tendency is displayed by both Annexin V staining of early apoptotic cells (Panel B) and Caspase3 activity values under the different experimental conditions (Panel C), indicating the triggering of an apoptotic pathway. Finally, also the occurrence of necrotic events is evident, as demonstrated by the marked increase of LDH release observed in HUVECs and HAECs incubated with rapamycin and TNFα (Panel D).

These results altogether demonstrate that a prolonged exposure to rapamycin hinders endothelial cell viability, as well as function, therefore suggesting a key role for mTOR kinase in the preservation of endothelial welfare and providing an explanation to clinical evidences of endothelial dysfunction in the area of DES implantation. The molecular mechanisms involved in such events remain however unclear.

In order to further investigate the molecular targets of rapamycin responsible for its effects on human endothelium, other compounds inhibiting mTOR in a rapamycin-independent way have been tested: AICAR, an AMPK-activator that selectively inhibits TORC1 complex, as demonstrated by the hypophosphorylation of p70S6K but not of Akt, and wortmannin, a widely employed inhibitor of PI3K that inhibits Ser473-Akt phosphorylation, without affecting p70S6K. The results of the Western Blot analysis, reported in Fig.24, confirm the expected effects of AICAR and wortmannin on TORC1 and TORC2 targets, Thr389-p70S6K and Ser473-Akt respectively. More interestingly, rapamycin, beyond causing a complete suppression of p70S6K phosphorylarion, as expected from TORC1 inhibition, also causes a decrease of Akt phosphorylation, a change only compatible with TORC2 inhibition, thus far considered rapamycin insensitive.

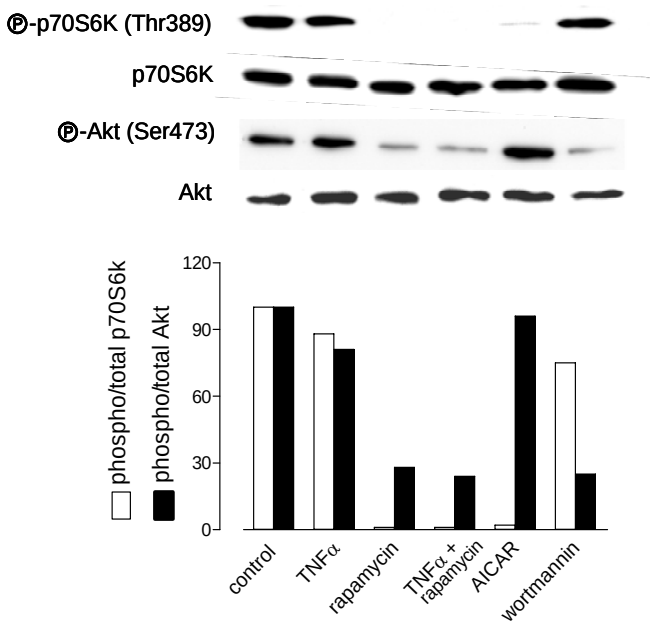


Fig.24 Effects of rapamycin on p70S6K and Akt phosphorylation.

The treatment of 24h with AICAR causes a hypophosphorylation of p70S6K but not of Akt, while wortmannin affects the only Ser473 phosphorylation. Rapamycin, both alone or combined with TNFα, similarly inhibits p70S6K and Akt phosphorylations, demonstrating its effects on both TORC1 and TORC2.

In agreement with these findings, also the changes in the organization of actin cytoskeleton observed in HUVECs upon incubation with rapamycin (Fig.25) support the assumption of mTORC2 sensitivity to the drug: rictor knockdown (and consequently TORC2 inhibition) is known to promote the formation of thick actin stress fibres throughout the cytoplasm[125]; consistently, the confocal images for the visualization of actin cytoskeleton in our endothelial model indicate that rapamycin, either in the absence or in the presence of TNFα (Panels C and D), markedly modifies the organization of the cytoskeleton, leading to

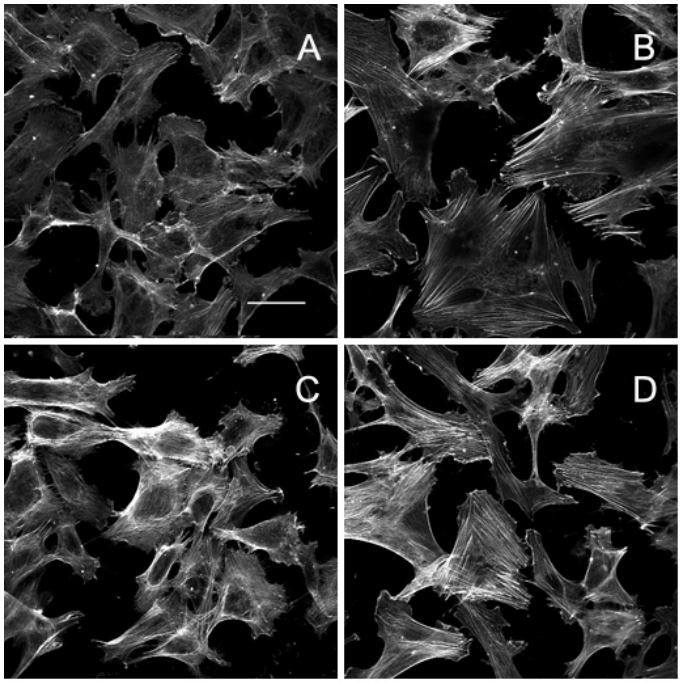


Fig.25 Effects of rapamycin on actin cytoskeleton

24h treatment with rapamycin, either in the absence or in the presence of TNFα (Panels C and D), leads to the formation of bundles of actin stress fibres, markedly thicker than those observed in control or TNFα-treated cells (Panel A and B).

the formation of evident bundles of stress actin fibers much thicker than those observed in control cells or in cells treated with the cytokine alone (Panels A and B).

We can conclude that rapamycin exerts its effects on human endothelium by inhibiting both mTOR complexes, as recently proposed also by Sarbassov et al.[126], and not only mTORC1, as thus far accepted.

Since the activation of the Akt pathway is known to mediate pro-survival signals[79], its inhibition by rapamycin via TORC2 could explain why cell death is promoted under our experimental conditions. In further support of this hypothesis, the assessment of AICAR and wortmannin effects on endothelial cell viability demonstrates that only the latter causes a significant cell loss, comparable to that caused by rapamycin, while AMPK-activator does not (Fig.26); this confirms the involvement of mTOR kinase in the maintenance of endothelial viability, underlying the specific role of mTORC2.

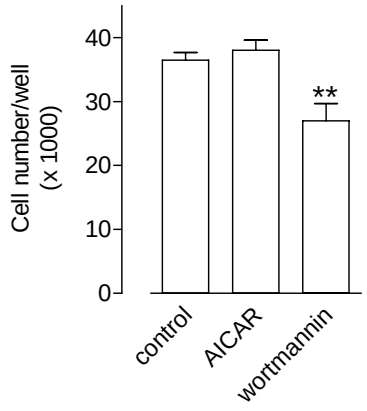


Fig.26 Effects of AICAR and wortmannin on HUVEC viability

24h treatment with AICAR doesn't affect endothelial cell number, while wotmannin causes a significant cell loss.
**P <0.01 treated *vs.* control, untreated cells.

The demonstration of the inhibitory effect of rapamycin on TORC2 can also help to explain the results of the wound healing assay. Indeed, as already described, a recent study has demonstrated that rapamycin impairs wound healing in vivo[124] and this effect is associated to a decrease of VEGF expression and of NO production in the wound area[127]: since it is widely accepted that Akt-dependent transduction pathway plays a pivotal role in VEGF-mediated endothelial cell migration[128], it is possible that also the inhibitory effect of rapamycin on cell motility depends on mTORC2 inhibition. This hypothesis would be also consistent with the findings that high doses of rapamycin hamper angiogenesis in vivo by inhibiting Akt phosphorylation[128,129].

Conversely, the specific contribution of TORC1, rather than TORC2, to rapamycin-dependent modulation of other endothelial functions (i.e. NO production and adhesion molecules expression) has not been fully elucidated thus far: in human aortic endothelial cells, AICAR has been demonstrated to activate eNOS and, consequently, to induce NO production[130], likely through the TORC2-dependent activation of Akt by AMPK and TSC1\2[131]; consistently, prolonged treatment with rapamycin, inhibiting both TORC1 and TORC2, is shown to decrease endothelial NOS expression, suggesting that TORC2 could effectively play a pivotal role in the modulation of endothelial nitric oxide production too. On the other hand, recent evidences demonstrate that the cytokine-induced expression of pro-inflammatory and adhesion molecule genes is inhibited by the AICAR-dependent activation of AMPK[132], while our data show that rapamycin stimulate them (see E-selectin, Fig.20); whether this effect is mediated by TORC1 rather than TORC2 remains however unclear.

In summary, this last part of the research has demonstrated that:

- ✓ a prolonged treatment with rapamycin, alone or even more clearly when employed in association with pro-inflammatory cytokines, impairs endothelial viability and

function, modifying adhesion molecules expression and cell mobility and impairing endothelial nitric oxide production;

- ✓ a 24h treatment with rapamycin leads to the inhibition of both mTOR complexes, TORC1, as already expected from literature data, and TORC2, previously considered rapamycin-insensitive;
- ✓ the molecular mechanisms underlying the effects of mTOR inhibition on cell viability and function are different: detrimental effects of rapamycin on cell viability are likely due to the inhibition of TORC2. Instead, as far as endothelial functions are concerned, the pathways involved seem to be function-specific: the modulation of nitric oxide production is probably due to the activity of TORC2, while it remains uncertain whether the stimulation of adhesion molecules expression occurs preferably via TORC1 or TORC2.

The precise understanding of the molecular mechanisms underlying rapamycin-dependent detrimental effects on human endothelium would contribute to explain the adverse consequences of rapamycin eluting stents, recently described in vivo[119,133]. In this context pharmacological treatments other than rapamycin or experimental conditions able to lower rapamycin cytotoxicity could also be pursued, so as to allow a safer employ of DES, avoiding endothelial damage and vascular dysfunction responsible for late stent thrombosis.

3. SUMMARY

Although cationic amino acids can be transported into cells by a number of differentially expressed transport systems (y^+, $b^{0,+}$, $B^{0,+}$, y^+L)[134], the influx of L-arginine in umbilical or saphenous vein-derived cells appears mainly referable to the activity of just y^+ and y^+L systems, as already described for other endothelial models[71]. More precisely, endothelial cells predominantly expresses CAT1 transporter[135], although, in cardiac microvascular endothelial cells and HUVECs, very low levels of CAT2B and CAT2A have also been found under basal conditions[68,136].

Consistently with previous contributions[137], the results of the studies here presented demonstrate that only system y^+ is involved in the endothelial response to inflammatory stimuli, while system y^+L is almost completely insensitive to both cytokines and bacterial LPS. In particular, the incubation of HUVECs or HSVECs with inflammatory compounds, such as TNFα or LPS, induces a significant stimulation of system y^+ activity, through the induction of the low affinity transporter SLC7A2/CAT2A and the increased expression of the high affinity SLC7A2/CAT2B. The molecular mechanisms responsible for such effects include the transcription process (specifically, the activation of NF-kB transcription factor) and likely involve the kinase mTOR, which seems to exert a repressive effect on SLC7A2 gene; indeed, its inhibition by the specific inhibitor rapamycin is associated to a significant induction of both CAT2A and CAT2B mRNAs, as well as to a straightforward huge increase of CAT2 protein levels.

Given the relevance of nitric oxide in cellular homeostasis and the prominent role of L-arginine for its biosynthesis, several contributions have thus far investigated the existence of a relation between the stimulation of cationic amino acid transporters upon incubation with inflammatory cytokines and the activation of NO's pathway. As a result, several conflicting contributions report a co-induction of arginine transporters and nitric oxide synthases in endothelial models upon proper stimulation or, rather, their complete independency. The aim of the present research project has been therefore to assess whether this possible correlation does exist also in human endothelial cells, where cytokines and bacterial LPS are known to induce a massive inflammatory response, as well as a huge stimulation of L-Arg transport. Both adult (HSVECs) and foetal (HUVEC) human endothelial cells have been employed, so as to assess possible tissutal specificities.

As expected for endothelial cells[17,113-115], cytokines-dependent stimulation of cationic amino acids transport and transporters do not produce in neither cell models a comparable induction of NO pathway: nitric oxide levels remain substantially unaffected, or rather lowered, by the cytokine, as well as the expression of endothelial nitric oxide synthase; iNOS isoform, undetectable under basal conditions, is still absent in TNFα-treated cells, while eNOS is expressed at low levels in control, untreated cells, and rather lowered by the cytokine, consistently with previous results[15]. These observations demonstrate that the induction of arginine transport and nitric oxide production are uncoupled in both HUVECs and HSVECs, although other contributions show the existence of a tight relation between these two events in different endothelial models[112].

In support of our findings, also the results of the experiments performed on rapamycin-treated cells confirm that CAT and NOS expressions are completely unrelated in HUVECs. Indeed, the treatment of endothelial cells with rapamycin, besides exerting the same stimulatory effects on L-Arg transport as TNFα, also induces the appearance of an activated phenotype in human endothelium, characterized by an impaired cell motility and by a modified expression of genes, markers of an inflammatory conditions, i.e. E-selectin and SLC7A2/CAT2A, usually associated to stress-conditions[47]; however, treatment with the drug, as well as with the cytokine, is ineffective in stimulating the synthesis of nitric oxide in the cell model employed, while even diminishes the expression of eNOS mRNA and protein. Taken together, these results demonstrate that, in human endothelium, an inflammatory condition, as that induced by rapamycin or TNFα, may not involve an alteration of NO levels, thus suggesting that an impaired metabolism of this molecule can not be considered a universal marker of inflammation, as well as human endothelial cells are concerned.

Given the relevance of the mTOR inhibitor in the clinical practice of coronary stenting and the adverse effects of rapamycin eluting stents, recently described on endothelium in vivo[119,133], the findings here reported also gain a clinical relevance. The results obtained have demonstrated, indeed, that mTOR inhibition by rapamycin exerts detrimental effects on human endothelial cells, thus pointing to a key role for the kinase in the preservation of endothelial homeostasis under physiological condition: its inhibition, indeed, leads to a significant cell loss, referable to the induction of both apoptotic and necrotic markers, as well as to the modification of endothelial cell motility and of other well known indicators of endothelial function. The precise molecular mechanism responsible for rapamycin effects remains substantially unclear, although the results here presented clearly demonstrate the involvement of bothmTOR complexes, mTORC1 and mTORC2, thus far considered

rapamycin-insensitive. Moreover, it looks apparent from our findings that mTORC2, rather than mTORC1, is involved in the maintenance of endothelial viability, likely through the activation of Akt pathway; indeed, an inhibitor of this kinase, but not AICAR (which specifically inhibits mTORC1), exerts detrimental effects comparable to those of rapamycin. Conversely, the pathways responsible for the control of endothelial function seem to be function-specific: the modulation of nitric oxide production is probably due to the activity of TORC2, while it remains uncertain whether the stimulation of adhesion molecules expression occurs preferably via TORC1 or TORC2.

Further investigations will be required to better define the mechanisms underlying rapamycin toxic effects, particularly in the area of stent implantation; their complete comprehension could then provide the basis for the development of pharmacological treatments alternative to rapamycin, still inhibiting mTOR, while minimizing detrimental effects on endothelial function and viability, and thus allowing a safer employ of DES.

4. CONTRIBUTIONS

ORIGINAL PAPERS:

1. Visigalli R., Bussolati O., Sala R., Barilli A., Rotoli B.M., Parolari A., Alamanni F., Gazzola G.C., Dall'Asta V. (2004) **The stimulation of arginine transport by TNFα in human endothelial cells depends on NF-κB activation.** *Biochim Biophys Acta,* 1664(1):45-*52.*

2. Visigalli R., Barilli A., Bussolati O., Sala R., Gazzola G.C., Parolari A., Tremoli E., Simon A., Closs E.I., Dall'asta V. (2007) **Rapamycin stimulates arginine influx through CAT2 transporters in human endothelial cells.** *Biochim Biophys Acta*, 1768(6):1479-87 (Epub 2007 Feb 24).

3. Barilli A., Visigalli R., Sala R., Gazzola G.C., Parolai A., Tremoli E., Bonomini S., Simon A., Closs E.I., Dall'Asta V., Bussolati O. **In human endothelial cells rapamycin causes mTORC2 inhibition and impairs cell viability and function.** Manuscript under revision.

CONGRESS COMMUNICATIONS:

1. Barilli A., Visigalli R., Sala R., Bussolati O., Dall'Asta V. **Effetto della rapamicina sull'aumento di trasporto di Arginina indotto da TNFα in HSVEC.** XXVII^{o} XXVIIo Congresso Nazionale della Società Italiana di Patologia (SIP). Modena, Italy, 2005.

2. Barilli A., Visigalli R., Bussolati O., Sala R., Gazzola G.C., Dall'asta V. **Rapamycin induces CAT transporters in human endothelial cells.** International Congress "Transporters 2006", *Acta Biomedica*, Vol.77 - Supplement 3/2006. Parma, Italy, 2006.

3. Barilli A., Visigalli R., Bussolati O., Sala R., Gazzola G.C., Dall'asta V. **Chronic exposure to rapamycin induces endothelial dysfunction in vitro.** International Congress "Experimental Biology – FASEB 2007" (Abstract No. 707.1). Washington DC, USA, 2007.

CHAPTER IV - ARGININE TRANSPORT IN HUMAN MONOCYTES AND MACROPHAGES

1. INTRODUCTION AND AIM OF THE STUDY

Macrophages, together with their circulating precursors monocytes, belong to the family of the mononuclear phagocytes, active in nonspecific defence (or innate immunity) as well as in specific defence (or cell-mediated immunity) of vertebrate animals.

Monocytes are produced by the bone marrow from a common myeloid progenitor, called monoblast, whose growth and differentiation depend on lineage-determining cytokines, such as macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), KIT, tumor necrosis factor (TNF)-family proteins, on key transcription factors, as PU.1 and on interactions with stroma in haematopoietic organs (Fig.27)[138].

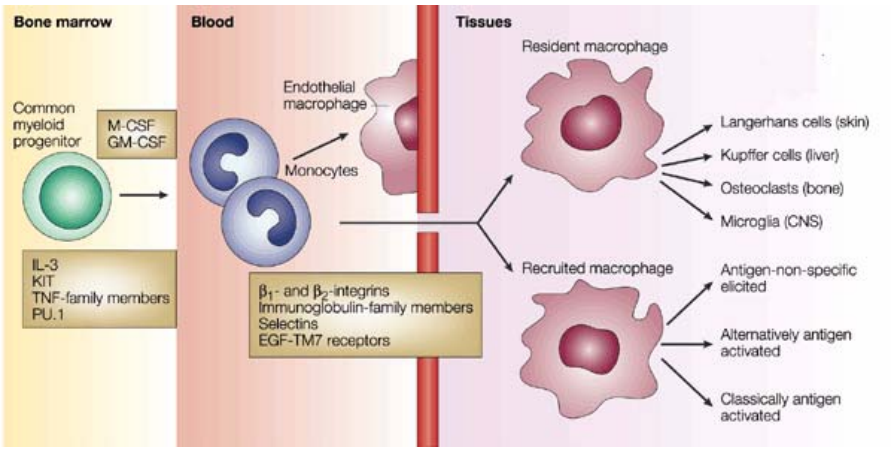


Fig.27 Simplified life history of the macrophage and closely related cells
(modified from Gordon, *Nature Reviews Immunology* (2003), 3, 23)

Monocytes circulate in the bloodstream (where they constitute between three to eight percent of the leukocytes in the blood) for about one to three days, and then typically move into tissues throughout the body. Once in the tissues, they undergo a series of morphological changes to evolve into mature macrophages, the so called *resident macrophages*, detectable at strategic points in almost every organ where their phagocytic activity is likely to be required; each of them, depending on its location, has tissue-specific name and function: connectival macrophages are also known as *hystiocytes*; in the spleen, resident macrophages begin the

process leading to the destruction of senescent red blood cells, which is kept on in the liver by the sinuisodal *Kupfer cells*; in the central nervous system, the so called *microglia* phagocytes apoptotic neurons, while a specialized population of macrophages, the *osteoclasts*, are involved in bone reabsorption; finally, lungs host the well known *alveolar macrophages* (AM), a group of phagocytes responsible for the first line defence of lower respiratory tract against airborne pathogens and involved in complex regulatory interplays with airway epithelial cells, as well as with endothelial cells, lymphocytes, and fibroblasts of the alveolar septa.

In the presence of proper stimuli, macrophages are rapidly recruited to enter the activation process, characterized by the blockade of cell proliferation[139] and by a more massive phagocytic activity, exerted through the induction of lysosomial enzymes and reactive oxygen species; activated macrophages also undergo other biochemical and morphological modifications that allow them to perform their functional activity[140], such as altered adhesion and migration, secretion of various products and antigen processing and presentation. Among the extracellular signals able to induce the activation of macrophages, cytokines play a fundamental role; in particular, Th1-type cytokines, such as IFNγ, together with bacterial lipopolysaccharide (LPS), induce the so called classical activation, while Th2-type cytokines, such as IL-4 and IL-13, are responsible of the induction of the alternative activation (Fig.28)[138].

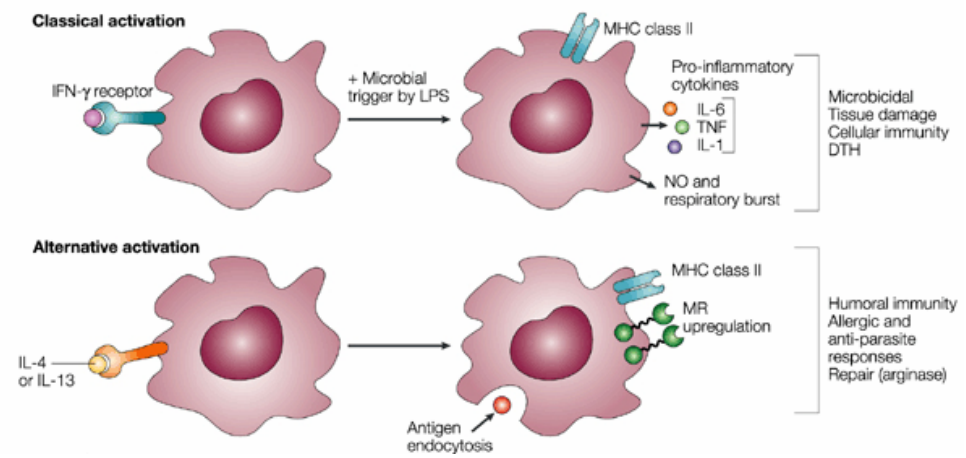


Fig.28 Classical and alternative activetion. (modified from Gordon, *Nature Reviews Immunology* (2003), 3, 26)

In murine systems the two types of activations are easily distinguishable on the basis of the metabolic pathway of L-arginine that is induced in response to either process: the stimulated expression of inducible nitric oxide synthase (iNOS, NOS2) and the consequent increased synthesis of nitric oxide (NO) are characteristic of classically activated macrophages; on the contrary, the alternative activation of murine macrophages is associated

to a marked increase of arginase pathway and of urea and ornithine production[140]. Given its requirement as substrate for the production of both nitric oxide, via classical activation, and polyamines and proline upon alternative activation, it is apparent the functional significance of the amino acid L-arginine in the macrophages activation process (Fig.29)[141]. The extracellular milieu is the main source of arginine required for cytokine-mediated activation; indeed, in rat and mouse models, it has been demonstrated that LPS/IFNγ-induced NO production does not occur in J774 macrophages (despite the induction of iNOS) if arginine is excluded from the culture medium or in the presence of lysine (which inhibits CAT-dependent arginine uptake by a competitive mechanism)[142]. It is then evident why also the characterization of the expression and activity of the cellular amino acid transporters appears of great interest for the definition of macrophages response to inflammatory cytokines.

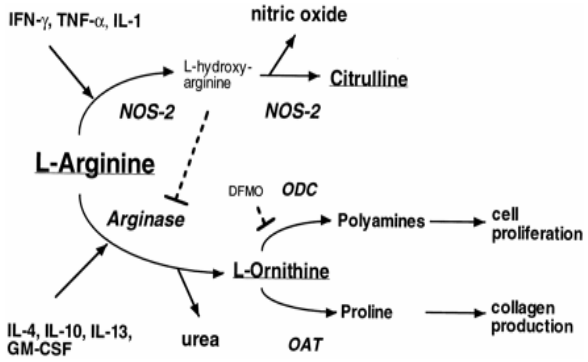


Fig.29 NOS2-Arginase pathway is controlled by Th1-Th2 cytokines.
(from Hesse et al., *The Journal of Immunology* (2001), 167(11), 6533)

In both monocytes and macrophages system y^+ is known to be the major transport system for cationic amino acids[143]; in particular, the best characterized transporters in these cell models are CAT-1 and CAT-2B: the first presumably accounts for the most basal rate of arginine influx, while the increased rate of arginine uptake observed upon incubation with inflammatory cytokines has been referred to the induction of CAT2B, whose expression is either very low or absent in normal tissues[144]. More in detail, Yeramian and colleagues have recently demonstrated that, in murine bone marrow-derived macrophages, SLC7A2/CAT2 is induced upon incubation with both Th1 and Th2 cytokines, i.e. during both classical and alternative activation, although only Th1-like cytokines stimulate NO production and only Th2-like ones induce arginase activity; on the contrary, SLC7A1/CAT1 expression is unaffected by any of these activating agents, thus suggesting that CAT2 is the main regulator of arginine transport under inflammatory conditions[145]. In the same paper, they also demonstrate that macrophages from SLC7A2/CAT2 knockout mice show a decrease of L-arginine transport in response to the two kinds of cytokines, associated to the reduced production of both nitrite and polyamines, even if neither NOS2 nor arginase expression and

the extracellular concentration of L-Arg are modified; they therefore conclude that SLC7A2/CAT2 can modulate and limit the function of macrophages, independently of L-Arg availability and of the expression of the enzymes that metabolize it.

However, the characterization of L-Arg transport under basal conditions as well as upon treatment with pro-inflammatory compounds has been mainly studied on murine rather than human models, and recent evidences suggest that the modulation of cationic amino acid transport in murine and human monocytes/macrophages may be profoundly different[146]. Therefore the first part of the research project has been intended to investigate the mechanisms of amino acid influx in human monocytes and alveolar resident macrophages at functional and molecular levels, under both basal and inflammatory conditions. Moreover, also the link between CAT2B-dependent arginine transport and nitric oxide production has been investigated in the same models: inflammatory cytokines-dependent induction of CAT2-mediated L-Arg transport has been shown to associate in murine models to a parallel increase of NOS2 protein expression, otherwise undetectable under basal conditions[142,144]; although thus far there are no data demonstrating CAT-2B-dependent NOS2 activity[71], the observed coinduction of the two genes has suggested that CAT-2B might provide substrate for NOS2, also in human cells. Also, the involvement of the other transport system for cationic amino acid, system y^+L, has been assessed in human monocytes and macrophages, since, thus far y^+LAT1 and y^+LAT2 transporters have been only scarcely studied in human leukocytes[147,148], without being referred to a particular cell type.

In resident alveolar macrophages the study of system y^+L activity appears particularly noteworthy also because of its involvement in Lysinuric Protein Intolerance (LPI). Indeed, system y^+L is known to be selectively altered in this rare autosomic recessive disease (LPI, MIM 222700), caused by a defect in the transport of cationic amino acids in the basolateral membrane of intestinal and renal epithelial cells. Because of this transport defect, LPI patients have an high renal clearance and a low intestinal absorption of cationic amino acids, whose plasma levels are consistently usually low[149]. Moreover, besides selective aminoaciduria, LPI patients also exhibit severe extra-renal alterations that are often the immediate cause of death. As far as airways are concerned, one of the best extra-renal complications observed in LPI patients is the increased frequency of pulmonary alveolar proteinosis (PAP), a rare disorder characterized by the accumulation of lipoproteinaceous material within alveoli, usually associated to susceptibility to pulmonary infections, also with opportunistic organisms[150]. There are three clinically distinct forms of PAP: the congenital disorder is due to mutations of genes encoding surfactant protein B or C or the beta chain of the receptor for granulocyte–

macrophage colony-stimulating factor (GM-CSF), an already described cytokine, involved in the process of macrophage differentiation; acquired (or idiopathic) pulmonary alveolar proteinosis is an autoimmune disease targeting GM-CSF; finally, LPI-associated PAP (or secondary pulmonary alveolar proteinosis) resembles autoimmune PAP with accumulation of surfactant lipids and proteins in the airspaces and the presence of enlarged alveolar macrophages that contain numerous phospholipid inclusions. The progresses in the definition of PAP pathogenesis have not yet shed light on the mechanisms responsible for LPI-associated PAP and, more in general, for LPI extra-renal alterations; in particular, it is not known if they derive from the low cationic amino acid levels available in plasma of LPI patients or, rather, from the consequences of the expression of LPI defect in extra-renal tissues. However, the observed accumulation of lipoproteins in LPI-associated PAP supports the latter hypothesis, suggesting that abnormalities of surfactant catabolism by the alveolar macrophages could contribute to the development of the disorder[151]. Consistently, a recent clinical evidence reports the case of an LPI patient who underwent a heart-lung transplantation for severe, PAP-associated respiratory insufficiency, relapsed and died of respiratory failure after a period of clinical remission [152], suggesting that circulating pathological cells, such as monocytes/macrophages, can re-colonize the transplanted lung and reproduce the pathological condition.

In 1999, SLC7A7, coding for y^+LAT1 subunit of system y^+L, has been identified by two groups independently[153,154] as the gene mutated in patients affected by LPI and likely responsible of the blockade of cationic amino acid efflux from kidney and intestine epithelial cells to the interstitium[46]; it is therefore apparent that the LPI-associated defect in SLC7A7 expression appears one of the best candidate to play an important role in the pathogenesis of PAP, thus rendering alveolar macrophages an interesting cell framework to build up a reliable in vitro model of LPI defect and open new field of investigation about surfactant metabolism.

Therefore, once defined the mechanisms responsible for the modulation of L-Arg influx in normal human alveolar macrophages, the second part of the project has been devoted to define the role of system y^+L and, specifically, of SLC7A7/y^+LAT1 in the regulation of L-Arg influx in pathological models, so as to verify whether a causal link exists that connects the alterations in arginine transport of alveolar macrophages to their defective response to surfactant, characteristic of LPI patients.

2. RESULTS AND DISCUSSION

2.1. CHARACTERIZATION OF L-ARG TRANSPORT IN HUMAN MONOCYTES AND MACROPHAGES

Since at least four distinct membrane transport systems can perform arginine transport in mammalian cells[134], a discrimination of the various components active in human monocytes and in freshly isolated (2h) macrophages (Fig.30) has been performed, according to the strategy already used in human endothelial cells, as well as in other cell models[15,155].

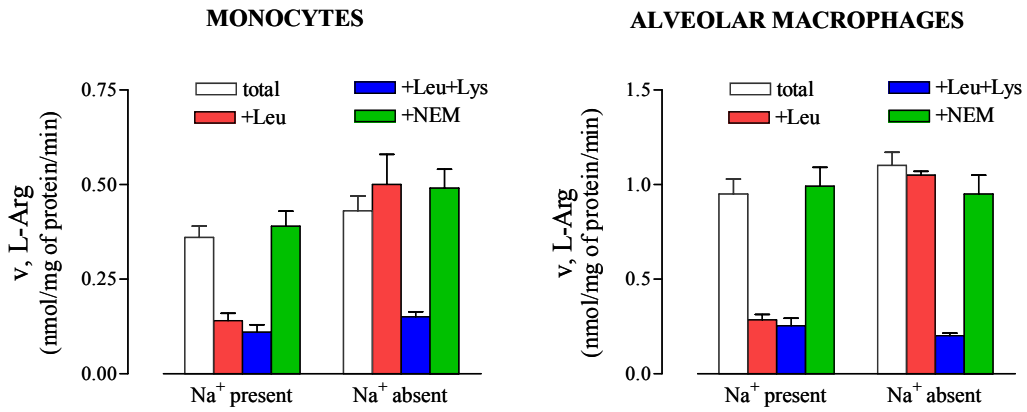


Fig.30 Characterization of arginine transport in human monocytes and freshly isolated macrophages.

In freshly isolated human monocytes system y^+ contribution to Arg transport is, at best, marginal, while system y^+L is the major transport system under basal conditions. Similarly, in adherent human alveolar macrophages system y^+L accounts for most of the saturable transport of arginine, while the contribution of system y^+ is negligible under the same conditions.

In detail, in human freshly isolated monocytes maintained in control medium, L-arginine uptake has been measured in the presence or in the absence of sodium, with or without the addition of 2 mM L-leucine; the assay has been performed with cells either treated or untreated with NEM, a well known inhibitor of system y^+ but not of system y^+L[57]. As shown in the plot, the substitution of extracellular sodium does not inhibit L-arginine uptake, thus excluding a significant contribution of sodium dependent transport systems, such as $B^{0,+}$; leucine inhibits L-Arg transport in the presence of sodium, a result compatible with system y^+L activity, but not in its absence, thus excluding a significant contribution of system $b^{0,+}$. Moreover, the pre-incubation of monocytes with NEM is ineffective in inhibiting L-arginine uptake under any of the experimental conditions adopted, confirming that system y^+ contribution is, at best, marginal under basal conditions.

Therefore, consistently with the results by Reade et al., which attribute only a minor role to system y^+ in a preparation of non stimulated human peripheral blood mononuclear cells, we can conclude that, while the contribution of system y^+ to the overall L-Arg influx in human monocytes appears marginal, system y^+L is the major transport system for arginine under basal conditions. The main characteristics shown by system y^+L in human monocytes are similar to those found in human mesenchymal models[15,155]; at variance with those models, however, human monocytes exhibit a significant stimulation of arginine transport by extracellular leucine under sodium-free conditions, which might be explained by a trans-stimulation of arginine influx by leucine entered into the cells through parallel pathways, other than system y^+L, during the transport assay.

Results comparable to those observed in monocytes have been obtained in human adherent alveolar macrophages (AM), after 2h-seeding: their arginine transport is similar in the absence or in the presence of sodium, thus excluding a significant contribution of sodium dependent transport systems, such as $B^{0,+}$. The addition of leucine is able to inhibit arginine influx by more than 70% only in the presence of sodium, indicating that most of the arginine uptake occurs through system y^+L; on the contrary, lack of inhibition by leucine in the absence of sodium excludes a significant contribution of sodium independent systems, such as $b^{0,+}$. Finally, either in the presence or in the absence of sodium, preincubation with NEM results ineffective in inhibiting arginine transport, thus pointing to system y^+L as the predominant cationic amino acids transporter also in human AMs.; consistently, system y^+L, identifiable as the leucine inhibitable portion of transport, accounts for most of the saturable component of arginine transport in all human AM isolated from 12 different subjects (Fig.31).

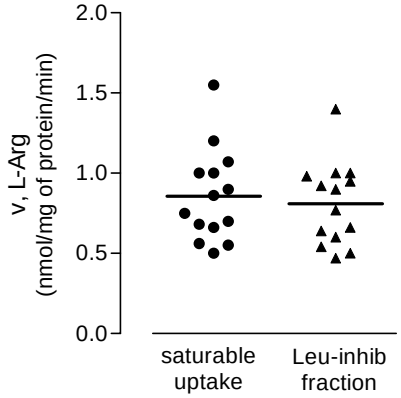


Fig.31 Dispersion plot of Arg transport in AMs.

The values of saturable arginine uptake of AMs isolated from 12 different subjects do not significantly differ from those of the leucine-inhibitable component.

Consistently with functional results, qualitative RT-PCR analysis of arginine transporters expressed in human monocytes (Fig.32, Panel A) shows that among system y^+-

related genes, only the expression of SLC7A1, coding for the CAT1 transporter, is detectable, whereas SLC7A2/CAT2B transcript is much less expressed and no expression of SLC7A2/CAT2A is detected; in alveolar macrophages (Fig.32, Panel B), not only SLC7A2/CAT2A, but even SLC7A2/CAT2B transcript is not detectable under basal conditions. In both cell models, the transcripts of SLC3A2, that code for system y^+L heavy chain 4F2hc/CD98, SLC7A7, coding for the light chain y^+LAT1, and SLC7A6, for the alternative light chain y^+LAT2, are all clearly detectable.

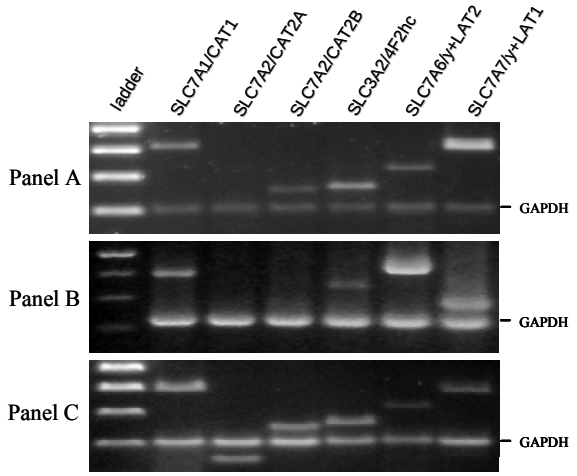


Fig.32 Expression of arginine transporters mRNAs.

RT-PCR products obtained with freshly isolated human monocytes (Panel A), human alveolar macrophages (Panel B) or human lung adenocarcinoma (Calu3, Panel C).

In particular, a clear-cut expression of the y^+L-related genes SLC7A6 and SLC7A7 that code for the two alternative light chains of the transporter, y^+LAT1 and y^+LAT2, is evident in alveolar macrophages, where, actually, arginine transport is accounted for by the activity of system y^+L (Fig.30).

Interestingly, a comparison between the levels of arginine transporters in human macrophages and in endothelial cell, where cationic amino acid transport preferentially occurs via system y^+, shows that the pattern of expression of the transcripts significantly differs in the two models (Table 5): in AMs the relative expression of SLC7A7 is enormously higher

	SLC7A1	SLC7A2 (CAT2B)	SLC7A6	SLC7A7
Alveolar Macrophages	132 ± 7	1.1 ± 0.6	18.2 ± 1.1	899 ± 92
HUVEC	178 ± 23	714 ± 59	35.3 ± 1.3	10.6 ± 0.6

Table 5. Relative expression of genes for L-Arg transport in human AM and endothelial cells (HUVEC).

The indicated gene mRNA is indexed to the housekeeping gene GAPDH, using the formula $1{,}000 \times 2^{\Delta Ct}$ ($\Delta Ct = Ct_{GAPDH} - Ct_{proband\ gene}$)

than in endothelial cells, suggesting that the expression of this transporter could be a good marker of cells of the monocyte/macrophage lineage; conversely, SLC7A2/CAT2B is definitely more abundant in HUVEC than in AM, consistently with L-Arg influx data.

In summary, the results of these preliminary studies clearly demonstrate that, at variance with murine models, human cells of the monocyte/macrophage lineage do not take up arginine through system y^+; in these models, instead, most of arginine transport (> 90% of the saturable uptake) occurs through system y^+L. In the absence of a functional system y^+, system y^+L-related genes SLC7A7 and SLC7A6 represent the most expressed transcripts for arginine transporter in human AMs, whereas, in the same model, SLC7A2/CAT2B is significantly lower, or rather undetectable, with respect to human endothelial cells.

2.2. EFFECTS OF INFLAMMATORY CYTOKINES ON L-ARG TRANSPORT

2.2.1. HUMAN MONOCYTES AND IFNγ

The differences observed between humans and animals as far as basal arginine transport is concerned prompted us to investigate the efficacy of pro-inflammatory cytokines in our human models, so as to evaluate whether differences are evident in activated cells too.

The first cytokine that we have tested on L-arginine transport in human monocytes and alveolar macrophages has been IFNγ, known to induce system y^+-mediated arginine influx in several animal models[145,156]. In contrast with murine models, a 24h-treatment (Fig.33, Panel A) with IFNγ produces in human monocytes a modest, although significant, increase of L-arginine transport fully attributable to an increase of system y^+L activity, rather than y^+; after

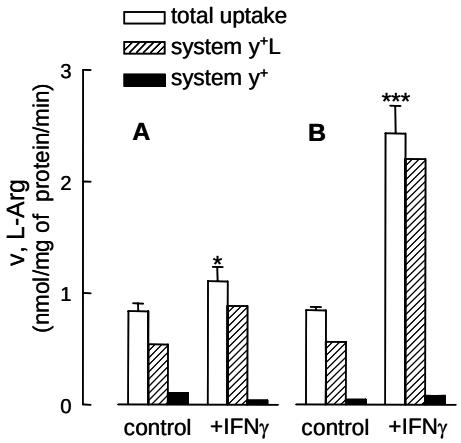


Fig. 33 Effects of INFγ on arginine transport in human monocytes.

After 24h-treatment with IFNγ (Panel A), system y^+L, but not y^+, activity is induced in freshly isolated monocytes; the stimulation appears even more marked after 48h (Panel B).

48h (Panel B), the stimulation of arginine influx by IFNγ becomes much more evident and, again, fully accounted for by a marked increase of system y^+L transport activity.

Consistently with functional measurement, the results of a semi-quantitative RT-PCR analysis of the expression of genes for cationic amino acid transporters upon treatment with IFNγ (Fig.34) demonstrate that the only gene significantly induced by the cytokine is SLC7A7. The correspondent densitometric analysis more precisely reveals that in IFNγ-treated monocytes the relative abundance of SLC7A7 expression, corrected for GAPDH, is increased more than three-fold at 48h compared with control, untreated cells. Under the same conditions neither the expression of SLC3A2, the gene for system y^+L heavy chain 4F2hc/CD98, and of SLC7A6, the gene for the alternative light chain y^+LAT2, nor the products of system y^+-related genes SLC7A1 and SLC7A2 (CAT2B transcript) are changed by the treatment with the cytokine.

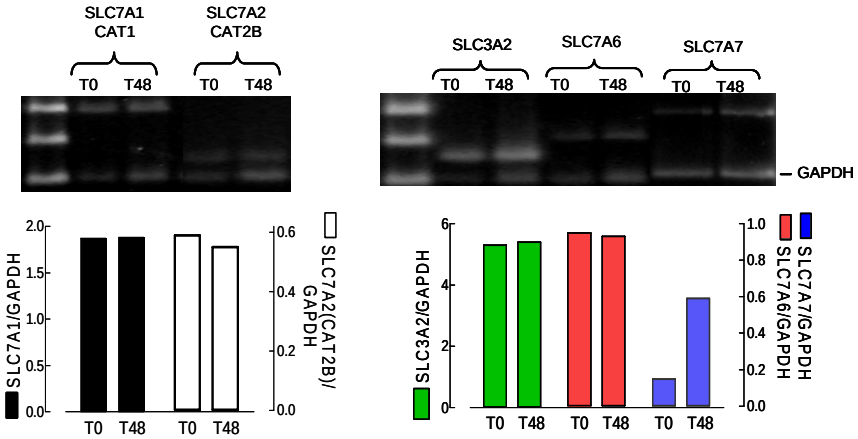


Fig. 34 Expression of arginine transporters in human monocytes upon stimulation with INFγ.

After 48h-treatment with IFNγ only the mRNA for SLC7A7 is markedly induced, while all other genes appear unaltered.

It is generally accepted that the y^+L light chain y^+LAT1 is the subunit expressed in absorbing epithelia, while the alternative isoform y^+LAT2 should be preferentially expressed in non epithelial tissues[46]; however, it is known that also y^+LAT1 is expressed in non epithelial cells, such as human fibroblasts[155], human endothelial cells[15], and leukocytes[147,148,157]. The results here reported, not only demonstrate that SLC7A7/y^+LAT1 is clearly expressed by human monocytes, but also show the stimulation of its expression by IFNγ, a thus far unknown effect of the cytokine. Indeed, although Mykkanen and coworkers have identified in the 5' regulatory region of the gene several putative transcription factor binding sequences, such as NFAT, that could be involved in IFNγ signal transduction[158], no previous report had indicated SLC7A7 as a target of the cytokine.

In summary, these data demonstrate that in human monocytes system y^+L activity, besides accounting for the major arginine transport system under basal conditions, is also

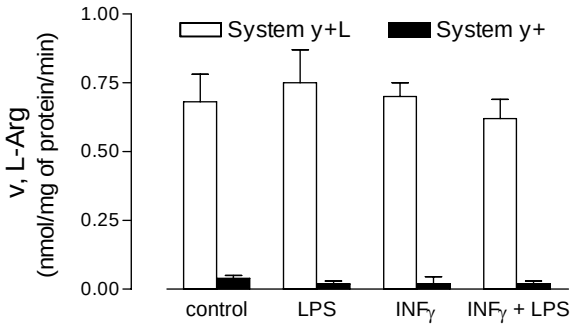
markedly stimulated by IFNγ, suggesting a role for it in the activation process induced by the cyotkine in these cells. Moreover, the clear-cut expression of SLC7A7/y^+LAT1 in human monocytes and its induction by IFNγ strengthen the hypothesis that monocytes may be a phenotypic target of LPI-associated mutations, since alterations of this gene are known to associate to this autosomal recessive condition[153].

2.2.2. HUMAN ALVEOLAR MACROPHAGES AND CYTOKINES

Human resident alveolar macrophages (AM) are even better candidate for the study of the pathogenesis of the severe LPI-associated alveolar proteinosis (PAP); therefore the effects of cytokines on amino acid transport have been assessed also in these monocyte-macrophage cells. Data in Fig.35 reports the discriminated activity of L-arginine transport routes in human alveolar macrophages, incubated for 24h in the absence or in the presence of LPS, IFNγ, or

Fig.35 Effects of IFNγ on arginine transport in AM.

A 24h-treatment with LPS or IFNγ doesn't affect either system y^+L or system y^+ activity in human alveolar macrophages.



both. Even in 24h-cultured alveolar macrophages, as well as in freshly isolated cells, system y^+L activity accounts for most of the arginine influx; interestingly its activity is modified neither by treatment with LPS nor with IFNγ. Consistently, no significant change is detectable

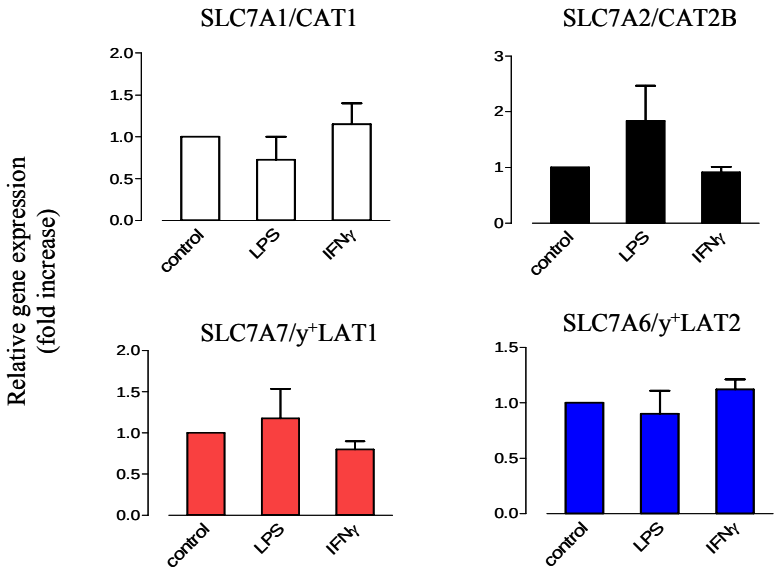


Fig.36 Effects of IFNγ on the expression of arginine transporters in AM.

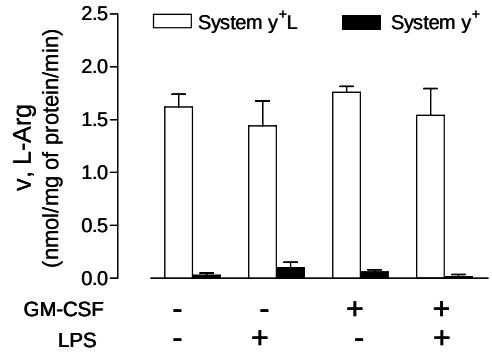
After 24h-incubation with LPS or IFNγ the expression of genes coding for arginine transporters remains substantially unaffected, although SLC7A2/ CAT2B appears slightly induced.

under any experimental condition for the genes coding L-Arg transporters, although LPS-treated cells exhibit an increase in abundance of SLC7A2/CAT2B transcript that, however, doesn't reach statistical significance (Fig.36).

Another stimulus known to induce arginine transport in macrophages from animal models is GM-CSF[156], a cytokine which promotes the survival and the differentiation of AMs and modulates their biological functions[159-161]. To evaluate the effect of GM-CSF on the expression and function of arginine transporters in AM, cells have been maintained in complete growth medium in the absence or in the presence of 10 ng/ml GM-CSF for 6 days; LPS was added in a subset of cells during the last 24h of incubation, either in the absence or in the presence of the colony-stimulating factor. Compared with arginine influx observed after 24h of culture, the activity of system y^+L in AM cultured for 6d is significantly higher (1.54 ± 0.154 (Fig.37) versus 0.72 ± 0.085 (Fig.35) nmol/mg of protein/min, $p < 0.05$) and constitutes, also at this time point, the predominant transport route for arginine, with only a marginal contribution of system y^+. However, neither the 6d-incubation in the presence of GM-CSF nor the further 24h treatment with LPS have any significant effect on either system y^+L or y^+ transport components.

Fig.37 Effects of LPS on Arg transport in the absence and in the presence of GM-CSF.

Neither the 6d-incubation in the presence of GM-CSF nor the 24h-treatment with LPS have any significant effect on L-Arg transport.



Consistently with functional assays, results comparable to those observed for 24h-treated cells have been obtained in 6d-treated cells, as far as the expression of transporters gene is concerned. Indeed, as shown in Fig.38, while SLC7A1 expression is not modified by the cytokine, a significant induction of SLC7A2/CAT2B expression is observed upon incubation with LPS. In GM-CSF–primed cells LPS produces a more evident induction of the transporter gene, while the colony stimulating factor alone does not produce a significant increase of SLC7A2B transcript. No significant change in the expression of either SLC7A6 or SLC7A7 genes, encoding for the system y^+L transporters y^+LAT2 and y^+LAT1, is observed in cells treated with GM-CSF, LPS, or both.

The discrepancy between the expression of CAT transporters at mRNA level and the lack of a detectable transport activity may be explained either by low levels of expression or by a lack of carrier protein production or membrane targeting. The first hypothesis is consistent with the exceedingly low levels of relative expression of SLC7A2/CAT2B detected in AM compared with human endothelial cells, as shown in Table 5. In contrast, as far as

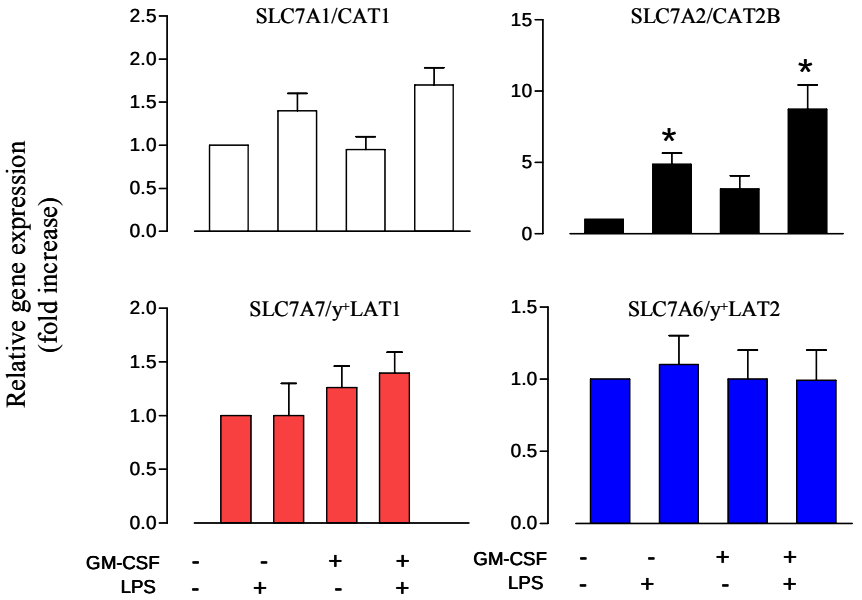


Fig.38 Effects of LPS on L-Arg transporters of AMs cultured in the absence and in the presence of GM-CSF.

No significant change of SLC7A6, SLC7A7 or CAT1 gene expression is observed in cells treated with GM-CSF, LPS or both; a significant induction of SLC7A2/CAT2B mRNA is conversely observed upon incubation with LPS.

SLC7A1/CAT1 is concerned, its expression, relative to that of GAPDH, is comparable in AM and in endothelial cells, where the operation of CAT transporters is readily detected[15]; therefore the same explanation could not to be valid for this transporter. Conversely, it seems more likely that in human AM CAT1 protein is expressed but remains in the intracellular compartment; consistently, literature evidence, obtained in non-macrophagic models, indicates that the trafficking of CAT transporters to the plasma membrane is regulated by PKC activity and suppressed when PKC is activated[162,163].

In any case, lack of transport activity of CAT transporters should not be referred to cell dedifferentiation during the culture procedure, since AM maintain the competence for Interleukin-1β (IL-1β) production in response to LPS treatment, as demonstrated by the results of the qRT-PCR analysis of cytokine's transcript (Fig.39, Panel A). The same pattern is observable in cells treated for 6d with GM-CSF and then exposed to LPS: treatment with LPS, but not with GM-CSF, produces a huge induction of IL-1β mRNA, which is nearly 50-fold more abundant in AM incubated with the endotoxin compared with untreated cells (Fig.39, Panel B).

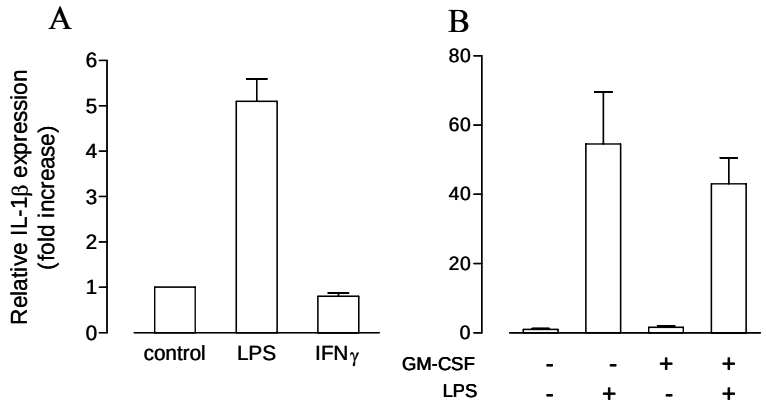


Fig.39 Effects of cytokines on IL-1β in AM.

24h treatment with LPS, but not with IFNγ (Panel A), produces a huge induction of IL-1β mRNA; the same LPS-dependent stimulation of IL-1β expression is detected after 6d-treatement with GM-CSF (Panel B).

In summary, the lack of system y^+ operation observed in freshly isolated AM, as well as in cells cultured for up to 6d, is maintained even if AM are incubated with GM-CSF, LPS, or IFNγ, stimuli that are able to induce this transport activity in macrophages from animal models[145,156]. These results confirm the hypothesis that monocytic/macrophagic cells from humans and animals present obvious differences as far as the regulation of arginine transport is concerned. However, what appears particularly noteworthy is that also differences among human cell types of the same monocyte/macrophage lineage may exist; for instance, our data demonstrate that, in human alveolar macrophages the treatment with inflammatory cytokines does not stimulate either SLC7A7 expression nor system y^+L activity, at variance with what happens in blood monocytes (Fig.33-34).

As already stated, mutations of SLC7A7/y^+LAT1 are responsible for LPI[148,153] and are associated with a severe, GM-CSF–insensitive form of pulmonary alveolar proteinosis (PAP) with apparent alterations in AM[151]. The high level of SLC7A7 expression found in human monocytes and alveolar macrophages suggests that system y^+L operation may be an absolute requirement for surfactant metabolism by alveolar macrophages and could therefore play an important role in the pathogenesis of LPI-associated PAP; it is possible, indeed, that the absence of y^+LAT1 expression and/or activity interferes with AM-dependent surfactant catabolism, thus mimicking the defects in GM-CSF signalling pathway that underlie most cases of PAP[150]. The definition of the functional role of system y^+L in human AM deserves, therefore, further studies, since it may contribute to the definition of LPI and PAP pathogenesis.

2.2. *EFFECTS OF INFLAMMATORY CYTOKINES ON NITRIC OXIDE PRODUCTION IN HUMAN MONOCYTES AND ALVEOLAR MACROPHAGES.*

As already described, the classical and alternative activations of macrophages in murine models are characterized by the induction of different L-Arg metabolic pathways: classically activated macrophages exhibit a stimulated expression of inducible nitric oxide synthase (NOS2) and the consequent increased synthesis of nitric oxide, while, the alternative activation implies a marked increase of arginase pathway and of urea and ornithine production. Conversely, in the human system, the production of nitrites by monocytes remains very low and substantially unaffected by the cytokine (Fig.40), although a high IFNγ-dependent stimulation of system y^+L-mediated arginine transport (Fig.33).

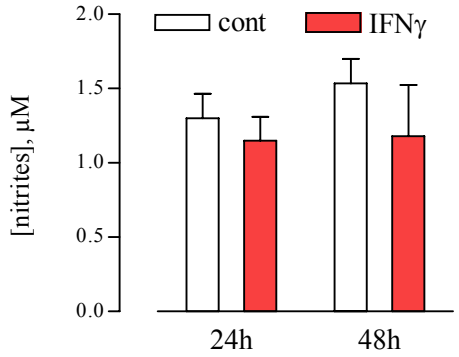


Fig.40 Effects of IFNγ on nitric oxide production in human monocytes.

IFNγ does not alter nitric oxide levels in human monocytes, after neither 24h nor 48h-treatment.

As far as alveolar macrophages are concerned, nitrite determination, performed under conditions of maximal stimulation of system y^+–related genes, indicates that also human AM have a very low, LPS- and GM-CSF–insensitive NO production (Fig.41, Panel A) and, consistently, do not express NOS2 at appreciable levels (Panel B).

These data are in agreement with the observations of other groups[164,165], indicating that

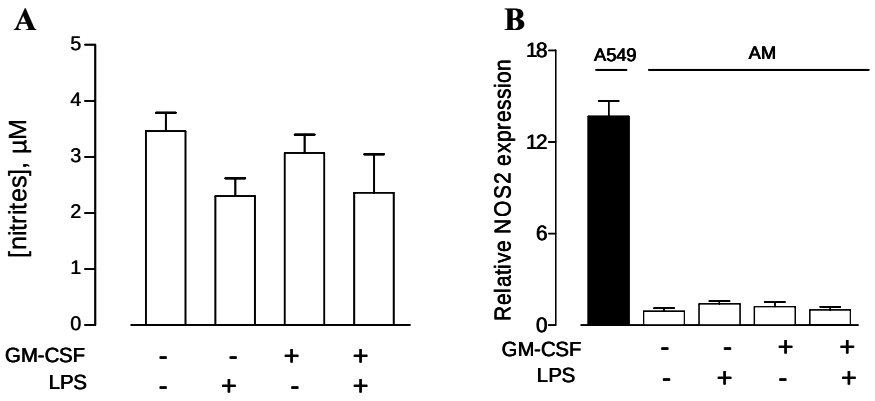


Fig.41 Effects of cytokines on nitric oxide production in AM.
Human AM produce a very low amount of nitric oxide, insensitive to LPS and GM-CSF (Panel A); consistently, they do not express NOS2 gene, under any of the experimental conditions adopted (Panel B)

arginine metabolism and NO production markedly differ between animal and human

macrophagic cells. For instance, at variance with what observed in murine cells, IFNγ fails to enhance growth inhibition of mycobacteria by human AM, the NOS inhibitor NMMA has no effect on macrophagic functions, and neither NOS2 mRNA nor NO can be detected in human AMs stimulated with LPS and IFNγ or even mycobacteria[166]. Consistently, our results show that IFNγ leaves human alveolar macrophages substantially unaffected, as far as NO production or NOS expression is confirmed.

As a consequence, the activation of macrophages in human cells is more difficult than in murine ones to be characterized, as monocyte-derived human macrophages from peripheral blood do not produce NO in response to the classical activating stimuli. The divergent behaviour of rodent and human AM has been suggested to depend on limited availability of extracellular L-arginine; however, the supplementation of the culture medium with the cationic amino acid does not trigger NO production in human AM[167], thus excluding this possibility. Another hypothesis is suggested by the finding that, in rodent model, at variance with other cells[168], NOS2-dependent NO synthesis markedly depends upon the transport of extracellular arginine to the intracellular compartment through the inducible transporter SLC7A2/CAT2B[169] and that, as a consequence, the expressions of NOS2 and CAT2B appear strictly linked; for instance, in rat alveolar macrophages, NOS2 and CAT2B are simultaneously induced in an NF-kB–dependent manner by LPS and IFN-γ[73] and are inhibited by polyamines[170], thus suggesting that arginine flux through CAT2B could be functionally linked to NOS2 activity. If this is the case, the absence of a significant activity of CAT transporters in human AMs would lead to an insufficient fuelling of NOS2 even in the presence of fairly high concentrations of extracellular arginine, thus explaining the different behaviour of human and rodent models, where, conversely, system y^+ is definitively effective.

In any case, it should be stressed that the results presented here may not be necessarily extendible to cells obtained from subjects affected by pathological conditions. Indeed, an effective NOS2 expression by AM has been reported in systemic sclerosis or acute respiratory distress syndrome[171]. In this last situation, although NO production by AM has not been directly demonstrated, high levels of nitrites/nitrates are detected in bronchoalveolar lavage and immunocytochemical positivity for protein nitrosylation has been observed in AM[171]. It will be, therefore, interesting to assess if, in these situations, system y^+ transport activity is expressed by human AM and, in this case, to define what kind of signals trigger its functional expression.

3. SUMMARY

In accordance with their role in the first line defence of the lower respiratory tract against airborne pathogens, alveolar macrophages (AM) are known to produce a wide array of mediators, such as cytokines, growth factors, reactive oxygen species and arachidonic acid derivatives; more controversial is if and under what conditions they also synthesize nitric oxide, although NO output from resting AM is believed to be very low[172]. L-arginine is the obliged substrate for NO production, and several contributions have demonstrated that in rodent monocyte/macrophage cells, NOS2-dependent NO production, induced by pro-inflammatory compounds such as LPS or IFNγ, mainly depends on the uptake of extracellular arginine[169,173].

Most of the studies have however concerned rodent cellular models, while thus far little is known about human macrophages and their circulating precursors, monocytes; moreover, recent evidences have also suggested that human and animal models may be profoundly different[146]. Therefore the aim of this research project has been to evaluate the production of nitric oxide in human monocytes and AM upon incubation with pro-inflammatory cytokines, as well as to define the mechanisms responsible for the modulation of arginine transport in these models, under both physiological and inflammatory conditions.

As for this last point, it is widely accepted that rodent monocyte/macrophage cells preferentially transport cationic amino acids through system y^{+}[143]; consistently, CAT2B expression has been documented in alveolar macrophages from animal models[73,174] and, most recently, CAT2B transporter has been found to play a critical role in regulating AM activity in the mouse[175]. Conversely, the results presented here clearly demonstrate that in both human monocytes and macrophages system $y^{+}L$ is the major transport system for cationic amino acids, while system y^{+} contribution is at best marginal. In accordance with functional results, also the analysis of the genes coding for the different transporters demonstrates a clear-cut expression of the $y^{+}L$-related SLC7A6 and SLC7A7, with respect to CAT genes. Consistently, also a comparison between human alveolar macrophages and human endothelial cells confirms that SLC7A7 gene is enormously expressed in human AM, where cationic amino acid transport is mainly accounted for by system $y^{+}L$, whereas, HUVECs exhibit a much evident expression of SLC7A2, consistent with the role of system y^{+} as the major route for L-arginine in this model.

Significant differences between human and animal models are also evident as far as the response to inflammatory stimuli is concerned: indeed, in human cells, at variance with

murine ones, pro-inflammatory compounds are ineffective in stimulating CAT-mediated L-Arg transport, while IFNγ rather stimulates system y^+L and the expression of its related gene, SLC7A7, in isolated monocytes; conversely, human AM are completely insensitive to LPS, IFNγ or GM-CSF, as far as either systems y^+ or y^+L-mediated cationic amino acid transport are concerned, thus suggesting that differences among cells of the same human monocyte/macrophage lineage may also exist.

Finally, as for the production of nitric oxide in human cells, the results presented here indicate that both human AM and monocytes synthesize a very low amount of this signalling molecule under resting conditions; interestingly, NO levels of nitric oxide remain unaltered even upon stimulation with cytokines able to induce arginine transport, as in the case of monocytes treated with IFNγ, thus suggesting that the two events must be, at least, uncorrelated. Similarly, in alveolar macrophages, the production of nitric oxide is almost undetectable and insensitive to GMCSF or LPS, the conditions of maximal stimulation of system y^+ activity.

Taken together these data confirm the hypothesis that animal and human monocytes and alveolar macrophages substantially differ, as far as the modulation of L-Arg transport under physiological or inflammatory conditions is concerned; however, also differences may exist, between cells of the same human monocyte/macrophage lineage, particularly upon stimulation with pro-inflammatory compounds. What also appears of great relevance is that neither monocytes nor AM produce a significant amount of nitric oxide, not only under basal conditions, but even under conditions of maximal stimulation of arginine transport; since comparable results have been obtained also in human endothelial cells, where inflammatory cytokines are ineffective in modulating NO production, besides stimulating arginine transport, it seems apparent that the hypothesis of a link between cationic amino acid transport and NO biosynthesis under inflammatory conditions may not be valid for human cells, although plausible in animal models.

4. CONTRIBUTIONS

ORIGINAL PAPERS:

1. Rotoli B.M., Bussolati O., Sala R., Barilli A., Talarico E., Gazzola G.C., Dall'Asta V. (2004) **IFNγ stimulates arginine transport through system y^+L in human monocytes**. *FEBS Letters*, 571:177-181.

2. Rotoli B.M., Dall'asta V., Barilli A., D'Ippolito R., Tipa A., Olivieri D., Gazzola G.C., Bussolati O. (2007) **Alveolar Macrophages from Normal Subjects Lack the NOS-Related System y^+ for Arginine Transport.** *Am J Respir Cell Mol Biol.*, 37(1):105-12 (Epub 2007 Mar 15).

CONGRESS COMMUNICATIONS:

1. Dall'Asta V., Bussolati O., Sala R., Rotoli B.M., Barilli A., Gazzola G.C. **Espressione di SLC7A7y+LAT1, il gene mutato nella LPI, in monociti e cellule dell'epitelio respiratorio.** XXVII° Congresso Nazionale della Società Italiana di Patologia (SIP). Modena, Italy, 2005.

2. Rotoli B.M., Barilli A., D'Ippolito R., Tipa A., Olivieri D., Gazzola G.C., Bussolati O., Dall'asta V. **Human alveolar macrophages from normal subject do not transport arginine through system y+: possible relationship with low NO production.** International Congress "Transporters 2006", *Acta Biomedica*, Vol.77 - Supplement 3/2006. Parma, Italy, 2006.

REFERENCES

1. **RH Boger and SM Bode-Boger.** The clinical pharmacology of L-arginine. *Annu Rev Pharmacol Toxicol* 41: 79, 2001.

2. **EI Closs, et al.** Plasma membrane transporters for arginine. *J Nutr* 134: 2752S, 2004.

3. **SM Morris, Jr.** Enzymes of arginine metabolism. *J Nutr* 134: 2743S, 2004.

4. **HH Krebs HA.** Untersuchungen uber die Harnstoffbildung im Tierkoerper. *Z. Physiol. Chem.* 210: 33, 1932.

5. **GL Foster.** Studies in protein metabolism. V. The utilization of ammonia for amino acid and creatine formation in animals *J. Biol. Chem.* 127: 319, 1939.

6. **HH Schmidt, et al.** Arginine is a physiological precursor of endothelium-derived nitric oxide. *Eur J Pharmacol* 154: 213, 1988.

7. **C Bogdan, et al.** The role of nitric oxide in innate immunity. *Immunol Rev* 173: 17, 2000.

8. **DS Bredt, et al.** Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* 351: 714, 1991.

9. **JS Pollock, et al.** Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc Natl Acad Sci U S A* 88: 10480, 1991.

10. **B Mayer, et al.** Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase. *FEBS Lett* 288: 187, 1991.

11. **NA Chartrain, et al.** Molecular cloning, structure, and chromosomal localization of the human inducible nitric oxide synthase gene. *J Biol Chem* 269: 6765, 1994.

12. **DA Geller, et al.** Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc Natl Acad Sci U S A* 90: 522, 1993.

13. **P Rosenkranz-Weiss, et al.** Regulation of nitric oxide synthesis by proinflammatory cytokines in human umbilical vein endothelial cells. Elevations in tetrahydrobiopterin levels enhance endothelial nitric oxide synthase specific activity. *J Clin Invest* 93: 2236, 1994.

14. **MS Zadeh, et al.** Regulation of ICAM-1/CD54 expression on human endothelial cells by hydrogen peroxide involves inducible NO synthase. *J Leukoc Biol* 67: 327, 2000.

15. **R Sala, et al.** Two-way arginine transport in human endothelial cells: TNF-alpha stimulation is restricted to system y(+). *Am J Physiol Cell Physiol* 282: C134, 2002.

16. **AR Rubio and MA Morales-Segura.** Nitric oxide, an iceberg in cardiovascular physiology: far beyond vessel tone control. *Arch Med Res* 35: 1, 2004.

17. **CD Searles.** Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression. *Am J Physiol Cell Physiol* 291: C803, 2006.

18. **G Wu and SM Morris, Jr.** Arginine metabolism: nitric oxide and beyond. *Biochem J* 336 (Pt 1): 1, 1998.

19. **KK McDonald, et al.** A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the "arginine paradox". *J Biol Chem* 272: 31213, 1997.

20. **D Fulton, et al.** Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399: 597, 1999.

21. **RM Palmer, et al.** Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327: 524, 1987.

22. **MW Radomski, et al.** An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc Natl Acad Sci U S A* 87: 5193, 1990.

23. **P Kubes, et al.** Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A* 88: 4651, 1991.

24. **UC Garg and A Hassid.** Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest* 83: 1774, 1989.

25. **AM Zeiher, et al.** Nitric oxide modulates the expression of monocyte chemoattractant protein 1 in cultured human endothelial cells. *Circ Res* 76: 980, 1995.

26. **RM Clancy, et al.** The role of nitric oxide in inflammation and immunity. *Arthritis Rheum* 41: 1141, 1998.

27. **E Peranzoni, et al.** Role of arginine metabolism in immunity and immunopathology. *Immunobiology* 212: 795, 2008.

28. **J Satriano.** Arginine pathways and the inflammatory response: interregulation of nitric oxide and polyamines: review article. *Amino Acids* 26: 321, 2004.

29. **S Pfeiffer, et al.** Protein tyrosine nitration in cytokine-activated murine macrophages. Involvement of a peroxidase/nitrite pathway rather than peroxynitrite. *J Biol Chem* 276: 34051, 2001.

30. **G Cirino, et al.** Endothelial nitric oxide synthase: the Cinderella of inflammation? *Trends Pharmacol Sci* 24: 91, 2003.

31. **RH Boger, et al.** Dietary L-arginine reduces the progression of atherosclerosis in cholesterol-fed rabbits: comparison with lovastatin. *Circulation* 96: 1282, 1997.

32. **JP Cooke, et al.** Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. *J Clin Invest* 90: 1168, 1992.

33. **W Aji, et al.** L-arginine prevents xanthoma development and inhibits atherosclerosis in LDL receptor knockout mice. *Circulation* 95: 430, 1997.

34. **PS Tsao, et al.** Enhanced endothelial adhesiveness in hypercholesterolemia is attenuated by L-arginine. *Circulation* 89: 2176, 1994.

35. **SM Bode-Boger, et al.** Chronic dietary supplementation with L-arginine inhibits platelet aggregation and thromboxane A2 synthesis in hypercholesterolaemic rabbits in vivo. *Cardiovasc Res* 37: 756, 1998.

36. **RH Boger, et al.** Dietary L-arginine decreases myointimal cell proliferation and vascular monocyte accumulation in cholesterol-fed rabbits. *Atherosclerosis* 136: 67, 1998.

37. **M Hecker, et al.** The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc Natl Acad Sci U S A* 87: 8612, 1990.

38. **HN Christensen and JA Antonioli.** Cationic amino acid transport in the rabbit reticulocyte. Na+-dependent inhibition of Na+-independent transport. *J Biol Chem* 244: 1497, 1969.

39. **HN Christensen and ME Handlogten.** Reactions of neutral amino acids plus Na(+) with a cationic amino acid transport system. *FEBS Lett* 3: 14, 1969.

40. **MF White, et al.** Cationic amino acid transport into cultured animal cells. I. Influx into cultured human fibroblasts. *J Biol Chem* 257: 4443, 1982.

41. **O Bussolati, et al.** Characterization of amino acid transport in human endothelial cells. *Am J Physiol* 265: C1006, 1993.

42. **R Deves, et al.** Identification of a new transport system (y+L) in human erythrocytes that recognizes lysine and leucine with high affinity. *J Physiol* 454: 491, 1992.

43. **LJ Van Winkle, et al.** Na+-independent transport of basic and zwitterionic amino acids in mouse blastocysts by a shared system and by processes which distinguish between these substrates. *J Biol Chem* 263: 3150, 1988.

44. **EI Closs.** Membrane transport of L-arginine and cationic amino acids analogs. in *Nitric Oxide: Biology and Pathobiology* (L. J. Ignarro) 225, 2000

45. **MP Sperandeo, et al.** The gene encoding a cationic amino acid transporter (SLC7A4) maps to the region deleted in the velocardiofacial syndrome. *Genomics* 49: 230, 1998.

46. **F Verrey, et al.** CATs and HATs: the SLC7 family of amino acid transporters. *Pflugers Arch* 447: 532, 2004.

47. **DK Kakuda, et al.** Stress differentially induces cationic amino acid transporter gene expression. *Biochim Biophys Acta* 1414: 75, 1998.

48. **N Vekony, et al.** Human cationic amino acid transporter hCAT-3 is preferentially expressed in peripheral tissues. *Biochemistry* 40: 12387, 2001.

49. **F Verrey, et al.** Glycoprotein-associated amino acid exchangers: broadening the range of transport specificity. *Pflugers Arch* 440: 503, 2000.

50. **BM Rotoli, et al.** The transport of cationic amino acids in human airway cells: expression of system y+L activity and transepithelial delivery of NOS inhibitors. *Faseb J* 19: 810, 2005.

51. **JL Sloan and S Mager.** Cloning and functional expression of a human Na(+) and Cl(-)-dependent neutral and cationic amino acid transporter B(0+). *J Biol Chem* 274: 23740, 1999.

52. **JL Sloan, et al.** Expression of the amino acid transporter ATB 0+ in lung: possible role in luminal protein removal. *Am J Physiol Lung Cell Mol Physiol* 284: L39, 2003.

53. **MH Dave, et al.** Expression of heteromeric amino acid transporters along the murine intestine. *J Physiol* 558: 597, 2004.

54. **EA Jaffe, et al.** Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 52: 2745, 1973.

55. **R D'Ippolito, et al.** Induced sputum in patients with newly diagnosed sarcoidosis: comparison with bronchial wash and BAL. *Chest* 115: 1611, 1999.

56. **GC Gazzola, et al.** The cluster-tray method for rapid measurement of solute fluxes in adherent cultured cells. *Anal Biochem* 115: 368, 1981.

57. **R Deves, et al.** N-ethylmaleimide discriminates between two lysine transport systems in human erythrocytes. *J Physiol* 468: 753, 1993.

58. **SA Bustin.** Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 25: 169, 2000.

59. **WC O'Neill and JD Klein.** Regulation of vascular endothelial cell volume by Na-K-2Cl cotransport. *Am J Physiol* 262: C436, 1992.

60. **TP Misko, et al.** A fluorometric assay for the measurement of nitrite in biological samples. *Anal Biochem* 214: 11, 1993.

61. **A Simon, et al.** Role of neutral amino acid transport and protein breakdown for substrate supply of nitric oxide synthase in human endothelial cells. *Circ Res* 93: 813, 2003.

62. **K Ishii, et al.** A simple and sensitive bioassay method for detection of EDRF with RFL-6 rat lung fibroblasts. *Am J Physiol* 261: H598, 1991.

63. **J O'Brien, et al.** Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem* 267: 5421, 2000.

64. **KS Weber, et al.** Expression of CCR2 by endothelial cells : implications for MCP-1 mediated wound injury repair and In vivo inflammatory activation of endothelium. *Arterioscler Thromb Vasc Biol* 19: 2085, 1999.

65. **GE Mann, et al.** Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. *Physiol Rev* 83: 183, 2003.

66. **CL MacLeod.** Regulation of cationic amino acid transporter (CAT) gene expression. *Biochem Soc Trans* 24: 846, 1996.

67. **PB Stathopulos, et al.** Increased L-arginine uptake and inducible nitric oxide synthase activity in aortas of rats with heart failure. *Am J Physiol Heart Circ Physiol* 280: H859, 2001.

68. **K Irie, et al.** Cationic amino acid transporter-2 mRNA induction by tumor necrosis factor-alpha in vascular endothelial cells. *Eur J Pharmacol* 339: 289, 1997.

69. **W Durante, et al.** Selective induction of a cationic amino acid transporter by tumor necrosis factor-alpha in vascular endothelium. *Proc Assoc Am Physicians* 108: 356, 1996.

70. **K Kawahara, et al.** Co-induction of argininosuccinate synthetase, cationic amino acid transporter-2, and nitric oxide synthase in activated murine microglial cells. *Brain Res Mol Brain Res* 90: 165, 2001.

71. **EI Closs, et al.** Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol Pharmacol* 57: 68, 2000.

72. **W Durante, et al.** Differential regulation of L-arginine transport and nitric oxide production by vascular smooth muscle and endothelium. *Circ Res* 78: 1075, 1996.

73. **R Hammermann, et al.** Nuclear factor-kappaB mediates simultaneous induction of inducible nitric-oxide synthase and Up-regulation of the cationic amino acid transporter CAT-2B in rat alveolar macrophages. *Mol Pharmacol* 58: 1294, 2000.

74. **AR Baydoun, et al.** Transmembrane signalling mechanisms regulating expression of cationic amino acid transporters and inducible nitric oxide synthase in rat vascular smooth muscle cells. *Biochem J* 344 Pt 1: 265, 1999.

75. **M Pan, et al.** TNF-stimulated arginine transport by human vascular endothelium requires activation of protein kinase C. *Ann Surg* 221: 590, 1995.

76. **LA Madge and JS Pober.** TNF signaling in vascular endothelial cells. *Exp Mol Pathol* 70: 317, 2001.

77. **TD Gilmore.** The Rel/NF-kappaB signal transduction pathway: introduction. *Oncogene* 18: 6842, 1999.

78. **M Spiecker, et al.** A functional role of I kappa B-epsilon in endothelial cell activation. *J Immunol* 164: 3316, 2000.

79. **LA Madge and JS Pober.** A phosphatidylinositol 3-kinase/Akt pathway, activated by tumor necrosis factor or interleukin-1, inhibits apoptosis but does not activate NFkappaB in human endothelial cells. *J Biol Chem* 275: 15458, 2000.

80. **J Surapisitchat, et al.** Fluid shear stress inhibits TNF-alpha activation of JNK but not ERK1/2 or p38 in human umbilical vein endothelial cells: Inhibitory crosstalk among MAPK family members. *Proc Natl Acad Sci U S A* 98: 6476, 2001.

81. **CC Chen, et al.** Tumor necrosis factor-alpha, platelet-activating factor, and hydrogen peroxide activate protein kinase C subtypes alpha and epsilon in human saphenous vein endothelial cells. *J Cardiovasc Pharmacol* 28: 240, 1996.

82. **ON Ozes, et al.** NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 401: 82, 1999.

83. **H Glantschnig, et al.** M-CSF, TNFalpha and RANK ligand promote osteoclast survival by signaling through mTOR/S6 kinase. *Cell Death Differ* 10: 1165, 2003.

84. **ON Ozes, et al.** A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. *Proc Natl Acad Sci U S A* 98: 4640, 2001.

85. **S Wullschleger, et al.** TOR signaling in growth and metabolism. *Cell* 124: 471, 2006.

86. **AC Gingras, et al.** Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev* 13: 1422, 1999.

87. **DA Guertin, et al.** Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Dev Cell* 11: 859, 2006.

88. **PT Bhaskar and N Hay.** The two TORCs and Akt. *Dev Cell* 12: 487, 2007.

89. **AC Gingras, et al.** Regulation of translation initiation by FRAP/mTOR. *Genes Dev* 15: 807, 2001.

90. **M Hidalgo and EK Rowinsky.** The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. *Oncogene* 19: 6680, 2000.

91. **RN Saunders, et al.** Rapamycin in transplantation: a review of the evidence. *Kidney Int* 59: 3, 2001.

92. **SO Marx and AR Marks.** Bench to bedside: the development of rapamycin and its application to stent restenosis. *Circulation* 104: 852, 2001.

93. **B Lagerqvist, et al.** Long-term outcomes with drug-eluting stents versus bare-metal stents in Sweden. *N Engl J Med* 356: 1009, 2007.

94. **PK Kuchulakanti, et al.** Correlates and long-term outcomes of angiographically proven stent thrombosis with sirolimus- and paclitaxel-eluting stents. *Circulation* 113: 1108, 2006.

95. **AV Finn, et al.** Vascular responses to drug eluting stents: importance of delayed healing. *Arterioscler Thromb Vasc Biol* 27: 1500, 2007.

96. **K Natarajan, et al.** Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. *Proc Natl Acad Sci U S A* 93: 9090, 1996.

97. **SM Yu, et al.** Inhibition of nitric oxide synthase expression by PPM-18, a novel anti-inflammatory agent, in vitro and in vivo. *Biochem J* 328 (Pt 2): 363, 1997.

98. **G Kothny-Wilkes, et al.** Interleukin-1 protects transformed keratinocytes from tumor necrosis factor-related apoptosis-inducing ligand. *J Biol Chem* 273: 29247, 1998.

99. **AW Wyatt, et al.** Early activation of the p42/p44MAPK pathway mediates adenosine-induced nitric oxide production in human endothelial cells: a novel calcium-insensitive mechanism. *Faseb J* 16: 1584, 2002.

100. **C Flores, et al.** Rapid stimulation of L-arginine transport by D-glucose involves p42/44(mapk) and nitric oxide in human umbilical vein endothelium. *Circ Res* 92: 64, 2003.

101. **TA Lane, et al.** Protein kinase C inhibitors block the enhanced expression of intercellular adhesion molecule-1 on endothelial cells activated by interleukin-1, lipopolysaccharide and tumor necrosis factor. *Biochem Biophys Res Commun* 172: 1273, 1990.

102. **KY Krotova, et al.** Classical isoforms of PKC as regulators of CAT-1 transporter activity in pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 284: L1037, 2003.

103. **M Minhajuddin, et al.** Inhibition of mammalian target of rapamycin potentiates thrombin-induced intercellular adhesion molecule-1 expression by accelerating and stabilizing NF-kappaB activation in endothelial cells. *J Immunol* 174: 5823, 2005.

104. **J Steffel, et al.** Rapamycin, but not FK-506, increases endothelial tissue factor expression: implications for drug-eluting stent design. *Circulation* 112: 2002, 2005.

105. **A Giordano, et al.** Rapamycin antagonizes NF-kappaB nuclear translocation activated by TNF-alpha in primary vascular smooth muscle cells and enhances apoptosis. *Am J Physiol Heart Circ Physiol* 290: H2459, 2006.

106. **EI Closs, et al.** Human cationic amino acid transporters hCAT-1, hCAT-2A, and hCAT-2B: three related carriers with distinct transport properties. *Biochemistry* 36: 6462, 1997.

107. **KV Prasanth, et al.** Regulating gene expression through RNA nuclear retention. *Cell* 123: 249, 2005.

108. **SN Bhattacharyya, et al.** Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* 125: 1111, 2006.

109. **WW Simmons, et al.** Cytokines and insulin induce cationic amino acid transporter (CAT) expression in cardiac myocytes. Regulation of L-arginine transport and no production by CAT-1, CAT-2A, and CAT-2B. *J Biol Chem* 271: 11694, 1996.

110. **K Inoki, et al.** Dysregulation of the TSC-mTOR pathway in human disease. *Nat Genet* 37: 19, 2005.

111. **K Inoki, et al.** Signaling by target of rapamycin proteins in cell growth control. *Microbiol Mol Biol Rev* 69: 79, 2005.

112. **W Durante.** Regulation of L-arginine transport and metabolism in vascular smooth muscle cells. *Cell Biochem Biophys* 35: 19, 2001.

113. **HD Anderson, et al.** Tumor necrosis factor-alpha inhibits endothelial nitric-oxide synthase gene promoter activity in bovine aortic endothelial cells. *J Biol Chem* 279: 963, 2004.

114. **BL Goodwin, et al.** Tumor necrosis factor-alpha reduces argininosuccinate synthase expression and nitric oxide production in aortic endothelial cells. *Am J Physiol Heart Circ Physiol* 293: H1115, 2007.

115. **N Makino, et al.** High serum TNF-alpha level in Type 2 diabetic patients with microangiopathy is associated with eNOS down-regulation and apoptosis in endothelial cells. *J Diabetes Complications* 19: 347, 2005.

116. **B Yang and V Rizzo.** TNF-alpha potentiates protein-tyrosine nitration through activation of NADPH oxidase and eNOS localized in membrane rafts and caveolae of bovine aortic endothelial cells. *Am J Physiol Heart Circ Physiol* 292: H954, 2007.

117. **J Davignon and P Ganz.** Role of endothelial dysfunction in atherosclerosis. *Circulation* 109: III27, 2004.

118. **CR Triggle, et al.** The endothelium in health and disease--a target for therapeutic intervention. *J Smooth Muscle Res* 39: 249, 2003.

119. **S Fuke, et al.** Impaired endothelial vasomotor function after sirolimus-eluting stent implantation. *Circ J* 71: 220, 2007.

120. **R Moreno, et al.** Drug-eluting stent thrombosis: results from a pooled analysis including 10 randomized studies. *J Am Coll Cardiol* 45: 954, 2005.

121. **N Clausell, et al.** Expression of tumour necrosis factor alpha and accumulation of fibronectin in coronary artery restenotic lesions retrieved by atherectomy. *Br Heart J* 73: 534, 1995.

122. **A Blum, et al.** Endothelial dysfunction and inflammation after percutaneous coronary intervention. *Am J Cardiol* 94: 1420, 2004.

123. **KF Montgomery, et al.** Activation of endothelial-leukocyte adhesion molecule 1 (ELAM-1) gene transcription. *Proc Natl Acad Sci U S A* 88: 6523, 1991.

124. **M Schaffer, et al.** Sirolimus impairs wound healing. *Langenbecks Arch Surg* 392: 297, 2007.

125. **DD Sarbassov, et al.** Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* 14: 1296, 2004.

126. **DD Sarbassov, et al.** Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell* 22: 159, 2006.

127. **M Morales-Ruiz, et al.** Vascular endothelial growth factor-stimulated actin reorganization and migration of endothelial cells is regulated via the serine/threonine kinase Akt. *Circ Res* 86: 892, 2000.

128. **TL Phung, et al.** Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. *Cancer Cell* 10: 159, 2006.

129. **NN Lang and DE Newby.** Emerging thrombotic effects of drug eluting stents. *Arterioscler Thromb Vasc Biol* 27: 261, 2007.

130. **VA Morrow, et al.** Direct activation of AMP-activated protein kinase stimulates nitric-oxide synthesis in human aortic endothelial cells. *J Biol Chem* 278: 31629, 2003.

131. **Q Yang, et al.** TSC1/TSC2 and Rheb have different effects on TORC1 and TORC2 activity. *Proc Natl Acad Sci U S A* 103: 6811, 2006.

132. **Y Hattori, et al.** Metformin inhibits cytokine-induced nuclear factor kappaB activation via AMP-activated protein kinase activation in vascular endothelial cells. *Hypertension* 47: 1183, 2006.

133. **SH Hofma, et al.** Indication of long-term endothelial dysfunction after sirolimus-eluting stent implantation. *Eur Heart J* 27: 166, 2006.

134. **R Deves and CA Boyd.** Transporters for cationic amino acids in animal cells: discovery, structure, and function. *Physiol Rev* 78: 487, 1998.

135. **P Graf, et al.** The transport activity of the human cationic amino acid transporter hCAT-1 is downregulated by activation of protein kinase C. *Br J Pharmacol* 132: 1193, 2001.

136. **WW Simmons, et al.** Glucocorticoids regulate inducible nitric oxide synthase by inhibiting tetrahydrobiopterin synthesis and L-arginine transport. *J Biol Chem* 271: 23928, 1996.

137. **EI Closs.** Expression, regulation and function of carrier proteins for cationic amino acids. *Curr Opin Nephrol Hypertens* 11: 99, 2002.

138. **S Gordon.** Alternative activation of macrophages. *Nat Rev Immunol* 3: 23, 2003.

139. **J Xaus, et al.** Interferon gamma induces the expression of p21waf-1 and arrests macrophage cell cycle, preventing induction of apoptosis. *Immunity* 11: 103, 1999.

140. **DM Mosser.** The many faces of macrophage activation. *J Leukoc Biol* 73: 209, 2003.

141. **M Hesse, et al.** Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol* 167: 6533, 2001.

142. **RG Bogle, et al.** L-arginine transport is increased in macrophages generating nitric oxide. *Biochem J* 284 (Pt 1): 15, 1992.

143. **AR Baydoun, et al.** Arginine uptake and metabolism in cultured murine macrophages. *Agents Actions* 38 Spec No: C127, 1993.

144. **M Caivano.** Role of MAP kinase cascades in inducing arginine transporters and nitric oxide synthetase in RAW264 macrophages. *FEBS Lett* 429: 249, 1998.

145. **A Yeramian, et al.** Arginine transport via cationic amino acid transporter 2 plays a critical regulatory role in classical or alternative activation of macrophages. *J Immunol* 176: 5918, 2006.

146. **V Venketaraman, et al.** Nitric oxide regulation of L-arginine uptake in murine and human macrophages. *Tuberculosis (Edinb)* 83: 311, 2003.

147. **A Noguchi, et al.** SLC7A7 genomic structure and novel variants in three Japanese lysinuric protein intolerance families. *Hum Mutat* 15: 367, 2000.

148. **D Torrents, et al.** Identification of SLC7A7, encoding y+LAT-1, as the lysinuric protein intolerance gene. *Nat Genet* 21: 293, 1999.

149. **O Simell, et al.** Lysinuric protein intolerance. *Am J Med* 59: 229, 1975.

150. **BC Trapnell, et al.** Pulmonary alveolar proteinosis. *N Engl J Med* 349: 2527, 2003.

151. **K Parto, et al.** Abnormal pulmonary macrophages in lysinuric protein intolerance. Ultrastructural, morphometric, and x-ray microanalytic study. *Arch Pathol Lab Med* 118: 536, 1994.

152. **F Santamaria, et al.** Recurrent fatal pulmonary alveolar proteinosis after heart-lung transplantation in a child with lysinuric protein intolerance. *J Pediatr* 145: 268, 2004.

153. **G Borsani, et al.** SLC7A7, encoding a putative permease-related protein, is mutated in patients with lysinuric protein intolerance. *Nat Genet* 21: 297, 1999.

154. **D Torrents, et al.** Identification and characterization of a membrane protein (y+L amino acid transporter-1) that associates with 4F2hc to encode the amino acid transport activity y+L. A candidate gene for lysinuric protein intolerance. *J Biol Chem* 273: 32437, 1998.

155. **V Dall'Asta, et al.** Arginine transport through system y(+)L in cultured human fibroblasts: normal phenotype of cells from LPI subjects. *Am J Physiol Cell Physiol* 279: C1829, 2000.

156. **L Martin, et al.** Granulocyte-macrophage colony-stimulating factor increases L-arginine transport through the induction of CAT2 in bone marrow-derived macrophages. *Am J Physiol Cell Physiol* 290: C1364, 2006.

157. **MC Reade, et al.** Increased cationic amino acid flux through a newly expressed transporter in cells overproducing nitric oxide from patients with septic shock. *Clin Sci (Lond)* 102: 645, 2002.

158. **J Mykkanen, et al.** Functional analysis of novel mutations in y(+)LAT-1 amino acid transporter gene causing lysinuric protein intolerance (LPI). *Hum Mol Genet* 9: 431, 2000.

159. **JJ Caulfield, et al.** Regulation of major histocompatibility complex class II antigens on human alveolar macrophages by granulocyte-macrophage colony-stimulating factor in the presence of glucocorticoids. *Immunology* 98: 104, 1999.

160. **MJ Thomassen, et al.** Differential effect of recombinant granulocyte macrophage colony-stimulating factor on human monocytes and alveolar macrophages. *Cancer Res* 49: 4086, 1989.

161. **TL Bonfield, et al.** PU.1 regulation of human alveolar macrophage differentiation requires granulocyte-macrophage colony-stimulating factor. *Am J Physiol Lung Cell Mol Physiol* 285: L1132, 2003.

162. **A Rotmann, et al.** Activation of classical protein kinase C reduces the expression of human cationic amino acid transporter 3 (hCAT-3) in the plasma membrane. *Biochem J* 395: 117, 2006.

163. **A Rotmann, et al.** Protein kinase C activation promotes the internalization of the human cationic amino acid transporter hCAT-1. A new regulatory mechanism for hCAT-1 activity. *J Biol Chem* 279: 54185, 2004.

164. **PG Jorens, et al.** Human and rat macrophages mediate fungistatic activity against Rhizopus species differently: in vitro and ex vivo studies. *Infect Immun* 63: 4489, 1995.

165. **NK Jesch, et al.** Expression of inducible nitric oxide synthase and formation of nitric oxide by alveolar macrophages: an interspecies comparison. *Environ Health Perspect* 105 Suppl 5: 1297, 1997.

166. **C Aston, et al.** Early inhibition of mycobacterial growth by human alveolar macrophages is not due to nitric oxide. *Am J Respir Crit Care Med* 157: 1943, 1998.

167. **RB Muijsers, et al.** L-Arginine is not the limiting factor for nitric oxide synthesis by human alveolar macrophages in vitro. *Eur Respir J* 18: 667, 2001.

168. **B Nicholson, et al.** Cat2 L-arginine transporter-deficient fibroblasts can sustain nitric oxide production. *Nitric Oxide* 7: 236, 2002.

169. **CK Manner, et al.** CAT2 arginine transporter deficiency significantly reduces iNOS-mediated NO production in astrocytes. *J Neurochem* 85: 476, 2003.

170. **J Mossner, et al.** Concomitant down-regulation of L-arginine transport and nitric oxide (NO) synthesis in rat alveolar macrophages by the polyamine spermine. *Pulm Pharmacol Ther* 14: 297, 2001.

171. **C Sittipunt, et al.** Nitric oxide and nitrotyrosine in the lungs of patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* 163: 503, 2001.

172. **MJ Thomassen and MS Kavuru.** Human alveolar macrophages and monocytes as a source and target for nitric oxide. *Int Immunopharmacol* 1: 1479, 2001.

173. **B Nicholson, et al.** Sustained nitric oxide production in macrophages requires the arginine transporter CAT2. *J Biol Chem* 276: 15881, 2001.

174. **TC Chou, et al.** The inhibition by dantrolene of L-arginine transport and nitric oxide synthase in rat alveolar macrophages. *Anesth Analg* 86: 1065, 1998.

175. **ME Rothenberg, et al.** Cationic amino acid transporter 2 regulates inflammatory homeostasis in the lung. *Proc Natl Acad Sci U S A* 103: 14895, 2006.