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CD8 T cells with constitutively active PI3Kγ induce hypertension in mice and increase myogenic tone of resistance arteries

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SOMMARIO

L'ipertensione è una condizione clinica caratterizzata da elevati valori di pressione arteriosa ed associata a danno cardiaco, renale e vascolare. Eziologicamente è definita come essenziale, se la causa non è ben identificabile, o come secondaria, se l'incremento di pressione ha un'origine nota. Il trattamento consiste nella modificazione dello stile di vita e nella somministrazione di trattamenti antipertensivi; nonostante ciò sono riscontrabili alcune forme di ipertensione farmaco-resistente. Ricercare i meccanismi alla base della patologia risulta, quindi, importante al fine di individuare nuovi bersagli farmacologici. Numerosi articoli scientifici hanno evidenziato la correlazione tra ipertensione ed attivazione del sistema immune, ma i meccanismi attraverso cui l'immunità agisce incrementando la pressione arteriosa sono sconosciuti. Questa tesi si pone l'obiettivo di chiarire come le cellule immunitarie sono implicate nell'insorgenza del fenotipo ipertensivo. È stato precedentemente studiato il coinvolgimento della fosfatidilinositolo-3-chinasi γ (PI3Kγ) nell'ipertensione ed è stato evidenziato che il modello murino non esprimente questo enzima (PI3Kγ KO) era protetto dall'incremento di pressione indotto dall'angiotensina II; inoltre, PI3Kγ è espressa nei linfociti regolandone la maturazione e la migrazione. Sulla base di queste premesse, mediante analisi FACS, è stato evidenziato che topi PI3Kγ KO angII mostravano un ridotto infiltrato immunitario renale rispetto a topi WT angII. Successivamente è stato osservato che il modello murino con espressione costitutiva di PI3Kγ (PI3K$\gamma^{CX/CX}$) era caratterizzato da ipertensione spontanea e l'analisi istologica effettuata sui reni di tali topi evidenziava i danni tipici del fenotipo ipertensivo, quali fibrosi, incremento delle dimensioni dei corpuscoli renali e della capsula di Bowman. In base a queste evidenze è stato valutato se i meccanismi immunitari sono coinvolti nel link tra PI3Kγ ed elevata pressione arteriosa. Analisi FACS hanno evidenziato che topi PI3K$\gamma^{CX/CX}$ presentavano un incrementato infiltrato immune renale caratterizzato da cellule $CD8^+CD69^+CD44^+$. Esperimenti di *adoptive transfer* hanno mostrato la capacità delle cellule $CD8^+$ spleniche isolate da topi PI3K$\gamma^{CX/CX}$ nel determinare l'incremento dei valori pressori in topi normotesi infusi con queste cellule. Poiché il tono miogenico, ovvero la

capacità di esplicare una risposta contrattile in funzione all'aumento della pressione intravascolare indipendentemente dalla regolazione neurormonale, risulta incrementato in diversi modelli animali di ipertensione, è stato studiato se le cellule $CD8^+$ sono determinanti di questa incrementata risposta miogenica. A tal fine è stato sviluppato un innovativo sistema sperimentale che permette la co-coltura di vasi di resistenza e cellule $CD8^+$. Grazie a ciò è stato dimostrato che cellule $CD8^+$ attivate da stimoli ipertensivi inducevano un significativo aumento della risposta miogenica in arteriole mesenteriche isolate da topi normotesi. Complessivamente questi dati suggeriscono che la segnalazione di PI3Kγ nelle cellule $CD8^+$ è cruciale nello sviluppo dell'ipertensione, nella migrazione di queste cellule verso i reni, dove contribuiscono al danno d'organo, e nella modulazione del tono miogenico che rappresenta un meccanismo chiave nella regolazione della pressione arteriosa.

Parole chiave: Ipertensione, sistema immune, fosfoinoside-3-chinasi γ, tono miogenico, sistema in coltura di vasi

ABSTRACT

Hypertension is a clinical condition characterized by elevated arterial pressure values associated with cardiac, renal and vascular damage. It can be defined etiologically as essential, whether the cause is not known, or secondary, whether the cause is well identifiable. The treatment consists in the lifestyle modification and in the antihypertensive therapy administration; despite this, some forms of drug-resistant hypertension can be found. Therefore, the research to understand the mechanisms underlying the pathology is important to identify new pharmacological targets. Numerous scientific articles showed a correlation between hypertension and immune system activation, but the mechanisms by which immunity acts to increase arterial pressure are unknown. This thesis aims to clarify how immune cells are involved in the onset of hypertensive phenotype. The involvement of phosphatidylinositol-3-kinase γ (PI3Kγ) in hypertension has been previously investigated showing that the murine model depleted of this enzyme (PI3Kγ KO) is protected from angiotensin II-induced hypertension; furthermore, PI3Kγ is expressed in lymphocytes regulating their maturation and migration. Based on these assumptions, trough FACS analysis, it is shown that PI3Kγ KO ang II mice are featured by reduced renal immune infiltrate compared to PI3Kγ WT angII mice. Subsequently, it is observed that the murine model with constitutive expression of PI3Kγ ($PI3K\gamma^{CX/CX}$) was characterized by spontaneous hypertension and histological analysis performed on kidneys of these mice highlighted the typical damages of the hypertensive phenotype, as fibrosis, increased dimensions of renal corpuscles and Bowman's capsule. Based on these evidences, it is investigated whether immune mechanisms are involved in link between PI3Kγ and high blood pressure. FACS analysis highlighted that $PI3K\gamma^{CX/CX}$ mice showed an increased renal immune infiltrate characterized by the presence of $CD8^+CD69^+CD44^+$ cells. Using adoptive transfer experiments, it is demonstrated the ability of $CD8^+$ splenic cells isolated from $PI3K\gamma^{CX/CX}$ mice to determine the increase in blood pressure in normotensive mice that was infused with these cells. Since the myogenic tone, ie the ability to perform a contractile response to counteract the increase in intravascular pressure independently of the

neurormonal regulation, is enhanced in different animal models of hypertension, it is investigated whether the immune cells were determinants of this increased myogenic response. To this end, it is developed an innovative experimental system that allowed the co-culture of resistance vessels with $CD8^+$ cells. Thanks to this system it was shown that $CD8^+$ cells activated by hypertensive stimuli induced a significant increase in myogenic response in mesenteric arterioles isolated from normotensive mice. Altogether these data suggest that the signaling of PI3Kγ in $CD8^+$ T cells is crucial in hypertension development, in the migration of these cells to the kidneys, where they contribute to organ damage, and in the modulation of myogenic tone which represents a key mechanism in regulation of arterial pressure.

Key words: Hypertension, immune system, phosphoinositide-3-kinase γ, myogenic tone, vessel culture system

1. INTRODUCTION

1.1 Hypertension: an overview

Hypertension (HTN or HT), also known as high blood pressure (HBP), is traditionally defined as a long-term medical condition characterized by the presence of elevated systemic arterial pressure above a threshold value.

The latest guidelines published by the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH) defined that hypertension is characterized by office systolic BP (SBP) values at least 140 mmHg and/or diastolic BP (DBP) values at least 90 mmHg (Williams B *et al*., 2018). For the American population, the 2017 American College of Cardiology/American Heart Association (ACC/AHA) guidelines delineated the new categories of BP, according to which patients with blood pressure greater than 130/80 mmHg are considered hypertensive; this new classification makes almost half of the adult population hypertensive (Table 1; Whelton PK *et al.*, 2017).

Worldwide trends in blood pressure highlighted that in 2015 1.13 billion of people suffered of high BP, with a prevalence of over 150 million in central and Eastern Europe (figure1). The overall prevalence of hypertension in adults is around 30 - 45%, with a global age-standardized prevalence of 24 and 20% in men and women, respectively. At the global level, the increase of hypertensive populations is attributable to population growth and ageing; moreover, in the high-income region the absolute number of people with high blood pressure is decreased compared to the past steadily, conversely in the low-income and middle-income region the number of people with raised blood pressure is still increasing (Zhou B *et al*., 2017).

In the light of the most recent discoveries, Giles T D and colleagues refined and updated the definition and classification of hypertension. This pathological condition can be defined as “a progressive cardiovascular syndrome arising from complex and interrelated etiologies” and its progression is associated with functional and structural injury to the heart, kidneys, brain and vasculature.

Many factors contribute to the raised blood pressure (BP), the most intensively studied are: salt intake, smoking, sedentary lifestyle, obesity and insulin resistance, elevated level of total cholesterol, genetic factors, endothelial dysfunction, low birth weight, early-onset menopause, intrauterine nutrition, neurovascular anomalies, renin-angiotensin system and sympathetic nervous system (Giles T D *et al.*, 2009).

Etiologically, HTN can be divided in two main groups: primary (or essential) and secondary hypertension. The majority of patients are affected by primary HTN in which HBP is not associated with any identifiable pathological causes. On the contrary, a minority of patients present secondary hypertension characterized by a specific cause of HBP, as obstructive sleep apnea, renal parenchymal disease, renal artery stenosis, primary aldosteronism, thyroid disease, Cushing's syndrome, pheochromocytoma, coarctation of the aorta (Carretero OA and Oparil S, 2000; Rimoldi SF *et al*., 2014).

Since HBP is the main factor risk for cardiovascular disease the treatment of this pathology is fundamental to limit the negative consequences induced in the short and long term. There are two established strategies to fight hypertension: lifestyle modifications and drug treatment. The lifestyle recommendations that have been shown helpful to reduce BP are: salt intake restriction, moderation of alcohol consumption, tobacco smoking cessation, diet rich in fruits and vegetables, maintaining an ideal body weight, and regular physical activity. Although healthy lifestyle contributes to lower BP, in many cases the pharmacological intervention is necessary. Major classes of antihypertensive drugs are represented by blockers of the renin-angiotensin system (angiotensin converting enzyme inhibitors and angiotensin receptor blockers), calcium channel blockers, thiazides and thiazide-like diuretics and beta-blockers. Furthermore, other drags for the treatment of this condition exist and are used in the drug-resistant hypertension where all other pharmacological options are not efficacy (Williams B *et al*., 2018).

People that suffer of HBP likely develop other pathologies as atherosclerosis, stroke, myocardial infarction, heart failure, chronic kidney disease and dementia. Since uncontrolled blood pressure remains a major threat to cardiovascular health, the prevention and management of hypertension are global public health issues (Lionakis N *et al*., 2012; WHO, 2013).

BP Category	Systolic (mmHg)		Diastolic (mmHg)
Optimal	<120	and	<80
Normal	120-129	and/or	80-84
High normal	130-139	and/or	85-89
Grade 1 hypertension	140-159	and/or	90-99
Grade 2 hypertension	160-179	and/or	100-109
Grade 3 hypertension	≥180	and/or	≥110
Isolate systolic hypertension	≥140	and	<90

Table 1. Categories of BP in Adults. Systolic and diastolic blood pressure values identify optimal, normal, high normal blood pressure conditions, grade 1, 2 and 3 of hypertension (Williams B *et al*., 2018).

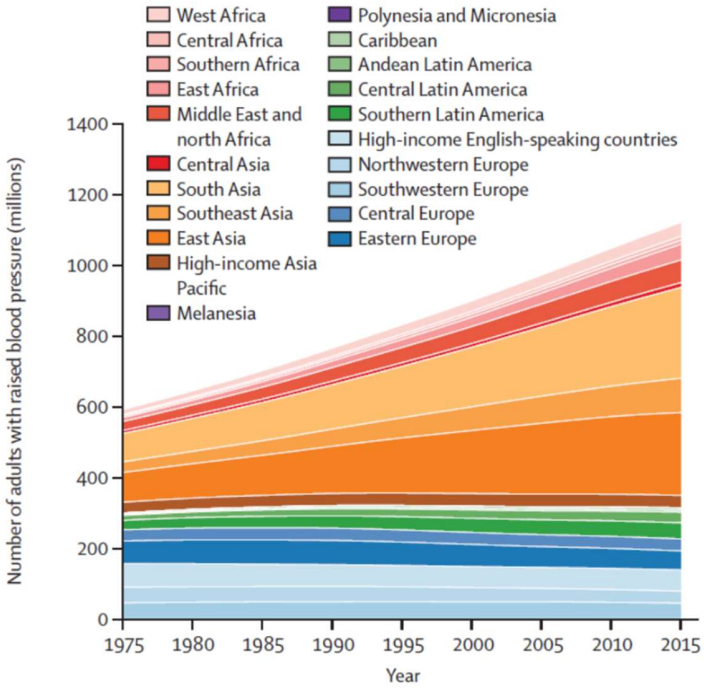

Figure 1. Trends in hypertension prevalence, 1975 - 2015. Trends by region in the number of adults aged 18 years and older with raised blood pressure. The estimated number of adults with raised blood pressure increased from 594 million in 1975 to 1·13 billion in 2015 (Zhou B *et al*., 2017).

1.2 Myogenic tone

Myogenic response is a smooth muscle cells (SMCs) hallmark of resistance arteries and arterioles to counteract to blood pressure changes. The first description of this vascular response is attributed to William Maddock Bayliss that described this phenomenon in 1902; the pressure-induced response has been demonstrated to occur independently of the endothelium and it does not require neurohumoral input (Bayliss WM, 1902; Schubert R *et al*., 1999). In particular, when an increase in transmural pressure occurs resistance arteries react by vasoconstriction; conversely, when a decrease in pressure takes place resistance vessels respond by vasodilatation (Davis MJ *et al*., 2012); therefore, vascular tone of resistance vessels is due to blood pressure within these vessels and it depends on the balance between vasoconstrictor and vasodilator signals (Davis MJ *et al*., 2011). There is an extensive literature showing that in experimental animal models of hypertension and in patients with high blood pressure occur an increase in peripheral vascular resistances that in turn contributes to the maintenance of chronic hypertensive status (Carnevale D *et al*., 2018; Goulopoulou S and Webb RC, 2014; Guzik T *et al*., 2007; Tang KM *et al*., 2003; Huang PL *et al*., 1995). The pressure-induced mechanosensory effects involve membrane depolarization, Ca^{2+} signaling, activation of contractile proteins via a myosin light chain kinase (MLCK)-mediated mechanism and remodeling of the cytoskeleton structure. **SMCs depolarization** can be ascribable mainly to voltage-gated Ca^{2+} channels (VGCC) opening, but also to closure of K^+ channels or opening of a Cl_2 channel. Electrophysiological approaches showed that SMC membrane deformation (e.g. due to stretch) causes activation of a cation current that presumably leads to membrane depolarization and a subsequent opening of VGCC. Stretch-activated channels (SACs), for which gating is modulated by physiological levels of stretch, participate in the membrane potential control. The opening of these channels results in a predominantly Na^+ current that causes the membrane depolarization. In addition, membrane stretch activates BK_{Ca} channels, producing a hyperpolarizing current that limits the extent of depolarization and, hence, myogenic constriction; this can be considered an important negative-feedback mechanism to limit the effects of additional myogenic contraction owing to pressure-induced vasoconstriction of

downstream arterioles. Others important cationic channels involved in myogenic signaling are represented by TRP (transient receptor potential) channels, that have been characterized as nonselective cation and Ca^{2+}- selective channels. In particular, it was demonstrated that the reduced expression of TRPC6 and TRPM4 resulted in marked attenuation of both pressure-induced depolarization and myogenic constriction. An alternate mechanosensory mechanism is represented by **integrins**, transmembrane receptors that facilitate cell-extracellular matrix (ECM) adhesion. It is demonstrated that the block of either $\alpha_v\beta_3$ or $\alpha_5\beta_1$ integrins abolishes myogenic constriction to step increases in intravascular pressure. According with the fact that integrin activation and cytoskeletal remodeling are coordinated processes, it is showed that myogenic activation of arterioles is associated with **cytoskeletal rearrangements** including globular to filamentous actin transformations, demonstrating the importance of the actin cytoskeleton in myogenic response. Myogenic contraction is also modulated by **second messengers**; in fact, the increase in transmural pressure leads to the generation of specific factors such as 20-HETE, sphingosine-1-phosphate (S1P) and diacylglycerol (DAG). Pressure activation also causes an **alteration in G_q/G_{11} activity** in a receptor-ligation-independent manner where activation of the trimeric G protein subsequently stimulates PLCβ and TRPC6 to cause membrane depolarization. Myogenic contraction is dependent on a global intracellular Ca^{2+}- calmodulin - MLCK regulated mechanism, as well as on **Ca^{2+} sensitization**. Ca^{2+} sensitization refers to processes that inhibit myosin phosphatase, thus blocking the dephosphorylation of myosin regulatory light chain. Candidate mechanisms for inhibition of the phosphatase include Rho-kinase-mediated phosphorylation of MYPT1 (myosin phosphatase target subunit 1) and PKC (protein kinase C)-mediated CPI-17 (C-kinase-activated protein phosphatase-1 (PP1) inhibitor, 17kDa) activation. As mentioned earlier, the opening of L-type Ca^{2+} channels is the principal event that follows the pressure stimulation. Moreover, there is a minor contribution from Ca^{2+} entry via non-voltage-gated Ca^{2+}-entry pathways. However, Ca^{2+} through these pathways may participate in the regulation of ion channels and **SR** (sarcoplasmic reticulum) **Ca^{2+} dynamics**. Despite the role of Ca^{2+} release from the SR in myogenic signaling is difficult to understand because of the technical limitations that do not allow a detailed study of this aspect, it is known that the entry of extracellular Ca^{2+} causes

the activation of RyR (ryanodine receptor) and, therefore, the release of calcium from the SR to the cytoplasm contributing to myogenic response. To the other hand the SR provides an inhibitory action trough the stimulation of β1 subunit of BK_{Ca} channels, giving rise to spontaneously transient outward currents (STOCs) and acting as a negative feedback mechanism to prevent excessive depolarization as pressure-induced constriction occurs. Moreover, the Ca^{2+} release from intracellular compartment can occurs by the binding of the second messenger inositol 1, 4, 5-trisphosphate (IP3) to IP3R/Ca^{2+} channels (Figure 2; Hill MA and Meininger GA, 2012). In summary, in the context of the myogenic response it is possible to identify three different phases (Osol G *et al*., 2002). The first phase, in which there is the development of myogenic tone or basal tone, is characterized by a significant elevation of intracellular calcium influx through the L-type voltage gated calcium channel (LTTC; Hill MA *et al*., 2001; Tajada S *et al*., 2013), cellular depolarization and deformation, followed by a reduction in vessel diameter. The second phase, named myogenic reactivity, is characterized by minor changes in membrane potential and intracellular calcium level but is present an intracellular calcium sensitization (Schubert *et al*., 2008). In this phase there is a further constriction in response to an intraluminal pressure increase. In the last phase, named dilatative force, is observable a complete loss of tone together with vasodilatation in response to high transmural pressure (Hill MA *et al*., 2006; Carnevale D and Lembo G, 2012).

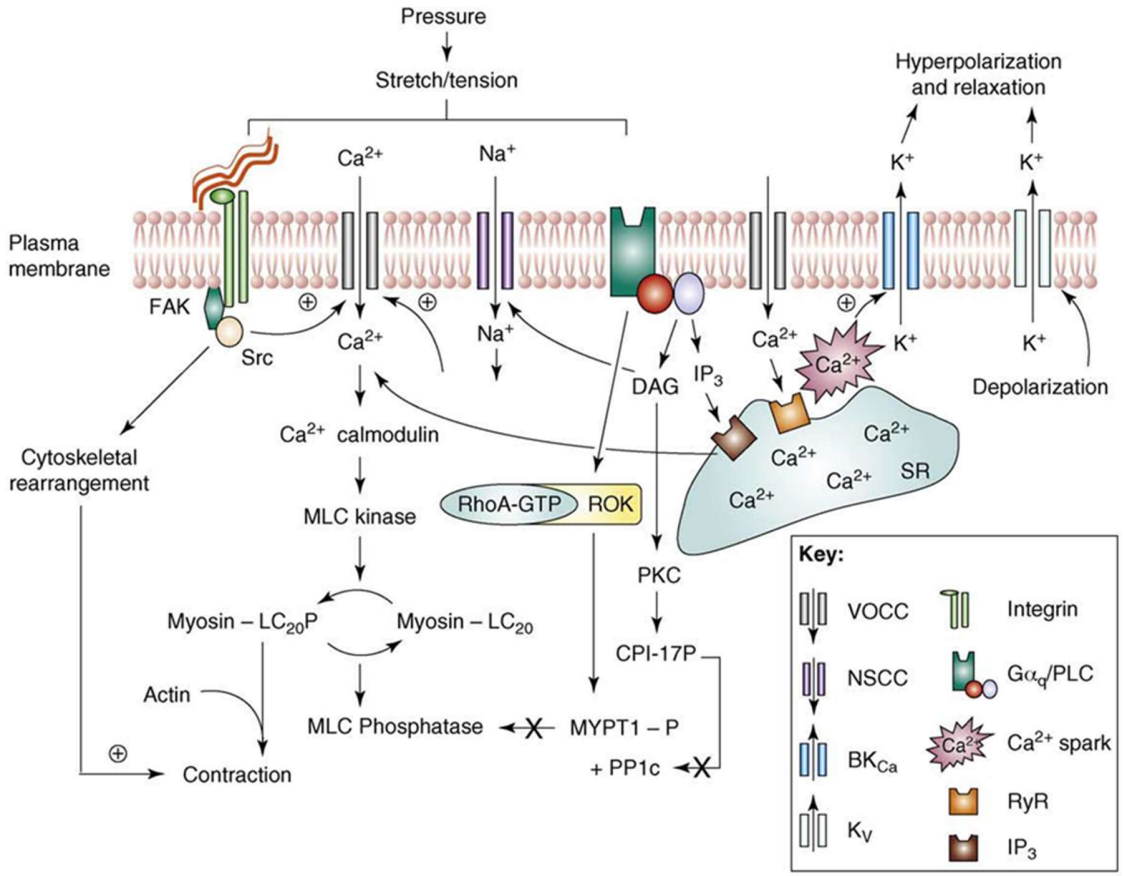

Figure 2. Schematic illustration of specific signaling mechanisms that mediate arteriolar myogenic vasoconstriction. Mechanical stimulus leads to membrane depolarization mainly mediated by VGCCs, increases in cyctosolic Ca^{2+} and contraction through calmodulin-mediated activation of MLCK. Phosphorylation of MLC_{20} ($MLC_{20}P$) allows acto-myosin interaction, cross-bridge cycling and contraction. The exact molecular events underlying this membrane depolarization are unclear but important candidates include direct activation of cation channels (for example, SACs, ENaC -Epithelial sodium channels- or TRP channels), second messenger-mediated (for example, DAG, PKC) activation of cation channels and integrin-mediated facilitation of VGCC opening. The mechanical stimulus activates cytoskeletal remodeling through mechanisms likely to be dependent on integrin-mediated activation of focal adhesions. A role of the SR in myogenic contraction has the potential for both stimulatory (through Ca^{2+} release to the bulk cytoplasm) and inhibitory actions (through both generation of Ca^{2+} sparks and activation of BK_{Ca}-mediated STOCs, and through its ability to sequester Ca^{2+} from the cytoplasm). Increased intraluminal pressure and cell stretch also activate tyrosine-kinase-mediated mechanisms (for example, involving FAK -focal adhesion kinase-, Src kinase and p42/44 MAPK -mitogen-activated protein kinase).

1.3 Immune system in hypertension: the early studies

Hypertension is generally attributed to perturbations of vasculature, kidney, and central nervous system (CNS). During the past several years, studies have shown consistent association between this disease, proinflammatory cytokines and cells of the innate and adaptive immune systems and have focused on defining the mechanisms linking the immune system to the hypertensive disease state.

Link between immune system and hypertension emerged in 1964 with the pioneering studies conducted by White and Grollman that described the importance of immunosuppressive therapy on regulation of BP levels in rats with partial kidney infarction (White FN and Grollman A, 1964). Subsequently, other studies demonstrated that the transfer of immune cells isolated from lymph nodes or spleen of hypertensive animals induced hypertension in normal recipient rats (Okuda T and Grollman A, 1967; Olsen F, 1980). In 1972 Olsen demonstrated that in arterioles and small arteries of hypertensive humans is observable an inflammatory mononuclear cellular infiltration (Olsen F, 1972). Studies performed by Svendsen revealed that in athymic nude mice hypertension did not maintain after renal infarction (Svendsen UG, 1976). Moreover, thymectomy performed in hypertensive mice, treated with deoxycorticosterone acetate (DOCA)-salt (Svendsen UG, 1976), or in genetically hypertensive rat model (Bataillard A *et al*.,1986) attenuated experimental HT. A further work demonstrated that in T cell-depressed spontaneously hypertensive rats (SHR) the thymus grafts reduced the blood pressure. Moreover, transplantation of compatible thymus tissues into neonatal SHR produced long-lasting recovery of immune functions and the complete immunologic restoration caused the suppression of HBP (Ba D *et al*., 1982). These first studies about the involvement of immune system and hypertension provided key elements for subsequent studies in this research field.

1.4 Immune cells and hypertension

The described discovers aroused much interest and the researchers focused on the investigation of immune cells subtypes that contribute to the modulation of hypertensive phenotype. To date, the involvement of adaptive and innate immunity has been demonstrated in the onset of hypertension (figure 3). The aim of subsequent paragraphs is that to provide a description of different types of immune cells involved in the development of HBP.

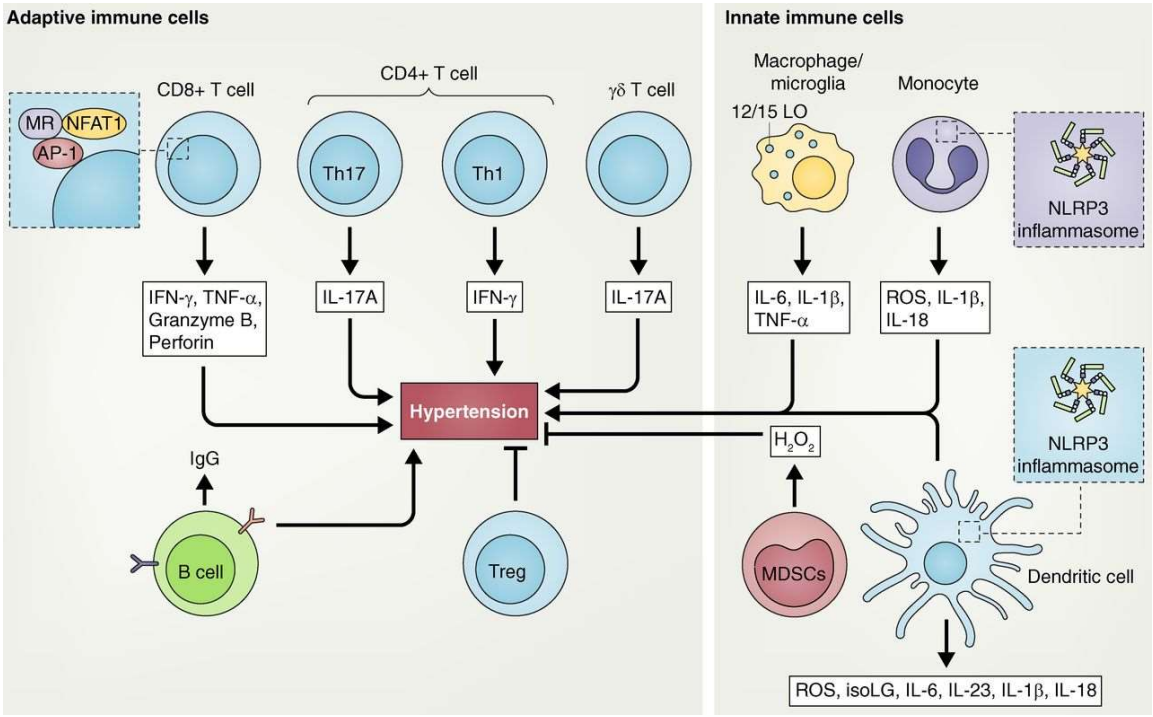

Figure 3. Innate and adaptive immune cells involved in hypertension. Adaptive immune cells that have been shown to play a role in high blood pressure are: $CD8^+$, $CD4^+$ T cells (Th1, Th17, and T reg cells) γδ T cells and B cells. These populations produce factors that promote or inhibit hypertension. Innate immune cells involved in hypertension are: macrophages, microglia, monocytes, DCs, and MDSCs that also produce cytokines and ROS, which promote or inhibit this pathological condition. The NLRP3 inflammasome in monocytes and DCs plays a key role in hypertension (Norlander AE *et al*., 2018).

1.4.1 Adaptative immunity in hypertension

Many researches highlighted the involvement of T cells in the onset of hypertension. It is described that pharmacological approaches that act with different mechanisms on immune cells are effective in the modulation of blood pressure. In particular, it is demonstrated that rats treated with mycophenolate mofetil (MMF), a selective T lymphocyte immunosuppressive agent, are protected to development of salt-dependent hypertension induced from angiotensin (Ang II) infusion (Rodríguez-Iturbe B *et al*., 2001). Additionally, it is showed that abatacept, a T cells costimulation inhibitor, is able to prevent angiotensin II and (DOCA)–salt induced hypertension (Vinh A *et al*., 2010). Important advances derived from the study of RAG1 knock out (KO) murine model. This model is characterized by a deletion of *Rag1* coding for recombinase protein involved in V(D)J recombination, a typically process that occurs in developing lymphocytes during the early stages of T and B cells maturation; therefore, this gene deletion causes the absence of mature B and T cells. It is observed that AngII or DOCA-induced hypertension is blunted in RAG1 KO mice (Figure 4) and that this phenotype is independent from angiotensin II receptors (AT1R and AT2R) expression. Moreover, many of the negative vascular consequences induced by angiotensin II, such as ROS production and impairment of endothelium-dependent vasodilatation, were prevented in these mice. Adoptive transfer of T lymphocytes, but not of B lymphocytes, restored the hypertensive phenotype, demonstrating that T cells play a critical role in the onset of hypertension. Moreover, the adoptive transfer of T lymphocytes isolated from AT1a KO mice partially restored hypertension, indicating that some hypertensive effects mediated by angiotensin II result from its action on T cells. Similarly, adoptive transfer of T cells that lack of NADPH oxidase p47 ^{phox} subunit, partially restored the hypertensive response to angiotensin II in RAG1 KO mice, suggesting a function of the T cell NADPH oxidase in hypertension (Figure 4; Guzik TJ *et al.*, 2007).

Subsequent study highlighted that genetic deletion of *Rag1* gene in Dahl sensitive salt rats reduced the degree of hypertension and renal damage (Mattson DL *et al.*, 2013). Further studies investigated the role of deletion of CD3 ζ chain (CD247), a gene involved in T-cell

signaling, in Dahl salt-sensitive rats and found that the mean arterial blood pressure and kidney injury were reduced in CD247$^{-/-}$, showing a similar phenotype to that observed in the rats lacking RAG1 (Rudemiller N *et al*., 2014). Moreover, other research group using a SCID (severe combined immunodeficiency) mice, that lack of lymphocytes activity, demonstrated the importance of lymphocyte responses in AngII-induced hypertension (Crowley S D *et al*., 2010).

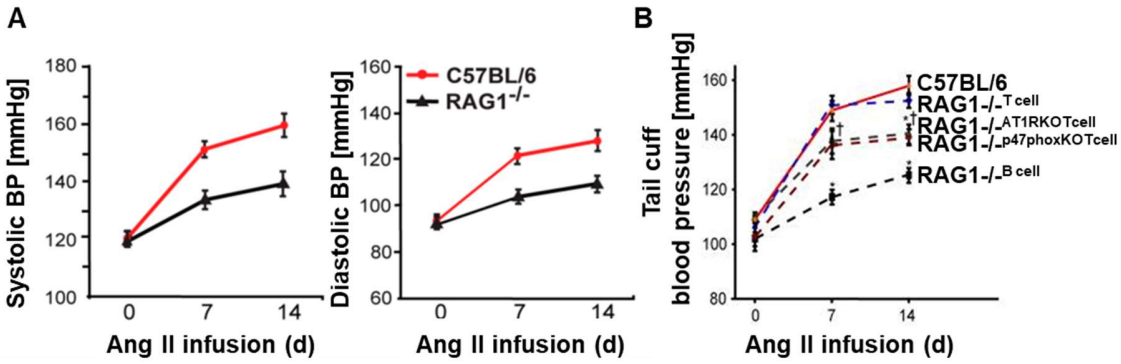

Figure 4. Blood pressure measurements in C57BL/6 and RAG1$^{-/-}$ mice. A. Invasive measurements of systolic and diastolic blood pressure at baseline and during angiotensin II infusion in C57BL/6 (red line) and RAG1$^{-/-}$ mice (black line). **B.** Blood pressures measurements at baseline and during angiotensin II infusion in C57BL/6 (red line), RAG1$^{-/-}$ that received T cells by adoptive transfer (RAG1$^{-/-\ T\ cell}$; dotted blue line), RAG1$^{-/-}$ that received T cells from AT1a$^{-/-}$ mice (RAG1$^{-/-\ AT1RKOT\ cell}$; dotted gray line), RAG1$^{-/-}$ that received T cells from mice lacking the oxidase subunit p47^{phox} (RAG1$^{-/-}$ p47$^{phoxKO\ T\ cell}$; dotted burgundy line), RAG1$^{-/-}$ that received B cells (RAG1$^{-/-\ B\ cell}$; dotted black line - Guzik TJ *et al.*, 2007).

Subsequently the subtypes of T cells involved in hypertension were analyzed: CD8, CD4, Treg, γδ T cells.

Different scientific articles highlighted the role of **CD8^{+} T cells** in the onset and development of hypertensive disease. Trott D W and colleagues analyzed the region Vβ of T cell receptor (TCR) as powerful indicator of clonal expansion of T cells that provide insight into adaptive immune responses. In this study, spectratyping profiles of TCRs revealed that in the kidney of angII-treated mice there was an increase in Vβ chain clonality

of $CD8^+$ cells, but no clonal skewing of $CD4^+$ T cells was observable. In the same way no clonal skewing of $CD4^+$ and $CD8^+$ T cells was present in mesenteric arteries and spleen of the hypertensive mice. The importance of $CD8^+$ T cell in the hypertensive contest was supported by experiments showing that $CD8^{-/-}$ mice were protected from hypertension induced from angII or DOCA salt, but this protection was missing in $CD4^{-/-}$ and $MHCII^{-/-}$ (major histocompatibility complex $II^{-/-}$, an alternate model of $CD4^+$ deficiency) mice. Moreover, adoptive transfer of $CD8^+$ cells from angiotensin II treated mice into RAG-$1^{-/-}$ mice caused an increase in blood pressure; this elevation was absent when RAG-$1^{-/-}$ mice was infused with $CD4^+/CD25^-$ cells. It is also observed that $CD8^+$ T cells exert an important role in endothelial disfunction, antidiuretic and antinatriuretic responses to angiotensin II (Trott D W *et al*., 2014).

It was also showed that $CD8^+$ T cells are crucial mediator of salt sensitive hypertension. In fact, these cells are able to up-regulate and activate the thiazide-sensitive sodium-chloride-co-transporter (NCC) in distal convoluted tubules (DCTs) of the kidney, which results in sodium retention and development of salt-sensitive hypertension. In particular, these immune cells stimulate NCC by upregulating the potassium channel Kir4.1 and subsequently the chloride channel ClC-K on the plasma membrane, thereby decreasing intracellular chloride. This last event leads to NCC activation and development of salt-sensitive hypertension (Liu Y *et al*., 2017).

An interesting human prospective study documented the pathogenic role of T cells in hypertension. Hypertensive patients showed a significant increase of senescent $CD28^-$ $CD57^+CD8^+$ T cells compared with normotensive subjects. In HTN patients these immunosenescent cells was characterized by cytotoxic and proinflammatory phenotype since they produced high levels of granzyme B, INFγ and TNFα. In the same study, the hypertensive condition is associated with renal infiltration of $CD4^+$ and $CD8^+$ T cells and increased circulating levels of CXCR3 chemokines, a tissue-homing chemokine for proinflammatory T cells (Youn J C *et al*., 2013).

There are evidences that also **$CD4^+$ T cells** are involved in hypertension. It is known that interleukin 17-A, mainly produced by a subset of $CD4^+$ T cells (Th17 cells), plays a critical role in hypertension and in others autoimmune disease associated with hypertensive

condition, as well as pre-eclampsia and systemic lupus erythematosus. Recent study demonstrated that IL-17 increases Rho-kinase-mediated eNOS phosphorylation leading impaired endothelium-dependent vasodilatation and hypertension in mice (Nguyen H *et al*., 2013).

Experiments performed in humanized mice model, in which the murine immune system is replaced by the human immune system, showed that after AngII infusion there was a significant increase of $CD4^+$ T cells in the kidney and lymph nodes. In these cells were observable an increase of the memory cell marker CD45RO but there were few cells expressing the activation marker CD69. Moreover, analysis on human blood revealed that hypertensive patients showed an increase of $CD4^+$ and $CD8^+$ circulating T cells than normotensive controls. It is found that production of IL-17A is increased in $CD4^+$ T cells from hypertensive subjects; in the same way production of IFN-γ and TNFα is augmented in $CD4^+$ and $CD8^+$ cells isolated from patients with high blood pressure (Itani HA *et al.*, 2016).

It is known that a regulatory T lymphocytes (**Tregs**), expressing CD4, CD25 and Foxp3, are able to suppress innate and adaptive immune responses, as well as proinflammatory effects of other lymphocytes, macrophages, dendritic cells, and neutrophils. It is demonstrated that adoptive transfer of Tregs prevents AngII-induced hypertension. Furthermore, AngII treatment impaired the vasodilatory response to ACh and increased wall stiffness of mesenteric arteries, increased NADPH oxidase activity in both the aorta and the heart, plasma levels of cytokines and T-cell infiltration in the aortic adventitia and periadventitial fat. It is verified that adoptive transfer of Tregs prevent all these events induced by AngII (Barhoumi T *et al.*, 2011).

Recently, it was demonstrated that complement receptors are necessary to prevent AngII-induced hypertension. In fact, C3aR and C5aR double deficiency enhances the functions of $Foxp3^+$ Tregs cells that have an immunosuppressive role and, consequently, are able to attenuate Ang II-induced inflammatory factors expression, target organ damage, and so BP elevation (Chen XH *et al.*, 2018).

A recent evidence suggests that another subset of T cells involved in hypertension is represented by **γδ T cells**. It is known that T cells can be divided in two families based on

TCR chain constitution: those expressing α and β TCR chains and those expressing γ and δ TCR chains. This second family is much smaller than the first one and represented only from 1 to 4% of total T cells. $CD4^+$ and $CD8^+$ αβ T cells recognize antigens presented by APC cells by their MHC complex-II and -I, respectively. On the contrary, γδ T cells recognize antigens without MHC restriction and without help from APC; for this reason, are unconventional T lymphocytes, mostly CD4 and CD8 double negative, with rapid innate like-responses that act in the initiation phase of the immune reaction. It was already observed that the number of aortic-infiltrating double negative T cells and the percentage of peripheral blood double negative T cells were increased in Ang II-infused mice (Guzik TJ *et al.*, 2007). Caillon and colleagues confirmed that AngII-induced hypertension determines an increase of γδ T cells and showed that these cells are CD69 positive and, so, activated. *Tcrδ*$^{-/-}$ mice, deficient in γδ T cells, are protected from AngII-induced hypertension and vascular dysfunction and showed a blunted spleen T-cell activation. Moreover, the importance of these cells in human hypertension is highlighted by an association between γδ T-cell frequency in the blood and systolic blood pressure (Caillon A *et al.*, 2017). The mechanism by which these cells behave in order to support hypertension need to be further deepen.

There are also suggestions that **B cells** and the antibodies that they produce participate to hypertension. In fact, BAFF-R$^{-/-}$ mice, deficient in B cells, showed attenuated pressor response to Ang II compared to wild-type mice. The magnitude of cardiac hypertrophy, that is a consequence of sustained elevations in BP, was lower in BAFF-R$^{-/-}$ mice than that in wild-type mice. The relevance of B cells in the hypertensive context is confirmed by adoptive transfer of these cells from WT in BAFF-R$^{-/-}$ mice; B lymphocytes introduction in mice deficient of these cells restored the pressor response to Ang II. It is demonstrated that AngII infusion causes activation of B cells, indicated as increase of $CD86^+$ expression in peripheral lymphoid organs and induces in the spleen a major differentiation of B cells in antibody-producing plasma cells and plasmablasts. In agreement with this, there was elevated level of IgG in serum and aortic wall of mice infused with AngII compared with saline-infused animals. The lack of mature B cells in BAFF-R$^{-/-}$ mice eliminated the increase in circulating IgG (Chan CT *et al.*, 2015).

1.4.2 Innate immunity involved in hypertension

There are ample evidences that innate immune system plays a role in hypertensive disease. The earliest demonstration of this became from the study of op/op mice, defective in production of functional colony-stimulating factor-1 (CSF-1), that have reduced **macrophages** number. It is showed that this condition is associated with blunted hypertension in response to chronic Ang II infusion, preserved vascular morphology, attenuated endothelial dysfunction, O_2^- generation, NAD(P)H oxidase activation, and vascular inflammation compared with WT littermates (De Ciuceis C *et al*., 2005).

The role of inflammatory myelomonocytic cells is evaluated using selective ablation of lysozyme M-positive myelomonocytic cells by low-dose diphtheria toxin in mice with inducible expression of the diphtheria toxin receptor ($LysM^{iDTR}$ mice). In this murine model the reduction of **monocytes** number in the circulation is correlated with prevention of hypertension, reduced vascular dysfunction and ROS formation. Adoptive transfer of wild-type $CD11b^+Gr\text{-}1^+$ monocytes into depleted $LysM^{iDTR}$ mice reestablished the effects induced by AngII (Wenzel P *et al.*, 2011).

The involvement of macrophagic component in the hypertension (Justin Rucker A and Crowley SD, 2017) is highlighted studying $Alox15^{-/-}$ mice lacking 12/15 lipoxygenase. This experimental animal model results protected from hypertension induced by DOCA-salt or NO synthase inhibitor nitro l-arginine methyl ester (L-NAME). A rescue experiment that restore the macrophagic functions by transfusing WT peritoneal macrophages into $Alox15^{-/-}$ mice showed a restoration of increased blood pressure after 5 days of L-NAME treatment (Kriska T *et al*., 2012).

In 2014 Kirabo and colleagues described a new pathway linked to hypertension disease in which **dendritic cells** play a critical role. It was showed that in DCs from angiotensin II-infused mice there was an increased production of ROS, isoketals accumulation, cytokines release, such as IL-6, IL-18, IL-1β, and IL-23 and increase in costimulatory proteins CD80 and CD86. These activated DCs promoted $CD8^+$ T cell proliferation, IFN-γ and IL-17A production and hypertension (Figure 5; Kirabo A *et al*., 2014).

Other cells involved in hypertension are the myeloid-derived suppressor cells (MDSCs). In different hypertensive models, these cells exert their role in the spleen where they interact with T cells. In particular, the role of MDSCs is to suppress inflammation, through hydrogen peroxide production, and to limit blood pressure increase (Shah KH *et al*., 2015).

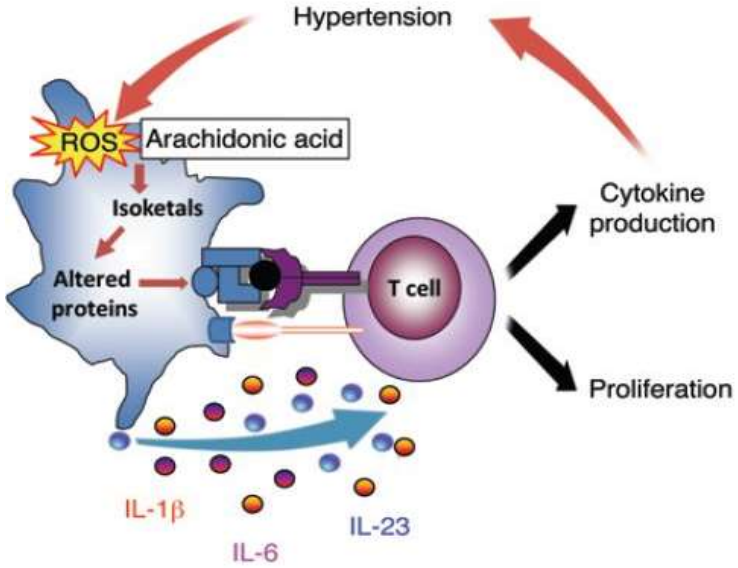

Figure 5. **Hypothesized pathway for dendritic cells activation.** Hypertensive stimuli increase ROS production, isoketals formation, cytokine release from DCs. Activated DCs promote T cell proliferation and cytokine production causing hypertension (Kirabo A *et al*., 2014).

1.5 The spleen: an important lymphoid organ involved in hypertension

The spleen is a secondary lymphoid organ surrounded by a capsule of connective tissue from which depart trabeculae. The splenic vessels are distributed in the thickness of the trabeculae and leave these structures to go through organ parenchyma. The central arterioles depart from the splenic artery branch and arrive in the white pulp (WP) area, which contains B cells in the follicles and T cells. The blood flows through the marginal zone (MZ), surrounding the WP, toward the red pulp (RP) area, where blood is directed to the venous sinuses. MZ represents a connection between circulating blood and immune cells and contains B cells together with subsets of neutrophils, DCs, and macrophages. RP is characterized for antibodies production and blood filtration process during which resident macrophages phagocyte old or damaged erythrocytes. In RP blood flows are conveyed to the splenic vein that converges in the portal vein and, in this way, blood leave the splenic organ (Mebius RE and Kraal G, 2005).

Recent papers highlight a central role of this organ in mediating hypertensive phenotype through a modulation of immune responses activated by nervous stimulation (Carnevale D *et al*., 2014; Lori A *et al*., 2017; Perrotta M *et al*., 2018).

As described in the previous paragraphs, different subpopulations of immune cells are involved in the development of high blood pressure. The figure 6 represents as these cells interact in order to elicit hypertensive phenotype. Vasoactive agents (for example Ang II, endothelin I -ET1-, aldosterone), genetic susceptibility, and high salt intake are important contributors in the hypertension. These mediators act increasing sympathetic nervous system (SNS) activity that is responsible of initial blood pressure elevation with subsequent renal and vascular damage. This tissue injury leads to DAMPs (damage-associated molecular patterns) and neoantigen formation that are responsible to innate immune activation through TLRs (toll like receptor) expressed on B cells, DCs and macrophages. Innate immunity contributes to inflammation and oxidative stress directly or through adaptive immune activation. All together these mediators promote progression of disease and cause end-organ damage (Caillon A and Schiffrin EL, 2016).

It is hypothesized that neoantigens are formed from modified endogenous proteins in the prehypertensive phase. These altered proteins activate T cells that infiltrate the vasculature and kidney, promote endothelial dysfunction, vasoconstriction and salt and water retention causing ultimately severe hypertension (Harrison DG *et al*., 2011).

It is described that the fibers of SNS innerve the spleen and that the synaptic endings are close to immune cells that populate this organ (Nance DM and Sanders VM, 2007). The neurotransmitters release from synaptic vesicles is able to regulate the immune responses through modulation of cytokines production. This represents the key point of neuro-immune communication, that is characterized by a bidirectional connection in which also the produced cytokines can affect neurons activity (Straub RH, 2004).

An important goal in the study of inflammation in the hypertension came from the discovery, made by the research group of Carnevale D and colleagues, that the **spleen is a key regulator in the pathogenesis of hypertension**. In particular, they showed for the first time the pivotal role of PlGF (placental growth factor), belonging to vascular endothelial growth factor (VEGF) family, as molecular pathway involved in the onset of hypertension by mediating immune response to the SNS activated by the AngII-induced hypertensive stimulus. Using adoptive transfer experiments, performed on mice expressing different allelic variants of CD45 (CD45.1 or CD45.2), it was demonstrated the splenic origin of T cells that infiltrate the target organs of hypertension. In particular, CD45.2 mice were splenectomized, transplanted with CD45.1 spleen and then infused with AngII to induce hypertension. The cytofluorimetric analysis revealed an increased number of donor (CD45.1) T cells in aortas and kidneys of AngII pre-HTN animals. Moreover, they showed the importance of splenic immune reservoir for the establishment of a hypertensive response to AngII. In fact, splenectomized mice infused with AngII were protected from BP elevation and T cells target organ infiltration (Figure 7; Carnevale D *et al*., 2104). Subsequent study showed that also in hypertension resulting from (DOCA)-salt PlGF exerts a pivotal role in immune response originating in the spleen (Perrotta ML *et al*., 2018).

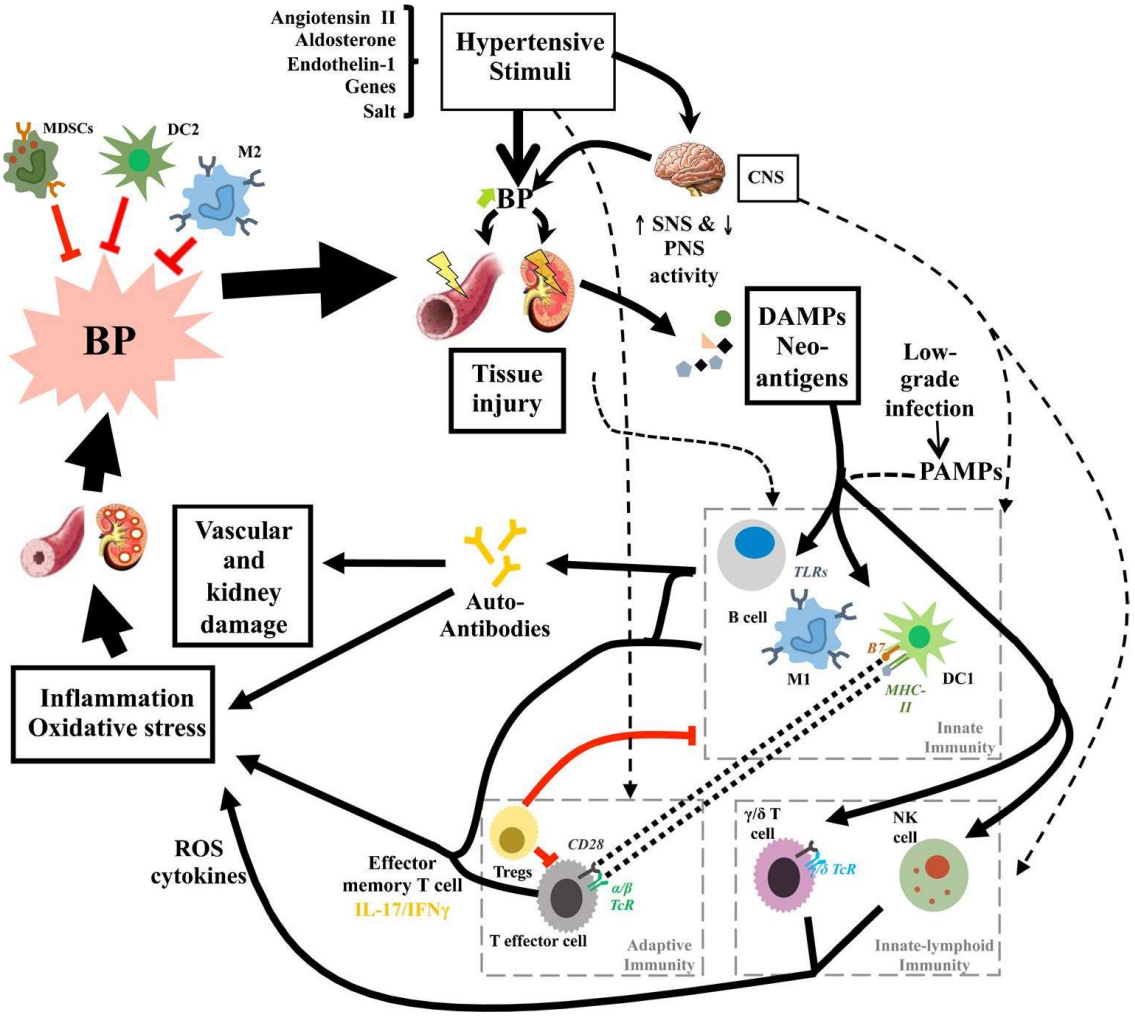

Figure 6. Schematic inflammatory mechanism in hypertension. Hypertensive stimuli may cause an initial elevation in BP due, in part, to increased SNS activation. This leads to mild tissue injury, formation of damage associated molecular patterns (DAMPs), and neo-antigens, promoting activation of innate immunity. Innate immune cells contribute to inflammation and oxidative stress directly or via the activation of adaptive immunity. The effects exercised by activated immune system lead to vascular and kidney injuries (Caillon A and Schiffrin EL, 2016).

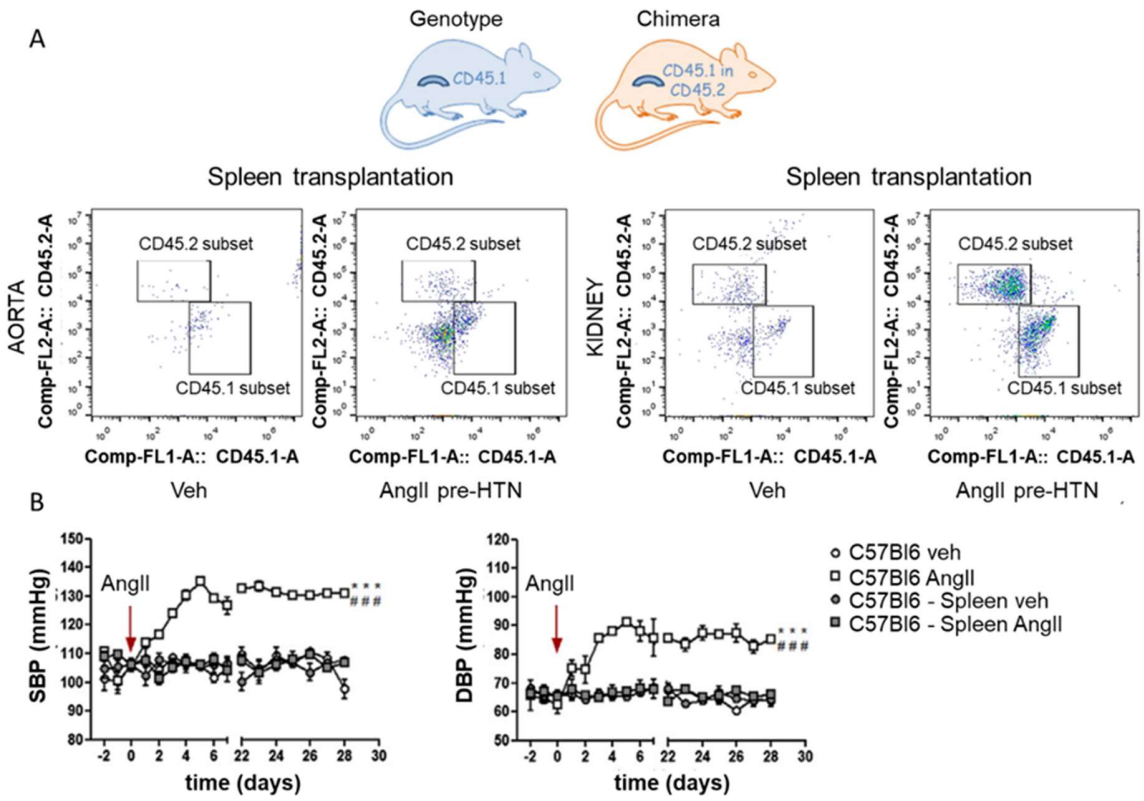

Figure 7. The spleen has a crucial role in deployment of T cell upon AngII and in hypertensive response. Accumulation of T cells originating exclusively from the donor spleen (CD45.1) as measured by flow cytometry (A) and BP response to chronic AngII, in splenectomized WT versus sham mice (B; Carnevale D *et al*., 2014).

1.6 PI3K family

The phosphoinositide 3-kinases (PI3K) is an enzyme able to phosphorylate the 3-hydroxyl group of the inositol ring of three species of phosphatidylinositol (PtdIns) lipid substrates, namely PtdIns, PtdIns4P and PtdIns(4,5)P2. PI3K signaling plays a key role in many processes, including cell cycle progression, cell growth, survival and migration, and intracellular vesicular transport, also if the specific contribution of several PI3K isoforms remains in part unknown.

These kinases have been assigned to three different classes (class I, class II and class III) based on structural features and lipid substrate preferences (figure 8).

Class I PI3Ks use phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) as substrate to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and they consist of catalytic subunit in complex with a regulatory subunit. In all PI3Ks the catalytic subunit includes a core structure that consists of a C2 domain (protein-kinase-C homology-2), a helical domain and a catalytic domain. Moreover, the catalytic subunit of class I and II PI3Ks also comprehends the Ras-binding domain (RBD); a subgroup of class I PI3Ks also presents the p85 binding domain. Regulatory subunit can include p85 isoform (for p110α, p110β and p110δ) or p101 or p87 (for p110γ). All p85 isoforms have two Src homology 2 (SH2) domains and are encoded by either *PIK3R1* (which, through differential promoter usage, encodes p85α, p55α and p50α), *PIK3R2* (which encodes p85β) and *PIK3R3* (which encodes p55γ). Roles of the individual p85 subunits are unknown. p101 and p87 lack SH2 domains, do not have homology to other proteins and have no identifiable domains. Relative to this, p110 subunits were historically divided into class IA, which binds the p85 type of regulatory subunit, and class IB, which do not. The SH2 domains bind phosphorylated tyrosine (pTyr) and the subdivision into IA or IB classes was correlated with the capacity to be activated through Tyr kinases or G protein coupled receptors (GPCRs), respectively. Most class I PI3K subunits might be activated by GPCRs directly through Gβγ protein subunits or indirectly, through a small GTPasi Ras. Ras can be activated by Tyr kinases and GPCRs, and it might engage all class I PI3Ks through their Ras-binding domain (RBD).

Therefore, class IA PI3Ks may be more responsive to GPCR stimuli than initially supposed (figure 9).

Class II PI3Ks use PtdIns as a substrate but might also use PtdIns-4-phosphate (PtdIns4P) under certain conditions. They lack regulatory subunits but have amino- and carboxy-terminal extensions to the PI3K core structure, which could mediate protein–protein interactions.

Class III PI3K has one catalytic member, vacuolar protein sorting 34 (Vps34; also known as PIK3C3 in mammals), which uses PtdIns as a substrate and binds Vps15 (also known as PIK3R4 in mammals). Vps15 consists of a catalytic domain (which is thought to be inactive), HEAT domains (which probably mediate protein–protein interactions) and WD (tryptophan-aspartic acid) repeats, which have structural and functional characteristics like a Gβ subunit. The WD repeats are essential for interaction with RAB5–GTP, the yeast guanine nucleotide-binding protein 1a (Gpa1; the homologue of the mammalian Gα of heterotrimeric G proteins) and autophagy-related protein 14 (Atg14; a potential Gγ protein; Vanhaesebroeck B *et al.*, 2010).

It is noted the importance of aberrations in PI3K signaling in the contribution to a broad spectrum of human diseases, such as cancer, immunological and neurological disorders, diabetes, localized tissue overgrowth, and cardiovascular disease (Fruman DA *et al.*, 2017).

In following paragraphs of this dissertation, the role of PI3Kγ isoform will be explored as key point that connects immune system and the pathogenesis of hypertension.

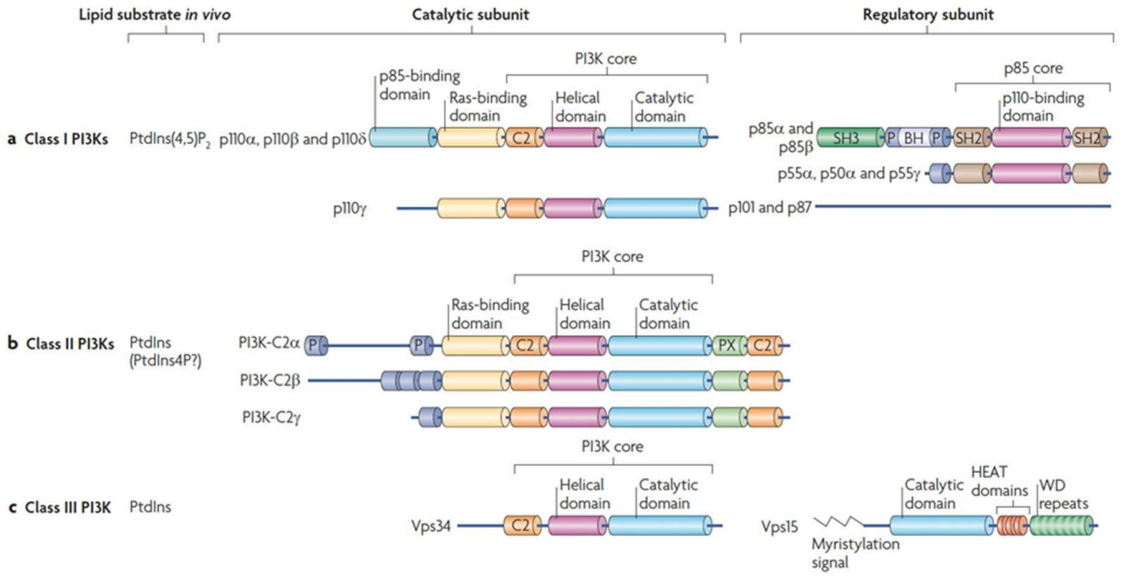

Figure 8. Classification and domain structure of phosphoinositide 3-kinases (PI3Ks). Three different classes of PI3Ks. A) Class I, B) Class II, C) Class III. BH, BCR homology domain; P, Pro-rich region; SH3, Src-homology 3 domain; SH2, Src-homology 2 domains; PX, phox homology domain (Vanhaesebroeck B *et al.*, 2010).

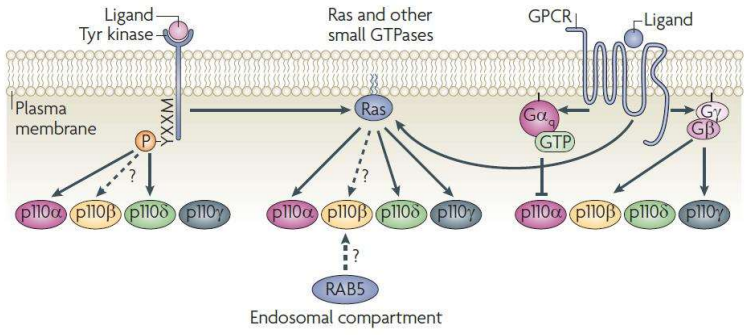

Figure 9. Activation mechanisms of class I PI3K. SH2 domains of the p85 protein bind with phospho-tyrosine generated by Tyr kinases in Tyr kinase receptors, resulting in the activation of p110α and p110δ, and probably also p110β (left). Tyr kinase pathways and GPCRs can also activate Ras, which then activates class I PI3Ks (middle). Direct interaction of PI3Ks with Gα or Gβγ subunits, which are downstream of GPCRs, can engage class I PI3Ks. p110α might be inhibited by Gα, whereas p110β and p110γ are activated by Gβγ subunits (right; Vanhaesebroeck B *et al*., 2010).

1.7 PI3Kγ in immune system

Several studies described that **PI3Kγ** is selectively enriched in leukocytes. This enzyme exerts a specific role in distinct leukocyte populations (figure 10) and it is involved in **inflammatory diseases** (Costa C *et al*., 2011). Neutrophils and macrophages represent the cellular populations that act as first defense line against an inflammatory insult. In order to effectively exert their function these cells need to migrate to the inflammatory sites through a process called chemotaxis. It was showed that PI3Kγ has an important role in **chemotaxis process** of neutrophils and macrophages, in fact PI3Kγ deficient mice presented an impairment of migration of these cells in response to GPCR-dependent stimuli (Li Z *et al*., 2000; Jones GE *et al*., 2003). It is demonstrated that the altered migration observed in PI3Kγ$^{-/-}$ neutrophils is due to the regulation of cell movement directionality exerted by PI3Kγ (Hannigan M *et al*., 2002). To study PIP3 function in leukocyte chemotaxis, Costa and coworkers generated a murine model characterized by constitutively expression of PI3Kγ. To generate these knock-in animals, the locus encoding the catalytic subunit of PI3Kγ was replaced with a chimeric minigene containing a mutant form of the human cDNA, in which the CAAX motif, derived from small GTPasi K-Ras, was added at its 5' end and followed by a neomycin resistance gene cassette sandwiched between loxP sequences. PI3Kγ fused to the specific sequences CAAX-box causes constitutive PIP3 production and its association with membrane fraction (figure 11). Mice with this mutation were viable, fertile and did not show any overt phenotype or alteration of life span. Furthermore, bone marrow-derived macrophages (BMDMs) homozygous for the mutation (PI3Kγ$^{CX/CX}$) compared with those PI3Kγ$^{+/+}$ presented the same pick of PIP3 production upon GPCR stimulation but, with the time, the amount of this phosphatidylinositol resulted higher in PI3Kγ$^{CX/CX}$ cells. It is observed that in PI3Kγ$^{CX/CX}$ mice leukocyte proliferation and survival are enhanced. The Authors showed that the constitutive expression of PI3Kγ induced a chemotactic defect in neutrophils and macrophages that fail to efficiently follow the chemotactic gradient because of a less persistent orientation toward the gradient,

indicating that modulation of PIP3 levels is crucial for efficient persistent directional movement (Costa C *et al*., 2007).

An important role of PI3Kγ is this to mediate the **endothelial cells (EC)-neutrophils interaction** in order to support the action of these cells of innate immunity in the inflamed vessel wall. In PI3Kγ$^{-/-}$ endothelial cells there was observable the decrease of selectin-mediated neutrophil attachment, an important event in the multistep process that enables neutrophils to migrate into inflamed tissues, and the increase of rolling speed of these cells in response to TNFα (Puri KD *et al*., 2005). Moreover, it is showed that the absence of PI3Kγ made the neutrophils unable to stably adhere to the endothelial wall in response to chemokine stimulation (Smith DF *et al*., 2006).

To fight the inflammatory process, neutrophils and macrophages exert phagocytic action and participate to the "oxidative burst" in order to generate ROS using the NADPH oxidase complex; ROS play a key role in microbial killing via a combination of direct ROS toxicity and indirect activation of proteases. It is demonstrated that PI3K activity is important in regulating **NADPH oxidase assembly and activation**. In particular, NADPH oxidase is composed by transmembrane proteins and soluble proteins, among the latter the small GTPase Rac. When Rac is activated it migrates from the cytosol to the membrane in order to form an active complex. PI3Ks act in this last process activating Rac protein, via regulation of guanine nucleotide exchange on Rac, and providing lipid anchoring sites for other complex components (Hawkins PT *et al*., 2007). It was showed that in mouse neutrophils ROS production is regulated only by PI3Kγ, whereas in human neutrophils, ROS generation has a biphasic regulation in which the first phase is dependent on PI3Kγ and the second phase is mediated by p110δ (Condliffe AM *et al*., 2005).

The analysis of hematopoietic lineage performed on peripheral blood and bone marrow showed that PI3Kγ$^{-/-}$ mice presented an increase in monocytes, neutrophils and basophils populations. In the spleen appeared an increase of myeloid cells whereas a decrease in thymocytes were observable. Thymocytes undergo different development stages in which the thymocytes precursors are represented by CD4^{-} and CD8^{-} cells (DN; double negative cells). These precursors are subdivided by the expression of CD44 and CD25 in DN1

($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3E ($CD44^-CD25^+FSC^{low}$), DN3L ($CD44^-CD25^{low}FSC^{high}$), and finally DN4 ($CD44^-CD25^-$) cells. DN4 cells give rise to immature thymocytes that are represented by $CD4^+$ and $CD8^+$ cells (DP; double positive cells) that finally mature in $CD4^+$ or $CD8^+$ single positive cells (SP). The first checkpoint in T cell development occurs at the DN3 to DN4 transition and is called β-selection because only cells that express a functional pre-TCR pass this checkpoint (Michie AM *et al*., 2002). In PI3Kγ$^{-/-}$ mice the proportion of SP cells did not result changed, only a slight decrease of DP cells and an increase DN cells was observable; also the maturation of DN and DP cells did not result altered, suggesting that PI3Kγ is not required for early thymocytes development. However, it was found that PI3Kγ plays an important role in the **thymus homeostasis**, controlling TCR and GPCR-induced thymocytes apoptosis. Moreover, in PI3Kγ$^{-/-}$ mice **proliferation and cytokines production** of T cells were impaired therefore, this enzyme exerts an important role in controlling these mechanisms and operates through secondary pathways resulting from TCR stimulation (Sasaki T *et al*., 2000).

A subsequent study analyzing the role of PI3Kγ and PI3Kδ in **T cell development** showed that it depends on the combined function of p110γ and p110δ. Analysis of DN thymocytes in p110γ/δ$^{-/-}$ mice showed that there was a reduction in the percentage of DN4 thymocytes, with normal numbers of DN3E and DN3L cells, providing evidences that T lymphocytes have a profound block of their development that occurs at the β-selection checkpoint. p110γ/δ$^{-/-}$ thymocytes presented a reduced pre-TCR signaling with a proliferative defect and increased apoptosis (Webb LM *et al*., 2005).

According with studies previously described it was determined the involvement of PI3Kγ in **TCR induced-T cell activation**. It was showed that p110γ is activated by the TCR and is involved in PIP3 localization at the **immunological synapse** (IS). Activated p110γ regulates in turn Rac activation and actin polymerization, which is fundamental for the stability of the IS. Therefore, p110γ affects the interaction between T cells and APCs, which could explain the defective activation of p110γ$^{-/-}$ T cells (Alcázar I *et al*., 2007).

Martin AL and coworkers demonstrated that CD8 effector T cells lacking p110γ have an impaired ability to migrate into an inflammatory site. This migration flaw is due to an

intrinsic defect of these cells since the adoptive transfer of this population in WT mice showed an impairment of p110γ$^{-/-}$ CD8^{+} migration into inflamed peritoneum. Moreover, these cells exhibit defective migration in response to a classic inflammatory chemokine, CCL5 (RANTES), as well as the proinflammatory lipid leukotriene B4 (LTB4). According to the cellular defects that occur in the absence of p110γ$^{-/-}$, the PI3Kγ KO mice are more susceptible to infection than control mice (Martin AL *et al*., 2008).

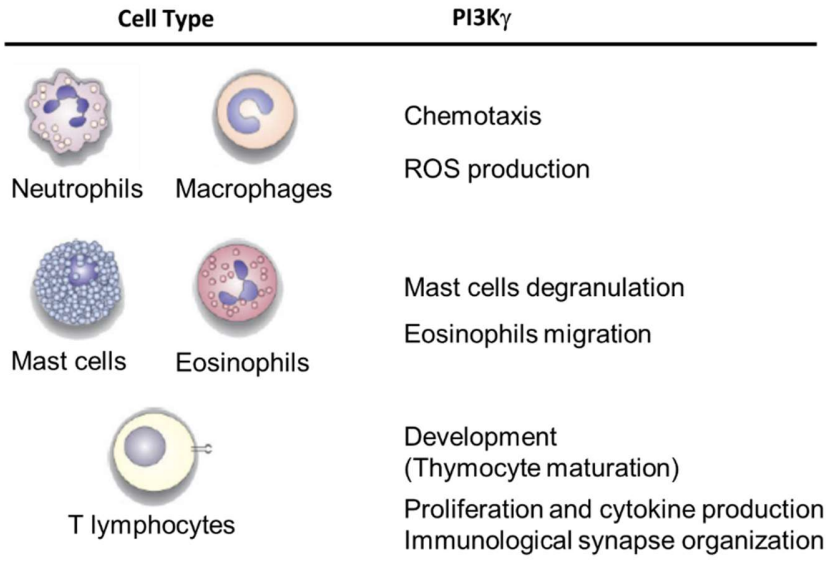

Figure 10. PI3Kγ function in inflammation. Role of PI3Kγ in different cell types of innate and adaptive immune system (Ghigo A *et al*., 2010).

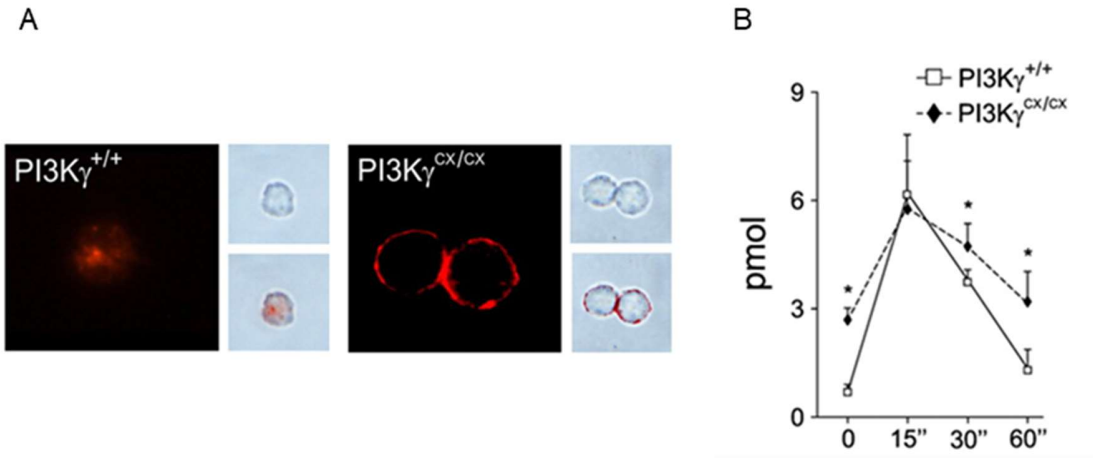

Figure 11. Effects of PI3Kγ constitutive expression. Immunofluorescence analysis revealed plasma membrane distribution of PIP3 in PI3K$\gamma^{CX/CX}$ bone marrow-derived macrophages (BMDMs) but not in PI3K$\gamma^{+/+}$ controls (left, A). Phase contrast images (right upper, A) merged with confocal images (right lower, A). ELISA measurement of intracellular PIP3 in PI3K$\gamma^{CX/CX}$ BMDM showed significantly increased PIP3 levels in resting conditions, before and after C5a stimulation (B; Costa C *et al*., 2007).

1.7.1 PI3Kγ in inflammatory diseases

Since p110γ orchestrates different functions in leukocyte populations, it has been investigated its potential involvement in various immune disorders. The results from these researchers indicate that this enzyme represents a pharmacological target for autoimmune diseases; for this reason, current studies are focusing on the identification of target molecules for PI3Kγ.

It was demonstrated that the block of this enzyme is able to reduce incidence and severity of pathological condition such as **systemic lupus erythematosus** (SLE) and **rheumatoid arthritis** (RA).

SLE is a chronic inflammatory condition characterized by deregulation of T cell-mediated B-cell activation, which causes glomerulonephritis and renal failure. In particular, it occurs an abnormal activation of $CD4^+$ T cells that accumulate as memory cells and that contribute to generalized B-cell expansion and hypergammaglobulinemia. This pathology is treated

with immunosuppressants, cytostatic agents and corticoids that involve numerous side effects. The abnormal activation of T cells and the consequent B cells expansion can be due to different mechanisms, for example cell-death defects such as Fas-Fas ligand deficiency in MRL-lpr mice and excess of lymphocyte activation signals including class I phosphoinositide-3 kinase (PI3K). Using mice homozygous for the lymphoproliferation spontaneous mutation (Fas^{lpr}) it was showed that intraperitoneal administration of a PI3Kγ selective inhibitor, AS605240 [5-(quinoxalin-6-ylmethylidene)-1,3-thiazolidine-2,4-dione], decreased pathogenic $CD4^+$ memory cells, reduced glomerulonephritis and increased lifespan in this mouse model of SLE limiting the adverse effects typical of classical treatments (Barber DF *et al*., 2005).

RA is a chronic systemic inflammatory disorder that affect mainly the joints that appear inflamed with infiltration of different population of leukocytes, including macrophages, neutrophils, mast cells and T cells that migrate to these sites thanks to chemokines and other chemoattractants. Since PI3Kγ is important in mediating mast cell degranulation, leukocyte chemotaxis and activation, pharmacological blockade of this molecule was proposed as a new therapeutic strategy for RA. It was demonstrated that oral administration of AS605240 inhibitor suppressed neutrophil chemotaxis and, in turn, the progression of joint inflammation and cartilage erosion in collagen-induced arthritis (CIA; Camps M *et al*., 2005).

It was also showed the therapeutic potential of PI3K inhibitors in **respiratory diseases**. The dual PI3Kγ/δ inhibition is showed to be effective in the allergic asthma and obstructive pulmonary disease (COPD) treatment. Intranasal administration of an aerosolized form of the double-selective compound TG100-115 to OVA-immunized mice results in a marked decrease of lung eosinophilia, accompanied by reduced production of IL-13, diminished perivascular and peribronchiolar leukocyte accumulation, and impaired mucin production. The same compound significantly reduces neutrophilia and TNF-α production in LPS or cigarette smoke exposure-induced murine model of CODP (Doukas J *et al*., 2009).

Since the role of PI3Kγ as an amplifier of mast cell activation, it takes a central role in the modulation of **inflammation and allergy**. In fact, PI3Kγ-derived PIP3 was a key element for external Ca^{2+} influx and release of histamine-containing granules from mast cells.

According to this, the absence of PI3Kγ disrupts **mast cell degranulation** (figure 10) and protects animals from edema formation induced by passive systemic anaphylaxis (Laffargue M *et al*., 2002). Finally, PI3Kγ exerts its role also in eosinophils, an important component of the inflammatory response in allergic asthma. It was showed that in chronic OVA-challenged PI3Kγ-deficient mice the airway responsiveness, the number of bronchoalveolar lavage (BAL), peribronchial eosinophils and peribronchial fibrosis were reduced than chronic OVA-challenged WT mice. It was proposed that the reduction in eosinophils number into the airway is likely due to reduced chemokine-induced **migration of eosinophils** (figure 10). On the other hand, the reduced peribronchial fibrosis can be attributed to reduced numbers of TGF-$\beta 1^{+}$ cells and reduced Smad 2/3 signaling, that are noted to play an important role in mediating this type of fibrosis (Lim DH *et al*., 2008).

1.8 PI3Kγ and cardiovascular diseases

There are studies that explored the role of PI3Kγ in the cardiovascular diseases in which there is an involvement of the immune reactions. Among these pathological conditions **atherosclerosis** and **myocardial infarction (MI)** were found.

Atherosclerosis is a chronic disease characterized by lesions formation in the arteries, known as plaques, with immune infiltration, lipid accumulation, cell death and fibrosis. These plaques, besides to cause stenosis, can break determining thrombotic occlusion of the artery. In the heart, it can occur myocardial infarction and heart failure, whereas in the brain atherosclerosis can cause ischemic stroke. If the pathology affects other arterial branches, they can occur renal impairment, hypertension and abdominal aortic aneurysms (Hansson GK and Libby P, 2006). The role of oxidized LDL (low density lipoprotein) and atherogenic cytokines/chemokines in trigger PI3K signaling and in Akt activation in inflammatory cells was already known (Biwa T *et al*., 2000). A paper of 2007 demonstrated that PI3Kγ is the specific isoform involved in this pathway and it is required for activation of Akt in macrophages in response to oxidized LDL, atherogenic cytokines, and angiotensin II *in vitro* and in atherosclerotic lesions of hypercholesterolemic mice *in vivo*. To study this pathological condition was used apolipoprotein E-deficient ($ApoE^{-/-}$) mice that develop spontaneous atherosclerotic lesions and are well characterized as a model for the early stage of atherosclerosis. Genetic deletion of PI3Kγ in the $ApoE^{-/-}$ mice correlated with the persistent reduction of plaque size. For this reason, p110γ was proposed as target for prevention of atherosclerotic disease (Chang JD *et al*., 2007). It was also demonstrated that AS605240-mediated PI3Kγ inhibition reduced lesion formation in $ApoE^{-/-}$ mice. This datum is also confirmed using $LDLR^{-/-}$ (low density lipoprotein receptors) mice, another model of atherosclerosis characterized by development of advanced lesion, in which the inhibition of PI3Kγ attenuated advanced atherosclerosis. The generation of mice lacking PI3Kγ exclusively in immune cells by bone marrow transplants from $PI3K\gamma^{-/-}$ mice to $LDLR^{-/-}$ recipient mice demonstrated that loss of PI3Kγ in immune cells is sufficient to reduce atherosclerosis. Therefore, PI3Kγ expression in immune cells drives the formation of atherosclerotic plaques. Moreover, in this model was observable a decrease of

macrophage and T-cell infiltration, and an increase of plaque stabilization that limits thrombosis and cardiac infarction (Fougerat A *et al*., 2008).

It was demonstrated the role of PI3Kδ and PI3Kγ in MI, that is a pathological condition that results from a biphasic ischemia/reperfusion (I/R) injury to the heart. In particular, it is showed that these kinases are involved in the inflammatory process responsible for the tissue damage that occurs upon reperfusion. TG100-115 inhibits edema and inflammation in response to mediators involved in myocardial infarction, including vascular endothelial growth factor (VEGF) and platelet-activating factor (PAF). Therefore, this inhibitor provided cardioprotection limiting infarct development and improving myocardial functioning in rodents (Doukas J *et al*., 2006). A subsequent paper showed the contribution of PI3Kγ to physiological and reparative angiogenesis in MI. Inhibition of PI3Kγ catalytic activity, using AS605240 or an adenoviral construct expressing a small interfering (si)RNA against p110γ, exerted negative effects on proliferation, migration, network formation and survival as underscored *in vitro* functional assay performed on human umbilical vein ECs (HUVECs).

It was also demonstrated the role of Akt in the PI3Kγ-mediated angiogenesis; inhibition of this enzyme resulted in reduced activation of Akt that is important to promote vascular cell survival and angiogenesis. Moreover, it was showed that PI3Kγ inhibition by AS605240 or PI3Kγ KO exacerbated MI-induced cardiac dysfunction. Indeed, the function of PI3Kγ KO hearts was more severely compromised than PI3Kγ WT or PI3Kγ KD hearts and infarct sizes resulted larger than control mice (Siragusa M *et al*., 2010).

1.8.1 PI3Kγ in hypertension

As described the p110γ isoform of Class I PI3K is expressed by immune system but also by cardiomyocytes and smooth muscle.

It is known the importance of this isoform in the regulation of voltage-gated L-type Ca^{2+} channels (LTCCs) activity (figure 12). The importance of Ca^{2+} fluxes regulation is highlighted by the fact that the control of this ionic flux has a central significance in different cardiac and vascular disease. For example, Ca^{2+} flux alterations in cardiomyocytes

cause contractile dysfunction and arrhythmias in heart failure; moreover, dysregulation of Ca^{2+} fluxes in smooth muscle cells can contribute to high blood pressure.

As previously described p110 catalyzes the production of PIP3 at the plasma membrane. This phospholipid acts as docking site for proteins carrying lipid-binding domains that include PKB/Akt, a downstream effector of PI3K, which is activated by phosphorylation on Thr308 and Ser473 by PDK1 (phosphoinositide-dependent kinase-1) and mTORC2 (mammalian target of rapamycin complex 2), respectively. The activated Akt exerts a central role in the organization of LTTC complex. In particular, the Akt-mediated phosphorylation of the accessory subunit of LTTC, $Ca_{V\beta 2}$, protects the pore-forming subunit $Ca_{V1.2}$ from the proteolytic degradation system. This event determines the increase of LTTC density and the modulation of the function of these channels. Relative to p110 isoform involved in this process, it should be noted that Akt-mediated phosphorylation of the LTCC accessory subunit is promoted by p110α and p110γ in cardiomyocyte and smooth muscle cells (SMCs), respectively. Moreover, in SMCs PI3Kγ can promote Ca^{2+} influx through yet unidentified PIP3-sensitive Ca^{2+} channels (Ghigo A *et al*., 2017; figure 12). Performing $[Ca2]_i$ measurements experiments and using specific antibodies against different PI3K isoforms, it is demonstrated that the γ isoform of the PI3K is specifically involved in the transduction pathway leading to calcium channel stimulation and the rise of $[Ca2]_i$ induced by AngII in rat portal vein myocytes (Quignard JF *et al*., 2001).

SMCs are important cellular components of vascular wall and are important players in the development of pathological conditions. Since these cells have a contractile potential, they are important regulators of vascular tone. Vecchione and colleagues showed that PI3Kγ KO mice were protected from AngII-induced hypertension and features of hypertrophic vascular remodeling were blunted significantly in these animals. The Authors also demonstrated that angiotensin II–dependent vasoconstriction resulted blunted in this animal model. Studying vessels that were derived from $PI3K\gamma^{KD/KD}$ mice, that expressed a catalytically inactive PI3Kγ, they showed that the enzymatic activity of PI3Kγ was required for angiotensin II–mediated vascular effects. Another important finding was that AngII-induced ROS generation required the activation of PI3Kγ. Moreover, it was showed that

PI3Kγ acted thought Akt activation because transfection of wild-type aorta with a dominant-negative Akt impairs Ca^{2+} mobilization induced by AngII. (Vecchione *et al*., 2005).

The pharmacological PI3Kγ inhibition, using AS605240 or GE21 molecules, caused hypotensive effect in PI3K$\gamma^{+/+}$, but not in PI3K$\gamma^{-/-}$ mice, treated with AngII or L-NAME. It was also showed that the mechanism through which PI3Kγ inhibition exerted antihypertensive effects involved the reduction of peripherical vascular resistance. In particular, in resistance vessels inhibition of PI3Kγ showed an independent-endothelium vasorelaxing effect, indicating the role of this kinase in the activation of a mechanism in SMCs that provokes vasodilatation. Moreover, the absence of this enzyme or its pharmacological inhibition caused a decrease of vascular myogenic tone in response to increased perfusion pressure steps. It was demonstrated that this effect is correlated to the inhibition of downstream Akt signaling that, as explained before, modulate calcium influx through LTCCs (Carnevale D *et al*., 2012).

It was reported that perfusion pressure can activate a Gβγ-dependent enzyme, such as PI3Kγ. To this propose the AT1R, the AngII receptor, can acquire activate conformation in presence of pressure stimulus also without its ligand; it allows G protein coupling and consequent βγ activation (Patel A *et al*., 2010). On these bases it was speculated that PI3Kγ could be activated thought induced-pressure AT1R/Gβγ pathway allowing the LTCC opening and finally myogenic response modulation (Carnevale D *et al.*, 2012).

The importance of PI3K in the regulation of blood pressure is highlighted by genome-wide association study that found six new loci influencing pulse pressure and mean arterial pressure. Among these, the single nucleotide polymorphism (SNP) rs17477177 on chromosome 7q22.3 was found to flank the region PIK3CG encoding for PI3Kγ (Wain LV *et al*., 2011).

This genetic evidence together with the data previously described support the idea that inhibition of PI3Kγ pathway could be considered as a novel tool to fight hypertension and damage in target organs.

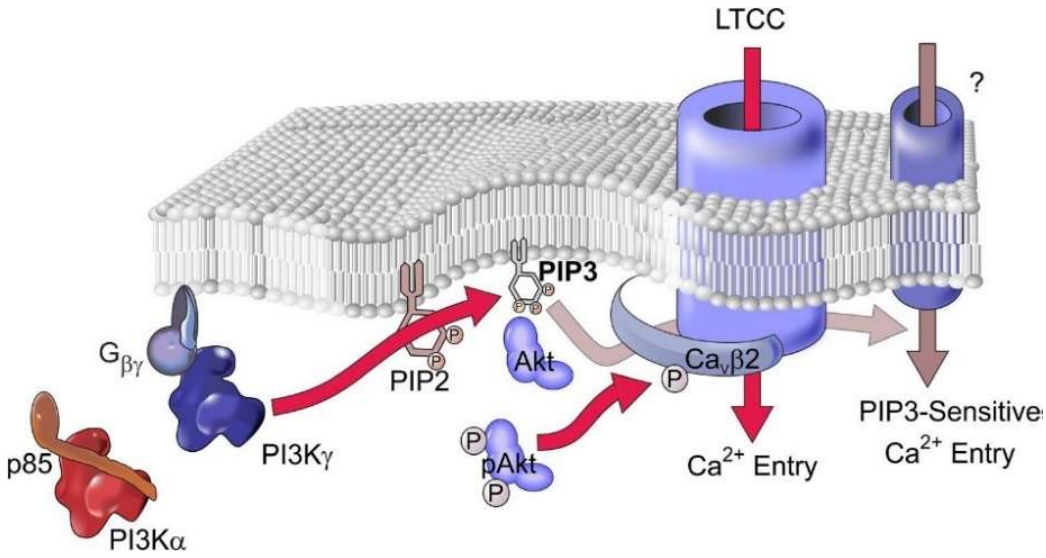

Figure 12. PI3K-mediated regulation of L-type Ca^{2+} channels in cardiomyocytes and in vascular smooth muscle cells. In cardiomyocytes, PI3Kα promotes Akt-mediated phosphorylation of $Ca_{V\beta}$ subunit, ensuing stabilization of $Ca_{V1.2\alpha}$ subunit on the plasma membrane. A similar mechanism, mainly involved PI3Kγ, controls the density of membrane LTCCs in vascular smooth muscle cells. Furthermore, PI3K can promote Ca^{2+} influx through yet unidentified PIP3-sensitive Ca^{2+} channels in smooth muscle cells and in T cells (Ghigo A *et al.*, 2017).

2. AIM OF THE STUDY

Hypertension is an important cause of morbidity and mortality worldwide, being the major risk factor for stroke, atherosclerosis, heart failure, myocardial infarction, kidney diseases and dementia. Nowadays, several therapeutic strategies are available for the cure of this pathology and the different developed pharmaceutical molecules act against the main components involved in blood pressure regulation. However, there are patients that are resistant to these antihypertensive drugs, suggesting that there are still unidentified mechanisms, which sustain the increase of BP. Thus, it is important to unrevealing new pathways underlying the pathology in order to develop novel treatments.

To this purpose, multiple reports recognized the involvement of immune system in the onset of hypertensive disease, although remained unclear how immunity participates to the more classical pathophysiological mechanisms that contribute to BP increase.

On this issue, as stated in introduction, it is interesting to notice that PI3Kγ is an important player in the HTN and it is a crucial intracellular signaling regulating the activation of CD8 T cells into effector cells. Since it was reported that the inhibition of PI3Kγ determined an antihypertensive effect in mice, one of the thesis aims was to characterize mice with constitutive expression of this enzyme, in order to assess whether immune mechanisms are involved in the link between PI3Kγ and high blood pressure. To this intent blood pressure levels and the renal immune infiltration were evaluated in PI3K$\gamma^{CX/CX}$ mice. Moreover, adoptive transfer experiments were used to clarify the role of CD8 cells in the onset of hypertension.

Since the blood vessels are a target organ of hypertension and it is known that high blood pressure caused immune cells recruitment in the vasculature, another purpose of this study was to investigate the vascular-immune interface. In particular, the aim was to understand whether CD8 effector T cells activated by hypertensive challenges were able to increase vascular myogenic tone. It is established a system of vessel organ culture, enabling the study of physiological properties of resistance arteries via a modified pressure myograph

which keeps vessels alive and functional for several days, while co-culturing CD8 immune cells.

3. MATERIALS AND METHODS

3.1 Murine Models

All animal handling and experimental procedures were performed according to European Community guidelines (EC Council Directive 2010/63) and the Italian legislation on animal experimentation (Legislative Decree 26/2014). All efforts were made to minimize suffering, and the principles of replacement, reduction, and refinement (i.e., the "three Rs") were applied to all experiments. Mice were housed in an air-conditioned room (temperature 21°C ± 1°C, relative humidity 60% ± 10%), with lights on from 06:00 to 18:00, and had sawdust as bedding, pellet food, and tap water *ad libitum*. Animals of 12–15 weeks were used in all experiments. Mouse strains used included PI3K$\gamma^{CX/CX}$, PI3K$\gamma^{-/-}$, RAG1$^{-/-}$. Wild-type (WT) mice with the same genetic background (C57Bl/6J) were used as the appropriate control strain. Where indicated, AngII (0.5 mg/kg/day) or vehicle (NaCl 0.9%) were delivered subcutaneously with osmotic mini-pumps (model 1007D, ALZET). At day 14 or 28, according to experimental design, mice were euthanized by overdose of sodium pentobarbital anesthesia and eventually used for following analysis.

3.2 Blood pressure measurements

Arterial BP monitoring was held in conscious mice with tail-cuff plethysmography (BP-2000 Series II, Visitech Systems, North Carolina, USA). Measurements of BP was performed daily, between 8 am and 1 pm, accordingly to previously described procedures (Perrotta M *et al*., 2018). Operators were blinded to the experimental group during blood pressure monitoring. Data are presented as mean values of systolic (SBP) and diastolic (DBP).

3.3 Isolation and preparation of single cell suspensions from spleen and kidney

Mice were anesthetized with ketamine/xylazine and spleen was isolated. Then, mice were exsanguinated and kidneys were collected.

Spleen was mechanically disrupted and then passed through a 40 μm sterile strainer (Falcon, BD) and centrifuged at 300x g for 10 min. After a treatment with Lysing Buffer (BD Pharm Lyse), the pellet was resuspended in PBS. Total splenic leukocytes were used to isolate CD8 positive lymphocytes by negative selection using a commercially available mouse CD8 T lymphocyte enrichment set - DM (BD IMag).

To check the purity of the isolated fraction of $CD8^+$ lymphocytes, cells were stained with appropriate antibodies and analyzed using flow cytometry. Subsequently, this population was used to perform other experimental procedures illustrated below.

Kidney cell suspension was obtained disrupting mechanically two decapsulated kidneys, and then passed through a 70 μm sterile strainer (Falcon, BD). The resulting cell suspension was centrifuged at 300x g for 10 min. To isolate lymphocytes from these cells, the pellet was then suspended in 36% Percoll (Sigma), gently overlaid onto 72% Percoll, and centrifuged at 1,000x g for 30 min at room temperature (RT).

Before proceeding with stainings for flow-cytometric analyses, lymphocytes from kidneys were enriched with mouse T lymphocyte enrichment set-DM (BD IMag).

Samples were resuspended in PBS and then the number of the cells was assessed using trypan blue and an automated counter (Countess, Life Technologies).

3.4 Flow Cytometry Analysis

$CD8^+$ splenic leukocytes (1×10^5) and total kidney T lymphocytes were preincubated with anti-CD16/32 Fc receptor (1:100; 2.4G2, BD Pharmingen) for 10 min at RT for blocking of nonspecific binding; then, various combinations of monoclonal antibodies (mAbs) were incubated, in the dark, together with the cells for 20 min at RT in FACS buffer, containing 0.5% BSA, 0.1% sodium azide and 2 mM EDTA. Cells were washed with PBS, centrifuged at 300x g for 5 min at 4°C, resuspended again with PBS and incubated for 10 min with

viability marker 7-AAD (1:20; BD Pharmingen). Data from the entire sample were acquired using a BD FACSCanto and BD FACSCelesta (BD Biosciences), with FACSDiva software (BD Biosciences) and analyzed using FlowJo software (V10.0.8, Tree Star).
To evaluate T cells infiltration in kidney the following fluorochrome-conjugated mAbs to mouse antigens were used: APC-Cy7 anti-CD45 (1:100; clone 30-F11), PerCP-Cy5.5 anti-CD8a (1:100; clone 53-6.7), FITC anti-CD4 (1:100; clone RM4-5), APC anti-CD69 (1:100; clone H1.2F3), PE-Cy7 anti-CD44 (1:100; clone IM7; BD Pharmingen).
To evaluate the $CD8^+$ fraction purity the following fluorochrome-conjugated mAb to mouse antigens were used: Buv395 anti-CD45 (1:100; clone 30-F11), Buv737 anti-CD8 (1:100; clone 53-6.7) and Bv605 anti-CD4 (1:100; clone RM4-5; BD Horizon).

3.5 Adoptive transfer of purified $CD8^+$ T cells

Spleen of donor mice WT or PI3K$\gamma^{CX/CX}$ was collected and cells were isolated as described above, obtaining sterile and highly enriched $CD8^+$ T cells population. Immediately after the cells isolation, 1 x 10^6 cells were resuspended in 100 µl of sterile PBS and injected into WT recipient mice via tail vein. Subsequently adoptive transfer blood pressure monitoring was performed for 14 days and then mice were anesthetized with xylazine/ketamine and sacrificed. Kidneys are isolated and used for FACS analysis.

3.6 Immunohistochemistry

For renal damage assessment, kidneys were explanted and embedded in paraffin for immunohistochemistry. Sections of 4 µm were deparaffinized and rehydrated before to perform staining.
Masson's trichrome and picrosirius red stainings were performed to analyze renal fibrosis. For trichrome stains were used nuclear, collagenous and cytoplasmic dyes. Nuclei were stained with Weigert's iron hematoxylin, cytoplasm and muscle were stained with Beibrich scarlet-acid fuchsin and collagen was demonstrated by staining with aniline blue.

Trichrome coloration was performed using a commercially available kit (Trichrome Stain, Masson Kit, Sigma-Aldrich).
Conversely, sirius red (Sigma-Aldrich) is an acidic hydrophilic staining that colors collagen fibers in red. The technique is based on the tight-binding of the stain sulfonic acid groups with the basic groups of collagen fibers that appear as bundles of pink to red fibers.
To analyze glomerular structure and, therefore, the renal damage periodic acid–Schiff (PAS) stain was performed. The PAS reaction in tissue sections is useful for the demonstration of polysaccharides, that appear magenta. When treated with periodic acid, glycols are oxidized to aldehydes, that are located following the reaction with Schiff's reagent. The PAS staining was performed using a commercially available kit (periodic acid-schiff (PAS) staining system, Sigma-Aldrich).
Images of kidney sections were used to calculate the size of renal corpuscle, glomerular area and Bowman's capsule, and the percentage of renal fibrosis, all using Image J software (NIH).
All images were captured using a DMI3000B Leica optical microscope provided by Leica Cameras (Leica Microsystems, Wetzlar, Germany) and processed with the Leica Application Suite (LAS V3.3).

3.7 Set up of a long-term vessel culture system

The methods described in this section are relative to the set-up of a long-term vessel culture system that allow to study vessels proprieties in chronic condition because it offers the possibility of maintaining vessel in vital and physiological condition for some days after explantation from mouse. A schematic representation of culture myograph system - 204CM is showed in figure 13. Briefly, this system is composed of a vessel culture chamber that contains inlet and outlet for the superflow medium and two accesses for the cannulas between which the vessel is mounting and secured using nylon sutures. The upper part of the chamber is closed with a cap to avoid the possibility of eventual contamination during the culture, conversely the lower part consists of a slide to allow vessel visualization using a microscope. The vessel image is processed by software that make possible to obtain

vessel parameters of interest. This chamber is located on a plate that is maintained at the controlled temperature of 37°C thanks to the presence of heat controller. Chamber is connected by the cannulas to two small bottles for input and output of medium that perfuses the vessel (inflow medium). This medium generates a flow inside vessel and it is needed to regulate the perfusion pressure, thanks to the presence of a compressor. The vessel is also connected with two other bottles, one containing the oxygenated superflow medium that surrounds the vessel and one in which the waste medium is collected. The presence of a peristaltic pump allows the superflow medium to be conducted in the chamber.

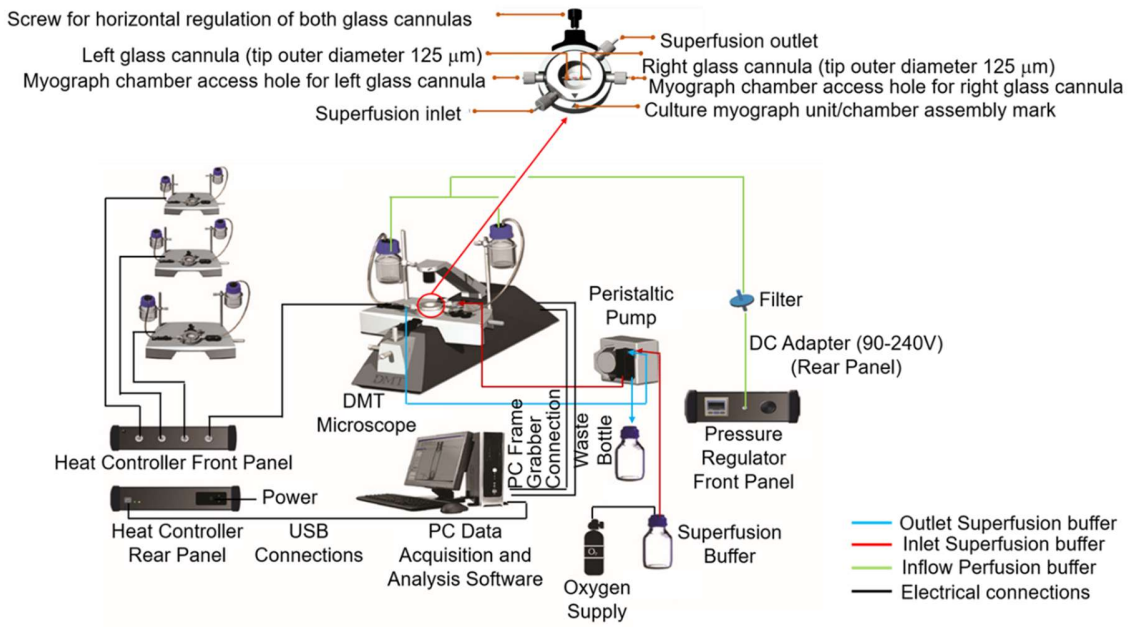

Figure 13. Schematic illustration of culture myograph system - 204CM. At the top of the figure is observable a magnification of the culture chamber with the indication of constituent elements described in the text. In the lower part of the figure are highlighted the connections among the various components of culture myograph system.

3.7.1 Vessel Culture Medium

Second branch of mesenteric artery was maintained for 3 days in culture system at 37°C, 60 mmHg and oxygenated with a gas mixture 95% O_2 and 5% CO_2. Vessel was perfused with

oxygenated inflow medium and surrounded by superflow medium, bubbled with gas mixture, with the following composition: Dulbecco's Modified Eagle Medium (DMEM) high glucose (4,5 gL^{-1}), supplemented with 1% penicillin-streptomycin, 4% L-glutamine and 10% dialyzed and heat inactivated fetal bovine serum (dialyzed FBS, Gibco). Heat inactivation was performed by heating serum to 56°C for 30 minutes. This procedure prevented the attenuation of endothelial function induced by complement proteins. Moreover, we added dialyzed FBS in order to remove small contractile factors that could affect our results (Bakker EN *et al*., 2000) and to improve endothelium-dependent relaxation (Morita T *et al*., 2013). The replacement speed of the superflow culture medium in culture chamber was 3 rpm, while the replacement rate of perfusion medium was dependent on the internal resistance of the vessel. Furthermore, culture medium was replaced every day in the superflow and inflow medium bottles.

3.7.2 Surgical Procedure and Mounting of Vessels on the Pressure Myograph

In order to avoid possible contaminants surgical procedures were performed in a cleaned room with strong disinfectant. For myogenic tone studies, second branch of mesenteric artery was excised from fasting mice after cervical dislocation, cleaned from surrounding adipose tissues, then cannulated and mounted on the culture myograph system - 204CM (DMT, Danish Myotechnology). The experiments were performed in Dulbecco's Modified Eagle Medium, with the composition previously described, at 37°C. After 30 minutes of equilibration, vessels were transilluminated under a microscope connected to a computerized system for continuous recording of measures. Evaluation of vessel functionality was performed testing KPSS (physiological salt solution with KCl; 60 mM KCl added to PSS solution with the following composition in mM: 118,99 NaCl, 4,69 KCl, 1,17 MgSO4*7H2O, 1,18 KH2PO4, 2,50 CaCl2*2H2O, 25 NaHCO3, 0,03 EDTA, 5,50 Glucose), norepinephrine (NE, 2μM) and acetylcholine (ACh, 10 μM) responses. To avoid eventual contaminants that could impair the culture, all chemical solutions are sterilized using 22 μm filter (Millipore). After mounting, only vessels that constricted to these chemicals were analyzed (minimum response: 70%). Every day, in order to perform a

vessel wake-up protocol and to test the vessel function, was performed KPSS stimulation; only vessels that constricted to this solution were studied.

3.8 Myogenic tone experiments

Myogenic tone was obtained measuring vessel inner diameters (IDs) in presence of DMEM-dialyzed FBS free (containing Ca^{2+} ions) or PSS-Ca^{2+} free. These measures were taken in increasing steps of intravascular (transmural) pressure from 0 to 125 mmHg, by steps of 25 mmHg (10 min per step). The percentage of myogenic tone (MT) was calculated using the following formula:

$$\mathbf{\%MT} = (ID_p - ID_a)/ID_p) \times 100$$

where ID_p is the passive diameter obtained in PSS-Ca^{2+} free solution and ID_a is the active diameter of the vessels obtained in DMEM-dialyzed FBS free, at a given intraluminal pressure value.

3.9 Statistical analysis

Sample size was pre-estimated on the basis of previously published research and from pilot experiments performed in our laboratory. Data distribution was assessed with D'Agostino Pearson test, and assumption of homogeneity of variance was tested using Levene's test of equality of variances. Statistical significance was assessed with the appropriate test according to each experimental design.

In detail, after assessing distribution of each data setting, we applied Student's t test for independent samples.

Multiple group analysis was performed with one-way ANOVA or two-way ANOVA followed by Bonferroni's *post hoc* for data with normal distribution. Analysis for repeated-measures was applied when required by the experimental setting.

Data are presented as mean ± SEM. $P < 0.05$ was considered significant.

Statistical analyses were performed with SPSS 23.0 (IBM Software) and graphs were made with GraphPad PRISM5 (GraphPad Software Inc, La Jolla, CA).

4. RESULTS

4.1 PI3Kγ KO mice are protected from AngII-induced infiltration of T cells in kidneys

To dissect the link between PI3Kγ and T cells infiltration in target organs of hypertension, cytofluorimetric analysis were performed on kidneys. It was used a murine model with a specific deletion of γ isoform of PI3K family, principally expressed in lymphocytes, cardiomyocytes and smooth muscle cells. To generate a hypertensive model, mice were treated with angII; on the contrary, control group received only vehicle. As expected, analysis of $CD4^+$ and $CD8^+$ T cells in kidneys isolated from PI3Kγ WT mice showed that 28 days later angII infusion there was an increase of these cellular populations than mice that received vehicle alone. Investigation of the same populations highlighted a lower amount of $CD4^+$ and $CD8^+$ cells in angII-infused mice lacking PI3Kγ than WT.

To explore cell migration and homing properties and the activation features of lymphocytes, the expression of the CD44 and CD69 markers was evaluated respectively. It was found that angII-infused WT mice showed a significative increase of $CD4^+CD44^+$, $CD4^+CD69^+$, $CD8^+CD44^+$ and $CD8^+CD69^+$ populations than vehicle-infused WT mice. Moreover, a reduction of these cellular populations was observed in angII-infused PI3Kγ KO mice compared with control (figure 14).

These data suggest the importance of PI3Kγ in target organs lymphocytes colonization in hypertension.

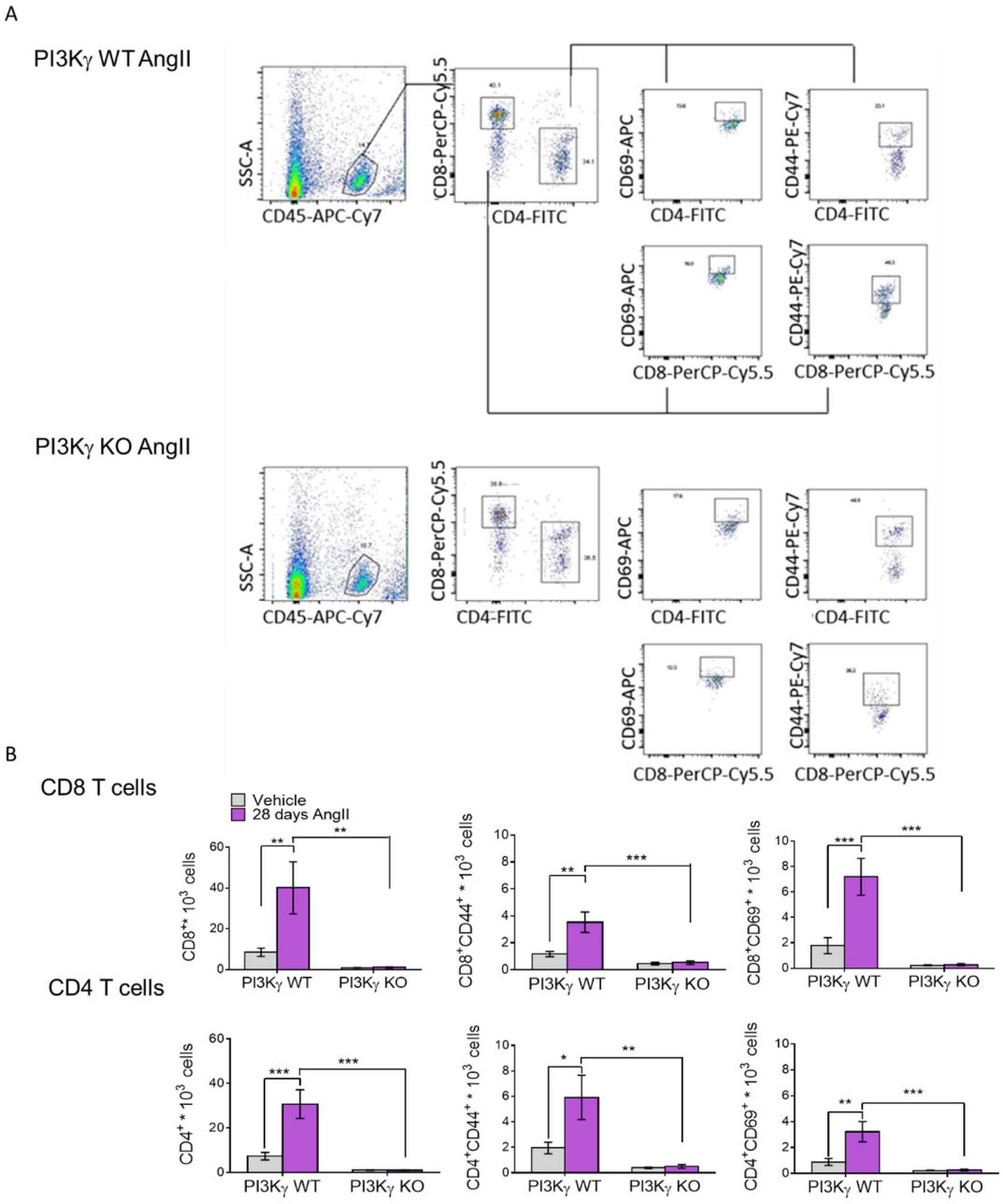

Figure 14. Deletion of PI3Kγ angII-treated mice protects from infiltration of T cells in kidney. Representative Plots of Flow Cytometry analysis of the immune infiltrate in kidneys. (A) Flow-cytometric gating in angII-treated PI3Kγ WT and PI3Kγ KO mice and (B) quantification of renal $CD4^+$, $CD8^+$, $CD4^+CD44^+$, $CD4^+CD69^+$, $CD8^+CD44^+$ and $CD8^+CD69^+$ leukocytes showed that PI3Kγ KO mice are protected from kidney immune infiltration. n= 6-9 for each group; *p<0.05, **p<0.01, ***p<0.001; two-way ANOVA.

4.2 Mice with constitutive expression of PI3Kγ showed a hypertensive phenotype

Since the inhibition of PI3Kγ produces an antihypertensive effect, the consequence of the constitutive expression of this kinase on the hypertensive phenotype has been evaluated. It is found that PI3K$\gamma^{CX/CX}$ mice showed systolic and diastolic values of blood pressure higher than PI3Kγ WT mice (figure 15 A).

To analyze the end-organ damage, it was assessed renal fibrosis and glomerular injury. It is found that mice with constitutive expression of PI3Kγ showed a significant amount of renal fibrosis compared with WT, evidenced by staining kidneys with Picrosirius Red and Masson's Trichrome (figure 15 B). This apposition of fibrotic tissue could deteriorate renal function, further contributing to the hypertensive phenotype. Accordingly, in PI3K$\gamma^{CX/CX}$ mice it was observed an enlargement of renal corpuscle size and an increase of Bowman's capsule size than the control, as represented by PAS staining (figure 15 C).

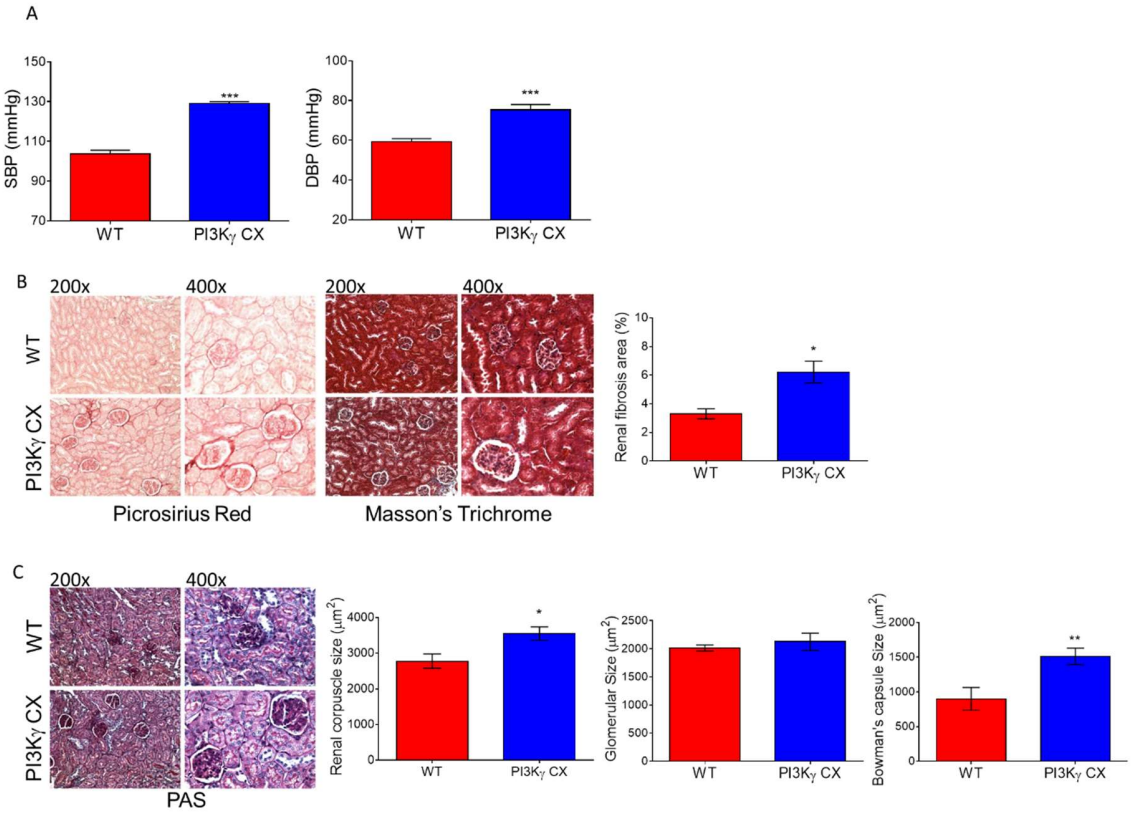

Figure 15. Hypertensive phenotype in PI3Kγ$^{CX/CX}$ mice. (A) Tail cuff blood pressure measurement in PI3Kγ$^{CX/CX}$ mice compared to WT control group. Panels represent mean ± SEM values of systolic BP (SBP) and diastolic BP (DBP; n=12 for each group; unpaired t-test; ***P< 0.001). (B) Picrosirius Red and Masson's Trichrome stainings of kidneys in PI3Kγ$^{CX/CX}$ and WT mice. Graph representing the quantitative analysis of fibrotic area. Images represent n=14 PI3Kγ$^{CX/CX}$ and n=6 WT mice. Mann-Whitney test, *P<0.05. (C) PAS staining showing the enlargement of renal corpuscle and Bowman's capsule in PI3Kγ$^{CX/CX}$ mice. Graphs representing quantitative analysis of renal corpuscle, glomerular and Bowman's capsule size. Images represent n=14 PI3Kγ$^{CX/CX}$ and n=6 WT mice. Unpaired t-test, *P<0.05 and **P< 0.01.

4.3 PI3Kγ$^{CX/CX}$ mice show a significant infiltrate of activated CD8^{+} T cells in kidney

To evaluate whether immune mechanisms are involved in link between PI3Kγ$^{CX/CX}$ and high blood pressure, the kidney immune infiltrate was analyzed in this murine model. Cytofluorimetric analysis of CD8^{+} T cells in kidneys isolated from PI3Kγ$^{CX/CX}$ mice showed an increase of this cellular population than WT mice. To explore the cell migration and homing properties and the activation features of lymphocytes, the expression of the CD44 and CD69 markers was evaluated respectively. It was found that CD8^{+}CD44^{+} and CD8^{+}CD69^{+} populations were increased in PI3Kγ$^{CX/CX}$ than the control mice (figure 16).

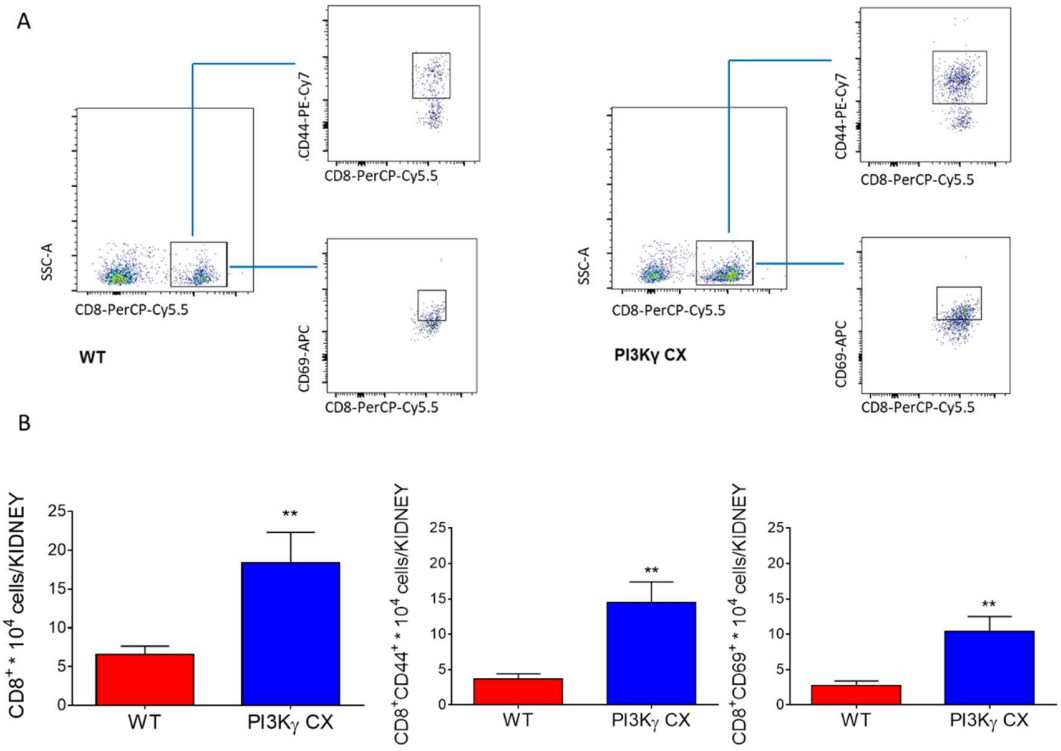

Figure 16. PI3Kγ$^{CX/CX}$ mice showed a CD8^{+} T cells infiltration in kidney.
Representative Plots of Flow Cytometry analysis of the immune infiltrate in kidneys. (A) Flow-cytometric gating in PI3Kγ WT and PI3Kγ$^{CX/CX}$ mice and (B) quantification of renal CD8^{+}, CD8^{+}CD44^{+} and CD8^{+}CD69^{+} leukocytes. Images represent n=8 for each group. Unpaired t-test, **P< 0.01.

4.4 The hypertensive phenotype depends on T lymphocytes

Data from previous experiments allowed us to hypothesize that hypertensive phenotype of PI3K$\gamma^{CX/CX}$ mice is dependent on lymphocytes. To test this assumption, it was crossed PI3K$\gamma^{CX/CX}$ with RAG1 KO mice and the obtained progeny consisted of PI3K$\gamma^{WT/WT}$ RAG1$^{WT/WT}$, PI3K$\gamma^{CX/CX}$ RAG1$^{WT/WT}$ and PI3K$\gamma^{CX/CX}$ RAG1$^{-/-}$ animals (figure 17 A). Blood pressure measurements in PI3K$\gamma^{CX/CX}$ RAG1$^{-/-}$ mice revealed that the lymphocytes depletion in mice with constitutively active PI3Kγ caused protection from the onset of hypertension, confirming the initial hypothesis (figure 17 B).

The role of immune cells in the onset of hypertension was evaluated also with adoptive transfer experiments (figure 18 A). CD8$^+$ T cells negatively selected from total lymphocytes were isolated from WT or PI3K$\gamma^{CX/CX}$ spleen and were infused in WT normotensive mice. Before the infusion in mice, cytofluorimetric analysis was performed in order to evaluate the purity of the isolated cellular fraction. It is observed that when CD8$^+$ PI3K$\gamma^{CX/CX}$ are injected in WT mice caused a significative increase of blood pressure compared to the injection of CD8$^+$ PI3K$\gamma^{WT/WT}$ in WT mice (figure 18 B), demonstrating that primed CD8$^+$ PI3K$\gamma^{CX/CX}$ have a role in the genesis of hypertension. Moreover, normotensive mice that received CD8$^+$ lymphocytes from PI3K$\gamma^{CX/CX}$ mice showed an increase of CD8$^+$CD44$^+$ and CD8$^+$CD69$^+$ populations (figure 18 C).

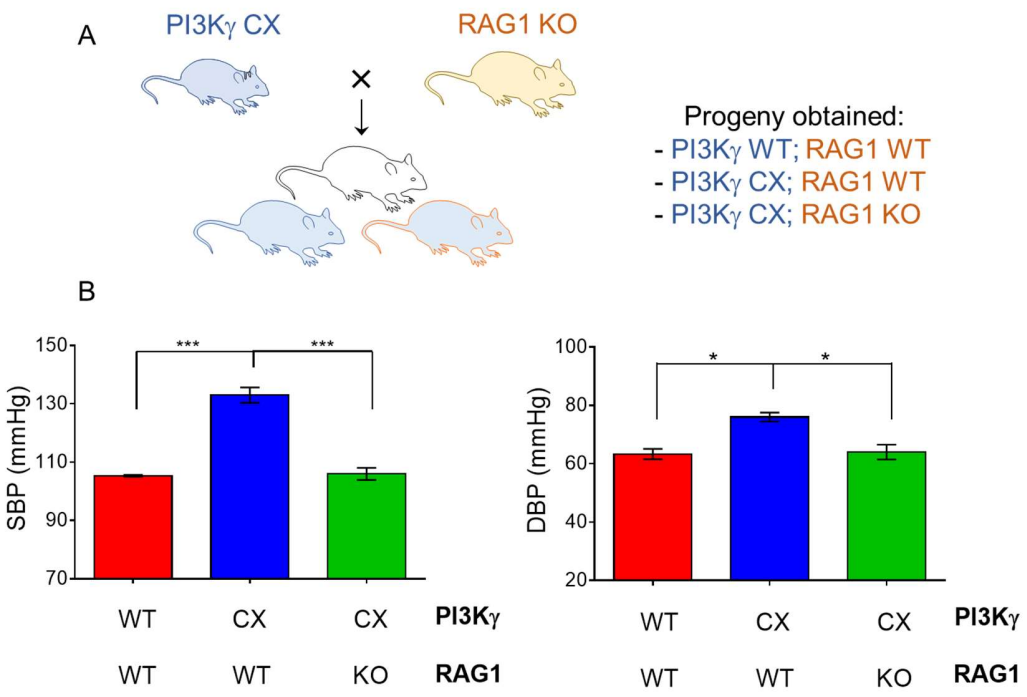

Figure 17. Lymphocytes involvement in hypertensive phenotype of PI3Kγ$^{CX/CX}$.
(A) Progeny obtained from PI3Kγ$^{CX/CX}$ and RAG1 KO mice crossing. (B) Blood pressure measurements in PI3Kγ$^{WT/WT}$ RAG1$^{WT/WT}$, PI3Kγ$^{CX/CX}$ RAG1$^{WT/WT}$ and PI3Kγ$^{CX/CX}$ RAG1$^{-/-}$ animals. Images represent n=4 for each group. ***P< 0.001, *P<0.05, one-way ANOVA.

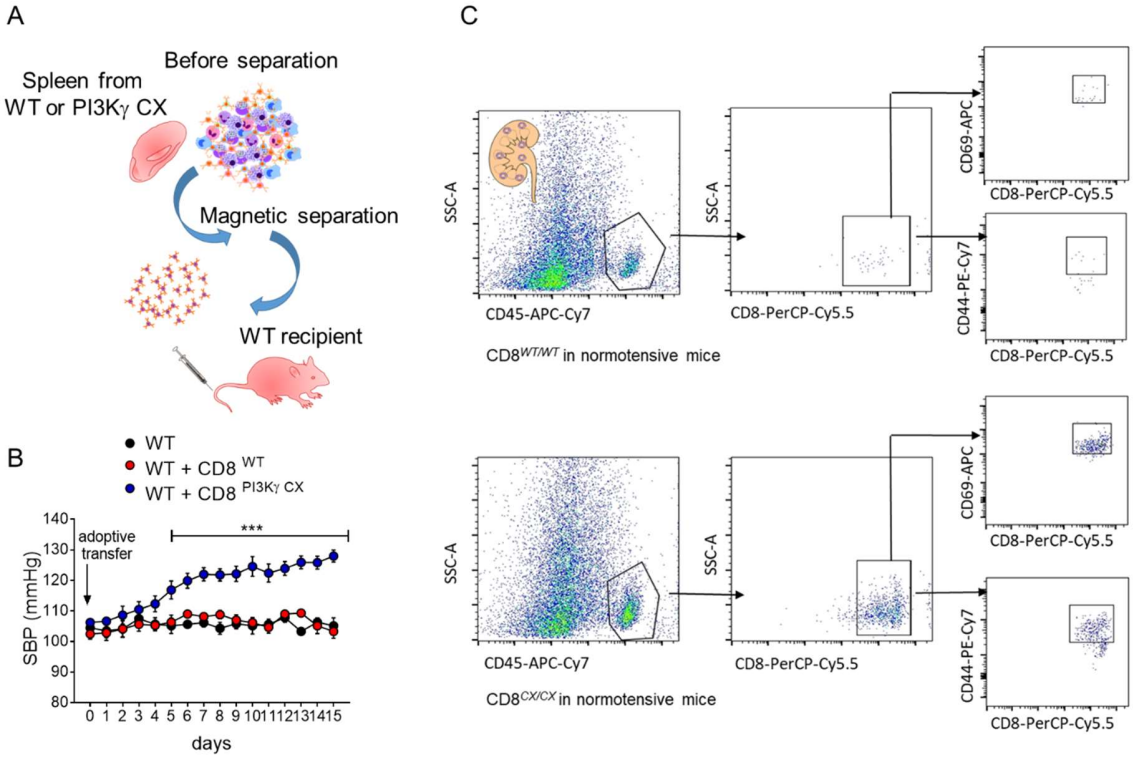

Figure 18. Hypertensive effect of adoptive transfer of CD8⁺ T cells from PI3Kγ$^{CX/CX}$ in WT mice. (A) Schematic representation of adoptive transfer of CD8⁺ cells isolated from WT or PI3Kγ$^{CX/CX}$ spleen in WT mice. (B) Tail cuff blood pressure measurements in WT mice that receive WT, PI3Kγ$^{CX/CX}$ CD8⁺ T cells or no cells. Panel represents mean ± SEM values of systolic BP (SBP). Graph represents n=15 for each group. 2-way ANOVA, ***P<0.001 WT + PI3Kγ$^{CX/CX}$ CD8⁺ vs WT + PI3Kγ$^{WT/WT}$ CD8⁺ or WT no cell. (C) Representative plots of flow cytometry analysis of renal immune infiltrate of PI3Kγ WT receiving WT or PI3Kγ$^{CX/CX}$ CD8⁺ T cells. The gated populations are CD8⁺, CD8⁺CD44⁺ and CD8⁺CD69⁺.

4.5 Innovative experimental approach development: culture myograph system

The results shown so far highlighted the importance of $CD8^+$ lymphocytes activated by hypertensive stimuli in the blood pressure increase. Since the vasculature is a known target of hypertensive disease, one of the objectives of this study is to understand whether $CD8^+$ T cells can affect vascular tone. To this aim it is developed a new experimental approach that allow to investigate vascular function after a long-term $CD8^+$ T cells-mesenteric artery coculture.

In order to evaluate the efficiency of this innovative approach, vessel viability assessment was daily performed using KPSS stimulation, as showed in figure 19 A. During culture period no significative differences in the contraction induced by stimulation with this salt solution was found. However, it was observable a slight reduction of the contraction in the second and third day of culture, probably due to the loss of K^+ conductance in cultured vascular SMCs (Manoury B *et al*., 2009) and to marked downregulation of some potassium channels (Thorne GD *et al*., 2002).

Given the relevance of α1-adrenergic receptors in the adrenergic sympathetic and humoral regulation of venous tone (Docherty JR *et al*., 2010), it was verified the integrity of this system with NE stimulation. It was found that no significant differences are noticeable in vascular constriction induced by this agonist (figure 19 B), thus demonstrating the correct working of analyzed vessel.

Moreover, it was showed that in the developed system the endothelial function was preserved. In fact, endothelium-dependent vasodilatation induced by ACh was not altered by three days of culture (figure 19 C).

Furthermore, it was verified the existence of eventual differences of myogenic response measurement between cultured or fresh isolated vessel and no significative differences between these two conditions was found (figure 19 D).

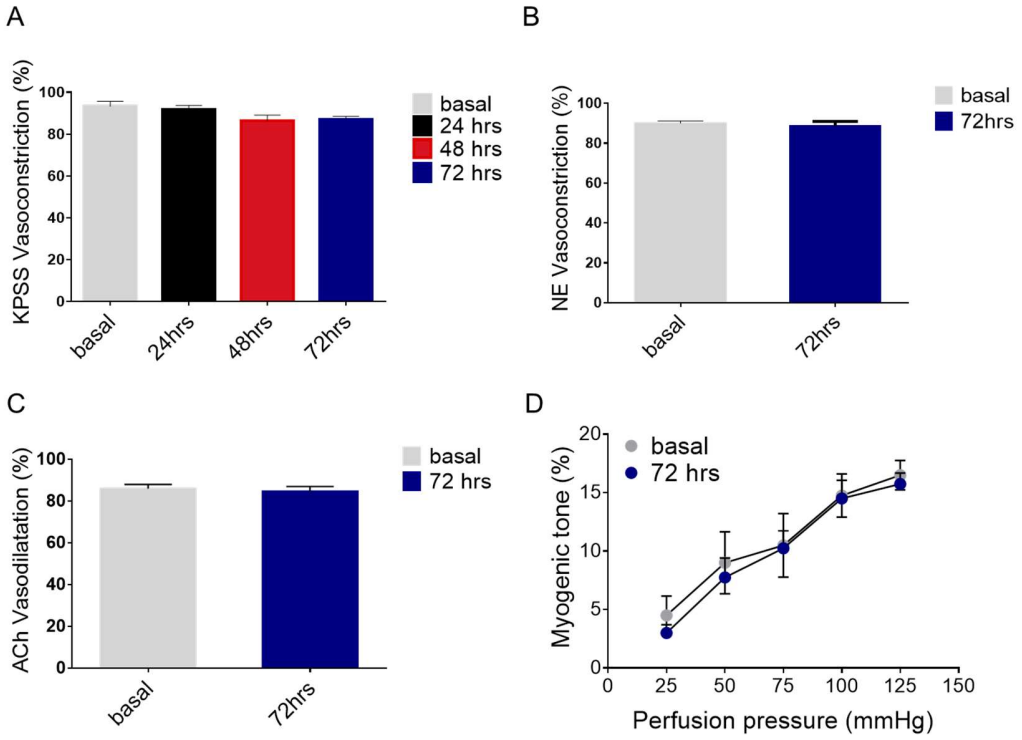

Figure 19. Vascular responsiveness after long-term culture. Vasoconstriction recording at different time of culture after stimulation with physiological salt solution containing KCl 60 mM (A). NE or ACh responsiveness before and after vessel culture (B and C). Myogenic tone measurements in fresh isolated vessel and in long-term culture condition (D). Graphs represent n=3 for each group, one-way ANOVA (A), unpaired t-test (B and C), two-way ANOVA (D).

4.6 Activated $CD8^+$ by hypertensive stimuli are capable to increase peripheral vascular resistance

In order to understand whether $CD8^+$ T cells isolated from hypertensive $PI3K\gamma^{CX/CX}$ mice are able to increase vascular tone, it was performed for 3 days a co-culture between these cells and second branch of mesenteric artery derived from normotensive mice; as control it were used $CD8^+$ T cells isolated from normotensive mice (figure 20 A).

To confirm that the purification procedure of $CD8^+$ lymphocytes was correctly performed, cells were stained with appropriate mAbs and analyzed using cytofluorimetric assay. In all experiments the purity of isolated cellular population was equal to or greater than 80%. This result and the selected strategy gaiting were represented in figure 20 B.

After incubation with the cells of interest, mesenteric arteries were maintained in the described culture conditions for 72 hours. Subsequently to this period, the myogenic response to different pressure values was evaluated. It was found that mesenteric arteries that were incubated with $PI3K\gamma^{CX/CX}$ $CD8^+$ T cells showed an increased myogenic tone than those incubated with $PI3K\gamma^{WT/WT}$ $CD8^+$ T cells (figure 20 C).

A similar approach was used to assess the effect of immune system primed by a different hypertensive stimulus; in fact, purified $CD8^+$ T cells (figure 21 A) were derived from a murine model of pre-hypertension. In particular, these cells were isolated from WT mice infused for three days with AngII or Vehicle and then incubated with mesenteric artery from WT normotensive mice. According to the previous showed data, in this experimental setting the resulting myogenic tone was increased (figure 21 B), highlighting and discovering one of the possible mechanisms by which these cells act determining the hypertensive phenotype.

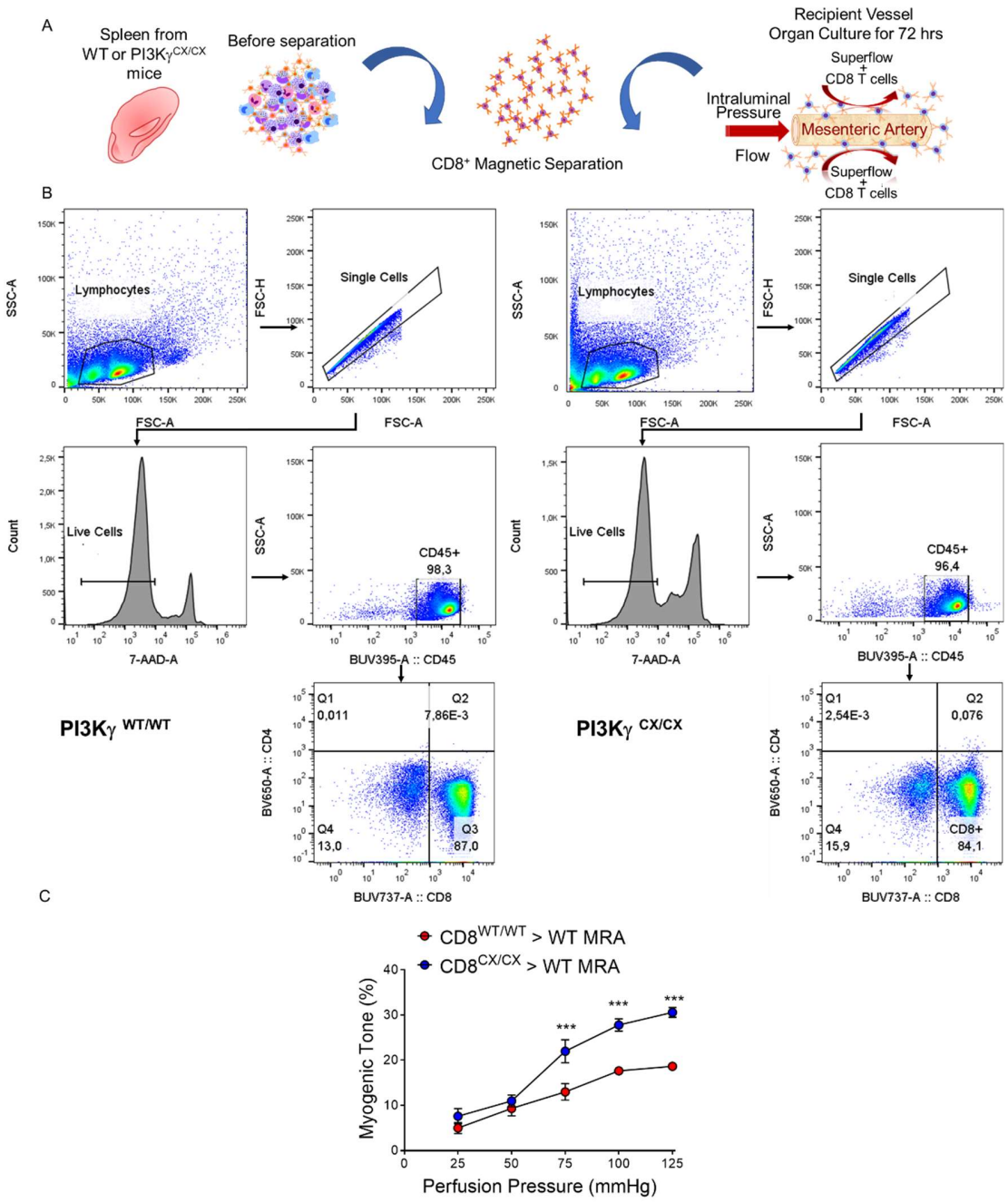

Figure 20. Myogenic response after co-culture between mesenteric resistance arteries and CD8⁺ cells constitutively expressing PI3Kγ. (A) Schematic representation of experimental design. (B) Evaluation of CD8 purity and illustration of gaiting strategy used to verify the correct isolation of this population. (C) Myogenic tone measurement in WT mesenteric arteries co-cultured for 3 days with CD8 cells from PI3K$\gamma^{CX/CX}$ or PI3K$\gamma^{WT/WT}$ mice. Graph represents n=5 for each group. Two-way ANOVA, ***P<0.001.

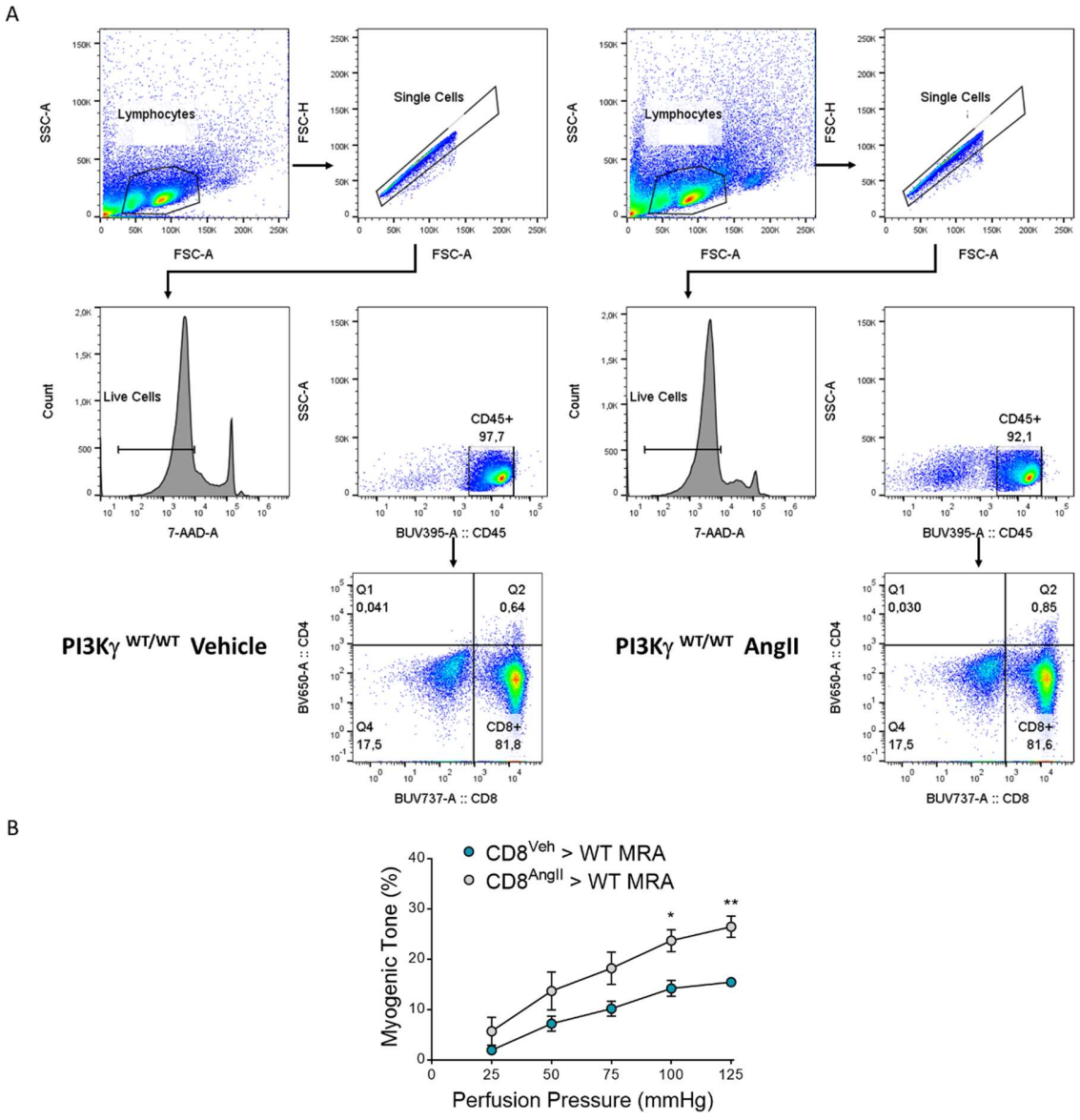

Figure 21. Myogenic response after co-culture between mesenteric resistance arteries and CD8^{+} cells isolated from infused-AngII or Vehicle WT mice. (A) Evaluation of CD8 purity and illustration of gaiting strategy used to verify the correct isolation of this population in this experimental setting. (B) Myogenic tone measurement in WT mesenteric arteries co-cultured for 3 days with CD8 cells from infused-AngII or Veh WT mice. Graph represents n=5 for each group. Two-way ANOVA, *P<0.05, **P<0.01.

5. CONCLUSIONS AND DISCUSSION

Arterial hypertension is an important cardiovascular risk factor and a worldwide healthcare problem. Several papers highlighted the correlation between high blood pressure and immune system and revealed the complexity of immune system interplay in HTN. However, it is not known what are the mechanisms through which immunity acts to modulate blood pressure.

In this dissertation it is showed that PI3Kγ KO mice, that are protected from hypertension, did not present kidney immune infiltration. Moreover, it is characterized an experimental murine model constitutively expressing PI3Kγ. This model appeared spontaneously hypertensive and showed the typical renal damage induced by this pathology. The immune infiltrate in the kidney of PI3K$\gamma^{CX/CX}$ mice leads to the hypothesis that probably immune mechanisms are involved in the link between PI3Kγ and high blood pressure. To evaluate this assumption, it was used a model mouse double mutant RAG1 KO PI3K$\gamma^{CX/CX}$ that allowed to clarify that lymphocytes have a role in the onset of hypertension. The importance of lymphocytes, and in particular of CD8 T cells, in the genesis of hypertension was demonstrated using adoptive transfer approach. This experiment showed that in normotensive mice receiving $CD8^{CX/CX}$ is observable an increase of pressure values, together with an increased kidney immune infiltrate. Moreover, thanks to an established system of vessel organ culture, it was showed that resistant arterioles isolated from normotensive mice presented an increased myogenic tone when co-cultured with $CD8^{CX/CX}$. The increased myogenic tone is observed also when arterioles from WT mice were incubated with $CD8^+$ isolated from infused-angII mice.

The presented results indicate that exist an involvement of immunity in linking between PI3Kγ and the pathogenesis of hypertension. In fact, starting from the evidence that PI3Kγ controls the migratory properties of CD8 effector T cells (Martin AL *et al*., 2008), here it was verified that splenic T cells migration to kidney in hypertensive conditions is regulated by the action of this kinase.

The reported results do the groundwork to elucidate a possible mechanism through which the immune cells regulate arterial pressure. Thanks to an established system of vessel organ culture it was demonstrated that activated CD8 lymphocytes by hypertensive stimuli are able to induce an increase of vascular myogenic response, thus contributing to the increase in peripheral resistances, a typic feature of hypertensive disease.

It were described the pressure, renal and vascular effects of $CD8^+$ cells derived from $PI3K\gamma^{CX/CX}$ or infused-angII mice, therefore, it should be interesting to study the same effects using immune cells from mice challenged with DOCA-salt and high fat diet to assess whether and how another hypertensive stimuli are able to modulate blood pressure, renal damage and myogenic response.

Renal failure analysis could be further investigated by monitoring renal function through metabolic cages in basal conditions or under hypertensive challenges; in this way it will possible to obtain data relative to urine creatinine, creatinine clearance and proteins/creatinine ratio.

It remains to understand whether exogenous T cells alone are able to induce gain in vascular contraction or whether resident immune cells are needed to obtain this effect.

RAG1 KO mice could allow to disclose this issue, since they are devoid of endogenous T cells. Adoptive transfer or co-culture system experiments performed using $CD8^+$ T cells primed by hypertensive challenges that are infused in RAG1 KO recipient mice or co-cultured with mesenteric artery from the same mice could help to elucidate this question.

It could be further investigated the phenotype of RAG1 KO $PI3K\gamma^{CX/CX}$ in order to assess whether these mice that presented normal BP are also protected from renal damage.

Since it's known that in mice expressing a kinase-dead PI3Kγ ($PI3K\gamma^{KD/KD}$) an impaired angiotensin II–evoked increase in blood pressure and vasoconstriction occur (Vecchione C *et al*., 2005), it would be interesting to evaluate also in this model the cells infiltrating kidneys.

Moreover, it will be important to understand how immune cells are capable to increase MT; this effect could be determined through vessel-cells direct contact or driven by cellular soluble mediators. To clarify this point, it would be useful to analyze the superflow

medium, that is in close contact with vessel, to evaluate whether in different culture time points a modification of cytokine production that could affect the vascular contractility occurs. In the same way ROS analysis could be interesting.

Here, it is described an innovative methodology for long-term culture that allows to maintain vessel in conditions close to that physiological. This was possible thanks to the ability to fine-tune the most favorable culture conditions for culture myograph system - 204CM. Moreover, ours goals have been to modify this system in order to allow the inflow medium oxygenation and the bubbler of gas mixture in super flow medium.
In literature there are different papers that performed vessel culture methodology (Ozaki and Karaki H, 2002), but no works have been able to study the murine vascular features following 3 days of culture in which the vessel is preserved in viability conditions under physiological pressure. Moreover, some developed systems are adapted only for to study vessel with big dimension (Zaniboni A *et al*., 2013; Bolz SS *et al*., 2000), while in our case we studied small resistance vessels. Furthermore, the majority of paper describes a vessel culture performed on arterial rings in a classical cells incubator to ensure optimal temperature and gas mixture conditions (Uddman E *et al*., 2003; Morita T *et al*., 2010), but the vessels are maintained in conditions without pressure, therefore more distant to *in vivo* situation.
The developed system in our laboratory could potentially be used also for other experimental applications, for example to evaluate the long-term effects on vascular system of pharmacologically active substances that have blood vessels as target.

In conclusion, it was demonstrated what role exerts the immunity in pathway that links PI3Kγ to hypertensive disease and in this context an innovative methodology with a broad-spectrum translational potential, due to its versatility and applicability in various fields of clinical research, was described.

6. ABBREVIATIONS

Ang II angiotensin II

ApoE Apolipoprotein E

AT1R, AT2R angiotensin II receptors

Atg14 autophagy-related protein 14

BK_{Ca}, large conductance, Ca^{2+}-activated K^+ channel;

BMDMs bone marrow-derived macrophages

BP blood pressure

CNS central nervous system

COPD obstructive pulmonary disease

CPI-17 17-kDA protein kinase C-potentiated inhibitory protein

CSF-1colony-stimulating factor-1

DAG diacylglycerol

DAMPs damage-associated molecular patterns

DBP diastolic BP

DCTs distal convoluted tubules

DC dendritic cells

DN double negative cells ($CD8^-$ $CD4^-$)

DP double positive cells ($CD8^+$ $CD4^+$)

EC endothelial cells

ECM cell-extracellular matrix

ET1 endothelin I

FAK/Src focal adhesion kinase and cSrc kinase

Gαq/PLC trimeric G-protein αq and phospholipase C

Gpa1 Guanine nucleotide-binding protein 1a

GPCRs G protein coupled receptors

HBP high blood pressure

HTN, HT Hypertension

HUVECs human umbilical vein ECs

Ins(1,4,5)P3R inositol trisphosphate receptor

IP3 inositol 1, 4, 5-trisphosphate

IS immunological synapse

KO knock out

L-NAME nitro l-arginine methyl ester

LDL low density lipoprotein

LTB4 lipid leukotriene B4

LTTC L-type voltage gated calcium channel

MI myocardial infarction

MLCK myosin light chain kinase

MLCP myosin light chain phosphatase

MMF mycophenolate mofetil

mTORC2 mammalian target of rapamycin complex 2

MYPT1 myosin phosphatase targeting subunit

MZ marginal zone

NCC sodium-chloride-co-transporter

NSCC nonselective cation channel

PAF platelet-activating factor

PDK1 phosphoinositide-dependent kinase-1

PIP_3 phosphatidylinositol (3,4,5)-trisphosphate

PI3K phosphoinositide 3-kinases

PlGF placental growth factor

PKC protein kinase C

PP1c protein phosphatase 1c

PtdIns phosphatidylinositol

RA rheumatoid arthritis

RBD Ras-binding domain

ROK Rho kinase

RP red pulp

RyR ryanodine receptor

SACs Stretch-activated channels

SBP systolic BP

SH2 Src homology 2

SLE systemic lupus erythematosus

SMCs smooth muscle cells

SNP single nucleotide polymorphism

SNS sympathetic nervous system

SP single positive cells ($CD8^+$ or $CD4^+$)

SR sarcoplasmic reticulum

STOCs spontaneously transient outward currents

TCR T cell receptor

TLRs toll like receptor

Tregs Regulatory T lymphocytes

TRP transient receptor potential

VEGF Vascular endothelial growth factor

VGCC voltage-gated Ca2+ channels

Vps34, Vps15 vacuolar protein sorting 34, 15

WP white pulp

7. APPENDIX

During my PhD program I had the possibility to participate in the development of another project that it was published on Immunity Journal on November 2017. In particular, I contributed in the realization of cytofluorimetric experiments reported in this paper. The abstract of the article is showed below.

TITLE: Targeting Interleukin-1β Protects from Aortic Aneurysms Induced by Disrupted Transforming Growth Factor β Signaling.

AUTHORS: Francesco Da Ros, Raimondo Carnevale, Giuseppe Cifelli, Dario Bizzotto, Manuel Casaburo, Marialuisa Perrotta, Lorenzo Carnevale, Iolanda Vinciguerra, Stefania Fardella, Roberta Iacobucci, Giorgio M. Bressan, Paola Braghetta, Giuseppe Lembo and Daniela Carnevale.

ABSTRACT: Aortic aneurysms are life-threatening conditions with effective treatments mainly limited to emergency surgery or trans-arterial endovascular stent grafts, thus calling for the identification of specific molecular targets. Genetic studies have highlighted controversial roles of transforming growth factor β (TGF-β) signaling in aneurysm development. Here, we report on aneurysms developing in adult mice after smooth muscle cell (SMC)-specific inactivation of Smad4, an intracellular transducer of TGF-β. The results revealed that Smad4 inhibition activated interleukin-1β (IL-1β) in SMCs. This danger signal later recruited innate immunity in the adventitia through chemokine (C-C motif) ligand 2 (CCL2) and modified the mechanical properties of the aortic wall, thus favoring vessel dilation. SMC-specific Smad4 deletion in Il1r1- or Ccr2-null mice resulted in milder aortic pathology. A chronic treatment with anti-IL-1β antibody effectively hampered aneurysm development. These findings identify a mechanistic target for controlling the progression of aneurysms with compromised TGF-β signaling, such as those driven by SMAD4 mutations.

KEYWORDS: aortic aneurysm, TGF-β, SMAD4, IL-1β, macrophages, smooth muscle cells, elastic lamellae, CCR2, MCP1, innate immunity.

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