Genetically Modified Macrophages and Renal Regeneration

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Tesis doctoral

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A mi familia

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ABBREVIATIONS

ARF	Acute renal failure
NO	Nitric oxide
CFU-GEMM	Granulocyte-erythrocyte-megakaryocyte-macrophage colony-forming unit
CFU-GM	Granulocyte- macrophage colony-forming unit
M-CFU	macrophage colony-forming unit
GM-CSF	Granulocyte- macrophage colony stimulating factor
M-CSF	macrophage colony stimulating factor
CD11b	cluster of differentiation 11b
CD18	cluster of differentiation 18
LPS	Lipopolysaccharides
CD14	cluster of differentiation 14
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL-1	Interleukin-1
TNF-α	Tumor necrosis factor alpha
IL-12	Interleukin-12
TGF-β	Transforming growth factor beta
IL-3	Interleukin 3
TLR	Toll like receptor
MHC-II	Major histocompatibility complex II
IL1-β	Interleukin-1 beta
FGF	Fibroblast growth factor
IL-1ra	Interleukin-1 receptor antagonist
PGE2	Prostaglandin E2
IL-8	Interleukin 8
IL-4	Interleukin 4
IL-13	Interleukin 13
IL-10	Interleukin 10

CD11a	cluster of differentiation 11 alpha
CD40	cluster of differentiation 40
CD54	cluster of differentiation 54
CD58	cluster of differentiation 58
CD80	cluster of differentiation 80
CD86	cluster of differentiation 86
ICAM-1	Intracellular adhesion molecule 1
iNOS	Inducible nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
MCP-1	Monocyte chemotactic protein 1
AP-1	Activator protein 1
PCNA	Proliferative cell nuclear antigen
BUN	Blood urea nitrogen
Ivns1abp	Influenza virus non structural binding protein 1a
bHLH-LZ	basic helix-loop-helix leucine zipper
PDGF	plateled-derived growth factor
bFGF	basic fibroblastic growth factor
PKC	protein kinase C
PKA	protein kinase A
MOI	Multiplicity of infection
МΦ	Macrophages

1. INTRODUCTION

1.1 THE KIDNEY

The urinary system plays a vital work of great importance, as it not only provides maintenance and body fluid homeostasis, but is also responsible for the removal of metabolic wastes from the body, potentially toxic, which can impair the normal functioning system. The vertebrate urinary system consists of two kidneys, which are the urine productive units, two ureters, which conduct the urine to a backup or store site, the bladder where the urine is temporary stored and the urethra which is the tube connecting the bladder to the outside to evacuate its contents.

Kidney functions are basically three (1):

- Excretion of waste products of metabolism such as urea, creatinine, phosphorus, etc...
- Regulation of the internal environment through the maintenance of electrolyte and acid-base balance, known as fluid homeostasis.
- Endocrine function by synthesis of active metabolites of vitamin D and rennin-angiotensin system as well as by synthesis of erythropoietin, kinins and prostaglandins.

With these functions, the kidneys, as well as other organs such as the lungs and the liver, recover essential components and eliminate waste substances generated as a result of metabolism. Therefore, play a crucial role in the regulation and maintenance of the composition and the volume of the extracellular fluid.

1.1.1 Kidney structure and organization

From an anatomical viewpoint, mammalian kidney is a red bean shaped organ located in the retroperitoneum on both sides of the spine. In the upper pole of each kidney an adrenal gland is located. Both kidney and adrenal gland are surrounded by two layers of fat, the perirenal and pararenal fat and the renal fascia. The entrance and exit of vessels, renal nerves and the uterers is called renal hilum and is located in the medial

concave part of the kidney. From a morphological point of view, three structures can be distinguished from the outside to the inside: the capsule, cortex and medulla. The most significant difference between cortex and medulla lies on the arrangement of their vascular network. In fact, approximately 90 to 95% of the blood flows through the kidney in the cortex, while only a 5% flows through the medulla.

The renal cortex consists mainly of renal corpuscles and a complex network of tubules that are forming part of the nephron. In the cortex are also distinguished a number of vertical striations, consisting of tubules and collecting ducts which extend to the core and are called medullary rays. The remaining regions between the medullary rays are called cortical cortex labyrinths.

In the renal medulla, tubules are distributed into conical structures called renal pyramids or pyramids of Malpighi. The base of said pyramids is orientated toward the cortex, while the apex is directed toward the renal sinus. Each renal pyramid is divided into outer medulla, near the renal cortex, and inner medulla (2).

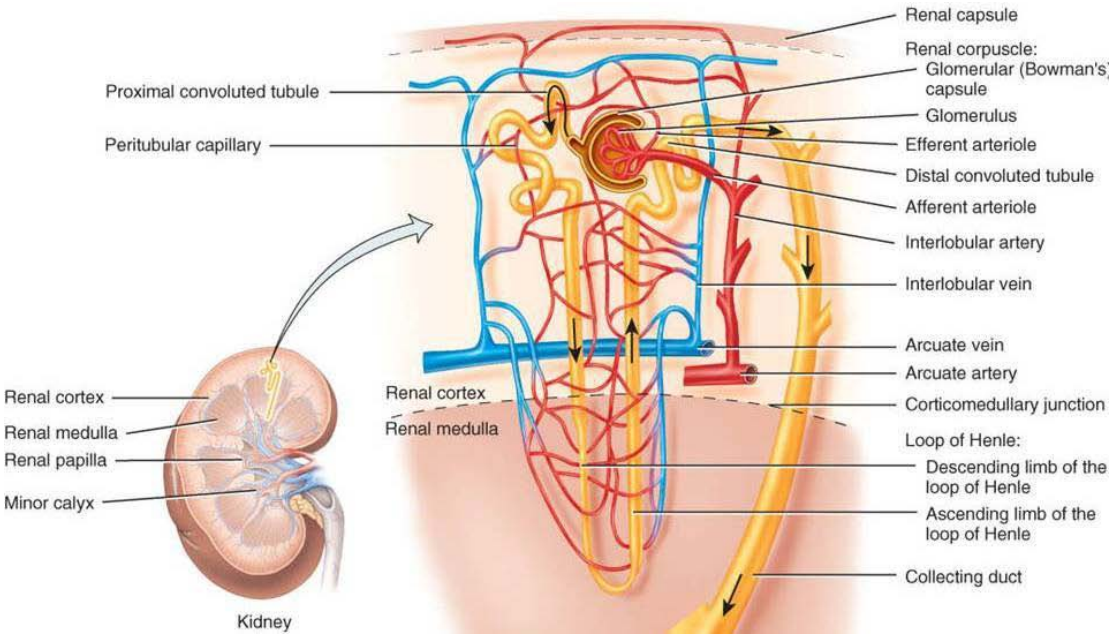


Figure 1. General diagram of the kidney and its functional unit (adapted from www.uaz.edu.mx/histo/TortorAna/)

1.1.2 The nephron

Each kidney in the human contains about 1 million nephrons, each capable of forming urine. The kidney cannot regenerate new nephrons. Therefore, with renal injury, disease, or normal aging, there is gradual decrease in nephron number. This loss is not life threatening because adaptive changes in the remaining nephrons allow them to excrete the proper amounts of water, electrolytes and waste products (1).

Each nephron contains a tuft of glomerular capillaries called the **glomerulus**, through which large amounts of fluid are filtered from the blood and a long tubule in which the filtered fluid is converted into urine on its way to the pelvis of the kidney. The glomerulus contains a network of branching and anastomosing glomerular capillaries that, compared with other capillaries, have high hydrostatic pressure (about 60 mm Hg). **The glomerular capillaries** are covered by epithelial cells, and the total glomerulus is encased in **Bowman's capsule**. Fluid filtered from the glomerular capillaries flows into Bowman's capsule and then into the **proximal tubule**, which lies in the cortex of the kidney. From the proximal tubule, fluid flows into the **loop of Henle**, which dips into the renal medulla. Each loop consists of a descending and an ascending limb. The walls of the descending limb and the lower end of the ascending limb are very thin and therefore are called the thin segment of the loop of Henle. After the ascending limb of the loop has returned partway back to the cortex, its wall becomes much thicker, and it is referred to as the thick segment of the ascending limb. At the end of the thick ascending limb is a short segment, which is actually a plaque in its wall, known as the **macula densa**. The macula densa plays an important role in controlling nephron function. Beyond the macula densa, fluid enters the distal tubule, which, like the proximal tubule, lies in the **renal cortex**. This is followed by the collecting tubule and the cortical connecting tubule, which lead to the **cortical collecting duct**. The initial parts of eight to ten cortical collecting ducts join to form a single larger collecting duct that runs downward into the medulla and becomes the medullary collecting duct. The collecting ducts merge to form progressively larger ducts that eventually empty into the renal pelvis through the tips of the **renal papillae**. In each kidney, there are about 250 of the very large collecting ducts, each which collects urine from about 4000 nephrons (1).

Although each nephron has all the components described earlier, there are some differences, depending on how deep the nephron lies within the kidney mass. Those nephrons that have glomeruli located in the outer cortex are called cortical nephrons and they have short loops of Henle that penetrate only a short distance into the medulla. About 20 to 30 percent of the nephrons have glomeruli that lie deep in the renal cortex near the medulla and are called juxtamedullary nephrons. These nephrons have long loops of Henle that dip deeply into the medulla, in some cases all the way to the tips of the renal papillae. The vascular structures supplying the juxtamedullary nephrons also differ from those supplying the cortical nephrons. For the cortical nephrons, the entire tubular system is surrounded by an extensive network of peritubular capillaries. For the juxtamedullary nephrons, long effered arterioles extend from the glomeruli down into the outer medulla and then divide into specialized peritubular capillaries called **vasa recta**. Like the loops of Henle, the vasa recta return toward the cortex and empty into the cortical veins. This specialized network of capillaries in the medulla plays an essential role in the formation of concentrated urine (2).

1.2 RENAL FAILURE

Acute renal failure (ARF) is defined as a rapid decline in kidney function that can occur in hours or days. This deterioration causes an inability of the kidneys to excrete nitrogen products derived from protein metabolism and subsequently to maintain hydro electrolytic homeostasis and acid-base balance. Electrolytes such as potassium, sodium and calcium, which are needed for normal functioning of the body, become harmful and sometime poisonous when they reach abnormally high or low values. In all cases there is a decrease in glomerular filtration rate, which in the clinic is measured by creatinine clearance.

Kidney damage, from etiological view point, can be divided into three categories (3):

1) **Prerenal (60-70% of cases)**: ARF characterized by decreased blood flow and is the physiological response to renal hypoperfusion in which is preserved the integrity of the kidneys.
2) **Intrinsic Renal:** ARF due to renal tissue damage (glomerular or tubulointerstitial causes, acute tubular necrosis, acute cortical necrosis). Directly affects the renal parenchyma. It can induce ischemia and/or toxicity to the kidney and is usually associated with necrosis of the renal epithelial cells called acute tubular necrosis (ATN). Constitutes 20-40% of cases.
3) **Postrenal (5-10% of cases):** ARF due to obstruction of the urinary tract (ureter / urethra).

Among the factors that increase the risk of ARF are advanced age, chronic renal failure, renal disease, diabetes, heart failure and obesity. Acute renal failure is a very common disease with important medical and socio-economic implications(4) , with mortality about 50%, which has not seen significant improvements in the last four decades(5) , even with nutritional and dialysis treatments. Complications of ARF may have an effect on the entire body, including the digestive system, heart, lungs and nervous system. Infections are one of the most common complications of ARF, because the immune system stops working properly(6). In addition, progression of the disease is highly variable, ranging from a transient loss of function in several days, often with complete recovery of renal function, to situations where the disease persists and requires dialysis, intensive care and transplantation. The outcome is recurrent hospital stays and/or reduced quality of life.

Typically, type of patients with clinical symptoms of ARF, are seen after the insult as transplanted patients(7).

Is urgently needed to cure the patient through stimulation of regenerative processes, since the disease is already developed when the patient reaches the hospital. Therapy should enhance recovery and reduce length of stay of patients, to improve the current situation, from the point of view of the patient's pain and medical resources consumed, such as visits, surgery, medical stocks, intensive care management and dialysis. Despite the progress and development of therapeutic strategies, there is no effective therapy for humans.

Although considered a multifactorial process, overlapping with various conditions (such as ischemia, sepsis or medication and nephrotoxicity), it is known that the leading cause of renal failure is the degree of acute or chronic ischemia(4).

1.3 ISCHEMIA/REPERFUSION

Ischemia is defined as blood flow stopping, resulting in a decrease in oxygen partial pressure, depriving ischemic tissues of oxygen and nutrients and causing a disruption of cell metabolism. When blood flow is restored another phenomenon called reperfusion is taking place. One might think that the restore of blood flowing and the supply of oxygen and nutrients to the organ, it would be able to restore it, leaving only the ischemic injury, however and paradoxically, reperfusion leads to a more serious injury than the ischemic one, called reperfusion injury. Together, the pathophysiologic event that occurs as a result of restoration of blood flow following a period of ischemia is called ischemia-reperfusion syndrome (I/R)(8). It's a complex process in which the injury begins during ischemia and increases during reperfusion.

Ischemia/reperfusion has been implicated in the pathogenesis of various renal disorders. Ischemia can cause ARF in transplant conditions, vascular surgery, sepsis, hypovolemic shock and plays an important role in the progression to a chronic disease. In close association with ischemic damage, a deep inflammatory response that is associated with overexpression of a variety of mediators such as cytokines and chemokines also occurs, triggering an inflammatory cascade(9). There is evidence that an important component of the pathophysiology of ischemic ARF is the core flow reduction, with the concomitant reduction of oxygen reaching the tubular structures, resulting in cell damage. In succession, this promotes hypoperfusion and thus more ischemia which leads in a self-destructive loop. Medullary vascular congestion leads to profound inflammatory response associated with intravascular leukocyte accumulation that has been identified for many years as an important component of the ARF. Ischemia is critical for multiple disease conditions affecting the kidney, including acute and chronic kidney disease in which an initial result leads to tissue damage and results in organ failure(10).

1.3.1 Physiopathology of renal damage

Ischemia and hypoperfusion can cause fundamental alterations in the kidney that can be divided into two categories: hemodynamic alterations and tubular damage.

One of the most important hemodynamic alterations is vasoconstriction, which causes changes in the distribution of persistent renal blood flow and oxygen delivery in a way that the outer medulla of kidney remains more ischemic than the rest of the renal tissue. Furthermore, the maintained ischemia in level of outer medulla preserves the injury at the proximal convoluted tubule and the ascending limb of loop of Henle. Those hemodynamic changes can be caused by multiple factors; the most important of them are listed bellow(11):

- Endothelin, which is released in excess during ischemic endothelial injury and provokes alterations in vasoconstriction.
- Decrease of nitric oxide (NO) by endothelial cells provoked by ischemia results in loss of maintenance of a normal blood vasodilatation.
- Congestion of medullar circulation results in reduction of oxygen release to the outer medulla.

Tubular injury can be mostly located at the proximal tubule and the thick ascending portion of the loop of Henle, probably due to their higher requirements of ATP and because the external core area, where those structures are found, has a worse vascular supply than other renal areas(11).

There are three fundamental types of tubular injury:

- Necrosis and tubular epithelium detachment, thereby tubule obstruction and increased pressure proximal to the obstruction.
- Loss of integrity of the tubular wall, resulting in communication between tubular lumen and interstitium.
- Escape of the ultrafiltrate circulating through the tubules to the intersistial tissue. The basis of all these morphological changes is formed by histological alterations such as tubular necrosis areas, tubular epithelial cell loss, distal tubule cylinders, etc…

1.3.2 Phases of Ischemia/Reperfusion

The clinical process of I/R can be divided into three phases: initiation, maintenance and recovery(11). The latter depends entirely on the severity of the injury. Each of the phases has different pathopshysiological characteristics and management.

- **Initiation phase**: Is the period of time between the exposure to the etiologic agent and the onset of parenchymal damage. This phase is characterized by the loss of cell polarity, swelling, loss of extracellular matrix adhesion and cell death (12). The glomerular filtration rate decreases due to the fall in renal blood flow and the loss of tubular epithelial integrity. The terminal portion of the proximal tubule and the medullary ascending portion of the loop of Henle, are the nephron segments most vulnerable to ischemia because they both have a high rate of active transport of solutes, ATP-dependent and therefore a high oxygen consumption.
- **Maintenance phase**: During this phase, which occurs when uremic complication starts to appear, the parenchymal damage is established. Despite the correction of the cause of renal failure (e.g. stabilization of hemodynamic status), the glomerular filtration rate remains low. Some of the possible reasons can be persistent renal vasoconstriction and medullar ischemia triggered by a change in the release of vasoactive mediators by damaged endothelial cells or medullary blood vessels congestion and reperfusion injury, caused by mediators released from leukocytes or from other parenchymal cells.
- **Recovery phase**: It's the period of time during which occurs renal tissue regeneration and repair and it is absolutely depended on the degree of the initial damage. This regeneration depends on the resilience of damaged cells, the removal of necrotic derbis and intratubular cylinders and the capacity for generation of tubular cells which results in continuity and normal function of tubular epithelium. At cellular level, an increase in mitotic and dedifferentiated cells in damaged areas is observed (13).

1.4 MACROPHAGES

1.4.1 Origin of macrophages

Macrophages are extremely diverse phagocytic cells involved in a huge numbers of complex functions both in disease and health due to their function as natural scavengers to rid the body of apoptotic and senescent cells and debris. They are critical to the establishment of proper defenses against invading pathogens and to the maintenance of homeostasis, by promoting angiogenesis, tissue remodeling and repair (14). Macrophages have pivotal roles in inflammation, injury and repair of damaged tissues because of their ability to secrete a large number of cytokines, chemokines and growth factors that recruit and activate a variety of cell types.

Macrophages, as every other cell of the immune system, have a common origin in the bone marrow. The process of the formation and development of mature blood cells resulting from the survival, proliferation, lineage-commitment and differentiation of early progenitors is called hematopoiesis (15).

The exact series of events to take place during mature blood cell development consists of an extremely coordinated complex of interactions with other cells, such as adipocytes, fibroblastic or endothelial cells, extracellular matrix components of the surrounding microenvironment and soluble factors that are produced locally or arise from circulation (16).

In the bone marrow, cytokines IL-1, IL-3 and IL-6 induce stem cell division that gives rise to a pluripotent myeloid cell defines as granulocyte-erythrocyte-megakaryocyte-macrophage colony-forming unit (CFU-GEMM). In the presence of IL-1 and/or IL-3, this precursor becomes a progenitor of both macrophages and granulocytes knows as the granulocyte-macrophage colony-forming unit (CFU-GM) which is then matured to the macrophage colony-forming unit (M-CFU), under the influence of the macrophage colony stimulating factor (M-CSF), the granulocyte-macrophage colony stimulating factor (GM-CSF) and interlekin IL-3. This differentiation of macrophage lineages assumes that the macrophage colony-forming unit (M-CFU) differentiates in the presence of the M-CSF to monoblast, promonocyte, monocyte and differentiated macrophages (Figure 2).

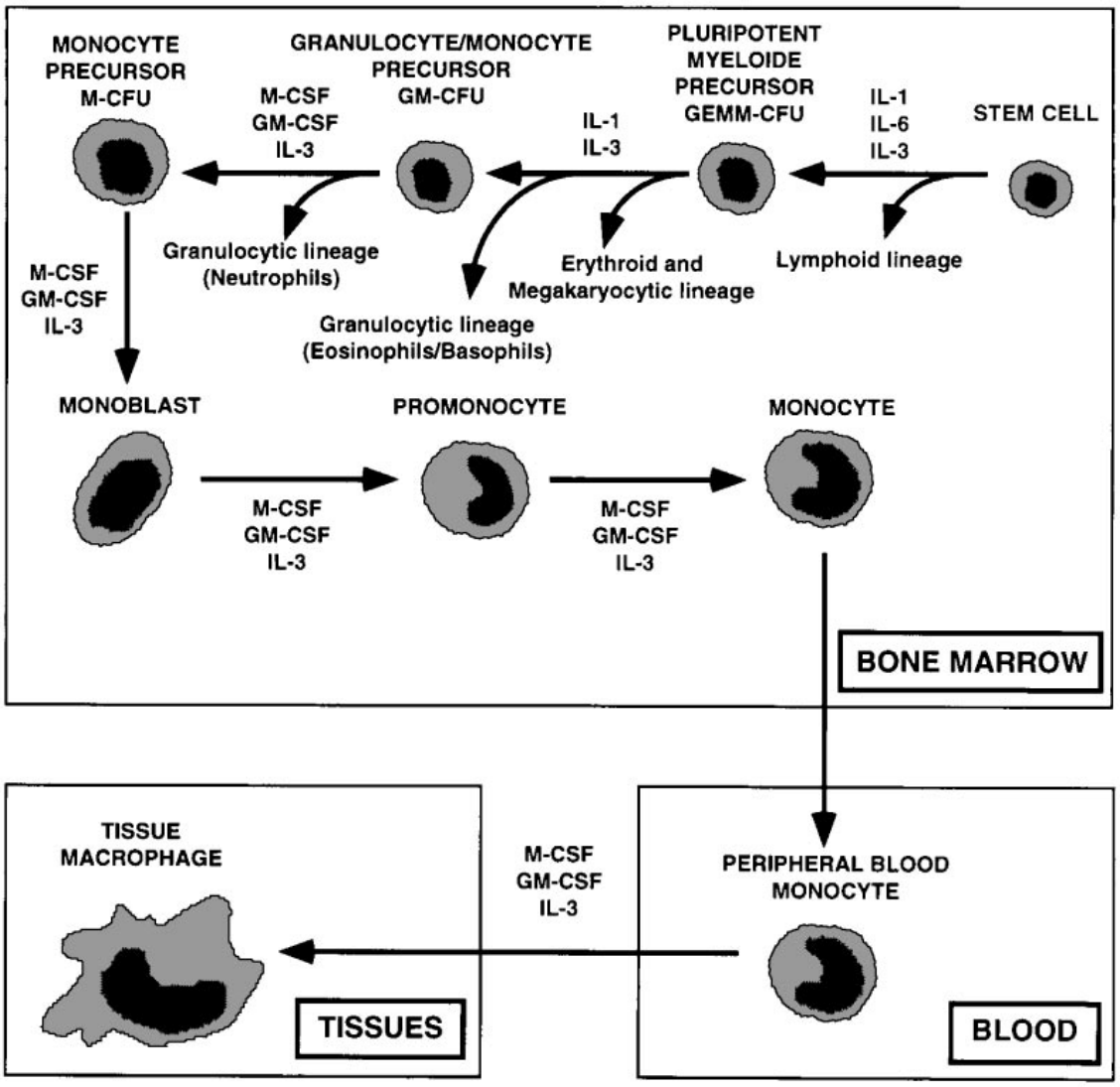


Figure 2.- Differentiation of stem cells to monocyte/macrophages (adapted from Celada A. Et al. Journal of Leukocyte Biology (1998) vol. 63 no. 4 405-417)

Monocytes have the capacity to leave the bone marrow and travel through the peripheral blood vessels, thus circulating to all parts of the body. To enter the tissue, monocytes interact with adhesion molecules, such as selectins and integrins found on the vascular epithelium, after being attracted by chemotactic chemokines. Furthermore, this process can also be regulated by other triggers of the organism, such as inflammation, ischemia, necrosis or apoptosis. In this case, the blood flow will be reduced in order to assure the interactions between monocytes and enthothelial cells, thus contributing to the recruitment of monocytes to tissues and their differentiation

into macrophages. This process is defined by an increase in cellular size, a major development of the lysosomal system and the content of hydrolytic enzymes and an augmented number of mitochondria and thus their energetic metabolism. The function of macrophages depends on the tissue in which they reside. Dendritic cells, microglia (central nervous system), osteoplasts (bone), Kupffer cells (liver), alveolar macrophages (lung), class A cells (joints) and Langerhans cells (dermis) have their origin in the macrophage cell lineage.

Macrophage development involves the regulation of several transcription factors regulating the lineage-specific expression of a number of genes and the subsequent establishment of the macrophage phenotype (17). Known genes that determine a macrophage phenotype are those that code for the M-CSF receptor, essential for efficient response to M-CSF, lysozyme, adhesion molecules CD11b (Mac-1) and CD18, LPS receptor (CD14), IFN-γ receptor, scavenger receptors and receptors for the constant region of IgG. Resident macrophages are also identified by specific F4/80 expression (18). Conversely, macrophages may regulate erythropoiesis by secreting several cytokines, including IL-1, TNF-α, IL-12, M-CSF and TGF-β, affecting cellular turnover (19, 20). Furthermore, tissue macrophages are committed to various fates. They have the capacity to proliferate under basal conditions in the presence of M-CSF produced in an autocrine manner or to differentiate under the control of other growth factors like GM-CSF and IL-3 (21). When macrophages are encountered in a focus of inflammation or recruited to a site of ischemia, necrosis or apoptosis, they stop proliferating and become activated, thus incrementing their specialized functions. On the contrary, in the absence of any stimulating factors, macrophages are committed to die through apoptosis, thus establishing a balance of production of their precursors in the bone marrow and the elimination of tissue macrophages (22).

Once macrophages reach the tissue of destiny, they implement a critical role of the outcome of immune responses, acting as mediators of a diverse number of mechanisms. Macrophages participate in innate and adaptive immune response recognizing, phagocytising and clearing of invading pathogens or apoptotic cells through the expression of pattern recognition receptors. Macrophages also participate in immune modulation / inflammation through the production of cytokines and chemokines, tissue repair, antigen presentation and the regulation of T cell activation

and differentiation. Under basal conditions, macrophages are prepared to fulfil a series of functions without the need of any type of stimulus. Nonetheless, macrophage specialized functions can only be realized after the appropriate stimulus, either by direct cell-to-cell interactions or by the secretion of diverse chemokines and/or cytokines depending on the microenvironment. So, macrophages comprise of a huge repertoire of autocrine, paracrine and endocrine factors which range from mitogens to chemotactic and apoptosis inducing agents (23).

1.4.2 Macrophage phenotypes and inflammation

In the context of innate immunity, macrophages phagocytose and eliminate invading particles such as bacteria, virus, parasites, other harmful macromolecules and even dead cells, thus preventing an uncontrolled inflammatory response.

Macrophages exhibit a great heterogeneity and functional plasticity, which is reflected in their expression of different phenotypes in response to micro-environmental signals (Figure 3) (24).

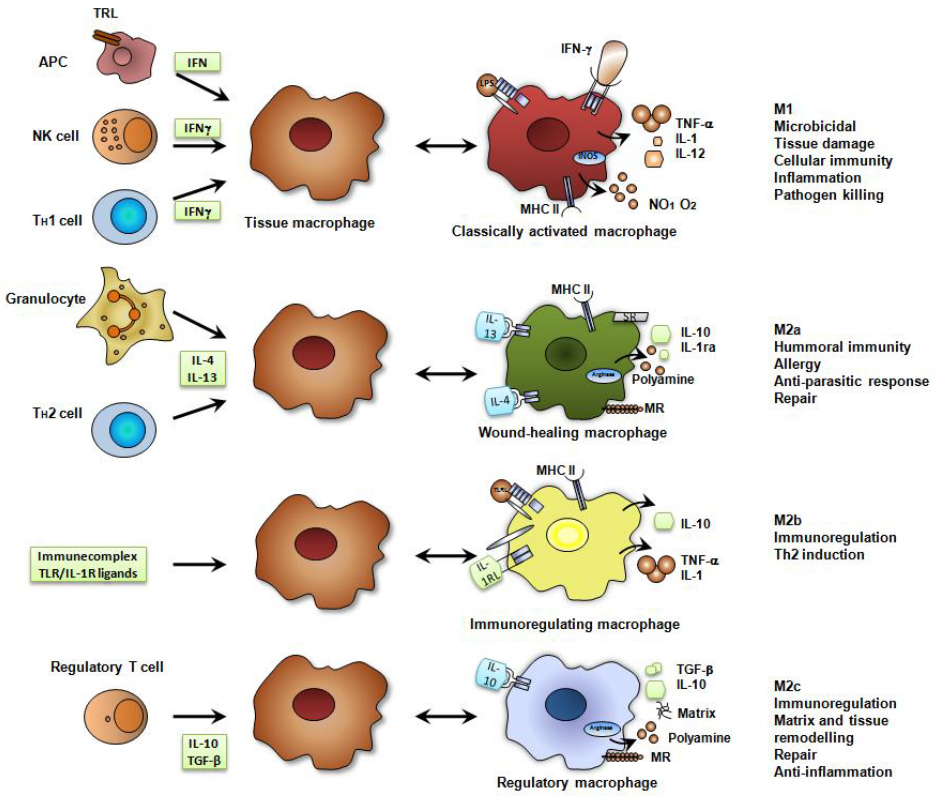


Figure 3.- Macrophage activation and phenotypes (adapted from Gordon S. Nature Reviews Immunology (2003) 3:23-35)

One of their major roles is the modulation of inflammation and its outcome, both regarding initiation and resolution. Nowadays, inflammation is considered a highly regulated response upon injury designed to restore normal function (25). Recently, heterogeneity of macrophage activation and especially their ability to curtail inflammation has been considered essentially crucial for the outcome of injury and highlights their pivotal role in maintaining tissue integrity. Inflammation is characterized by the rapid infiltration of leukocytes, in particular neutrophils, macrophages and lymphocytes, which are key players in the induction of injury. The pathogenesis of inflammation is characterized by a complex interplay of chemokines, adhesion molecules, cytokines and diverse cell types. In this sense, macrophages contribute to the initiation phase of inflammation dealing with microbial cell wall components such as lipopolysaccharides (LPS), a range of receptors, such as toll-like receptors (TLRs) on the cell surface and T helper 1 (Th1) lymphocyte-derived IFN-γ. This in turn leads to the activation of intracellular signalling pathways in the macrophage and the production of a range of cytotoxic mediators like nitric oxide (NO), superoxide (O_2-) and TNF-α, the expression of major histocompatibility complex II (MHC II) as well as a variety of pro-inflammatory chemokines and cytokines (26). These macrophages, which are called classically activated or M1, promote antigen presentation, elimination of pathogens and activation of the adaptive immune system (27). Additionally, some of the factors produced by macrophages like TNF-α, IL-1β and IL-6, enhance the recruitment of other cells of the immune system, mostly neutrophils, to the site of infection or inflammation. Still, resident cell destruction is one of the major site effects of the action of M1 macrophages and the persistence of inflammation often results in tissue damage and the immune system faces a permanent challenge for developing anti-inflammatory mechanisms (Figure 4) (26).

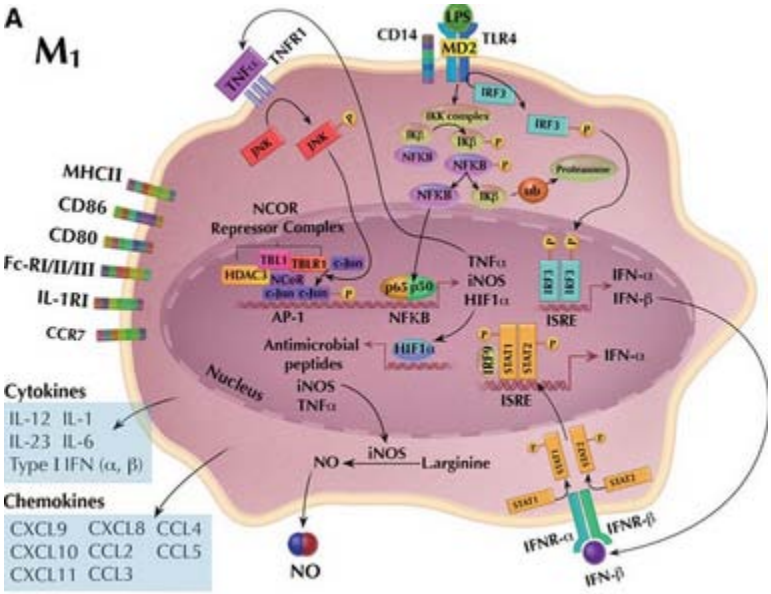


Figure 4.- Classically activated (M1) macrophage (adapted from Muckesh K. Jain et al. Arterioscler Thromb Vasc Biol.(2013)2013;33:1135-1144)

But, macrophages also participate in resolution of inflammation and in the promotion of healing through induction of matrix synthesis, fibroblast proliferation, angiogenesis and clearance of cellular debris (28). The induction of apoptosis in immune cells like neutrophils and lymphocytes normally determines the end of the initiation phase in the pathogenesis of inflammation and induces the resolution phase (29). Apoptotic cells are recognized and removed by macrophages, which in turn represents a powerful biological stimulus on reprogramming the macrophage phenotype towards anti-inflammation. Such macrophages produce growth factors like FGF and TGF-β in order to augment fibroblast proliferation and the activation of vascular endothelium, thus favouring the blood flow and the repair of damages tissues. Alternatively activated or M2 macrophages show increased expression of mannose receptor and MHC II, increased endocytosis, decreased NO production and augmented expression of IL-1 decoy receptor and IL-1 receptor antagonist (IL-1ra) (30). Additionally, engulfment of apoptotic cells provokes the release of growth factors, anti-inflammatory cytokines, pro-angiogenic and matrix remodelling factors (31). The ingestion of apoptotic cells is a complex series of events involving a range of cell surface receptors and mediators(Figure 5) (32).

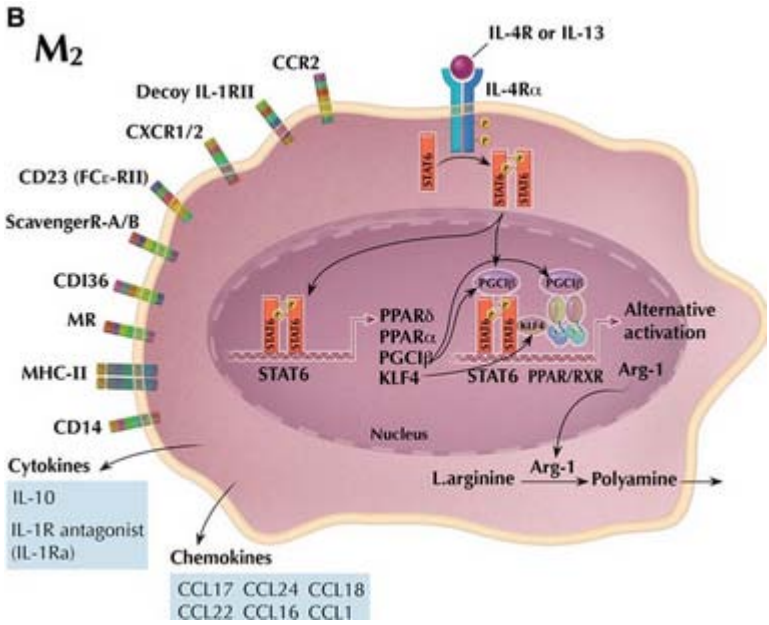


Figure 5.- Alternatively activated (M2) macrophage (adapted from Muckesh K. Jain et al. Arterioscler Thromb Vasc Biol.(2013)2013;33:1135-1144)

The uptake of apoptotic cells activate macrophages to develop anti-inflammatory properties, incrementing the production of anti-inflammatory mediators such as transforming growth factor-β (TGF-β) and prostaglandin E_2 (PGE_2) in line with a decreased expression of IL-8, IL-1β, and TNF-α (33). In the presence of T helper 2 (Th2) and lymphocyte mediated production of anti-inflammatory cytokines, such as IL-4, IL-13, and IL-10, macrophages are stimulated to express scavenger receptors, to enhance IL-10 production, to block NO production and to dampen O_2- release (26).

Additionally, M2 macrophages fail to generate NO from L-arginine and do not efficiently limit the growth of intracellular pathogens. The elevated production of L-arginine leads to the accumulation of prolines, glutamate and polyamines. These molecules have been associated to accelerated tissue repair, as proline acts as precursor of collagen and polyamines have been related to proliferation. Moreover, alternatively activated macrophages exhibit enhanced endocytic and phagocytic ability, increased expression of class II MHC molecules and can perform antigen presentation (26). Interestingly, M2 macrophages express similar levels of CD11a, CD40, CD54, CD58, CD80 and CD86 co-stimulatory molecules as M1 macrophages.

In summary, macrophages have the ability to eliminate pathogens either directly by phagocytosis or indirectly by activation of T lymphocytes. Furthermore, the M1

macrophage plays a key role in the initiation phase of inflammation by orchestrating the outcome of injury by stimulating diverse cell types and the production of inflammatory cytokines and chemokines. In the following phase of inflammation resolution, it is the M2 macrophage the responsible for tissue repair and remodelling by eliminating apoptotic cells and by activation of anti-inflammatory cytokine expression. As such, macrophages are pivotal in protecting organs and surrounding tissues against detrimental immune responses promoting tissue remodelling. Macrophage types are shown in Figure 3.

1.4.3 Macrophages and tissue repair

As previously mentioned, the macrophage phenotype and its biological function ultimately determine the outcome of inflammation and the development of irreversible tissue damage. Despite of the general anti-macrophage therapy by complete depletion, a more selective approach would point out the different roles of functionally distinct macrophage subpopulation in different phases of injury and remodeling. Conditional depletion models demonstrated that tissue associated macrophages play a key role in recovery phase after liver injury (34). This study underlines the role of macrophages in matrix regression during the recovery phase of experimental **hepatic fibrosis**. Moreover, Kupffer cells have been shown to be essential to liver regeneration using an approach of macrophage depletion by injection of liposome-encapsulated dichloromethylene diphosphate at day 3 after partial hepactomy (35). Devely et al. propose a key role for hemeoxygenase-1 (HO-1) in macrophage mediated protection from **liver ischemia/reperfusion** injury (36). The researchers clearly show both ¨in vitro¨ and ¨in vivo¨ that HO-1 is essential for the differentiation of macrophages, further suggesting that HO-1 inhibits inflammatory monocyte expansion. Using an approach of selective macrophage depletion, the role of Kupffer cells and not circulating monocytes, in hepatocellular survival was confirmed. Additionally, Friedmann et al. have shown a direct implication of macrophages on the regulation of **stellate cell proliferation** (37), thereby confirming a key role of macrophages in proliferation and regeneration.

Another study discovered similar results for **muscle cell growth** (38). Macrophages have been shown to enhance myogenic growth by activating myogenic precursor cells within the muscle. Arlnold et al. confirmed these results by ¨¨in vivo¨¨ tracing of macrophages (39), thereby discovering that inflammatory macrophages initially recruited to the site of injury may rapidly switch their phenotype towards anti-inflammation. Moreover, lens injury leads to a massive influx of macrophages into the eye, which is determent to **optic nerve regeneration** (40). Infiltrated macrophages are activated and secrete diverse factors implicated in axon regeneration. Furthermore, Vinuesa et al. determined that macrophages are involved in the **kidney repair** phase depended on the administration time point in a mouse model of ischemia / reperfusion injury (41). The most interesting finding was that the administration of macrophages was unable to promote regeneration at time points of injury and inflammation, thus confirming the fact that the microenvironment plays a critical role to macrophage function.

In this sense, macrophages and the trophic factors influencing their recruitment and phenotype determination are thus implicated in injury resolution and cellular restoration in a number of organs. Concluding, injury and repair compromise a critical balance between cell loss and proliferation, both processes depending on infiltrating macrophages.

1.5 INFLAMMATION ASSOCIATED TO RENAL I/R

In recent years, more and more information is available showing that the cellular and molecular pathogenetic mechanisms of ischemia / reperfusion resemble an acute inflammatory disease (42). The I/R generates an inflammatory response which causes renal tissue damage associated with severe impairment of epithelial cells, endothelial activation, protease activation, cytokine production and adhesion molecule expression with subsequent leukocyte infiltration in kidney (43) polymorphonuclear entrapment and microvascular hemodynamic compromise ((44, 45). These interactions between endothelium and leukocytes are further evident in the outer medulla, because of the increased vascular congestion typically seen in this region of kidney.

Is important to understand the signals that initiate inflammation in response to I/R. The complement system, for example, is an important initiator of the immune response, but is also involved in tissue damage (46) and sometimes in the regeneration (47).

Some researchers have suggested that inflammatory cells, particularly lymphocytes, macrophages and neutrophils have an important role in ischemic ARF. Cellular components of the inflammatory response, such as neutrophils (48) and macrophages (49), should contribute to the tissue destruction during the injury but then provide the signals needed for resolution. Although the role and kinetics or neutrophil infiltration into the ischemic ARF is controversial (50, 51), the infiltration of neutrophils seems to be an early event in mouse (9) and in biopsies from patients with NTA were also found in neutrophil vasa recta (52, 53). The T cells and macrophages infiltrate later in the course of the disease and persist in the recovery phase (51). Has been also observed lymphocyte infiltration posterior to ischemia in human kidney as well as the expression of the adhesion molecule ICAM-1, also related to the infiltration of neutrophils in ischemic renal tissue (54, 55, 56, 57). Another route which contributes to damage by I/R is the cytotoxic component of infiltrating cells like macrophages, lymphocytes and neutrophils involving reactive oxygen species and nitric oxide. Thus, in projects in which the enzyme inducible nitric oxide synthase (iNOS) was inhibited by antisense oligonucleotides, proximal tubular cell are protected from ischemic / hypoxic injury ¨in vivo¨ (58, 59). Previous group studies have shown that during the renal I/R process, the enzyme endothelial NO synthase (eNOS) is over expressed, which in turn induces the expression of the inducible form (iNOS), leading in the subsecuent production of NO, capable of inducing apoptosis (60). Moreover, over expression of neuronal NOS (eNOS) inside mitochondria, results in increased production of NO, which together with the oxygen free radicals (OFR) produce peroxynitrite (ONOO-), which acts nitrating actin cytoskeleton, inducing its disorganization and causing the release of mitochondrial cytochrome c to the outside, inducing caspace 3 activity and apoptosis (61, 62).

The interest in nontraditional mediators of acute renal failure, such as inflammatory pathways and microvascular events, has generated new paradigms and research lines. Recently there have been outlining various leukocyte subsets involved in this complex inflammatory process.

In the early stages of ischemia / reperfusion renal microvascular hemodynamic changes are occurring characterized by congestion and red cells slowdown along with platelets and leukocytes.

Blocking leukocyte-endothelium interactions showed significant protection against renal ischemia in experimental models. However, the experiments focused on the role of neutrophils in acute renal failure have provided poor results despite expectations. Although it is known that if the accumulation of neutrophils is obstructed, tissue damage is improved, is not clear that these depletion models are capable of discerning the involve of neutrophils compared to T lemphocytes and macrophages (63).

There is a general consensus which supports that in early stages there is a predominance of polymorphonuclear cells, but at later stages predominate macrophages and especially T cells. CD4+ cells, through the path of both IFN-γ and co stimulatory molecules, appear to be important modulators of acute renal failure, in which mechanism, B cells has also been recently implicated.

Kidney resident cells are also contributing to the pathogenesis of this inflammation, generating pro-inflammatory mediators such as cytokines TNF-α, IL-1β and TGF-β and the chemokine MCP-1. The monocyte chemoattractant protein (MCP) and its receptors are crucial in the development of the inflammatory response and recruitment of immune cells to sites of inflammation. Proximal tubular epithelium may also modulate the activity of T lymphocytes and its subsequent repair, but how and to what extend are involved is not fully defined.

Although the presence of macrophages in the ischemic tissue is well defined, little is known about their actual participation in the injury. The presence of macrophages in the renal tissue has classically been associated with the pathogenesis of chronic morphological damage. However, some recent evidence is questioning this data and instead, relates macrophages to a beneficial effect on the chronic inflammatory process. The new tools for modulating inflammatory cells constitute a promise for future clinical trials in acute renal failure.

1.5.1 Renal Inflammation and Macrophages

One of the events taking place in kidney disease and more specifically in the progress of Ischemia / Reperfusion is macrophage infiltration (51), which plays a role by the production of cytokine and chemokines (64, 65).

The classic model that defines I/R injury involves macrophages in proinflammatory damage and suggests that the increasing number of macrophages in the tissue is essentially due to the infiltration of monocytes that differentiate into macrophages, but also the result of increased local cell division (66, 67). Infiltrated macrophages, initially heterogeneous, adapt to the local microenvironment and develop a number of attributes that allow them to perform a particular function (68).

The properties of macrophages in damaged tissues vary with the nature and evolution of damage (69, 70). The rapid increase in the number of cytokines and growth factors that alter macrophage function, shows broad complex activation, in fact, many of the cytokines involved are also produced by macrophages and are likely to have an autocrine effect.

It is now accepted that the process of inflammation is a highly regulated response to injury, designed to restore the normal function of the tissue with minimum damage to it. In this regard, it has been demonstrated in different models that macrophages contribute to the healing process and they have the ability to control, manage and occasionally stop when the recovery process is complete (71). In addition, many researchers argue that the repair process, which can occur even with a reduction in the number of neutrophils, is not performed if macrophages are eliminated pharmacologically (72). This is probably due to that macrophages act as director cells, promoting the migration of macrophages themselves, as well as inducing the formation and stimulation of fibroblasts (73). But while this process seems clear in other models of injury, not enough data is available about the injury by I/R in the kidney. Several studies have shown that macrophages injected to animals, accumulate in the kidney and act as biologically functional cells that can accelerate or attenuate renal injury (74, 75, 76, 77), and in some cases macrophages may induce renal injury in terms of proteinuria and mesangial cell proliferation in rats with glomerulonephritis (76, 77).

Monocytes and macrophages can alter their phenotype in response to changes in the local environment of cytokines (78). Macrophages can be activated conventionally by microbial wall lipopolysaccharides (LPS) and Th1-type cytokines (like those secreted by activated lymphocytes by IFN-γ or IL-12), triggering the activation of pro-inflammatory phenotype or M1, characterized by the production of TNF-α and IL1-β as well as antiproliferative agents and microbicides, such as lysozyme or NO (induction of iNOS).

In the presence of Th-2 type cytokines (secreted by lymphocytes activated by IL-4) such as IL-4, IL-13 or IL-10, in the macrophage is induced an activation known as alternative or M2 phenotype. M1 and M2 phenotypes are specifically distinguished by the cytokines they produce and the way in which arginine is processed. Unlike macrophages M1, M2 phenotype is not able to produce NO from L-arginine and also fails to control the growth of intracellular pathogens. In contrast, M2 macrophages show high endocytic and phagocytic ability and are capable of producing large amounts of L-arginine obtaining as final product proline, glutamate and polyamines. These metabolites promote tissue repair, thus proline is acting as a collagen precursor and polyamines have been linked to proliferation and differentiation processes and to the inhibition of NO production by macrophages activated with LPS. Thus, these alternatively activated macrophages are able to protect the surrounding tissues from prolonged inflammatory processes or aggressive immune responses, inducing repair and/or tissue remodeling.

However, the exact functional properties of classical or alternatively activated macrophages ¨in vivo¨ still remain an open field of study. Therefore, a better understanding of the mechanisms that contribute to the generation of these phenotypes and their function ¨in vivo¨ could provide important information regarding the development of new therapies for the treatment of inflammatory diseases

In summary, macrophage as an effector and phagocytic cell is capable of performing the elimination of pathogens either directly by phagocytosis or indirectly through the activation of T lymphocytes. Subsequently, the macrophage itself is responsible for inducing the repair of damage caused during the inflammatory response produced by removing any cell derbis (such as apoptotic cells and cell remains) and completing the remodeling of the affected tissue.

1.5.2 Inflammatory mediators

Once leukocytes have arrived at a site of infection or inflammation, they release mediators which control the later accumulation and activation of other cells. However, in inflammatory reactions initiated by the immune system, the ultimate control is exerted by the antigen itself, in the same way as it controls the immune response itself. For this reason, the cellular accumulation at the site of chronic infection, or the autoimmune reactions (where the antigen cannot ultimately be eradicated), is quite different from that at sites where the antigen stimulus is rapidly cleared.

Inflammatory mediators are soluble, diffusible molecules that act locally at the site pf tissue damage and infection and at more distant sites. They can be divided into **exogenous and endogenous mediators**.

Bacterial products and toxins can act as **exogenous mediators of inflammation**. Notable among these is microbial wall lipopolysaccharides (LPS) of Gram-negative bacteria. LPS can trigger complement activation, resulting in the formation of anaphylatoxins C3a and C5a which cause vasodilatation and increase vascular permeability. LPS also activates the Hageman factor, leading to activation of the coagulation and fibrinolytic pathways as well as the kinin system.

Endogenous mediators of inflammation are produced from within the (innate and adaptive) immune system itself, as well as other systems. For example, they can be derived from molecules that are normally present in the plasma in an inactive form, such as peptide fragments of some components of complement, coagulation and kinin system. Mediators of inflammatory responses are also released at the site of injury by a number of cell types that either contain them as preformed molecules within storage granules, e.g. histamine, or which can rapidly switch on the machinery required to synthesize the mediators when they are required, for example to produce metabolites of arachidonic acid.

Macrophages are central in these cases and the capacity to generate and secrete diverse inflammation-regulating molecules is enormous and the repertoire of secreted molecules is diverse, ranging from lipid mediators, enzymes, cytokines and chemokines to a variety of soluble receptors.

Moreover, soluble receptors may be secreted into the microenvironment and can participate in regulating signal transduction in an active manner. Since receptors can be released into the microenvironment, they may interact with potential ligands, thereby competing with membrane-bound receptors and affecting the concentration of active ligand, which in turn modulates inflammatory response signaling. Additionally, soluble receptors can render cells of diverse tissues to be able to respond to ligands for which they are normally unresponsive due to lacking receptor molecules.

1.6 RENAL REGENERATION AND ISCHEMIA / REPERFUSION

Ischemia / reperfusion is a syndrome that at the moment it has not have a specific treatment, reflecting in part the relatively poor understanding of the disease (79).

Depending on the severity of ischemia and the susceptibility of the tissue, ischemic injury may result in permanent impairment of renal function attributable to cell death or in a transient compromise of function by sub lethal damage of renal cell, with subsequent recovery (45, 80, 81).

In any case, cell recovery after ARF is the only way to improve the damage, although the mechanisms that mediate the recovery are complex and still poorly understood. This process requires replacement or regeneration of tubular epithelial cells, which is accompanied by changes in the expression of genes that modulate the growth (82, 83).

Biological processes, classically attributed to the development of renal ischemic injury are concomitant with the different phases which lead to regeneration. These can be divided into at least three major cellular processes: apoptosis of renal epithelial cells of the proximal tubule (84), formation of pro-inflammatory mediators versus anti-inflammatory / macrophage phenotype (85, 86) and proliferation (87) for renal epithelial cells followed by the reorganization of the tubular structure (tubulogenesis). In this regard, there is a parallelism between the prevalence of apoptosis and regeneration (88) and also accumulation of macrophages at sites where the proliferation takes place (43).

Obviously, the three biological processes are subject to complex genetic programs which affect irreversibly the damage as well as kidney regeneration and recovery when

they are properly coordinated. Each of these biological processes (apoptosis, macrophage infiltration / inflammation and proliferation) is interlinked by activation of crossed signals (89). But although it is well established that the processes are linked and some single markers are known, the mechanism of how genes are expressed and coordinate regeneration is not yet known.

Among the therapeutic approaches aimed at recovering the ARF kidney, only disappointing results have been obtained in clinical trials of therapies with growth factors (e.g. EGF, HGF, IGF-1) (90, 91). These regenerative proposals probably failed due to lack of knowledge in basic biological processes, genes involved and the functions of these genes in the evolution from the damaged kidney to cure / recovery.

1.6.1 Regeneration markers

In the last decade several important cell markers, related to cell proliferation and associated with cancer, have been identified and used in the in the study of renal regeneration after ischemic insult. Prominent among these are markers such as stathmin, PCNA and Ki-67.

Stathmin: Statmmin is a cytosilic protein of 19 kDa, highly conserved and ubiquitously expressed. Is a member of a group of proteins that binds and disrupts the microtubule network (92). Stathmin´s ability to bind to tubulin and destabilize the microtubule structure is modulated by its phosphorylation at multiple serine residues by protein kinases (93, 94, 95). This protein, whose expression is increased during regeneration (96), acts on the transition from G2 to M phase during the cell cycle and plays an important role in regulating spindle (97). These observations suggest how important stathmin in the regulation of cell proliferation and tissue regeneration is after injury. The association between increase of stathmin and tubular recovery was confirmed in kidney biopsies of patients recovered from NTA.

PCNA: The proliferating cell nuclear antigen (PCNA) is a nonhistone nuclear acidic protein of 36 kDa and 261 amino acids with a high content of glutamic acid and

aspartate (98), which functions as adjuvant protein to DNA synthesis (99) and plays a critical role in the initiation of cell proliferation (100). PCNA is considered necessary but insufficient to complete the process of cell proliferation (101). PCNA synthesis is very low at the initial phase of the G1 phase, increases during the late G1 phase and reaches its maximum at the beginning of S phase (102). Besides its rate of synthesis decreases to baseline levels during the end of phase S2. Is expressed, therefore, in all proliferating cells, but not in quiescent or resting cells (phase G0).

Ki-67: The exact nature of this nuclear antigen is unknown although it has been established similarity with the DNA polymerase alpha. The existing monoclonal antibodies anti Ki-67 detect two non-histone proteins of 395 and 345 kDa respectively. These nuclear proteins are associated with cell proliferation and are expressed in all cell cycle stages except in the G0 phase (resting phase) and the initial phase G1, in other words, are present in late G1 phase, S phase, G2 and M phase. Its expression has been used as marker of cell proliferation in tissues.

1.6.2 Genes upregulated during regeneration depending of macrophages and or cytokines

Ischemic acute renal failure (ARF) is the most common form of ARF in the adult population. The molecular mechanisms of tubular regeneration after ischemic renal injury remain largely unknown. An understanding of the mechanisms that lead to renal cell proliferation and regeneration will be necessary for the exploration of novel therapeutic strategies for the treatment of ARF. It has been suggested that regeneration processes may recapitulate developmental processes in order to restore organ or tissue function. The adult tubular epithelial cells have a potent ability of regenerate after cellular damage.

Activated macrophages are a more heterogenous group of cells than originally appreciated, with different physiologies and performing distinct immunological functions. These cells can also be considered as the centre of a complex regulatory

network receiving and distributing signals from all biological process involved from renal injury to recovery. Several experiments where carried out in order to define and identify set of genes/proteins associated with functional recovery from renal injury and to follow the basic biological process involved in renal regeneration. Those experiments where based on genomics, functional genomics and recombinant protein synthesis SMEs in fundamental biological process associated to the renal ischemia/reperfusion and regeneration processes. Results showed differentiation in the patterns of expression of several genes in terms of renal regeneration and injury. One of the most important genes implicated in macrophage depended renal regeneration is the gene named Influenza Virus Non Structural 1A binding protein (Ivns1abp).

1.7 Micro RNAs

Micro RNAs (miRNAs) are small RNAs (18-22 nucleotides) that do not code for proteins and regulate gene expression at a post-transcriptional stage (103). miRNAs regulate their targets by inhibiting translation and destabilization or degradation of messenger RNA (mRNA) by their union mainly with the 3 ´non translated region (3 ´UTR) of the mRNA (104, 105).

miRNAs play an important role in the processes of cell differentiation, proliferation, apoptosis and carcinogenesis(106).

Under normal conditions miRNA act as a regulator of gene expression in a moderate manner, but under conditions of stress or illness it presents more intense action.

MicroRNAs are produced from either their own genes or from introns. The majority of the characterized miRNA genes is intergenic or oriented antisense to neighboring genes and is therefore suspected to be transcribed as independent units. However, in some cases a microRNA gene is transcribed together with its host gene; this provides a mean for coupled regulation of miRNA and protein-coding gene (107). As much as 40% of miRNA genes may lie in the introns of protein and non-protein coding genes or even in exons of long nonprotein-coding transcripts (108). These are usually, though not

exclusively, found in a sense orientation (109, 110), and thus usually are regulated together with their host genes (108, 111, 112).

miRNA genes are usually transcribed by RNA polymerase II (Pol II) (113, 114). The polymerase often binds to a promoter found near the DNA sequence encoding what will become the hairpin loop of the pre-miRNA. The resulting transcript is capped with a specially modified nucleotide at the 5’ end, polyadenylated with multiple adenosines (a poly(A) tail), and spliced(109, 113).

Pre-miRNA hairpins are exported from the nucleus in a process involving the nucleocytoplasmic shuttle Exportin-5. This protein, a member of the karyopherin family, recognizes a two-nucleotide overhang left by the RNase III enzyme Drosha at the 3' end of the pre-miRNA hairpin (115).

In the cytoplasm, the pre-miRNA hairpin is cleaved by the RNase III enzyme Dicer (116). This endoribonuclease interacts with the 3' end of the hairpin and cuts away the loop joining the 3' and 5' arms, yielding an imperfect miRNA:miRNA duplex about 22 nucleotides in length (116). Overall hairpin length and loop size influence the efficiency of Dicer processing, and the imperfect nature of the miRNA:miRNA pairing also affects cleavage (116, 117). Although either strand of the duplex may potentially act as a functional miRNA, only one strand is usually incorporated into the RNA-induced silencing complex (RISC) where the miRNA and its mRNA target interact.

The mature miRNA is part of an active RNA-induced silencing complex (RISC) containing Dicer and many associated proteins (118). RISC is also known as a microRNA ribonucleoprotein complex (miRNP) (119); RISC with incorporated miRNA is sometimes referred to as ¨miRISC¨.

Dicer processing of the pre-miRNA is thought to be coupled with unwinding of the duplex. Generally, only one strand is incorporated into the miRISC, selected on the basis of its thermodynamic instability and weaker base-pairing relative to the other strand (120, 121, 122). The position of the stem-loop may also influence strand choice (123). The other strand, called the passenger strand due to its lower levels in the steady state, is denoted with an asterisk (*) and is normally degraded. In some cases, both strands of the duplex are viable and become functional miRNA that target

different mRNA populations (124). The biogenesis and function of microRNAs is resumed in Figure 6. In this study we will focus on mir-9 microRNA which is LPS regulated and takes part on a regulatory circuit controlling cell activation acting at the level of NFKB1, a transcriptional regulator with a key role in the inflammatory response (125).

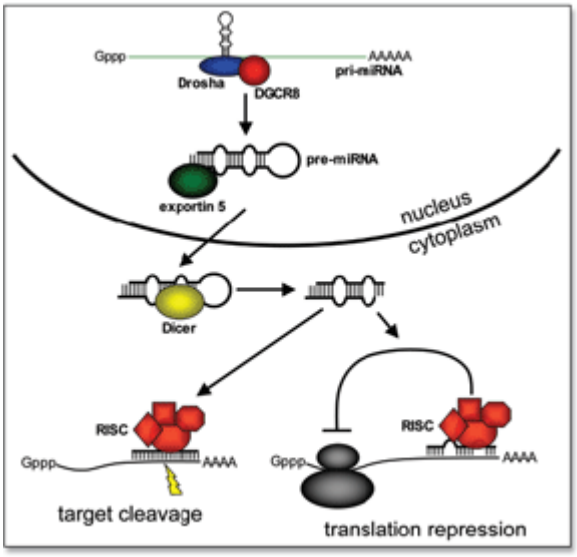


Figure 6.- microRNA biogenesis and function (adapted from www.biocat.com)

1.8 PROTEASOME

Proteasomes are protein complexes inside all eukaryotes and archaea, and in some bacteria. In eukaryotes, they are located in the nucleus and the cytoplasm (126). The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds. Enzymes that carry out such reactions are called proteases. Proteasomes are part of a major mechanism by which cells regulate the concentration of particular proteins and degrade misfolded proteins. The degradation process yields peptides of about seven to eight amino acids long, which can then be further degraded into shorter amino acid sequences and used in synthesizing new proteins. Proteins are tagged for degradation with a small protein called ubiquitin. The tagging reaction is

catalyzed by enzymes called ubiquitin ligases. Once a protein is tagged with a single ubiquitin molecule, this is a signal to other ligases to attach additional ubiquitin molecules. The result is a polyubiquitin chain that is bound by the proteasome, allowing it to degrade the tagged protein (127).

In structure, the proteasome is a cylindrical complex containing a "core" of four stacked rings forming a central pore. Each ring is composed of seven individual proteins. The inner two rings are made of seven β subunits that contain three to seven protease active sites. These sites are located on the interior surface of the rings, so that the target protein must enter the central pore before it is degraded. The outer two rings each contain seven α subunits whose function is to maintain a "gate" through which proteins enter the barrel. These α subunits are controlled by binding to "cap" structures or regulatory particles that recognize polyubiquitin tags attached to protein substrates and initiate the degradation process. The overall system of ubiquitination and proteasomal degradation is known as theubiquitin-proteasome system.

The proteasome most exclusively used in mammals is the cytosolic 26S proteasome, which is about 2000 kilodaltons (kDa) in molecular mass containing one 20S protein subunit and two 19S regulatory cap subunits. The core is hollow and provides an enclosed cavity in which proteins are degraded; openings at the two ends of the core allow the target protein to enter. Each end of the core particle associates with a 19S regulatory subunit that contains multiple ATPase active sites and ubiquitin binding sites; it is this structure that recognizes polyubiquitinated proteins and transfers them to the catalytic core.

The proteasomal degradation pathway is essential for many cellular processes, including the cell cycle, the regulation of gene expression, and responses to oxidative stress.

1.9 IVNS1ABP

Ivns1abp belongs to the kelch family proteins that contain 4-7 kelch motifs that form a propeller-like structure (128, 129). In addition to the kelch motifs, the majority of the kelch proteins contain a BTB/POZ domain (broad complex, tram track and brick a brac/Pox virus and zinc finger) that mediates protein-protein interactions (130).

Bioinformatics analysis identified at least 71 kelch proteins in the human and mouse genome, which reveals that the kelch motif is widely distributed (131). Many kelch domain containing proteins interact with actin and are important mediators of fundamental cellular functions, such as regulation of cellular architecture, cellular organization, and cell migration (132, 133, 134). More recently, it has been suggested that the kelch repeats may also bind to proteins other than actin. It has also been shown that some BTB-kelch proteins serve as a substrate specific adaptor for cullin 3 ubiquitin ligases (135, 136). Gene Ivns1abp codes for a novel actin binding protein, Nd1, which belongs to the Kelch family of proteins (137).

The Ivns1abp (Nd1) gene expresses two forms of splicing variants of the primary transcript. The long form of Nd1 (Nd1-L) contains a BTB/POZ domain in its N terminus and 6 kelch repeats in the C terminus (137). The short form (Nd1-S) has a BTB/POZ domain but lacks kelch repeats. Nd1-L mRNA is ubiquitously expressed in normal mouse tissues, most abundantly in the heart. Promoter analysis revealed that the expression of the Nd1 gene was regulated as a housekeeping gene (138). The Nd1-L mRNA level did not change throughout the cell cycle or after activation in lymphocytes. One of the basic functions of Nd1 protein is the interaction with the NS1 protein of influenza A viruses. In non infected cells, Nd1 or NS1-BP is accumulated in discrete nuclear domains which are enriched with pre-mRNA splicing factors. However, in influenza A virus infected cells, Nd1 is relocalizes throughout the nucleoplasm (139).

The human cDNA isolated through the interaction trap encoged amino acids 347 to 619 of NS1-BP, which suggests that this region contains the binding site for the NS1 protein. This part of the NS1-BP consists almost entirely of five imperfect repeats of 47 to 49 amino acids that are homologous to the kelch repeat motif (140). Based on phylogenetic sequence comparisons, it was suggested that kelch repeats take on a conserved three-dimensional fold that was previously identified in procaryotic and eucaryotic enzymes (140). A high-resolution Xray diffraction analysis for one of these enzymes, galactose oxidase of *Dactylium dendroides*, revealed that each repeat

element folds into a blade-like domain of four-stranded antiparallel b sheets. The blade-like domains are circularly arranged, resulting in a b propeller structure (141). The sequence homology suggests that the five repeats of NS1-BP may also adopt a similar three-dimensional fold. Nd1 protein also plays an important role in cell division and in the organization of the actin cytoskeleton dynamics as a stabilizer of actin filaments by the association with F-actin through Kelch repeats (137). Studies of Nd1 overexpression in the heart of transgenic mice indicated that Nd1 protects cardiomyocytes from apoptosis and improves survival index after doxorubicin injection (142).

1.9.1 Stabilization of actin skeleton

Dynamics of the actin cytoskeleton regulate a wide range of structures and functions of eukaryotic cells. Purified actin exists as a monomer and spontaneously assembles into actin filaments in the presence of salt and ATP. In the cortex of cells, actin molecules continually polymerize and depolymerize to generate cell surface protrusions such as lamellipodia and microspikes. Polymerization is regulated by extracellular signals binding to cell surface receptors that act through G proteins and the small GTPases Rac and Rho (143). Thus, the actin-based cytoskeleton is responsible for a wide range of cellular functions such as the generation and maintenance of cell polarity and cellular motility. These properties of actin filaments depend on a large retinue of actin-binding proteins that bind to actin filaments and modulate their properties and functions. Actin-binding proteins cross-link actin filaments into loose gels, bind them into stiff bundles, attach them to the plasmamembrane, or forcibly move them relative to one another. Sets of actin-binding proteins are thought to act cooperatively in generating events on the cell surface, including cytokinesis, phagocytosis, and cell locomotion.

It has been reported that BTB/POZ domains mediate homo- and heterodimerization ¨in vitro¨ and the formation of multimeric complexes ¨in vivo¨ (144, 145, 146). Is demonstrated that kelch repeats of Nd1-L directly associate with F-actin. Double immunofluorescent staining data also indicates that Nd1-L colocalizes with actin filaments in cytoplasm ¨in vivo¨. Thus, Nd1-L is an actin-associated protein (actin). It is

also shown that overexpression of Nd1-L protected against the actin disorganization induced by cytochalasin D. Cytochalasin D binds to the end of actin filaments like capping proteins and inhibits polymerization resulting in nucleation and shortening of actin filaments (147, 148).

Since Nd1-L plays an important role in cell division and stabilization of actin filaments and is ubiquitously expressed, Nd1-L may play a critical role in fundamental cellular functions such as cell division by coordinating with the dynamics of actin cytoskeleton as a housekeeping gene.

1.9.2 Ivns1abp/c-myc association

The c-Myc gene is located on human chromosome 8q24, consisting of three exons. Its transcription may be initiated at one of three promoters. Translation at the AUG start site in the second exon produces a major form of 439 amino acids; the 64 kDa c-Myc protein (also termed Myc2). Alternative translational initiation start sites result in both longer and shorter forms of the protein, termed p67 Myc (or Myc1) and MycS, respectively (149). The c-Myc sequence contains three conserved N-terminal domains, termed Myc boxes (Mb), which are also found in closely related proteins, N-myc and L-myc. The C-terminal region of c-Myc contains a dimerization motif, termed the basic helix-loop-helix leucine zipper (bHLH-LZ). The bHLH-LZ domain mediates homotypic or heterotypic dimerization with other bHLH-LZ proteins. The c-Myc dimerization domain is necessary for cellular transformation, and in 1991 the bHLH-LZ protein Max was identified as a c-Myc ob ligate partner protein (150, 151). DNA-bound Myc-Max complexes activate transcription through the amino terminal 143 amino acids of c-Myc, termed the transcriptional activation domain (TAD). A small segment of this region is also required for c-Myc mediated transcriptional repression, although the mechanism is not well understood.

In normal cells, c-Myc expression is tightly regulated in response to growth signals. It is ubiquitously expressed during embryogenesis and in tissue compartments of the adult possessing high proliferative capacity. In fact, mouse embryos in which both alleles of c-Myc have been deleted die in utero around E10.5 with severe defects in

vasculogenesis and angiogenesis (152). Non-proliferating or quiescent cells generally express non-detectable levels of c-Myc, but the gene is rapidly induced following mitogenic stimulation and, thereafter, continues to be expressed (at very low levels) in proliferating cells. Deregulated c-Myc expression is often associated with aggressive, poorly-differentiated tumours. Activation of c-Myc gene occurs in several ways. In many tumours, c-Myc activation can be attributed to structural alterations affecting the c-Myc gene, including chromosomal translocations, retroviral promoter or enhancer insertions, and gene amplification.

Expression of the c-Myc proto-oncogene is induced as a consequence of signalling pathways downstream of a variety of mitogens and cytokines such as 49 platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) (153, 154, 155). The components of the signal transduction pathways which drive c-Myc transcription have not been fully elucidated, yet studies have identified a few of the players such as protein kinase C (PKC), protein kinase A (PKA), src, E2F, ets, and abl (156, 157, 158, 159, 160, 161, 162). It can be concluded that c-myc is a gene regulated by cytokines and transcription factors.

The Ivns1abp gene promoter is regulated by a series of transcription factors and miRNAs. By using common internet sources such as ¨Genecards¨ (www.genecards.org) we were able to identify the most important of those. According to this particular data base, the most relevant transcription factors that bind to the promoter of the Ivns1abp gene were: c-myc, AhR, Max1, C/EBPbeta, etc. There are also certain miRNAs that can interact and regulate Ivns1abp mRNA such as miR-4291, mir-9 and let-7a-2.

Since our experiments are based in the regulation of the expression of Ivns1abp in macrophages, which is mainly carried out by cytokines, we decided to focus on the interaction of c-myc transcription factor with the Ivns1abp promoter, since this transcription factor is regulated by the presence of certain cytokines.

2. HYPOTHESIS AND OBJECTIVES

2.1 HYPOTHESIS

After ischemic damage in kidney, alternatively activated or M2 macrophages are key players in resolving inflammation and participating in kidney repair. Obtaining this particular phenotype is a process controlled by deregulation of a series of genes. Modulation of those genes and in particular of the Ivns1abp gene results in controlling macrophages phenotype and functions, aiming new approaches in resolving ARF.

2.2 OBJECTIVES

1. Identifying a set of repair genes are related to the presence of macrophages M2, involved in tissue repair.
2. Demonstrate that modulation of Ivns1abp gene, the inflammation and renal repair could be modulated.
3. Define pathways regulating the transcription of Ivns1abp gene.

3. MATERIALS AND METHODS

3.1 EXPERIMENTAL MODELS

3.1.1 Models Description

Both the experiments and the pretreatment of the animals were carried out according to the standards set by the Council of the European Union for the use of experimental animals (86/609/ EU) and the ministry of the Presidency (Royal Decree 1201/2005, BOE No. 252 of 21/10/2005 on the protection of animals used for experimental and other scientific purposes). The animals used were male mice of the Swiss strain CD1 (Charles River) weighing between 25 and 30 grams, housed in the Faculty of Medicine of the UB a week before the operation. The environmental conditions were kept constant, the temperature was between 21-22 ^{0}C, relative humidity of 70% and the alternative cycles of light / dark were of 12 hours each. The animals were fed a standard feed AO4 (Panlab) and water from the Barcelona network ad libitum throughout the experiment.

3.1.2 Ischemia/Reperfusion surgical procedure

- **Anesthesia**

The operations were performed under inhalation anesthesia (Fluorane, Abbot Laboratories). The anesthesia was induced with 4% isoflurane with an oxygen flow of 2.5 to 3 l/min. Anesthesia in the animals was maintained by inhalation of 1.5-2% of isoflurane and oxygen flow of 2-2.5 l/min. Oxygen flow was kept constant throughout the operation (41).

- **Ischemia / Reperfusion**

Once anesthetized, the animals were placed in supine position on the operating table. The surgical field was washed with povidone-iodine and transverse laparotomy was performed 1cm below the xiphoid appendix. The intestinal mass was moved aside in order to have a clear view of the kidneys and bilateral ischemia was induced by occlusion of the renal arteriovenous pedicle using non-traumatic microvascular clamps (Aesculap). Ischemia was verified by the blackening of the kidneys, which represents

the disruption of blood flow. Flow was restored after 45 minutes of ischemia, with the removal of the clamps (reperfusion) and it was checked visually. Control animals were treated the same way but without occluding the renal pedicles. During the process, hydration and body temperature of the animals was monitored by covering their abdominal areas with gauze with saline at 37 ^{0}C and plastic in order to minimize dehydration of exposed tissues. After ischemia the animals were kept under observation (41).

3.1.3 Sample collection

After the reperfusion period (varies according to the study group), the kidneys were removed and divided in four parts. Three of them were introduced directly on dry ice for storage at -80 ^{0}C for biochemical and molecular processing, the fourth part was induced in buffered 4% paraformaldheyde for subsecuent histological processing. Blood samples were collected with a heparinized syringe and kept at 4 ^{0}C until being centrifuged 10 minutes at 3000 rpm to obtain plasma which was stored at -80 ^{0}C (41).

3.1.4 Macrophage depletion / macrophage administration

CD1 Swiss mice were anesthetized with isoflurane prior to administration of liposomes and RAW 264.7 macrophages.

- **Liposome preparation**

The clodronate (dichloromethylene diphosphate, CL2MBP), a biphosphate commonly used for metabolic or bone diseases, is a nontoxic drug itself. Free clodronate can not pass easily through the phospholipid bilayers of liposomes and cell membranes, but the liposomes are phagocytoced by macrophages, which implies that clodronate once introduced into the phagocytic cells via liposomes, it cannot escape. Once inside the macrophage, lipid bilayer is digested by lysosomal phospholipases and the drug is released and dissolved in the aqueous compartment between the bilayers. Clodronate is accumulated intracellularly and when it exceeds a certain consentration, the cell is irreversibly damaged and dies by apoptosis (163). Free clodronate, released from dead

macrophages, has a very short half-life in the circulation and it is eliminated by the renal system.

Clodronate containing liposomes were prepared according to the method of van Rooijen et al (1994), which consists of a mixture of phosphatidylcholine (Sigma) and cholesterol (Sigma), resuspended in 0.7 M solution of clodronate (Sigma) that is sonicated for producing multiamellar liposomes. The liposomes were washed several times by ultracentrifugation to remove unencapsulated clodronate and resuspended in sterile PBS for intravenous injection. For PBS liposomes, the lipids are mixed with sterile PBS (41, 163).

- **Macrophage administration**

Mice received 100µl / 10g of weight of clodronate or PBS liposomes 24 hours prior to ischemia (163). For the injection are used the lateral tail veins after having anesthesized the mouse. These are dilated with alcohol or heat, <25 G needles are used and inoculated volume is 0.3 ml or less.

3.1.5 Femur extraction procedure

Mice were sacrificed by inhalation of isofluorane. The abdomen and hind legs were sterilized with antimicrobial wipes and 70% ethanol. Upon an incision in the midline of the abdomen, the hind legs were exposed. All muscle tissue was removed from the bones by the use of scissors. Afterwards, femurs were cut on both ends to free them and were maintained in Hank's buffered solution on ice. Isolated bones were transferred to a tissue culture plate and were cut on both ends under the hood in order to expose the bone marrow. Bones were then flushed into a sterile non-adherent culture pot with GM-CSF supplemented complete culture medium using a 10-ml syringe and a 25-gauge needle. Bone marrow cells were pipeted up and down in order to bring the cells into single cell suspension. Bone marrow-derived cells from mice were then cultured as described (1.2.1.). For the adoptive transfer studies, bone marrow-derived macrophages were transduced with adenoviral vectors to express either Ivns1abp or the control gene β-gal (164).

3.2 CELL CULTURES

3.2.1 Bone Marrow Derived Macrophages (BMDMs) culture

Mouse bone marrow derived cells were isolated by aspiration of femur and resuspended in DMEM 1:1 F-12 nutrient supplement with high glucose, supplemented with 10% heat-inactivated fetal bovine serum, 10ng/ml Granulocyte-macrophage colony-stimulating factor (GM-CSF), 100U/ml penicillin and 100 µg/ml streptomycin and were kept in non-adherent culture flasks (Roland Vetter Laborbedarf, Ammerbuch, Germany) to mature for 7 days. Macrophages were subsequently isolated by adherence to normal plastic cell culture flasks. Cells were kept in a humidified atmosphere of 5% CO2 in air at 37°C (165).

3.2.2 RAW 264.7 culture

RAW 264.7 murine macrophages (obtained from ECACC, Sigma) where cultured in bottles (25 cm^2) in Dulbecco's Modified Eagle Medium (DMEM), F-12 nutrient 1:1 with glucose, 15 mM Hepes and L-glutamine, supplemented with 100 units / ml penicillin, 100 µg / ml streptomycin and 10% (v / v) fetal bovine serum (FBS) (Invitrogen). The cells where maintained in an atmosphere of 5% CO_2 on air, at 37 ^{0}C. Cells were left to reach 70-80% of confluence.

Before the intravenous injection, cells were collected, counted and kept in PBS until injection into the animal. 100 x 10^6 cells / animal were administrated by the tail, one day before sacrifice.

3.2.3 NRK–52e culture

The rat renal epithelial cell line NRK-52e (a kindly gift from Prof. Dr. Josep Maria Grinyó, Ciutat Sanitaria i Universitaria de Bellvitge (ICS), Hospital de Bellvitge, Servei de Nefrología, Barcelona) was used for *¨in vitro¨* experiments. This cell line was cloned from a mixed culture of normal rat kidney cells and has epithelial-like morphology (166). NRK-52e cells were passaged at 90% confluence. Usually, the cells were split two to three times per week by removing the consumed medium and the addition of 1mM

EDTA/0.025% trypsin (Invitrogen, Barcelona, Spain) for 5 minutes to detach the cells from the flask. To stop the reaction, a determined amount of serum containing fresh medium was added and afterwards the cells were separated by pipetting. The cells were centrifuged at 1200 rpm for 5 minutes at a sparing manner. The resulting pellet was then resuspended in culture medium and transferred to a new flask.

3.2.4 NRK–52e damage assay

We use colchicine as a tool for reducing cell proliferation. This substance was selected by its characteristic in reducing proliferation by inhibiting S-phase DNA synthesis without compromising the viability of the culture neither dramatically increase cell death (for results, see additional data). In order to decrease proliferation of NRK-52e cells without compromising their viability, 50 µM colchicine (Sigma, Madrid, Spain) was added for 24 hours. After this time and verification of cell culture viability, colchicine was removed and replaced by fresh medium. Different stimuli were then applied for another 24 h (164).

3.2.5 RAW 264.7/NRK-52e co-culture

In co-culture experiments, previously damaged NRK 52e renal epithelial cells (bottom) were separated from macrophages (top) by 0.4-µm pore Polyester Membrane Transwell inserts (Corning, Madrid, Spain). Cells were seeded at a ratio of 1.5 to 1 macrophages to epithelial cells and co-cultured for 24 hours. At the end of the culture, the number of live cells was determined by trypan blue exclusion and Alamar Blue viability assay. Cells were then processed separately for gene expression determination, and supernatants were collected to assess protein release.

3.3 STUDY GROUPS

3.3.1 Study 1: Identification of genes implicated in renal regeneration after Ischemia / Reperfusion and related to macrophages.

- **Control (C):** In control group, animals undergo identical manipulation as the I/R group, but without clamping the renal pedicles. The kidney was removed from the animal without undergoing any type of surgical procedure or drug and it was immediately frozen with dry ice or included in formalin for further processing
- **Ischemia / Reperfusion (I/R):** Animals subjected to 45 minutes of bilateral ischemia followed by 24, 48 or 96 hours of reperfusion.
- **Ischemia/ Reperfusion + clodronate liposomes (I/R + Lipo-Cl_2MBP):** The same procedure as in I/R groups, but with macrophage depletion by clodronate liposomes administrated by tail one day before ischemia.
- **Ischemia / Reperfusion + clodronate liposomes + Macrophages RAW264.7 (I/R + Lipo-Cl_2MBP + MΦ):** Animals with the same treatment as group I/R + Lipo-Cl_2MBP but treated with RAW 264.7 cells administrated by the tail 24 hours before sacrifice. RAW 264.7 cells were either control (not treated at all) or silenced for certain genes.

3.3.2 Study 2: Role of Ivns1abp gene in the modulation of the inflammation and renal repair

- **Sham:** Control group where animals undergo identical manipulation as the I/R group, but without clamping the renal pedicles.
- **Sham + MΦ - Ivns1abp:** Animals control treated with RAW 264.7 cells , over-expressing Ivns1abp, administrated by the tail 24 hours before sacrifice.
- **I/R :** Animals subjected to 45 minutes of bilateral ischemia followed by 48 hours of reperfusion.

- **I/R + MΦ - Ivns1abp:** Animals subjected to 45 minutes of bilateral ischemia followed by 48 hours of reperfusion and treated with RAW 264.7 cells, transfected with a vector that enhances the expression of Ivns1abp gene, administrated by the tail 24 hours before sacrifice.
- **I/R 48h + MΦ - GFP :** Animals subjected to 45 minutes of bilateral ischemia followed by 48 hours of reperfusion and treated with RAW 264.7 cells, transfected with a vector that expresses GFP, administrated by the tail 24 hours before sacrifice.
- **Resting MΦ:** Untreated Macrophages
- **MΦ + LPS / cytokines:** Macrophages treated with LPS or cytokine cocktail (TNF-α, IFN-γ)
- **MΦ - Ivns1abp:** Macrophages over-expressing Ivns1abp
- **MΦ - GFP:** Macrophages expressing GFP
- **MΦ siIvns1abp :** Macrophages transfected with a vector that silences Ivns1abp expression
- **MΦ control shRNA:** Macrophages transfected with an empty vector
- **NRK:** Untreated renal epithelial cells NRK52-e.
- **NRK+Col:** Epithelial cells were treated with 50 µM of colchicine for 24 hours.
- **NRK+Col+MΦ:** Damaged NRK-52e cells were co-cultured with unstimulated macrophages for 24 hours.
- **NRK+Col+MΦ+shRNA:** Damaged NRK-52e cells were co-cultured with macrophages transfected with an empty vector.
- **NRK+Col+MΦ siIvns1ab:** Damaged NRK-52e cells were co-cultured with macrophages transfected with a vector that silences the expression of Ivns1abp gene.

3.3.3 Study 3: Regulation of the transcription of the Ivns1abp gene

- **Resting MΦ:** Untreated Macrophages

- **IFN-γ / IFN-γ+TNF- α / LPS / LPS+ IFN-γ / LPS+ IFN-γ+ TNF- α:** Macrophages treated with those specific cytokines or cytokine cocktails.
- **MΦ + cytokines:** Macrophages incubated for 24 hours with TNF-α and IFN-γ.
- **MΦ + IL-10:** Macrophages incubated for 24 hours with IL-10
- **MΦ mir-9:** Macrophages over-expressing microRNA mir-9
- **MΦ + F4 100 µm:** Macrophages treated for 24 hours with 100 µm of the c-myc inhibitor 10058-F4
- **MΦ - Ivns1abp + F4 100 µm:** Macrophages over-expressing Ivns1abp treated for 24 hours with 100 µm of the c-myc inhibitor 10058-F4
- **MΦ - Ivns1abp + cytokines:** Macrophages over-expressing Ivns1abp treated for 24 hours with TNF-α and IFN-γ.

3.4 CELLULAR TRANSFECTION

3.4.1 Adenoviral vectors

Adenoviral vectors were applied in order to transduce macrophages *ex vivo*. Viral vector systems are based on their ability to deliver genetic information into the host cell. Adenoviral vectors in particular offer the most effective means of *ex vivo* gene transfer of transient gene transfer and have the advantage that they can be produced in high titers, incorporate transgens up to 30kb, and transduce both quiescent and proliferating cells.

The adenoviral vectors carrying cDNA encoding recombinant Ivns1abp, β-Gal and GFP were elaborated, amplificated, and purified by ViraQuest, Inc (North Liberty, IA, USA).

3.4.2 Adenoviral transfection

Cells were collected, counted and seeded to obtain $1x10^6$ cells per well in 6-well plates (Sarstedt) and cultivated for 24 hours. After this time period different eppendorf tubes were prepared with 800µl of culture medium at 2% FBS and 1µl of poly-l-lysine was added to each tube. Ivns1abp, β-Gal or GFP adenovirus were added compared to the group. Control groups were treated only with poly-l-lysine. The virus- poly-l-lysine

solution was incubated for 45 minutes at room temperature on a shaker. Meanwhile, wells were washed with PBS solution and in each one was added 200 µl of culture medium at 2% FBS. After the 45 min incubation was over, the solution was added to the previously prepared cells. Virus transduction was induced by centrifugation at 1000xg for 30 minutes at 4ºC. After 24 hours of incubation (37ºC, 5% CO_2 supernatant was discarded and replaced with complete DMEM medium (165).

3.4.3 mir-9 transfection

In order to increase the miRNA copies of mir-9 microRNA in BMDMs, the following nucleotides where transfected into the cells (Table 1).

	Sequence 5'→3'		**Final concentration**
hsa-miR-9	5'-AUAAAGCUAGAUAACCGAAAGU-3'	Applied Biosystems	100 nM

Table 1.- Oligonucleotides used for the immitation of let-7 and mir-9 miRNAs

Oligonucleotides were administrated to cells using Lipofectamina 2000TM (Invitrogen) following the protocol recommended by the manufacturer. Briefly, oligonucleotide and lipofectamine were incubated separately during 15 min at 37 ^{0}C in standard culture medium without FBS or antibiotics in order to prevent interference with the efficiency of the transfection. Then, oligonucleotides and lipofectamine solutions were mixed and incubated for another 15 minutes at room temperature. After this period, supernatant of the cells was removed and it was added the lipofectamine-oligonucleotide complex followed by 6 hours incubation at 37 ^{0}C and 5% CO_2. After that, the medium was replaced with a complete standard culture medium (167).

3.5 BIOCHEMICAL DETERMINATIONS

3.5.1 Protein concentration

The proteins were determined by Bradford colorimetric method with a commercial reagent from Bio-Rad. This assay is based on the reaction of an acidic solution of Coomassie blue dye in response to various concentrations of protein. Protein concentration in the sample is directly proportional to the absorbance observed at a wavelength of λ= 595 nm. As a calibration curve was used an albumin solution which its highest concentration point was 4.375 μg / ml. Starting from this concentration, 6 serial dilution were made by dilution in half of each solution (167).

3.5.2 Creatinine and BUN levels

The levels of both substances in plasma were measured with the multichannel analyzer ADVIA 2400 (Siemens Medical Diagnostics) in Biomedical Diagnostic center of Hospital Clinic of Barcelona (165).

3.6 HISTOLOGY

3.6.1 Hematoxylin and Eosin stain

This histological staining applies mainly in order to describe the state of the tissue from a morphostructural point of view. Eosin is a basophyl dye which stains pink the cytoplasmatic components, while hematoxylin because of its acidophilus nature, stains the nuclei in blue-purple. Immediately after extraction, the samples were fixed in 4% buffered formaldehyde and subsequently embedded in paraffin. 4 sections were prepared with an ultramicrotome and stained with H & E (41).

3.6.2 Immunofluorescence

- **¨in vivo¨ samples**

The stathmin and PCNA immunofluorescence were prepared as previously described (210-211). 4 μm sections were deparaffinized, washed in PBS and unmasked in sodium citrate pH 6.0. The sections were blocked with 10% goat serum and 0.3% of Triton 40X in PBS. Sections were incubated with polyclonal anti-stathmin (Calbiochem), which binds to de-differentiated epithelial cells, mitotically active (212,213) and monoclonal anti-proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology), which marks cells in G1, S and G2 phases of cell cycle, followed by incubation with secondary antibodies Rabbit anti-goat IgG Alexa Fluor 488 (Molecular Probes) for anti-stathmin and Goat IgG anti-mouse Alexa Fluor 546 (Molecular Probes) for anti-PCNA, for 2 hours at room temperature.

Immunofluorescent staining of macrophages was obtained by overnight incubation at 4 ^{0}C with monoclonal anti-mouse CD68 (Serotec) which binds to monocytes and macrophages. The sections were washed in PBS and as secondary antibody was used goat anti-mouse IgG conjugated with Alexa Fluor 546 (molecular probes) for 2 hours at room temperature. The sections were washed three times for 5 minutes with PBS and finally mounted with Mowiol (Calbiochem). Sections were examined with a Leica Nt laser microscope (Leica Microsystems) with 40X objective under oil inmersion. Visual examination was performed (in an average of 8 fields x400) for the quantification of cell proliferation, by counting the number of PCNA and stathmin positive cells per 100 cells counted and the density of macrophages infiltrated in the outer medulla (41).

- **¨in vitro¨ samples**

Matured bone marrow-derived macrophages from both strains were seeded at a density of 1x106 cells on a coverslip. Cells were then fixed in 3.7% paraformaldehyde for 10 minutes, washed with PBS, permeabilized with acetone for 5 minutes on ice, and blocked with goat serum (Sigma, Madrid, Spain) for one hour at room temperature. Cells were washed twice with PBS and incubated with Alexa

Fluor 568 phalloidin staining (Invitrogen) 0,5 Units/100ul in 1% PBS/BSA buffer for 30 minutes at room temperature. After washing with PBS, samples were incubated with

DAPI solution (0.2 μg/mL) (Sigma-Aldrich) for 5 minutes. After that time is passed, cells were washed three times with PBS. The preparation was mounted with mowiol (Calbiochem, Madrid, Spain) and cells were viewed using a Leica TCS NT laser microscope (Leica Microsystems, Wetzlar, Germany).

3.7 QRT-PCR

3.7.1 Primer design

Primer design is a critical step in the application of PCR-based technologies in gene expression analysis. Primers were designed according to site specific exon-intron sequences in order to obtain an amplicon between 150 and 200 bp, each specific for the gene of interest and thus avoiding the amplification of unspecific, similar sequences.

By the use of serial dilutions of a target DNA, specific conditions for maximal efficiency of each primer were tested, including concentration of primers, optimum annealing temperature, rounds of amplification cycles, and exclusion of the formation of primer-dimers or unspecific amplifications. These parameters were analysed by studying the melt curve of each PCR product.

Common internet sources for the design of the primers were used:

♦ NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez)

♦ Primer 3 (http://frodo.wi.mit.edu/)

♦ Operon (http://www.operon.com/default.aspx)

Primers were synthesized from Invitrogen (Barcelona, Spain).

Additionally, commercially available pre-validated primers from Qiagen (Madrid, Spain) and Applied Biosystems (Madrid, Spain).

Additionally, commercially available pre-validated primers from Qiagen (Madrid, Spain) and Applied Biosystems (Madrid, Spain). Table 2 shows selected primers used in the study.

Gene (Accesion number)	Source	Primer Sequence	Amp. Length, bp
GAPDH (NM_008084)	Invitrogen	For. 5'-TGA-AGC-AGG-CAT-CTG-AGG-C-3'	102
		Rev. 5'-CGA-AGG-TGG-AAG-AGT-GGG-AG-3'	
Stathmin (NM_019641)	Invitrogen	For. 5'-CTT-GCG-AGA-GAA-GGA-CAA-GC-3	200
		Rev. 5-CGG-TCC-TAC-ATC-GGC-TTC-TA-3'	
PCNA (NM_011045)	Invitrogen	For. 5'-AAT-GGG-GTG-AAG-TTT-TCT-GC-3	173
		Rev. 5'-CAG-TGG-AGT-GGC-TTT-TGT-GA-3'	
KI-67 (NM_001081117)	Invitrogen	For. 5'-CAG-TAC-TCG-GAA-TGC-AGC-AA -3'	170
		Rev. 5'-CAG-TCT-TCA-GGG-GCT-CTG-TC-3'	
TNF-α (NM_013693)	Invitrogen	For. 5'-AAC-TCC-CAG-AAA-AGC-AAG-CA-3'	212
		Rev. 5'-CGA-GCA-GGA-ATG-AGA-AGA-GG-3'	
Rn_Mannose Receptor (NM_001061231)	Invitrogen	For. 5' - CGA-GGT-GGT-TTA-TGG-GAT-GT- 3'	111
		Rev. 5' - GGG-TTC-AGG-AGT-TGT-TGT-GG- 3'	
iNOS (NM_010927.3)	Qiagen	QuantiTect Primer Assay for SybGreen detection	
IL-10 (NM_010548)	Qiagen	QuantiTect Primer Assay for SybGreen detection	103
Ivns1abp (NM_001039511.1)	Applied biosystems	TaqMan Gene ExpressionAssay	91
GAPDH (NM_008084.2)	Applied biosystems	TaqMan Gene ExpressionAssay	98
mir-9 (AJ459704.1)	Applied biosystems	TaqMan Gene ExpressionAssay	
c-myc (AFO76523)	Applied biosystems	TaqMan Gene ExpressionAssay	138

Table 2.- Primers used for RT-PCR

3.7.2 Total RNA extraction

Total RNA from cells was isolated using the RNeasy mini kit following the manufacturer's protocol (Qiagen, Barcelona, Spain). Briefly, cells were first lysed and homogenized with a highly denaturating buffer, which inactivates RNases to ensure purification of intact RNA. Afterwards, ethanol is added in order to obtain optimum binding conditions to the silica-based membrane Mini spin column, which contains very selective binding properties. Cell lysate was added to the column, RNA binds to the membrane, and contaminants are efficiently washed out. Thereafter, RNA was eluted in 50µl of RNase-free water. Total kidney RNA was isolated from homogenized

tissue with TRIzol Reagent (Invitrogen) according to manufacturer's instructions. Briefly, approximately 50 mg of renal tissue was homogenized in 1 ml of TRIzol Reagent using a high power homogenizer. Following homogenization, insoluble material was removed by centrifugation at 12000xg for 10 minutes at 4ºC. The resulting pellet contains extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernatant contains RNA. Afterwards, phases were separated adding chloroform and centrifuged at 12000xg for 15 minutes at 4ºC. The mixture separates into three phases, and RNA remains exclusively in the aqueous phase. RNA was then precipitated by the addition of isopropyl alcohol. After centrifugation, RNA was visible as a gel-like pellet. Supernatant was removed and RNA was washed once with 75% ethanol at 7500xg for 5 minutes at 4ºC. The resulting pellet was briefly dried and then resuspended in 50µl of RNase-free water.

RNA concentrations were calculated from A260 determinations using a Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). As there could be any impurities and contaminations of proteins in the sample, we also determined A280 in order to calculate A260/A280. A number of 1.8-2.0 was determined as optimum. Purity and quality of extracted RNA were further evaluated using the RNA 6000 LabChip and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Only high-quality RNA with RNA integrity numbers (RINs) greater than 7.5 were used for the experiments (167).

3.7.3 cDNA synthesis

- **cDNA synthesis from mRNAs**

Complementary DNA (cDNA) was synthesized by using the iScript cDNA synthesis Kit from Bio-Rad (Barcelona, Spain) according to the recommend protocol from the manufacturer table 3 (165).

cDNA Synthesis	Final Volume (20 µl)
5x iScript Reaction Mix	4 µl
iScript Reverse Transcriptase	1 µl
Nuclease-free water	15 µl
RNA template (500 ng)	15 µl

Table 3.- Protocol for cDNA synthesis

Briefly, reverse transcriptase is used to generate a DNA copy of a range of different input RNAs. This enzyme is an RNA-dependent DNA polymerase isolated from a retrovirus (MMLV) and is pre-blended with RNase inhibitors. By adding the iScript Reaction Mix, which should contain all four deoxynucleotidetriphosphates, magnesium ions, and at neutral pH, the reverse transcriptase synthesizes a complementary DNA according to the mRNA target. Conditions were optimized to yield 1 µg of cDNA using a conventional thermocycler (MyCycler; Bio-Rad, Barcelona, Spain) applying the protocol described in table 4.

Cycle	Temperature, ^{0}C	Time, min	Function
1	25	5	
2	42	30	Retrotranscription
3	85	5	Deactivation RT
4	4	∞	

Table 4.- Protocol for RT-PCR

- **cDNA synthesis from miRNAs**

Complementary DNA (cDNA) of our interest was synthesized from total RNA using the TaqMan microRNA Assay (Applied Biosystems) following the protocol recommended by this commercial (table 5) (167).

cDNA synthesis	Final Volume (15 µl)
dNTP mix (100 mM total)	0.15 µl
MultiscribeTMRT enzim (50 U/µl)	1 µl
10 x RT Buffer	1.5 µl
Inhibidor RNase(20 U/µL)	15 µl
RNAse free water	4.16 µl
Primer (hsa-let-7e / SU6)	3 µl
RNA (10 ng)	5 µl

Table 5.- Protocol for cDNA synthesis from miRNA

The synthesis of cDNA from miRNA is different compared to the synthesis explained to the previous section (from mRNA) since in this case a small primer with a loop that facilitates union, specific for each RNA of interest is used.

The reaction conditions were optimized using a Mycycler thermocycler (Bio-Rad) following the procedure described in table 6.

Cycle	Temperature, ^{0}C	Time, min
1	16	30
2	42	30
3	85	5
4	4	∞

Table 6 .- Protocol for RT-PCR for miRNA

3.7.4 Real Time PCR

Quantitative RT-PCRs were performed on a Bio-Rad iCycler iQ Real-Time-PCR detection system using either SYBR Green RT-PCR detection Kit (Bio-Rad, Madrid, Spain) or pre-validated TaqMan probes (Applied Biosystems, Madrid, Spain) according to manufacturer's instructions and following the below described protocol for amplification (Table 7). Accuracy of reproducibility of real-time PCR data is given due

to collection of quantitative data at a point in which every sample is in the exponential phase of amplification. The analysis of reactions at a given number of cycles provides several orders of magnitude of dynamic range. We used two of the currently four available detection methods for real-time PCR, all based on the generation of the generation of a fluorescent signal (165).

Cycle	Cycle Description	Repeats	Step	Temperature, ^{o}C	Time
1	Denaturalization	1	1	95	3 min
2	Hybridization and				
	Extension	30-40	1	95	15 sec
			2	48-60	20 sec
			3	72	15 sec
3	Melt curve	80	1	55	10 sec
				+ 0.5 ºC/cycle	

Table 7 .- PCR protocol

TaqMan probes are specific oligonucleotides with a fluorescent reporter dye attached to the 5' end and a quencher moiety coupled to the 3' end. The technique is based on the 5'-nuclease activity of the DNA polymerase which hydrolyzes oligonucleotides that are hybridized to the target amplicon, thus decoupling the fluorescent dye. Consequently, the fluorescent signal increases with each cycle, proportional to the amount of cleaved probe. Samples were prepared as shown in table 8.

TaqMan	
Taqman (IQ supermix)(Bio-Rad)	10 µl
TaqMan primer and probe	1 µl
RNAse free water	8 µl
cDNA	1 µl
Final Volume	20 µl

Table 8 .- Reaction mix for TaqMan probes

Detection with SYBR Green instead is based on binding of double-stranded DNA, which, upon excitation emits light. Therefore, if a PCR product accumulates, fluorescence will increase. A disadvantage of this technique may be that SYBR Green binds to any double-stranded DNA, including primer-dimers and other non-specific reaction products. For this reason, primers should be very well design, the melting curve should be interpreted with caution, and a previous optimization study should be conducted. Samples for SYBR Green detection were prepared as described in Table 9.

SYBR Green	
2 x SYBR Green Supermix	10 µl
10 nM forward primer	1 µl
10 nM reverse primer	1 µl
RNAse free water	7 µl
cDNA	1 µl
Final volume	20 µl

Table 9 .- Reaction mix for SYBR Green detection

3.8 Phagocytosis Assay

- **Microscope**

In order to examine the phagocytic function of BMDM cells, phagocytosis assay tests were performed. Briefly, BMDM cells after being differentiated for 7 days and seeded to 6-well plates, were stimulated during 24 hours, either with DMSO (1:1000) or with TNF-a (60ng/ml, 3µl/ well from Stock 20µg/ml), IFN-γ (100U/ml, 1µl/ Stock 100U/µl) or c-myc Inhibitor (1:1000, 100µM, 1µl/well). Then medium was removed and FCS free media with 10µM CMRA was added and incubated with the cells for 15-45 min. Then, the dye working solution is replaced with fresh, prewarmed medium and cells were incubated for another 30 min at 37°C. Fluorescent carboxylate beads (2µm diameter, Invitrogen, Carlsbad, CA) were incubated for 1 hour with the cells. After the incubation was ended, cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 15 minutes at room temperature. Cells were washed with PBS and incubated with Hoechst Farbstoff (0.2 µg/ml 1:5000) for 7 min. After washing with PBS and water,

samples were mounted with vectashield and viewed using a Leica TCS NT laser microscope (Leica Microsystems, Wetzlar, Germany) (168).

- **FACS**

Samples, after being incubated with the fluorescent carboxylate beads as dicribed above, were washed extensively with PBS and 500 µl of Accutase per well was added during 5 min in order to detach the cells. Detachment of the cells was complete using a 1ml pipet. Samples were placed in FACS tubes containing 1-2 ml of PBS to stop accutase reaction. 500 µl of the sample was used for RNA analysis while the rest of it was centrifuged (5 min, 500 g, 4 ^{o}C) to remove PBS and accutase and blocked during 15 min using Fc Block MIX (0.5µl FcBlock in PBS). After blocking, F4/80 Antibody PBS mix (1µl/ sample in 100µl PBS) was added and cells were analyzed by flow cytometry (168).

3.9 Apoptosis measurements

3.9.1 Proteasome

10 µg of total protein extract was used in order to measure proteasome 26S activity ATP-dependent chymotrypsin activity of the proteasome was measured using the substrate N-Suc-Leu-Leu-Val-Tyr-aminomethylcoumarin (ENZO Life Sciences, Madrid, Spain). The cleavage products AMC were analyzed with a fluorimeter (excitation/emission 380/460 nm). Product formation was linear with time (at least for 60 min). Background activity (caused by nonproteasomal degradation) was determined by addition of the proteasome inhibitor epoxomicin at a final concentration of 20 µM (ENZO Life Sciences, Madrid, Spain) (169).

3.10 Microarrays

To identify signatures in gene expression profiles associated to a specific treatment in order to prevent renal ischemia-reperfusion associated injury, we used the microarray technique to examine the gene expression patterns in kidney tissue homogenate from

different experimental groups. Preparation of digoxigenin-labelling, hybridization, and microarray imaging were performed at the Genomic Unit of the "Centro Nacional de Investigaciones Cardiovasculares Carlos III" (CNIC, Madrid, Spain) under the supervision of Ana Dopazo.

3.10.1 Preparation of digoxigenin-labelled ¨in vitro¨ transcribed cDNA

All RNA targets were labelled using the Applied Biosystems RT-IVT Labelling Kit Version 2.0. Briefly, 1.5 µg of the total RNA sample was reverse transcribed via 2 h incubation at 42ºC with ArrayScript RT enzyme (Ambion, Austin, TX, USA) and oligo dT-T7 primer. Double-stranded cDNA was produced following 2 h incubation with E. coli DNA polymerase and RNase H at 16ºC. Double-stranded cDNA was purified according to the RT-IVT kit protocol. *¨in vitro¨* transcription was performed by incubation of the cDNA product with T7 RNA polymerase, 0.75 mM digoxigenin-11-UTP (Roche Applied Science, Indianapolis, IN, USA) and all other NTPs for 9 h. Labelled cRNA was purified according to the RT-IVT kit protocol and analysed for quality and quantity using standard UV spectrometry and the Bioanalyzer(167).

3.10.2 Hybridization of labelled cDNA to microarrays and microarray imaging

Digoxigenin-labelled cRNA targets were hybridized to Applied Biosystems Rat Whole Genome Survey Microarrays using the Applied Biosystems Chemiluminescent Detection Kit. Briefly, 15 µg of labelled cRNA targets were fragmented via incubation for 30 min at 60ºC with fragmentation buffer provided in the kit. Fragmented targets were hybridized to microarrays during 16 h of incubation at 55ºC with buffers and reagents from the Chemiluminescent Detection Kit. Post-hybridization washes and anti-digoxigenin-alkaline phosphatase binding were performed according to the kit protocol. Chemiluminescence detection, image acquisition and analysis were performed using the Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer following the

manufacturer's protocols. Images were autogridded and the chemiluminescent signals were quantified, corrected for background and, finally, spot- and spatially-normalized using the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer software version 1.1. Gene expression profiles were obtained from all experimental groups in three biological replicates (167).

3.10.3 Data Analysis

All data analyses were carried out by Genedata AG (Basel, Switzerland). Data were imported into Genedata Expressionist® Pro Analyst and were filtered by setting the S/N ratio ≥ 2. The medians of the expression values of the experiments range between 3792 and 6926. Data were therefore normalized to a median of 5000 excluding the controls on the microarray and using only the transcripts with a S/N ratio ≥ 2. The principal component analysis showed that experimental groups are clearly separated from each other. The evaluation of increases or decreases in gene expression was performed using an unpaired two class t-test. Results for comparison pairs are expressed as a fold change. Statistical significance was assigned at a minimum 1.5-fold change and $p < 0.05$. Hierarchical clustering of samples was performed by calculating the Spearman correlation coefficient within the filtered set of transcripts. Differences were considered to be significant at a P value <0.05 according to the two way ANOVA test.

4. RESULTS

STUDY 1: Identification of genes implicated in renal regeneration after Ischemia / Reperfusion and related to macrophages.

- **Determination of Injury and Recovery phase**

Ischemia/reperfusion injury is an important cause of acute renal failure in native kidneys and allografts but cellular and molecular or even genetic responses of the kidney to recover from acute ischemic injury are complex and not fully understood. In this study we aimed to identify set of genes associated with functional recovery from Ischemic / Reperfusion renal injury.

The first experiments in this study reinforce previous data reported by our group that previously demonstrated an early injury phase characterized by massive macrophage recruitment and a later recovery phase with a low recruitment. Therefore was clear that the I/R insult presents two different phases; phase of injury and phase of recovery. The start point data of this study (Figure 7) confirms that during the phase of 24 hours of reperfusion after ischemia, markers of renal function such as BUN were significantly increased compared to control levels while levels of regeneration were kept low. This phase can be considered as injury. On the other hand, during the late phase of 96 hours of reperfusion, the level of the BUN is reaching the level of the control group whereas the level of regeneration markers reaches its highest expression point. Therefore, this phase is considered as recovery.

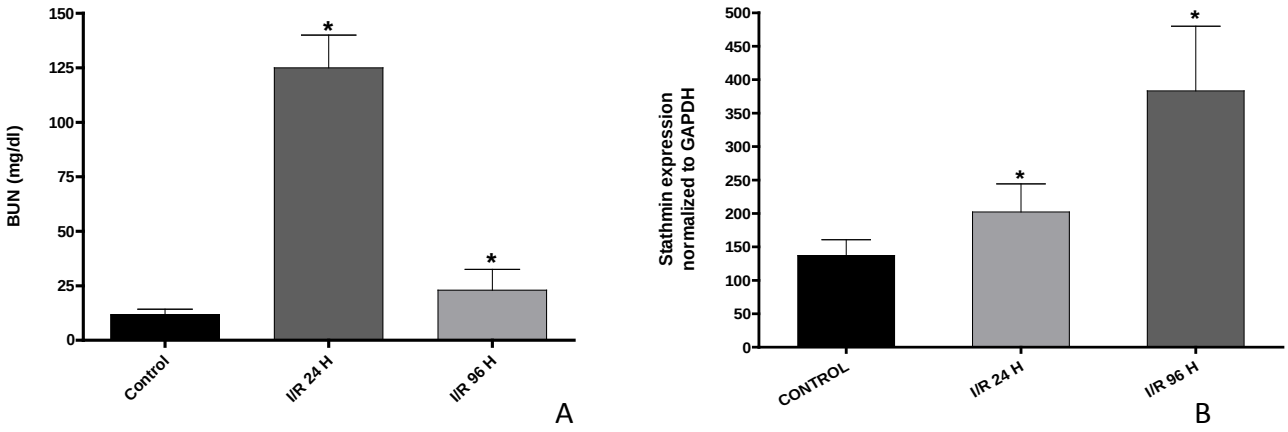


Figure 7. level of BUN (renal function marker) and Stathmin (regeneration marker) in 24 and 96 hours of reperfusion after 45 min of ischemia in mice. BUN levels in plasma increase at 24 hours of reperfusion and return to the levels of control group at 96 hours of reperfusion, while in stathmin levels occur the opposite effect. Data is represented as mean ± SEM. $*p < 0.05$ versus C; n = 5

To confirm the implication of macrophages during those two phases we used the techniques of macrophage depletion and/or macrophage transfer in mice (Figure 2).

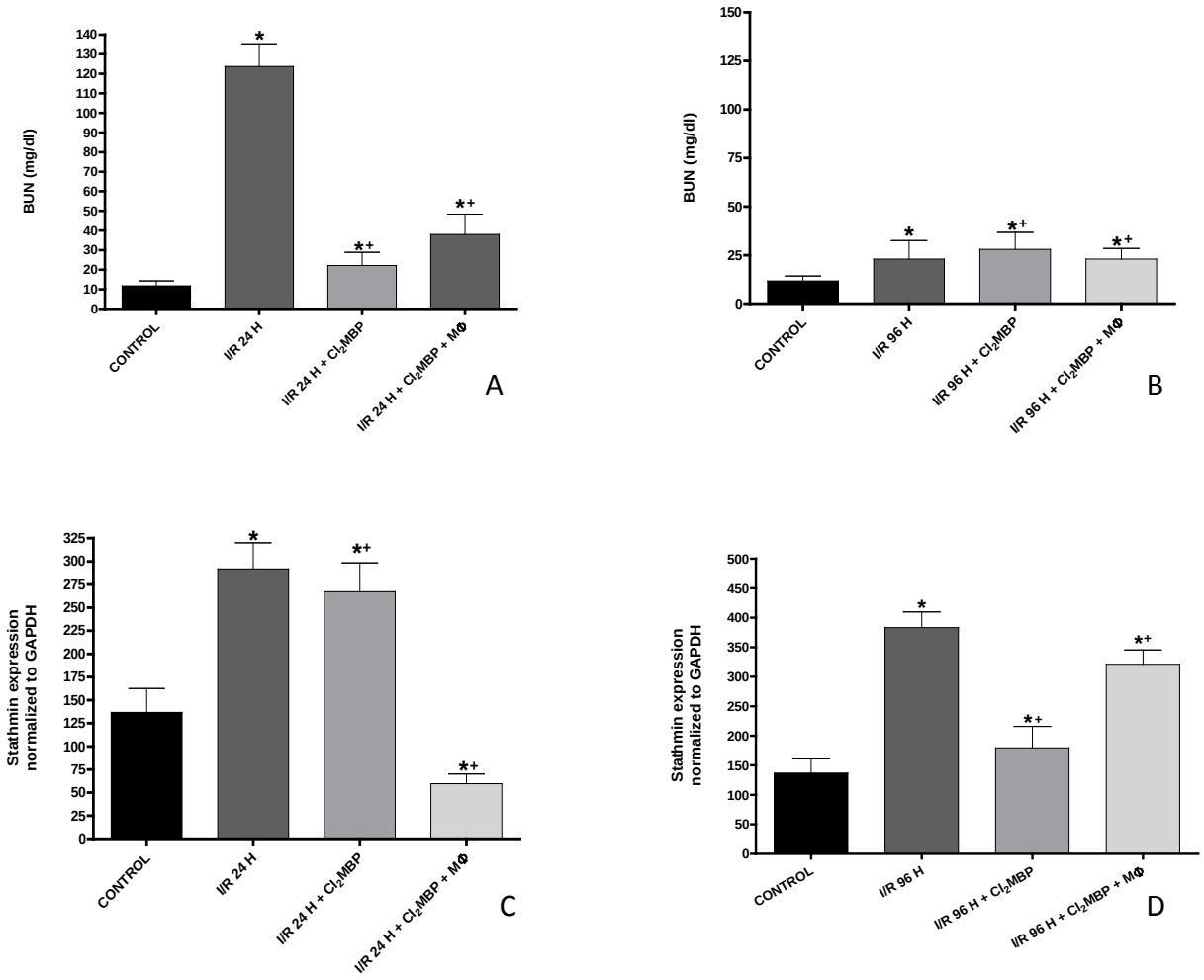


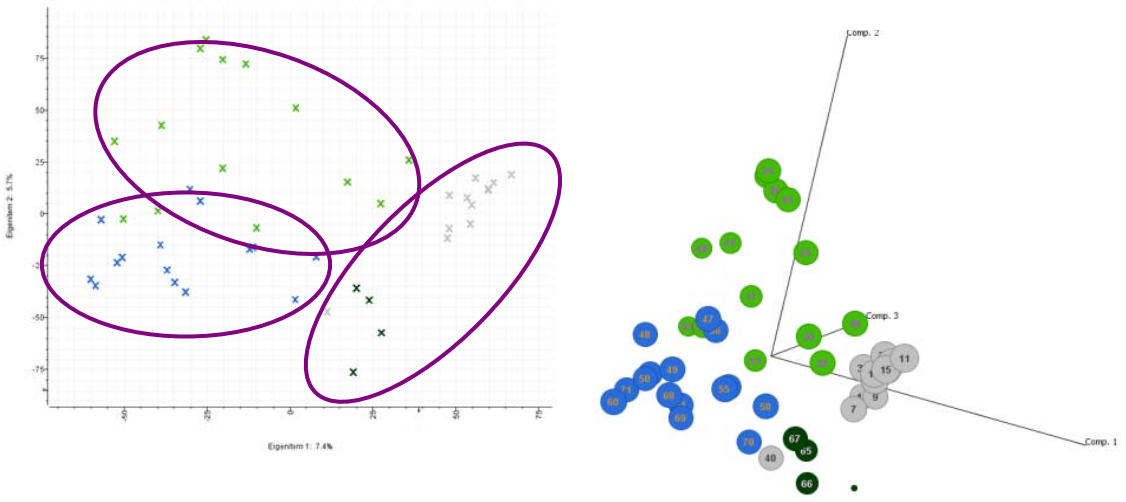
Figure 8. Macrophage participation in renal function (BUN) in control and I/R injured kidneys in mice A) sacrificed 24 hours or B) 96 hours after reperfusion. Stathmin mRNA expression in control and I/R injured kidneys in mice C) sacrificed 24 hours or D) 96 hours after reperfusion. Data is represented as mean ± SEM. ∗$p < 0.05$ versus C; †$p < 0.05$ versus I/R 24 h n = 5

The results indicated that serum BUN increased in I/R groups with respect to control animals and macrophage depletion by lipo-Cl2MBP decreased urea levels (Figure 8A). On the other hand, depletion and addition of macrophages during the phase of recovery (96 h time point) doesn't have any particular effect on the BUN level (Figure 8B) but the absence of macrophages during this phase has an important effect on decreasing regeneration (Figure 2D) while the depletion of macrophages during the 24 hour time point has no particular effect on the levels of tissue regeneration measured by stathmin (Figure 8C).

- **Microarrays**

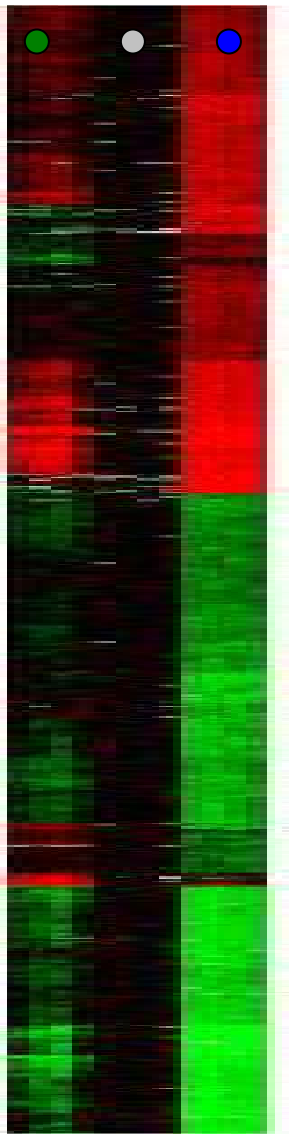
To identify gene expression profiles in each of the previous groups, we used the technique of microarray. Gene expression profiling was performed in kidney tissue homogenate in each of the experimental groups through the system ¨Applied Biosystems 1700 Chemiluminescent Microarray analyzer¨. The data obtained was processed by ´Genedata¨, an experienced company in experimental design, statistical analysis and interpretation of molecular profiling studies. Overall, the experiment included groups treated with clodronate liposomes in order to deplete macrophages, groups in which macrophages were added after depletion, groups treated with PBS liposomes and finally, groups not treated at all.

The above conditions were applied in mice control, and in mice that a 45 min ischemia was performed followed by 24 or 96 hours of reperfusion. Figure 9A, 9B and 9C provides us with a representative image of the gene expression profiles.



A B

C



Control groups

24h ischemia/reperfusion group

96h ischemia/reperfusion group

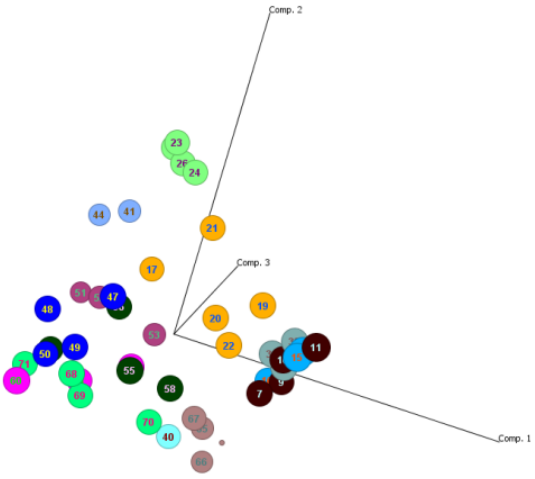
Figure 9. Coloring the experiments according to the treatments (control, 24 hours of reperfusion and 96 hours of reperfusion) shows a very clear treatment-specific separation of gene expression.

Results demonstrate that the all the subgroups that undergo the same treatment, present a similar pattern of gene expression, corresponding to this particular treatment, without any significant deviation.

Figure 10 shows a representative image of the analysis of the genetic data of each sub-group of every category and comparing its expression profile to the other sub-groups of the same group and the sub-groups of the different groups. The groups that we performed this data analysis are shown in table 10:

	Study Groups
	Control
	Control + liposomes
	I/R 24 hours
	I/R 96 hours
	I/R 24 hours + liposomes
	I/R 96 hours + liposomes
	I/R 24 hours + liposomes + macrophages
	I/R 96 hours + liposomes + macrophages
	Control + PBS liposomes
	I/R 24 hours + PBS liposomes
	I/R 96 hours + PBS liposomes

Table 10. Study groups used for 2D hierarchical clustering



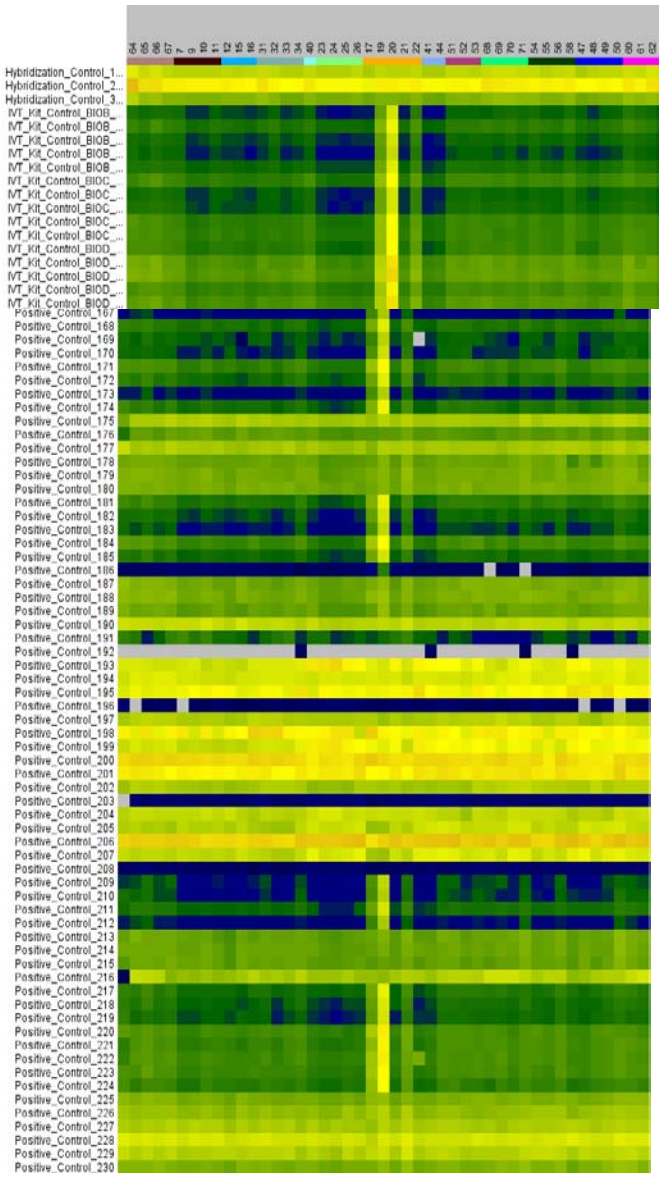


Figure 10. 2D hierarchical clustering of control groups and groups of 24h and 96 h of I/R after adding liposomes and/or macrophages

Clustering the transcripts from the combined results of the above groups after normalizing the data against the median the control groups, 2D hierarchical clustering of the groups described above, showed a large number of deregulated genes among them. As a result of the informatic processing of the data 7313 genes were found deregulated.

Based on the characterization of the model in terms of injury and regeneration and after the analysis and comparison of the data performed by ¨Genedata¨, which is an experienced company in statistical analysis, we focused on choose a number of genes that were upregulated or downregulated as a function of macrophage presence. Therefore we established four ¨blocks¨ of study for further comparison seen in table 11.

Injury Phase	
A. Effects of internal macrophages at 24 hours	Deregulated genes: **110**
B. Effect of external macrophages at 24 hours	
Recovery Phase	
C. Effects of internal macrophages at 96 hours	Deregulated genes: **38**
D. Effects of external macrophages at 96 hours	

Table 11. Study blocks of groups during injury and recovery phase.

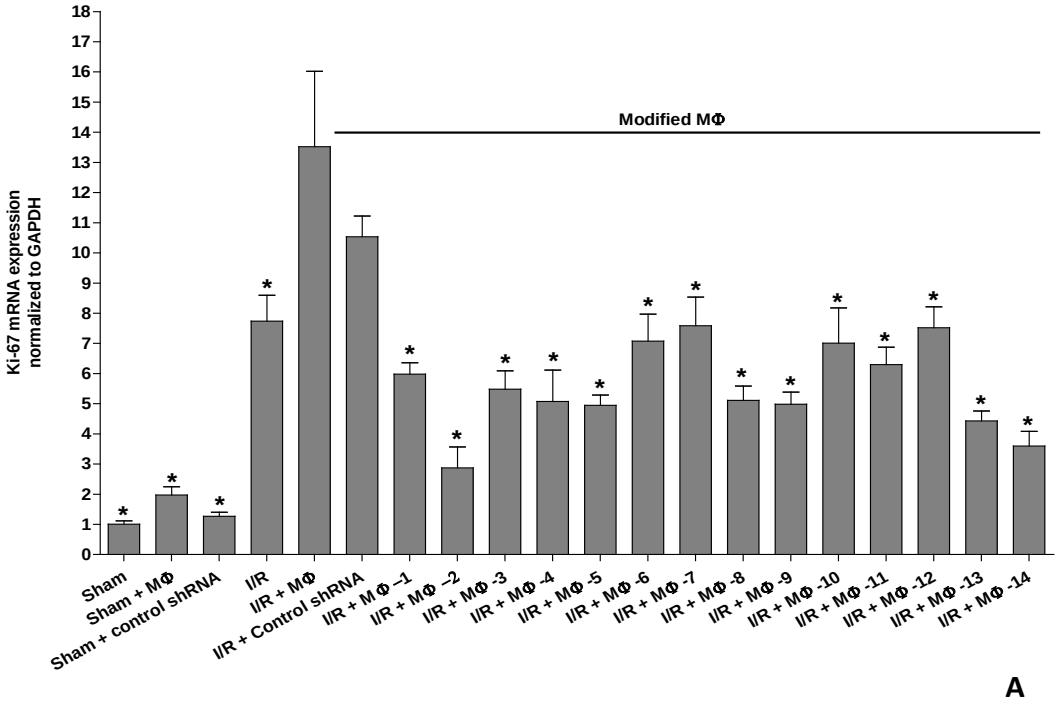
Finally, between the 148 deregulated genes we selected the genes that were inhibited in groups A and B but at the same time, overexpressed in groups C and D. We then reached a number of 14 genes (Table 12).

	Gene name		Gene name
1	BC036333	8	Ivns1abp
2	Nrp1	9	Casp3
3	Ricen cDNA 2610204 k14	10	Ino80D
4	Sfrs1	11	LOC676347
5	Ubtf Cpt1a	12	Malat1
6	Zfp820	13	Hnrpa3
7	Cpt1a	14	Nedd4

Table 12. Transcripts that were inhibited during the phase of injury but also upregulated during the phase of recovery.

The last step was performing an Assay test to validate the effect of those 14 genes to tissue proliferation. To this end, we performed cell therapy experiments using silenced macrophages for each one of the genes into control mice as well as in mice that undergo ischemia and reperfusion. In order to have a clearer result, we decided to administrate macrophages in a time point that corresponds in the beginning of the regeneration phase and not in the point that this phase is already well established. The selected time-point of study was 48 hours of reperfusion.

Results showed us that the silencing of those 14 genes provokes a significant decrease in renal regeneration, when compared any of them to the effect of untreated macrophages (Figure 11A, 11B).



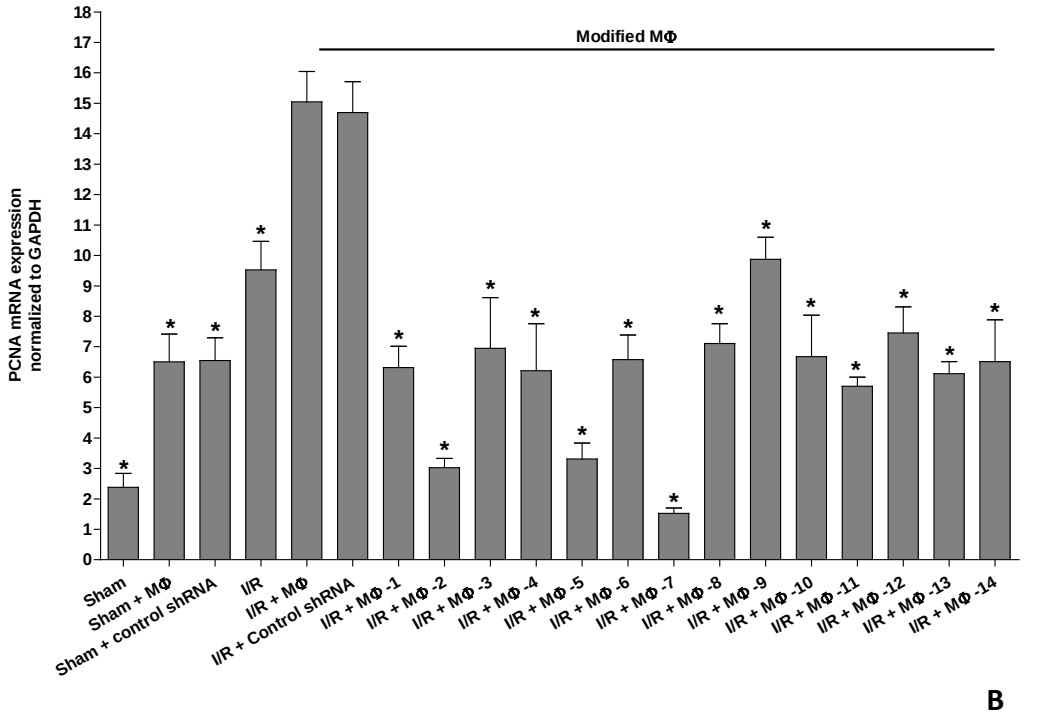


Figure 11. Tissue proliferation was evaluated by the expression levels of Ki-67 A) and PCNA B) mRNA using Real-Time RT-PCR. The Figure shows the effect of the administration of each of the genetically modified macrophages on proliferation and renal regeneration. Data is represented as mean ± SEM. ∗p < 0.05 versus I/R+Mϕ; n = 5

After the validation of these 14 genes, the first one selected for deeper study in the implication in renal regeneration was the gen Ivns1abp which is the subject of this thesis.

STUDY 2: Role of Ivns1abp gene in the modulation of inflammation and renal repair

In order to study whether this gene plays a role in renal regeneration through transformation of macrophage phenotype from M1 to M2 we proceeded in manipulating this gene in macrophages, study its behavior and its reparative capacity.

For that reason we decided to use as a tool for our study, macrophages that overexpress the Ivns1abp gene. Mouse Ivns1abp cDNA ORF – GFP tagged was cloned into a CMV vecror and it is expressed under the control of a CMV promoter. The vector was produced by ¨Viraquest¨ which is a biotechnology company specializing in the production of adenoviral vectors.

Before proceeding in any experiment, we established the conditions that this specific vector functions the best. In order to achieve that, we used a vector that carries GFP instead of Ivns1abp. Firstly, in order to measure transfection efficiency, macrophages were transfected with this control virus carrying the gene coding for the GFP protein. After trying different virus concentrations (MOIs), results show that the most effective concentration was the 250 MOI (Figure 12).

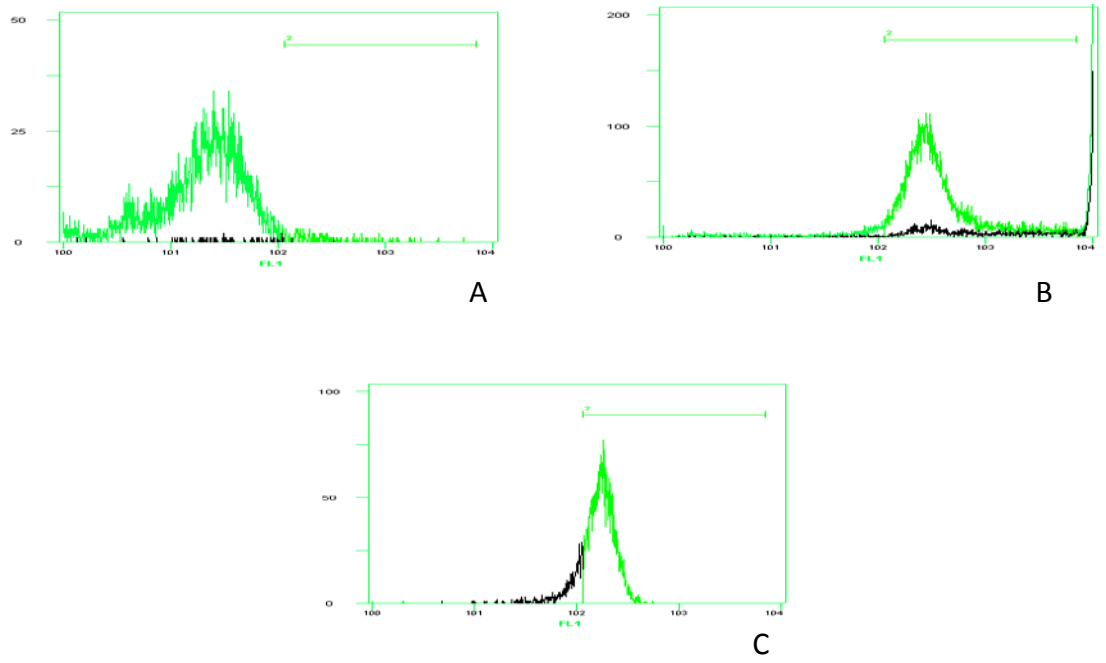


Figure 12. GFP transfection efficiency on macrophages measured by flow cytometry. A. MOI 0, 1.3% GFP positive, B. MOI 80% GFP positive, C. MOI 250 84% GFP positive

Results show that the most effective concentration for the GFP vector is the MOI of 250. After defining that, same experiment was held but this time using the adenovirus carrying Ivns1abp in order to establish the most appropriate MOI for carrying on our experiments. The efficiency of over-expression of each group was measured by determining the mRNA by RT-PCT and flow cytometry (Figure 13A, B and C).

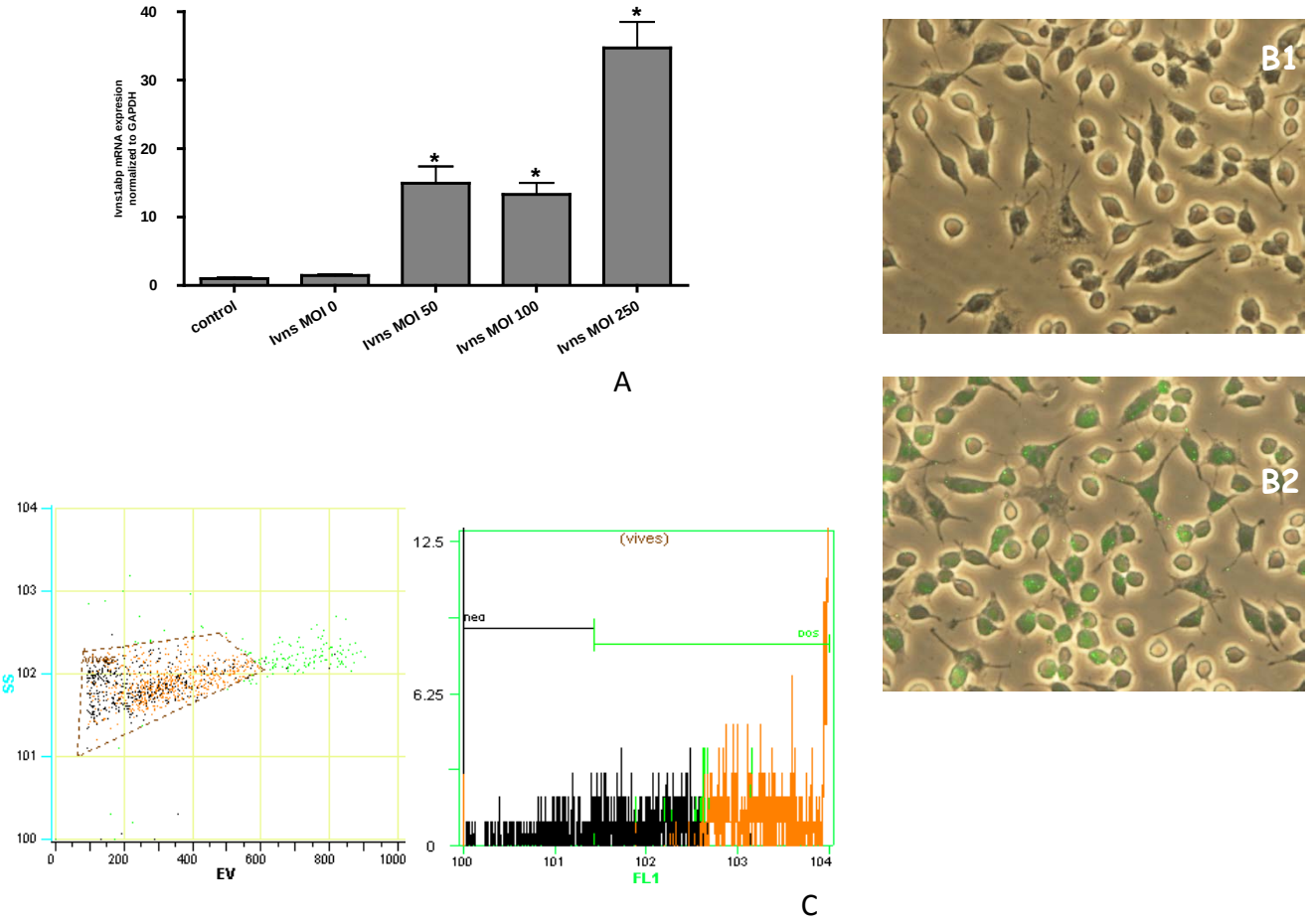


Figure 13. A) Expression of Ivns1abp in macrophages transfected with different MOI of adenovirus measured by RT-PCR. Control cells (B1) and cells transfected with Ivns1abp vector MOI 250 (B2). Ivns1abp transfection efficiency on macrophages measured by flow cytometry (MOI 250, 85% GFP positive) Data is represented as mean ± SEM. ∗p < 0.05 versus Control; n = 5

Same as before, the most efficient adenovirus treatment was the one in which was used a MOI 250. In this group expression of Ivns1abp gene was almost 35-fold

upregulated compared to the control group and almost an 85% of the total cell number is transfected with their vector.

Next, we also determine the most effective time point of Ivns1abp transfection. In this line we carried out a 6, 12, 24 and 48 hour transfection experiment in RAW264.7 and murine bone marrow derived macrophages (BMDMs). Data showed that the most efficient time point for RAW264.7 cells is 12 hours of transfection (Figure1 4A), while for BMDM cells the time point changes to 24 hours of transfection (Figure 14B). Since both cell types were presenting a higher transfection efficiency at 24 hours, we decided to equalize to this time point for further experiments.

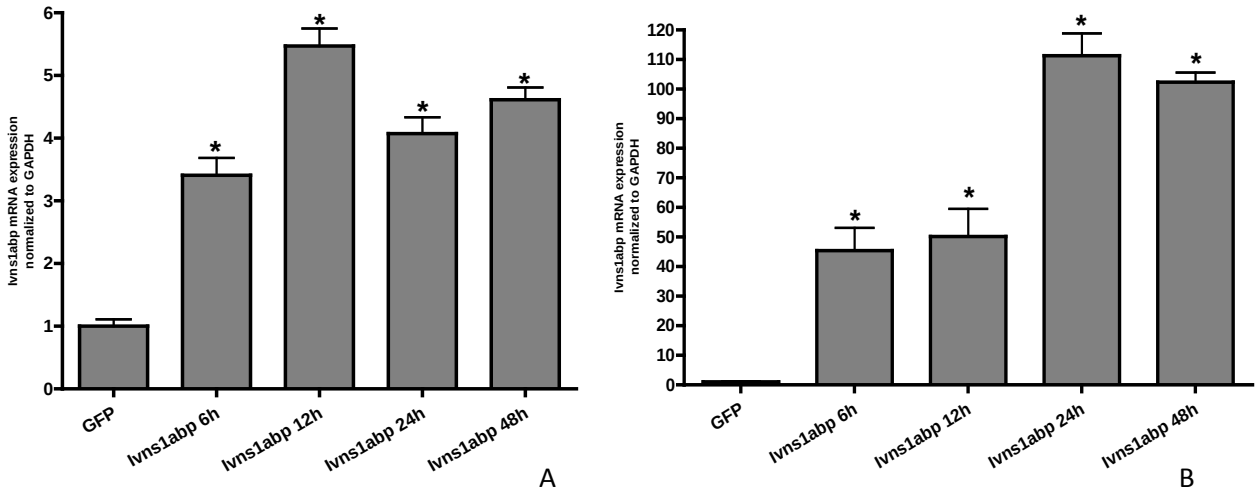


Figure 14. Ivns1abp expression in different time points in A) RAW264.7 cells and B) in BMDM cells Data is represented as mean ± SEM. ∗p < 0.05 versus GFP; n = 5

- **Cell therapy with Ivns1abp overexpression enhances tissue proliferative markers.**

Once established the conditions under of which our tool, macrophages overexpressing Ivns1abp, can function the best we proceeded in performing an assay test in order to study whether Ivns1abp gene upregulation plays a role in renal regeneration through transformation of macrophage phenotype from M1 to M2. That fore we proceeded in its overexpression in macrophages and its administration in mice.

As seen in Figure 15A, administration of macrophages overexpressing Ivns1abp, increases alone the level of regeneration measured by stathmin, while macrophages

that carry the control GPP vector cannot affect the level of the regeneration in the tissue. The same result is obtained when the experiment is repeated using different markers of regeneration such as Ki-67 (Figure 15B) and PCNA (Figure 15C), indicating the importance of this concrete gene during tissue regeneration.

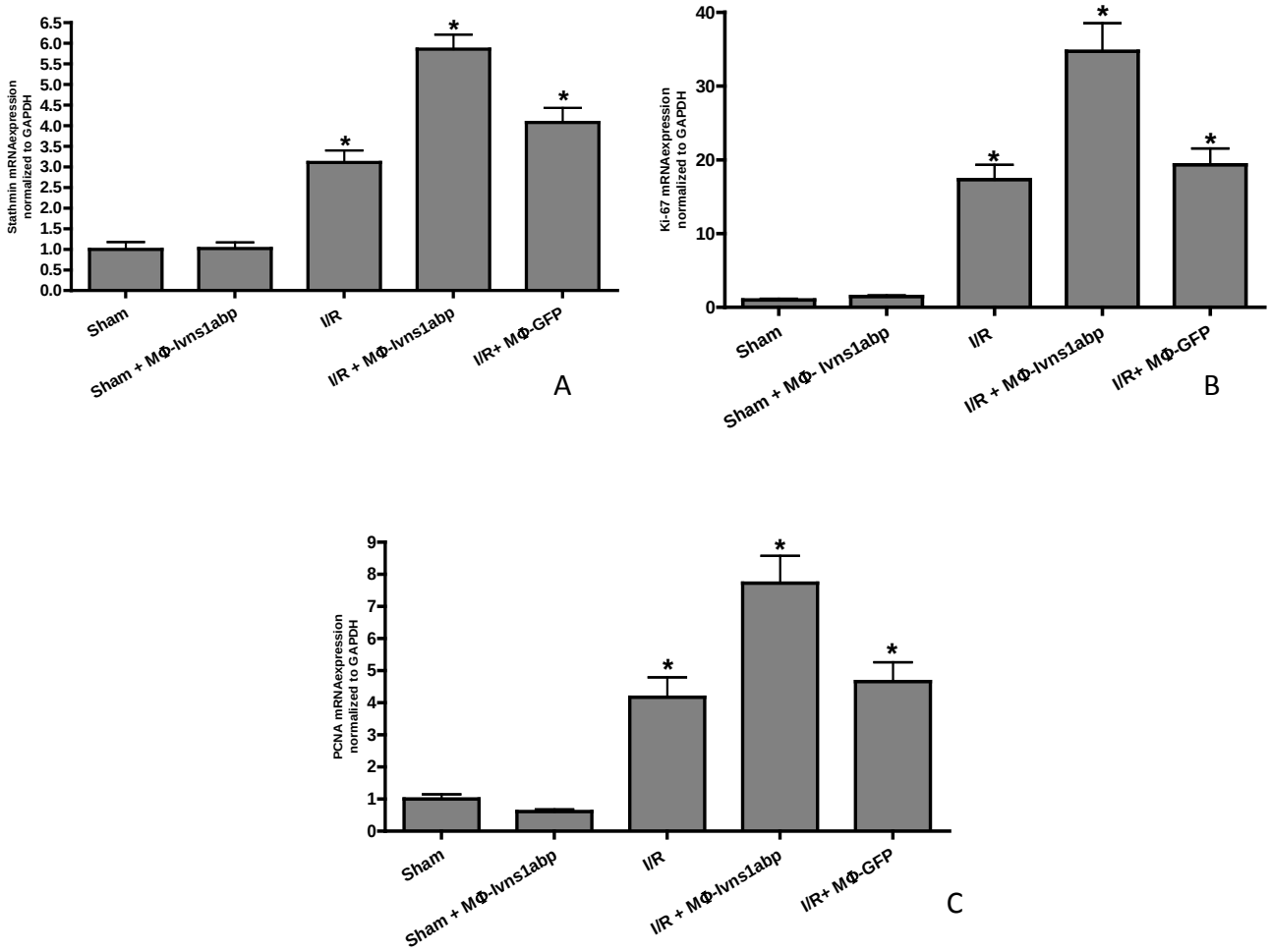


Figure 15. Expression of regeneration markers in animals control and in animals injected with modified macrophages that over expresses Ivns1abp and/or GFP. A) Stathmin, B) Ki-67 and C) PCNA Data is represented as mean ± SEM. ∗$p < 0.05$ versus Sham; n = 5

- **Ivns1abp upregulation modulates macrophage inflammatory state**

In order to exam the inflammatory state of the macrophages over-expressing Ivns1abp, we examined through RT-PCR the levels of expression of cytokines produced

by macrophages. Figure 16A shows the expression of TNF-α, a pro-inflammatory cytokine in control groups and macrophages that over-express Ivns1abp. A significant downregulation of TNF-α is observed in macrophages overexpressing Ivns1abp compare to control group and to macrophages overexpressing GFP. After measuring the expression iNOS, which is an enzyme that is highly expressed in pro-inflammatory environments, same results are obtained (Figure 16B). On the other hand, when IL-10, an anti-inflammatory molecule is measured, macrophages that over-express Ivns1abp present higher levels of expression of this particular cytokine (Figure 16C). The same result is obtained when we measured manose receptor, an other anti-inflammatory indicator (Figure 16D).

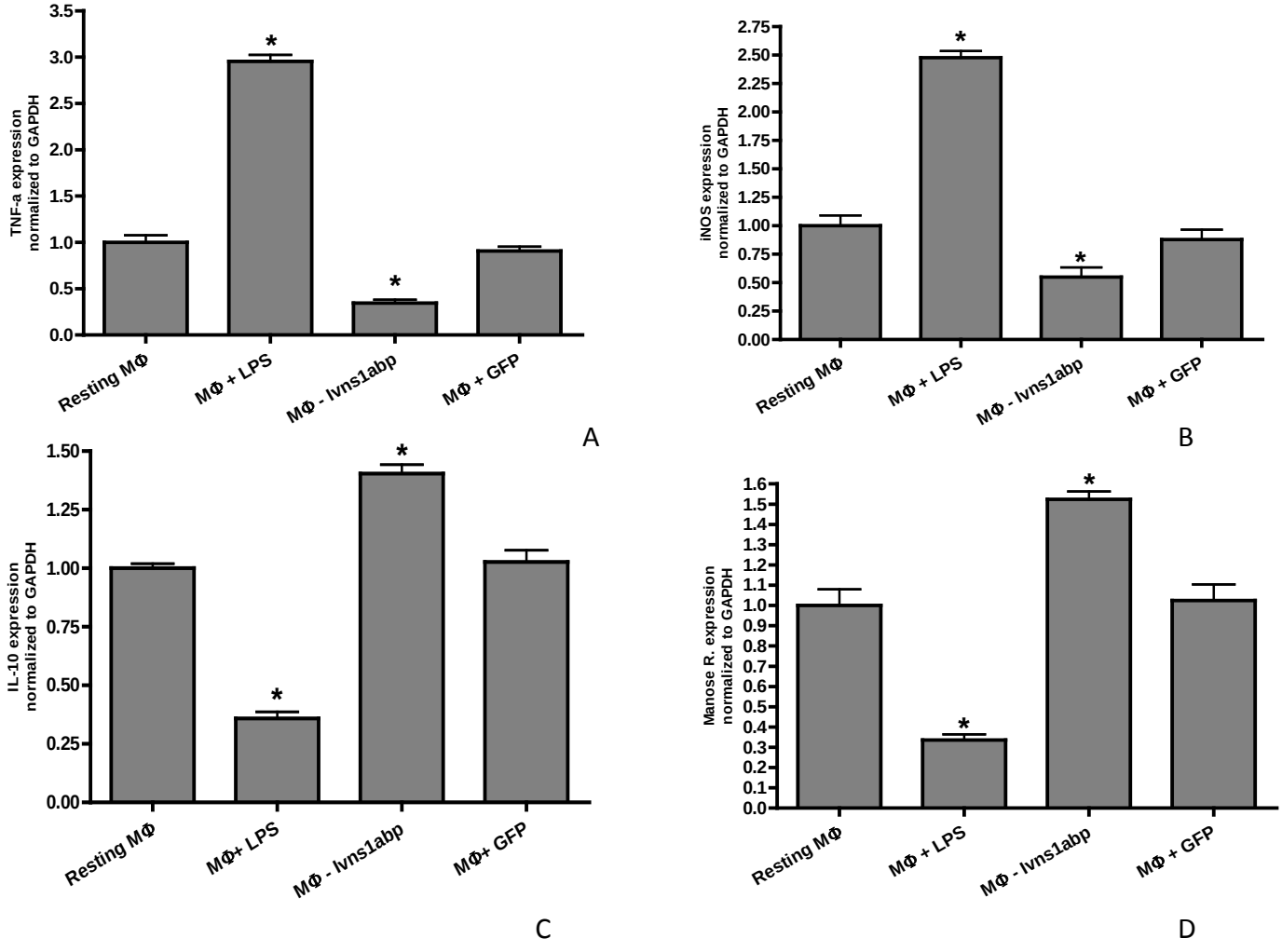


Figure 16. Expression of A) TNF-α, B) iNOS, C) IL-10 and D) Manose Reseptor in macrophages control and macrophages transfected with Ivns1abp or GFP adenovirus Data is represented as mean ± SEM. ∗p < 0.05 versus Resting Mϕ; n = 5

After the above, we performed phalloidin staining in the same groups. Figure 17A shows control macrophages having a rather round phenotype with actin all around the cytoplasm, same as macrophages transfected with GFP adenovirus (Figure 17B).

On the other hand, macrophages over-expressing Ivns1abp adopt a more extended morphology with actin forming filaments throughout the whole cytoplasm,morphology which is characteristic of the M2 macrophages (Figure 17C).

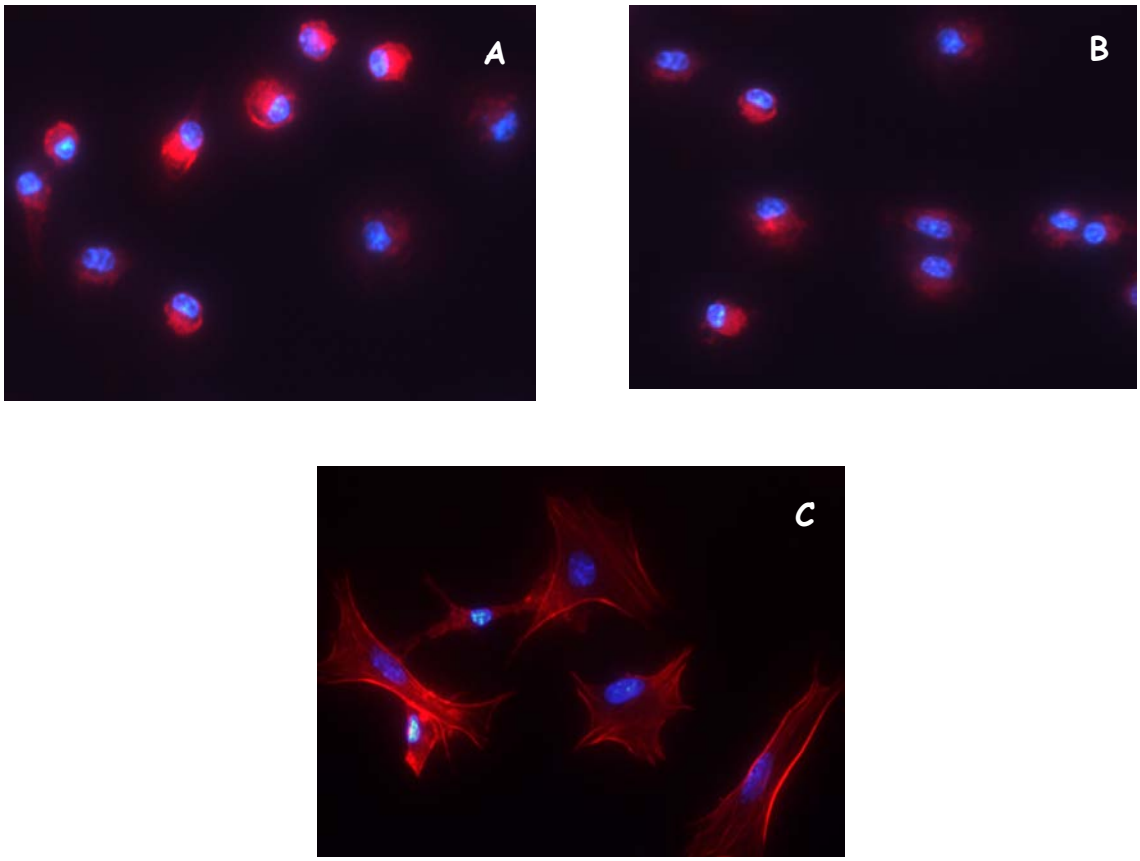


Figure 17. Phalloidin staining of A) control macrophages, B) macrophages transfected with GFP adenovirus and C) macrophages transfected with Ivns1abp adenovirus.

- **Ivns1abp increases macrophage resistance against inflammation**

We next aimed to unravel the ability of resistance against inflammation of the modulated macrophages. To this end we added a pro-inflammatory cocktail and we determined the expression of of TNF-α, iNOS, IL-10 and Manose Reseptor (Figure 18).

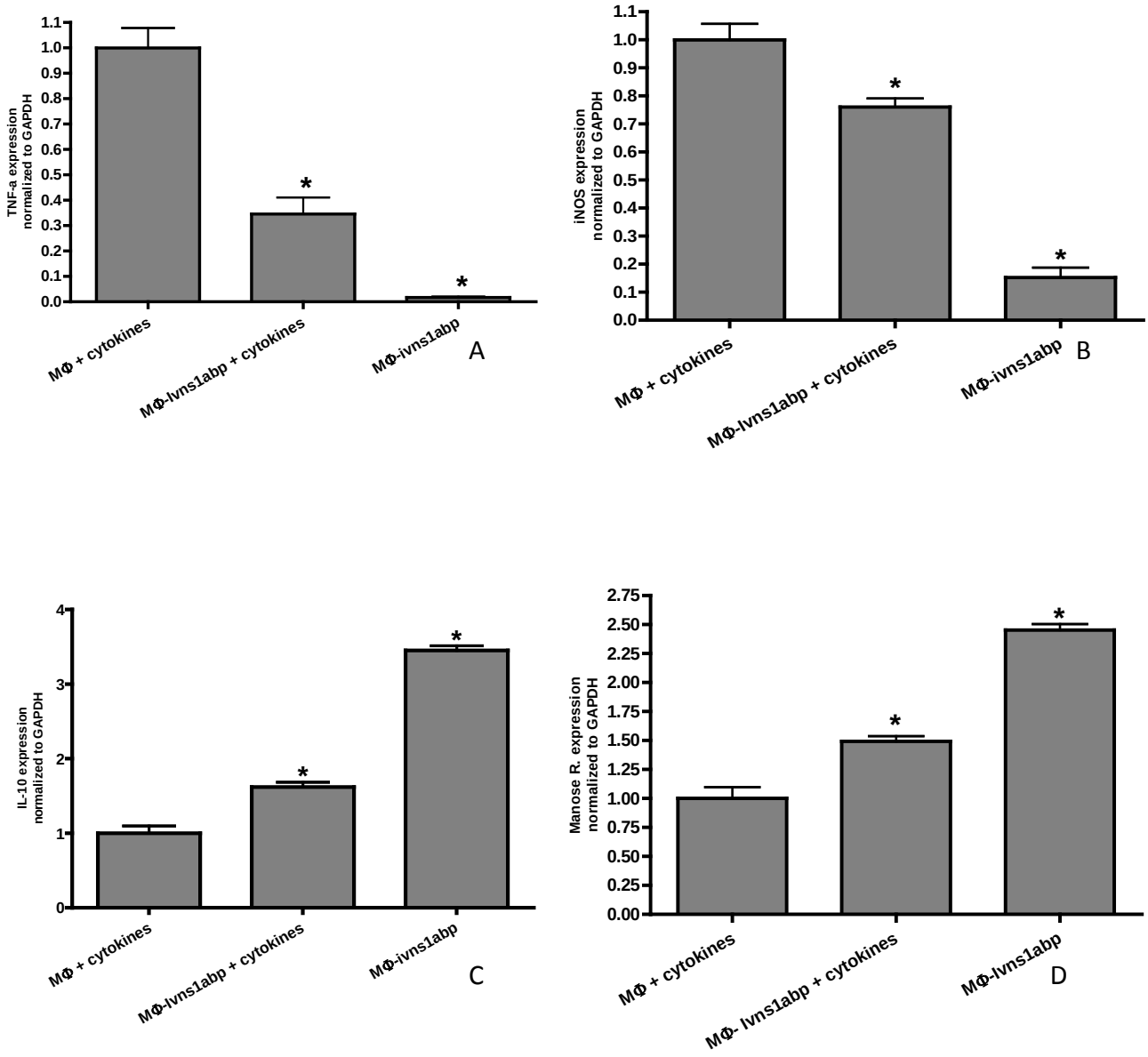


Figure 18. Expression of A) TNF-α, B) iNOS, C) IL-10 and D) Manose Receptor in macrophages control and/or transfected with Ivns1abp treated with cytokines Data is represented as mean ± SEM. ∗p < 0.05 versus Mϕ+cytokines; n = 5

As we can see in Figure 18A, a significant downregulation at the levels of the expression of TNF-α is observed in the group of macrophages over-expressing Ivns1abp compared to control macrophages treated with cytokines. The same effect is observed when the level of the expression of iNOS is measured. Macrophages

overexpressing Ivns1abp reduce the levels of the expression of the enzyme compared to the normal macrophages treated with cytokines (Figure 18B). On the other hand, modified macrophages present higher levels of expression of IL-10 and manose receptor compared to macrophages treated with cytokines (Figure 18C and D).

We also evaluated phalloidin staining to check the morphology of the macrophages between the groups.

In groups treated with cytokine cocktail we can observe that the actin filaments form clusters around the nucleus and the cells obtain characteristically round morphology witch correspond to the morphology of M1 macrophages (Figure 19A). On the other hand, macrophages transfected with Ivns1abp, adopt a more extended morphology with actin forming filaments throughout the whole cytoplasm, morphology which is characteristic of the M2 macrophages (Figure 19B). Macrophages over-expressing Ivns1abp and treated with LPS preserve their extended form while actin forms clusters next to the nucleus (Figure 19C). As a conclusion we can point that the over-expression of Ivns1abp in macrophages inhibits their conversion into pro-inflammatory or M1 macrophages and probably leads them towards the M2 phase (anti-inflammatory).

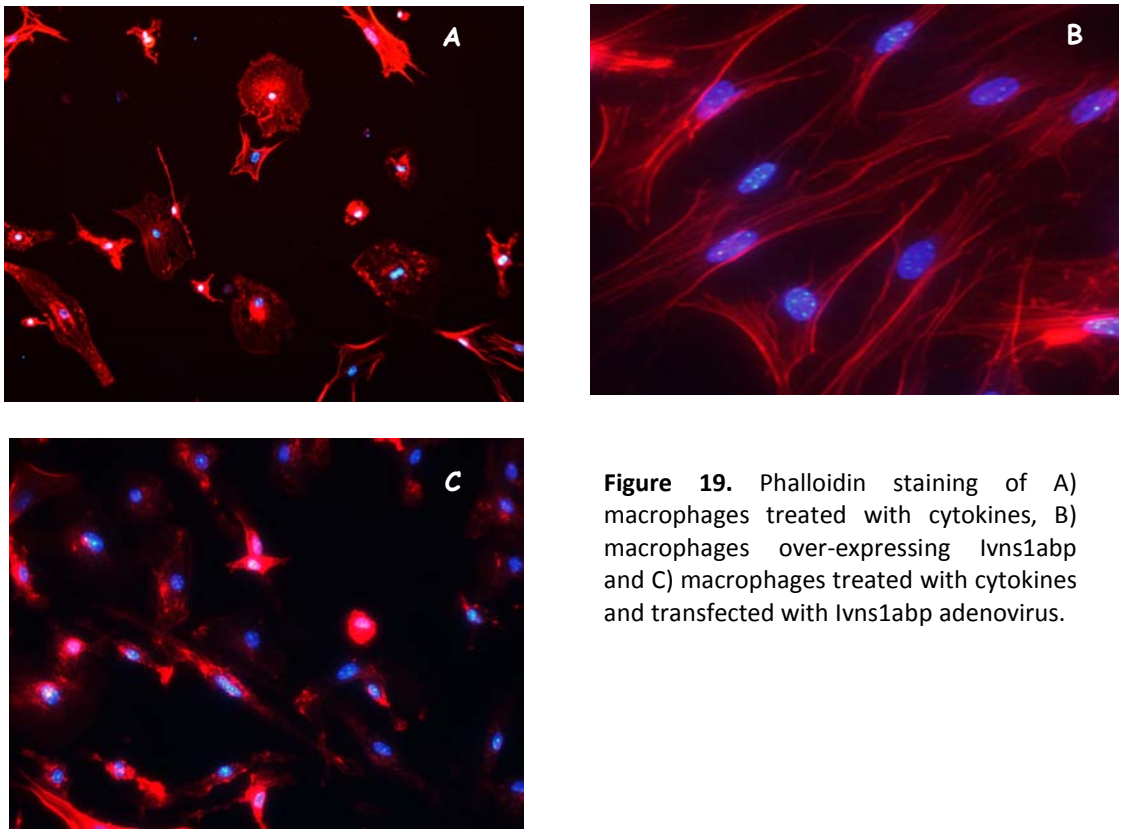


Figure 19. Phalloidin staining of A) macrophages treated with cytokines, B) macrophages over-expressing Ivns1abp and C) macrophages treated with cytokines and transfected with Ivns1abp adenovirus.

Results from previous studies indicate that Ivns1abp expression in macrophages is crucial in determining their phenotype, modulating their inflammatory state and increasing their resistance against inflammation. In order to confirm those results, we performed experiments silencing Ivns1abp gene expression in macrophages.

- **Effects on silencing in gene expression and cell phenotype**

In order to silence Ivns1abp gene, we used a specific adenovirus provided by ¨Galapagos Genomics¨, an innovative company that it has developed a RNA interference-based gene silencing technology, based on an adenovirus that is able to knock out gene expression in a range of cell types. Firstly, we determined the ability of the virus in downregulating the Ivns1abp gene in macrophages (Figure 20).

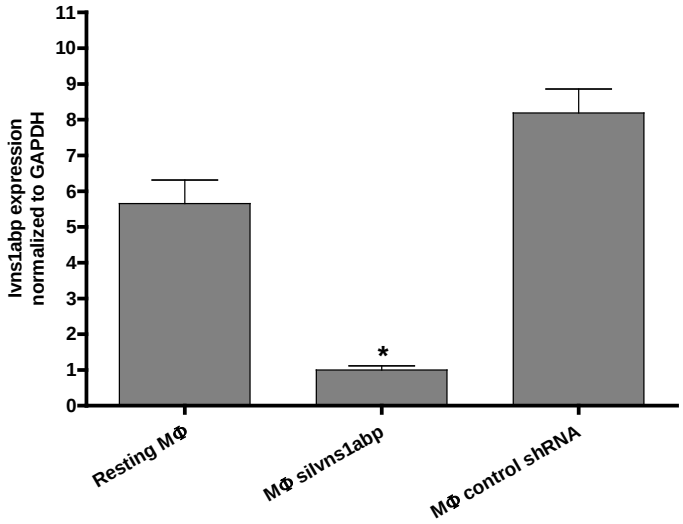


Figure 20. Ivns1abp gene expression in untreated macrophages and macrophages silenced for the expression of the above gene Data is represented as mean ± SEM. ∗p < 0.05 versus Resting Mϕ; n = 5

Experiment results showed that silenced macrophages were expressing only 12-15% of the Ivns1abp gene compared to the level of expression of untreated macrophages. In order to examine the effect of the Ivns1abp gene silencing in macrophage's state, the expression of the TNF-α cytokine was measured through RT-PCR (Figure 21).

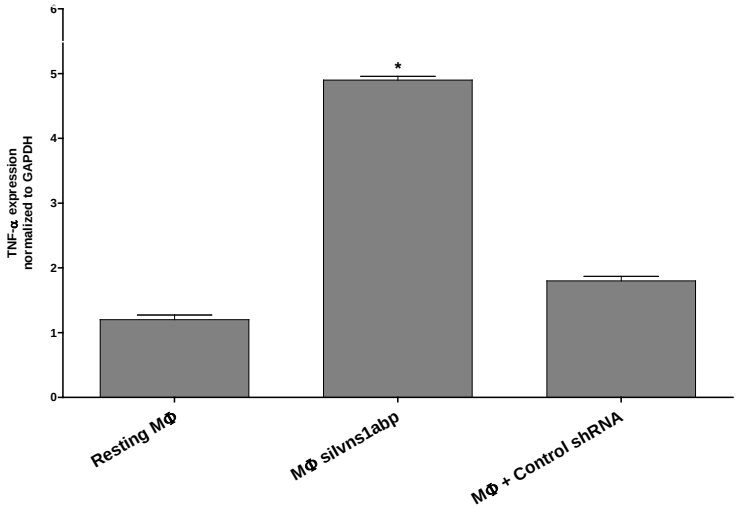


Figure 21. CytokineTNF-α expression in untreated macrophages and macrophages silenced for the expression of the Ivns1abp gene. Data is represented as mean ± SEM. ∗p < 0.05 versus Resting Mϕ; n = 5

The results showed that in the groups that Ivns1abp was down-regulated, the expression of TNF-α increased significantly compared to the control group, demonstrating that the absence of Ivns1abp gene causes conversion of macrophage into its pro-inflammatory phenotype. Scrambled siRNA did not affect the level of TNF-α. As a confirmation, we stained the cytoskeleton fibers with Phalloidin. Results showed a clear transformation of the macrophage phenotype from a round form (control) to an activated form where actin filaments form clusters around the nucleus and the cells obtain characteristically round morphology witch correspond to the morphology of M1 (absence of Ivns1abp)(Figure 22A and B).

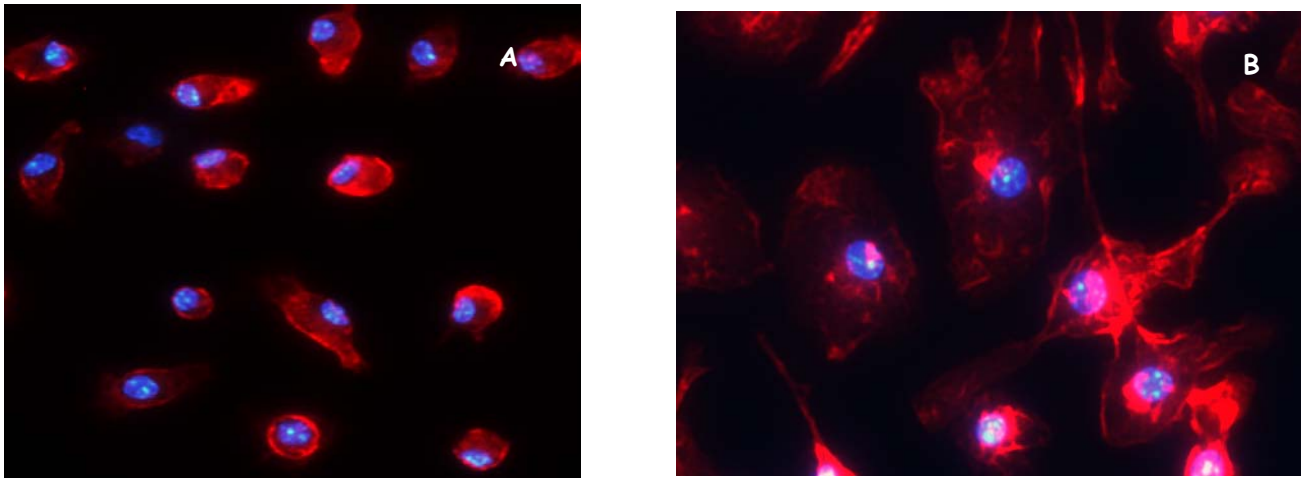


Figure 22. Phalloidin staining of macrophages control (A) and macrophages silenced for the Ivns1abp gene (B).

- **Silencing of Ivns1abp provokes injury ¨in vivo¨ and decreases regeneration**

After obtaining these results, we decided to perform an assay test in order to confirm the results in a ¨in vivo¨ model. In the I/R mice, macrophages were administrated (untreated y silenced for the Ivns1abp gene expression) 24 hours before sacrificing the animals. After that, renal function markers were measured. As seen in Figure 23A, the blood urea nitrogen (BUN), a marker of functional renal injury is significantly increased in the group of ischemia / reperfusion. Macrophages administration decreases this damage parameter. In contrast, Ivns1abp gene silenced macrophages don't have any effect on the levels of BUN. Same results are obtained when we measure creatinine levels, another marker of renal function (Figure 23B)

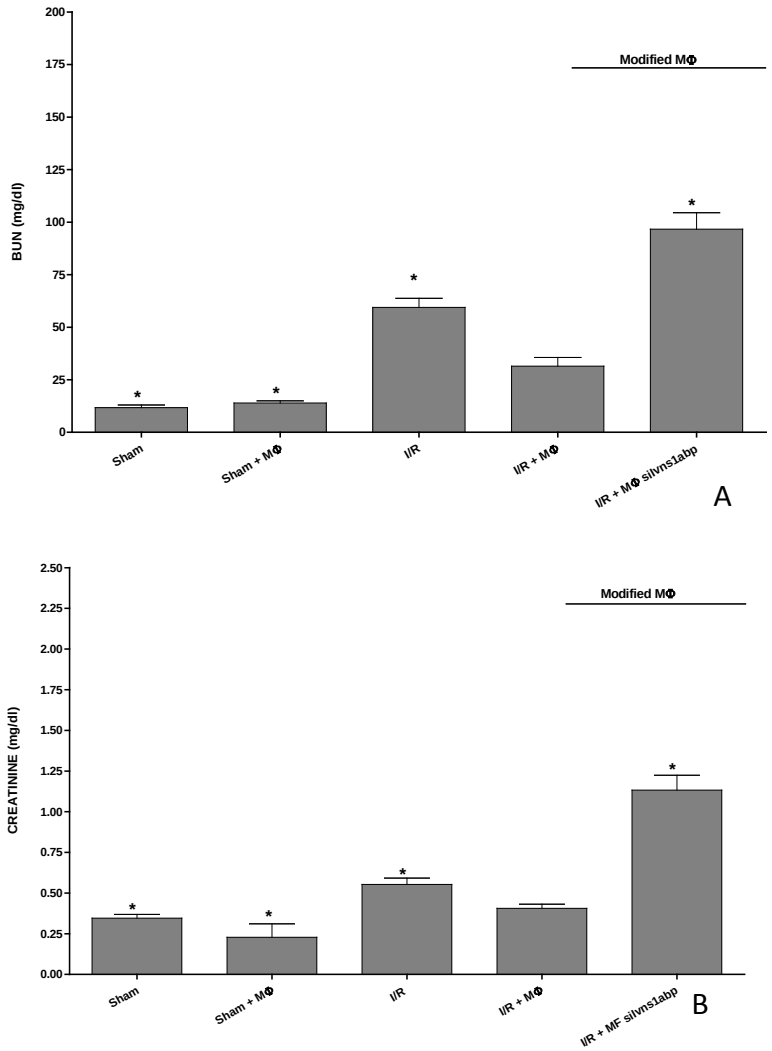


Figure 23. Effect of macrophages silenced for Ivns1abp on the levels of kidney function markers BUN (23A) and creatinine (23B). Data is represented as mean ± SEM. ∗p < 0.05 versus I/R+Mϕ; n = 5

Histological analysis of the tissue reveals that the most affected area is located in the medulla of the kidney (representative pictures in Figure 24). At 48 hours of reperfusion is observed severe interstitial edema, cellular infiltrate, tubular cell balloonization, and necrosis, (Figure 24B). However, treatment with macrophages preserves renal tissue integrity (Figure 24C). On the contrary, silenced macrophages lose their protective effect and cause further tissue damage (Figure 24D).

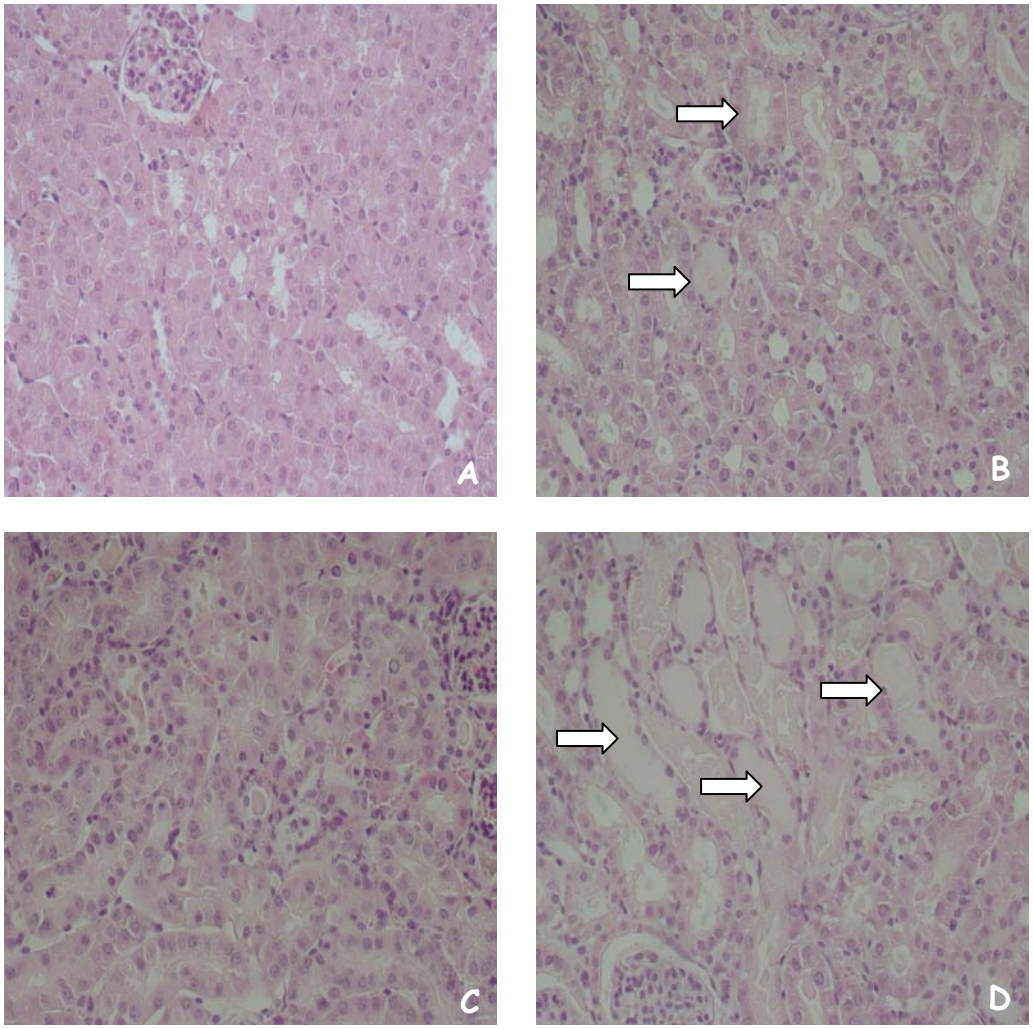


Figure 24. Histological analysis of renal tissue. A) control, B) I/R 48 hours, tissue starts loosing its integrity presenting tubular cell balloonization, dilation, and proteinaceous casts. I C) I/R 48 hours + macrophages, tissue is starting to assemple to the control group, D) I/R 48 hours + silenced macrophages. Macrophages that don't express Ivns1abp are incapable of reverting the degradation of the renal tissue.

Groups	Epithelial necrosis	Epithelial ballooning	Tubular dilatation	Intratubular detachment	Group media	Error
Control	1	0.5	0	1.5	3	0.5
I/R 48	1.5	0.5	2.5	2.5	7	0.5
I/R 48+MΦ	1	0	2	1.88	4.88	0.37
I/R 48+MΦ siIvns1abp	2	0.5	2	2	6.5	0.6

Figure 24 E. Histological analysis of renal tissue

Figure 25 shows a regeneration marker set of Ki-67, PCNA and stathmin.

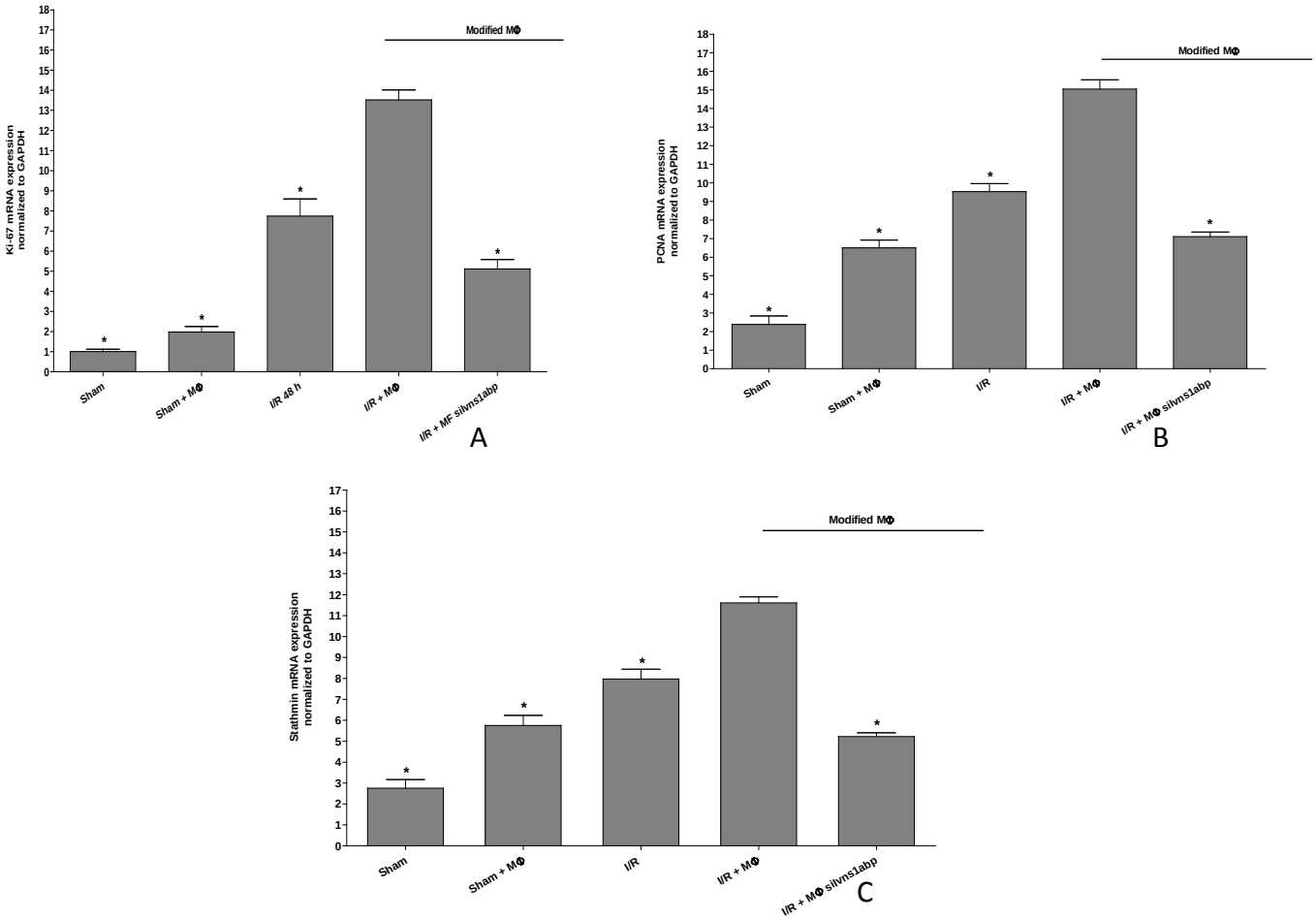
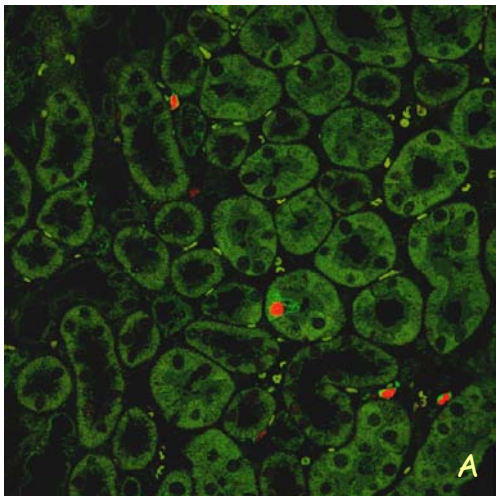
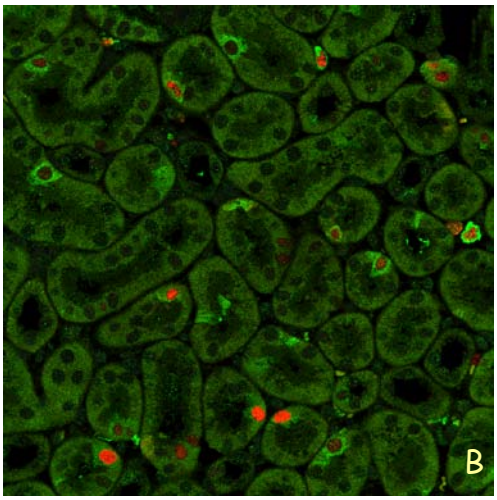


Figure 25. Effect of the administration of genetically modified macrophages on the expression of the proliferation and renal regeneration marker Ki-67 (A), PCNA (B) and Stathmin (C). Data is represented as mean ± SEM. ∗p < 0.05 versus I/R+Mϕ; n = 5

The results of the RT-PCR indicate that at 48 hours of reperfusion the expression of Ki-67 is increased compared to control animals (Figure 25A). By administrating non modified macrophages 24 hours before sacrifice, we detected a significant increase in cell proliferation in the tissue and therefore an advanced regeneration time after 48 hours of reperfusion. On the other hand, administration of modified macrophages did not have the ability to induce proliferation in the renal tissue, which indicates that the gene is responsible for promoting macrophage depended renal regeneration. Same results are obtained when we measured the levels of PCNA (Figure 25B) and stathmin (Figure 25C).

In order to confirm the previously obtained results, was performed immunofluorescence of stathmin and PCNA in renal tissue (Fig. 26) which reveals that when macrophages where administrated, the expression was further increased compared to the ischemic group(Figure 26C). With the application of macrophages that do not express the Ivns1abp gene the result is reversed (Figure 26D).





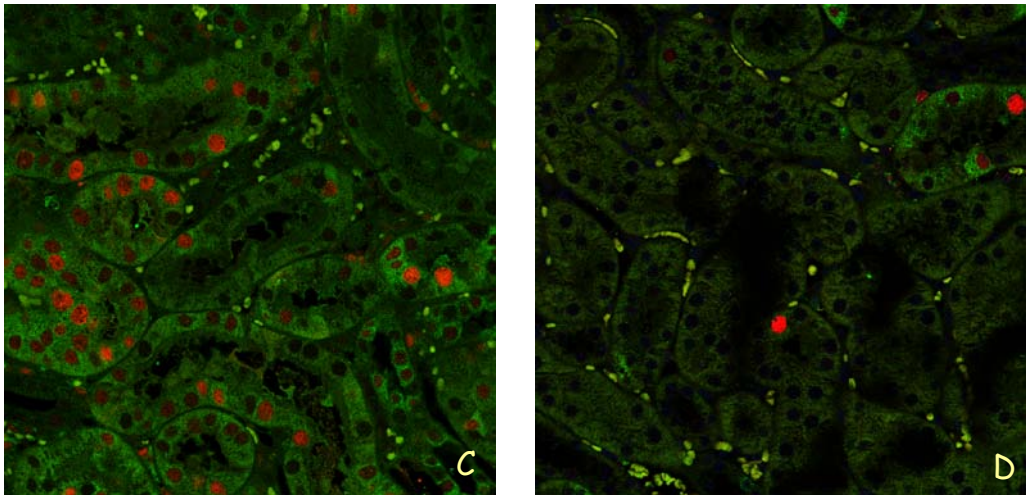


Figure 26. immunofluorescence of stathmin (green) and PCNA (red) in renal tissue, A) control, B) I/R 48 hours, C) I/R 48 hours + macrophages, D) I/R 48 hours + macrophages silenced for Ivns1abp gene expression (level of proliferation decreases).

All the above results reveal that the proliferative capacity of macrophages is linked to the presence of the Ivns1abp gene and that the mechanism through which Ivns1abp provokes proliferation could be the ability of induction of the anti-inflammation phenotype of macrophages by this gene.

- **Co-culture test**

Afterwards, we performed co-culture experiments in order study the effect of Ivns1abp silencing in the reparative and not proliferative function of the macrophages. To this end we added renal epithelial cells (NRK-52e). Colchicine was added to NRK-52e cells in order to reduce cell proliferation without compromising the viability of the culture and macrophages in order to restore the proliferation level.

Figure 27 shows the expression of proliferation markers Ki-67 and PCNA. It can be observed a decrease in the expression of those markers in the group treated with colchicine regarding to control groups. After cultivating treated NRK cells with non modified macrophages or macrophages transfected with a control virus (shRNA), the expression of Ki-67 and PCNA increases reaching the untreated cells level. On the other hand, when treated NRK cells are co-cultivated with macrophages that don't express Ivns1abp, levels of proliferation are decreasing compared to the group that is co-cultivated with untreated macrophages, indicating that the proliferative capacity of macrophages is linked to the presence of the Ivns1abp gene.

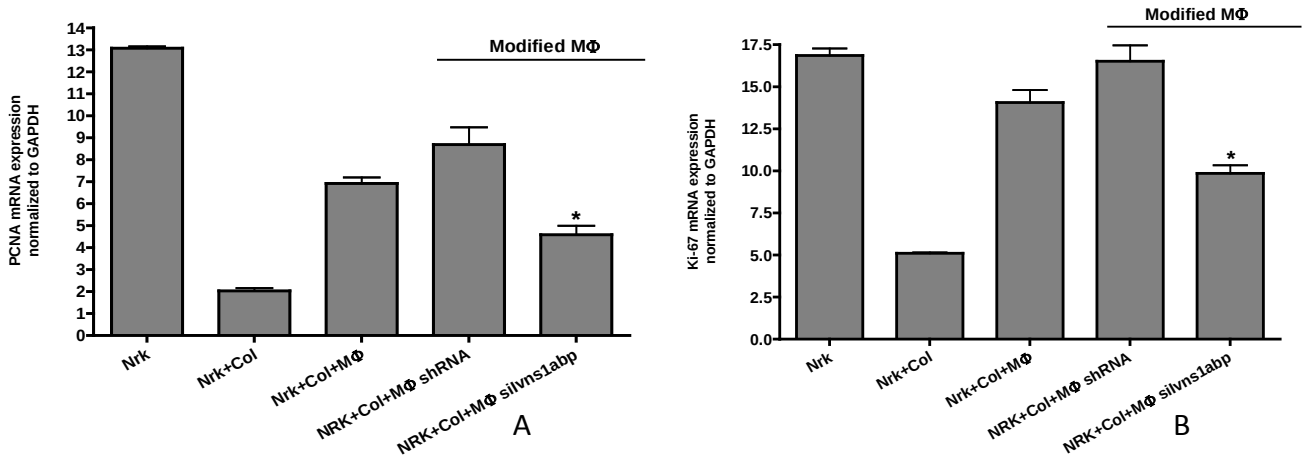


Figure 27. Expression of the proliferation markers Ki-67 and PCNA in NRK-52e renal epithelial cells after co-cultivation with untreated and treated macrophages. Data is represented as mean ± SEM. ∗p < 0.05 versus NRK+Col+Mϕ; n = 5

In order to visualize the previous result, we performed the same co-culture experiment but instead of using colchicine, we provoked damage by a mechanical way and we added macrophages control and/or macrophages silenced for Ivns1abp (Figure 28) .

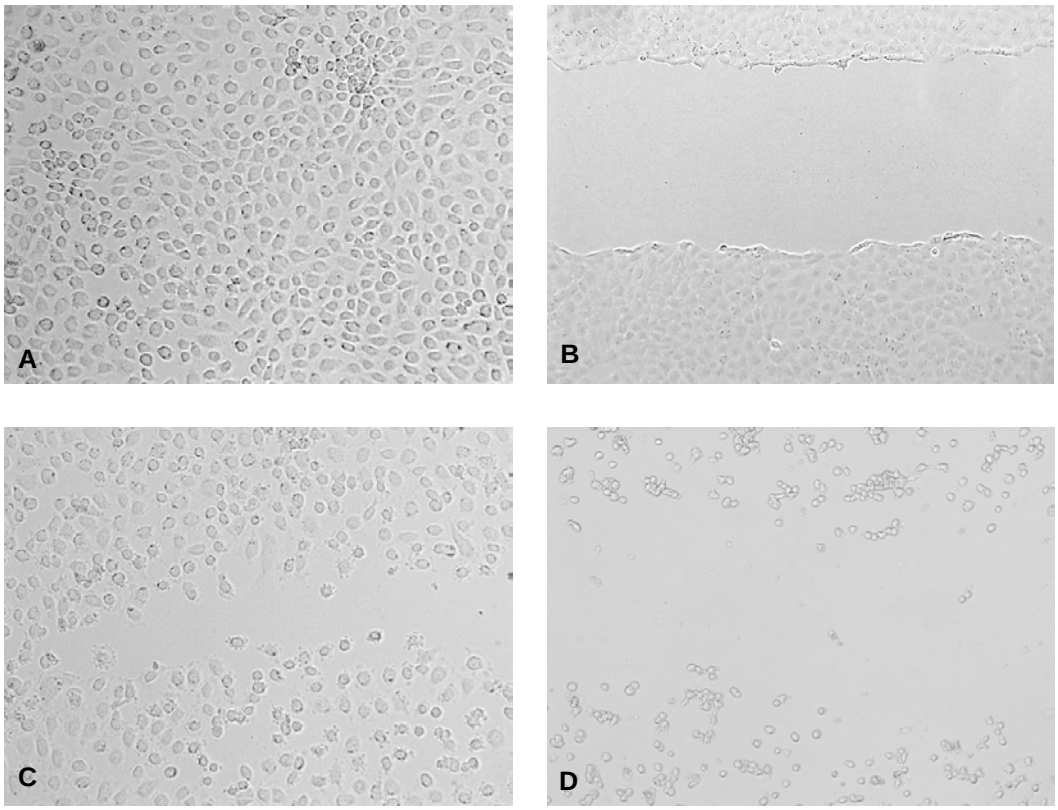


Figure 28. A) NRK-52e control cells, B) control cells after mechanical damage, C) control cells co-cultivated with untreated macrophages 24 hours after mechanical damage and D) control cells co-cultivated with silenced macrophages after 24 h of mechanical damage

Results indicate that macrophages unable to express Ivns1abp gene are loosing their ability to proliferate damaged cells, indicating the importance of this Ivns1abp gene on the proliferative state of macrophages.

STUDY 3: Regulation of the transcription of the Ivns1abp gene

Previous results indicate that Ivns1abp expression is crucial, by determining the type of phenotype that macrophages acquire (M1 or M2). The next object of our study is to determine the possible factors that regulate Ivns1abp gene expression. As we already show in study 1, Ivns1abp was downregulated in macrophages during the period of injury, which is the phase that is characterized by the presence of pro-inflammatory cytokines. To confirm that result, we measured the levels of Ivns1abp expression in control macrophages and macrophages treated with several combinations of pro-inflammatory cytokines (Figure 29).

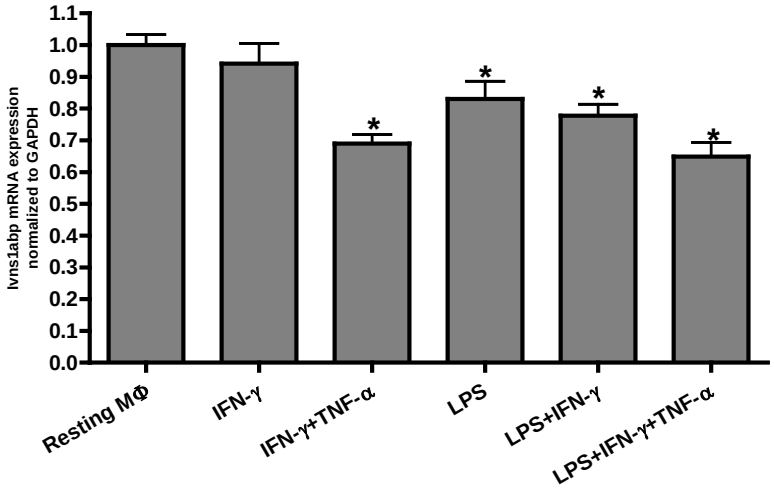


Figure 29. Ivns1abp expression in macrophages control and treated with pro-inflammatory cytokines cocktails (TNF-α and IFN-γ) Data is represented as mean ± SEM. ∗p < 0.05 versus Resting Mϕ; n = 5

Results show that Ivns1abp expression decreases in the groups treated with pro-inflammatory cytokines. In order to study the mechanism through which Ivns1abp gene is downregulated by cytokines, we decided to focus on specific microRNAs and/or transcription factors that control the expression of the Ivns1abp gene and are regulated by cytokines. Focusing on microRNA candidates we used a specific microRNA data web searcher (www.microrrna.org/microrna/) and we found that one of the microRNAs highly aligned with Ivns1abp, is mir-9 microRNA. The next step was establishing the connection between this specific micro RNA and cytokines. For that reason we carried out RT-PCR in macrophages control and macrophages treated with

pro-inflammatory (TNF-α and IFN-γ) and/or anti-inflammatory cytokines (IL-10) (Figure 30).

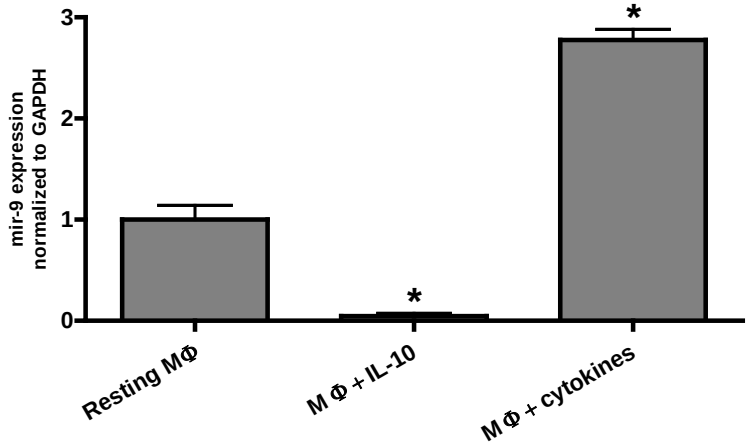


Figure 30. mir-9 expression in macrophages control and macrophages treated with pro-inflammatory and anti-inflammatory cytokines. Data is represented as mean ± SEM. ∗p < 0.05 versus Resting Mϕ; n = 5

Results show a direct connection of mir-9 with the inflammatory state of the environment transforming it in a good candidate for our hypothesis.

After that, we examined the connection in the regulation of Ivns1abp by mir-9 microRNA. For that reason, we transfect macrophages with the adenovirus that over-expresses Ivns1ap and we measured the levels of Ivns1abp expression (Figure 31).

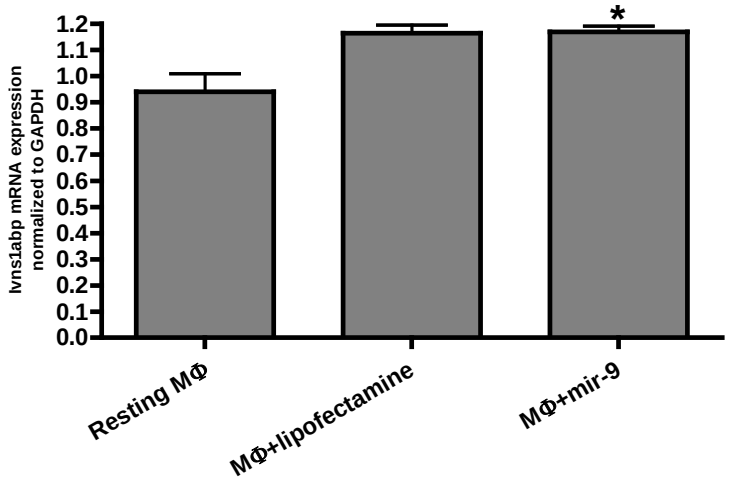


Figure 31. Ivns1abp expression in macrophages over-expressing mir-9 microRNA Data is represented as mean ± SEM. ∗p < 0.05 versus Resting Mϕ; n = 3

Results indicate that when mir-9 micro-RNA is overexpressed in macrophages, there is no difference in the level of expression of the gene Ivns1abp, indicating that there is no possible connection between mir-9 and Ivns1abp regulation. After this result we decided to focus more on Ivns1abp transcription factors that are regulated by cytokines.

By using common internet sources such as ¨Genecards¨ (www.genecards.org) we were able to identify the most important of those. According to this particular data base, one of the most relevant transcription factors that bind to the promoter of the Ivns1abp gene was the transcription factor c-myc.

Figure 32 showed us that both the cytokine cocktail and LPS also downregulat c-myc expression confirming that the expression of this transcription factor is cytokine depended.

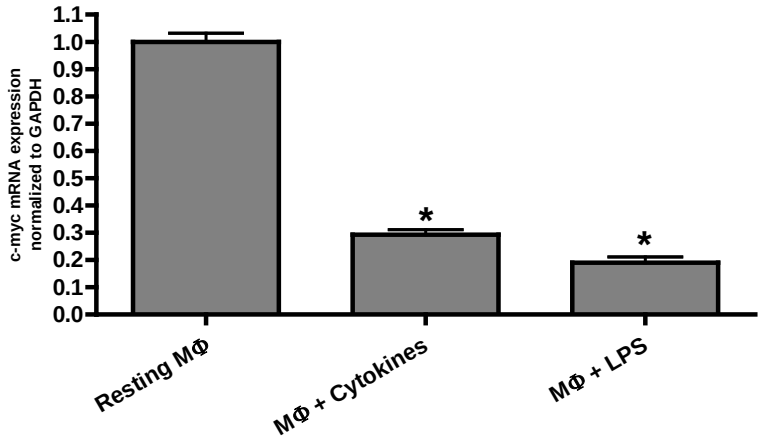


Figure 32. c-myc expression in macrophages treated with cytokine cocktail and LPS Data is represented as mean ± SEM. ∗p < 0.05 versus Resting Mϕ; n = 5

In order to examine the connection between Ivns1abp expression and c-myc we carried out RT-PCR of macrophages under different conditions. Figures 33 shows that macrophages treated with c-myc inhibitor, present a significant decrease at the levels of Ivns1abp expression compared to the control group. The same result is observed after treating macrophages that over-express Ivns1abp with cytokines and c-myc inhinitor, levels of Ivns1abp decrease compared to the group of macrophages transfected with Ivns1abp, indicating the implication of c-myc in Ivns1abp regulation.

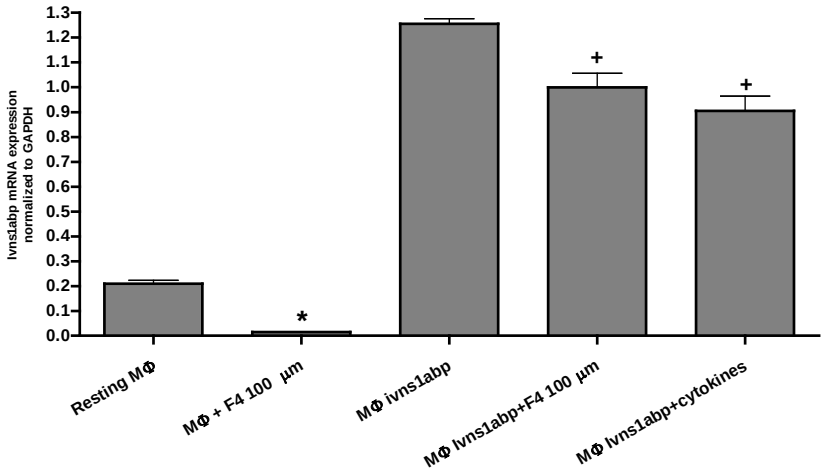


Figure 33. Expression of Ivns1abp in macrophages treated with cytokines and c-myc inhibitor Data is represented as mean ± SEM. ∗p < 0.05 versus Resting Mϕ; †p < 0.05 versus Mϕ Ivns1abp n = 5

After showing that c-myc transcription factor, modulates Ivns1abp gene expression we focused on studying how this modulation alters the functions of macrophages.

Firtly, we studied the results of the c-myc Inhibitor at macrophage phenotype. We performed phalloidin staining in control macrophages and macrophages treated with this particular c-myc inhibitor. Figure 34A shows control macrophages having a rather round phenotype with actin all around the cytoplasm. On the other hand in groups treated with c-myc inhibitor we can observe that the actin filaments form clusters around the nucleus and the cells obtain characteristically round morphology witch correspond to the morphology of M1 macrophages (Figure 34B), same as macrophages treated with cytokines, indicating the role of c-myc in the regulation of Ivns1abp gene by cytokines.

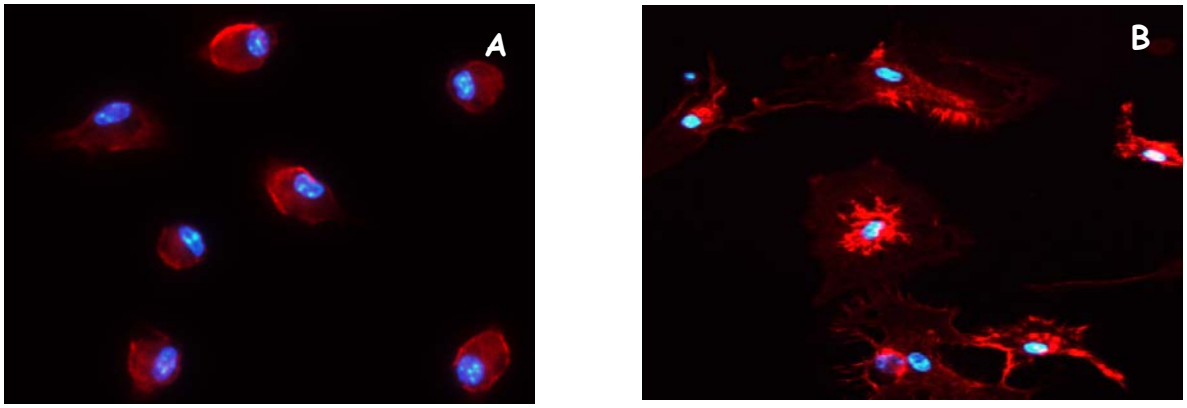


Figure 34. Phalloidin staining of A) control macrophages, B) macrophages treated with c-myc inhibitor

- **Phagocytosis**

Previous results showed that over-expression of Ivns1abp in macrophages can alter their phenotype towards the characteristic phenotype of M2 macrophages, through its interaction with the actin filaments. In order to find out if the change in phenotype also affects the functions of the macrophage we performed a phagocytosis experiment. Phagocytosis is one of the main functions of alternatively activated or M2 macrophages, while in M1 or classically activated macrophages level of phagocytosis is decreased.

Phagocytosis assay was performed using FluoSpheres Carboxylate-Modified Microspheres, 2.0 µm for different time points. Macrophages were stained with an F4/80-PE-Cy7 Antibody and analyzed by flow cytometry. Figure 35 shows the difference in the percentage of phagocytoced beads. Percentage of phagocytoced beads is decreased in macrophages treated with pro-inflammatory cytokines such as TNF-α and IFN-γ compared to control levels. Same results are obtained in cells treated with 10058-F4, by blocking Ivns1abp expression through the inhibition of its main transcription factor, c-myc. The experiment was followed by Hoechst Farbstoff and Phalloidin staining in order to confirm the previous results (Figure 36). Results showed us that inhibiting expression of Ivns1abp through the blockage of the main transcription factor implicated in its expression (c-myc) provokes the same functional change to macrophages as treatment with pro-inflammatory cytokines, indicating the importance of Ivns1abp in the process of macrophage activation towards an anti-inflammatory phenotype.

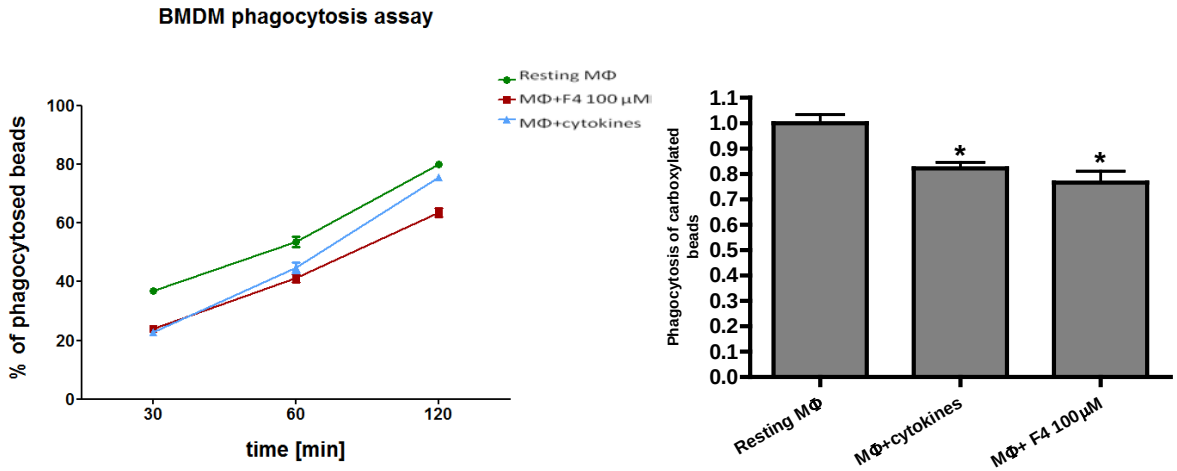
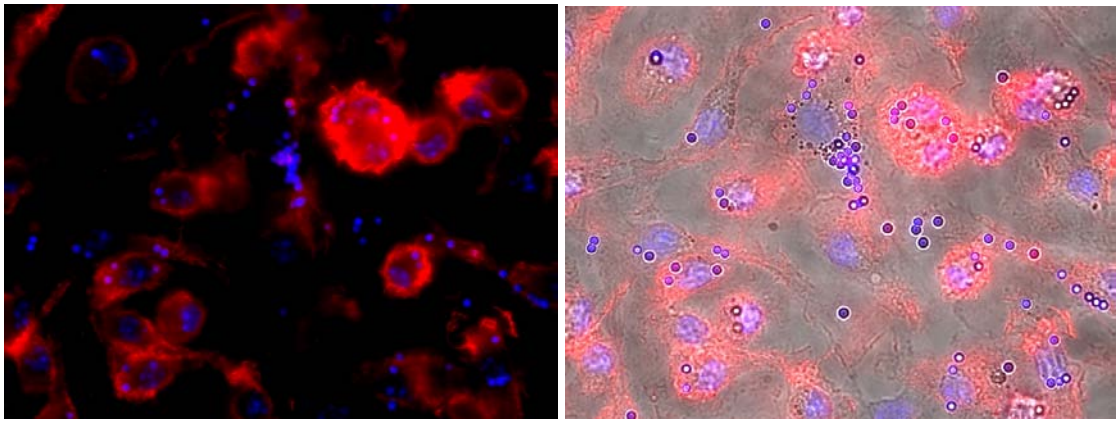


Figure 35. Phagocytosis assay using Carboxylate-Modified Microspheres in macvrophages treated with cytokines and c-myc inhibitor (macrophages control and macrophages overexpressing Ivns1abp). Data is represented as mean ± SEM. ∗p < 0.05 versus Resting Mϕ; n = 5



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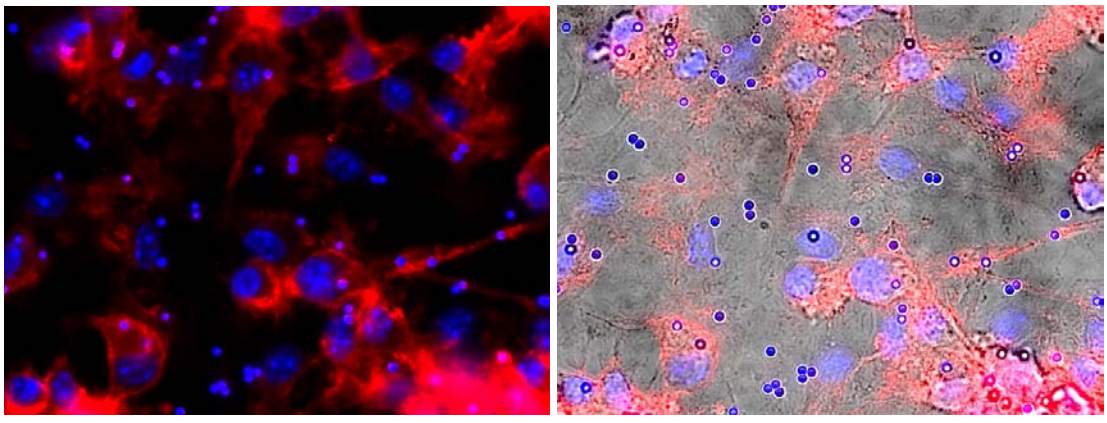

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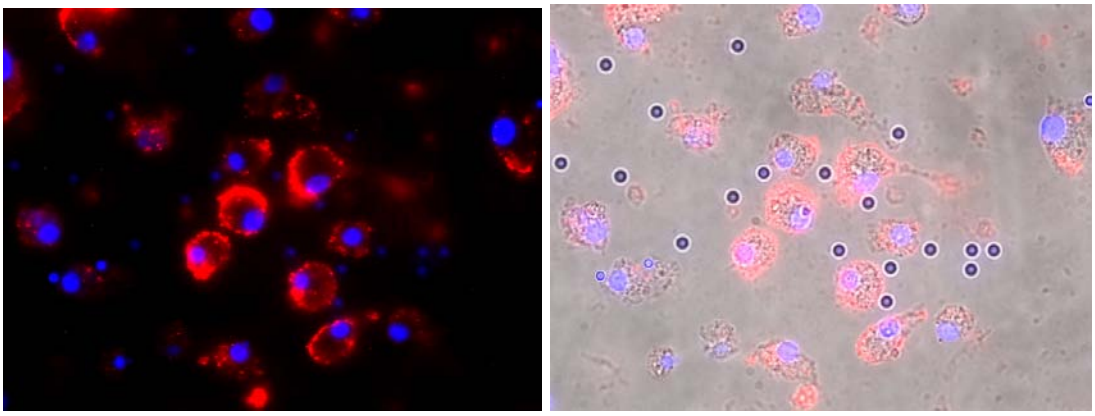

C

Figure 36. Phagocytosis assay using Carboxylate-Modified Microspheres in A) control macrophages, B) macrophages treated with cytokines and C) macrophages treated with c-myc inhibitor.

- **Proteasome activity**

Most proteins which are going to be degraded in the cell are first tagged with several moieties of a small, 76-amino acid polypeptide called ubiquitin. Proteins tagged with polyubiquitin chains are recognized and bound by the 26S proteasomes, which subsequently degrade them into small peptides. Our next step in this study was to study the protein degradation profile of macrophages in pro-inflammatory environment and to compare it with the same profile when Ivns1abp expression is blocked by blocking transcription factor c-myc. Results shown in Figure 37.

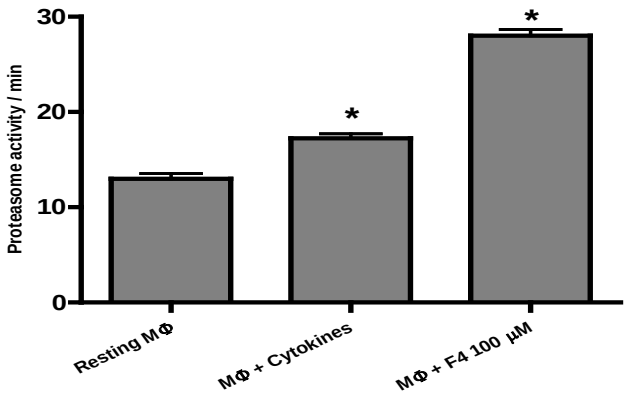

Figure 37. Proteasome activity in macrophages control and treated with cytokines and/or c-myc inhibitor. Data is represented as mean ± SEM. ∗p < 0.05 versus Resting Mϕ; n = 5

Results not only indicate the importance of Ivns1abp function in preventing cell protein degradation and apoptosis, but also that this regulation occurs through the transcription factor c-myc.

5 . DISCUSSION

Acute renal failure remains a major critical problem that still offers many challenges to the researchers. It should be noted that patients with ARF reach mortality rates between 50 and 70%. This high mortality has not decreased in decades (5).

One of the therapeutic approaches aimed to heal ARF, has been clinical trials using growth factors such as EGF, HGF and IGF-1, but the results were disappointing (90, 91). These regenerative proposals maybe failed due to lack of knowledge in basic biological processes, genes involved and functions of these genes.

It is known that the degree of acute or chronic ischemia is the main cause of renal failure (4), and that the regeneration of dysfunction that occurs after ischemic insult is part of the same response to injury. For example, there is a parallelism between the prevalence of apoptosis and regeneration and between macrophage accumulation and cell proliferation.

Macrophage infiltration is a classical feature of inflammatory processes, ranging from simple skin wounds to complex and severe, even chronic inflammation-associated pathologies. Many renal diseases are a clear example of this.

Macrophages exhibit a great heterogeneity and functional plasticity which is reflected in their expression of different phenotypes in response to micro-environmental signals (24).One of their major roles is the modulation of inflammation and its outcome, both regarding initiation and resolution. In the case of the kidney, the general view of macrophage implication was long focused on injury development, by releasing a huge amount of chemokines, inflammatory cytokines, and other cytotoxic mediators. But also is pointed out the importance of macrophages in facilitating repair and the down-regulation of both injnury and inflammation processes.

It is clear that the ability of macrophages to ingest inflammatory cells and other apoptotic cells of the injured tissue dampens their pro-inflammatory activity and changes their phenotype towards anti-inflammation and pro-repair. In this case, the anti-inflammatory M2 macrophage is the responsible for tissue repair and remodelling by eliminating apoptotic cells and the activation of anti-inflammatory cytokine expression. As such, macrophages are pivotal in protecting organs and surrounding tissues against detrimental immune responses, promoting tissue remodeling.

Thus, due to their versatility and heterogeneity in biological functions, the use of macrophages, both as therapeutic targets in regenerative medicine and vectors in cellular therapy, has become of interest to the scientific board.

Knowing the mechanisms which control the M1 to M2 macrophage phenotype transition and modulating the genes implicated in this process could result as a powerful tool and an alternative approach for treating ARF and to develop specifically therapeutic targets and therapies to treat diseases, such as kidney failure.

Ischemia / Reperfusion injury is an important cause of acute renal failure in native kidneys and allografts but cellular and molecular or even genetic responses of the kidney to recover from acute ischemic injury are complex and not fully understood.

The first place of tissue injury after an ischemic insult, which occurs in the first 24 hours of reperfusion, has been well characterized in various organs by previous studies and includes mainly an increased turgor, loss of adhesion and polarity and cell death. These changes are accompanied by an increase in oxidative stress and inflammatory mediators. But eventually, the injury phase is followed by a recovery phase, in which the mitotic cells de-differentiated cells are increasing in damaged areas (170). It is also demonstrated by previous studies of our group that the early injury phase is characterized by massive macrophage recruitment, and the later recovery phase with low macrophage recruitment.

After using mice that undergo 24 hours and 96 hours of reperfusion after 45 minutes of ischemia, we measured BUN, in plasma, which is a marker of renal function and stathmin expression, which is a marker of tissue regeneration.

Results indicate that the levels of BUN are highly increased at the 24 hour reperfusion time point compared to the levels of control or / and the levels at the 96 hour time point, indicating an altered kidney function at this time point and confirming the 24 hours of reperfusion can be considered as the injury phase.

On the other hand, stathmin's expression, which is a specific renal regeneration marker (171), remains low in control groups and the first hours after reperfusion, showing its highest expression at the 96 hour time point of reperfusion, indicating this particular time point as the recovery phase.

We next confirmed that macrophages are implicated and are also playing a critical role in the determination of those two different phases by using the technique of adoptive

transfer of macrophages both during the damage and the regeneration phase, after depleting animal endogenous macrophages by using clodronate liposomes. Regarding the injury phase, results indicate that levels of BUN in plasma are highly reduced after depletion of macrophages and after adding exogenous macrophages BUN levels are rising up again indicating the importance of the presence of macrophages in order the injury phase to be established. Focusing on the recovery phase and on stathmin expression we can also observe a connection between this particular regeneration marker and macrophage presence or absence. Those results confirm the dual role of macrophages depending on the nature of the environment that they are found (pro-inflammatory or anti-inflammatory environment).

After confirming the two different phases of injury and recovery in the ischemic kidney, we focused on the identification of genes that are implicated in renal regeneration and are also related to macrophages. To this end and under the framework of the European project PROLIGEN, we proceeded in profiling the gene expression of each one of the above experimental groups by microarray analisis through the system “Applied Biosystems 1700 Chemiluminescent Microarray Analyzer” performed in CNIC (Centro Nacional de Investigaciones Cardiovasculares, Carlos III, Madrid, Spain). The data obtained was then processed and analyzed by “Genedata” a Swiss bioinformatic company with great experience on statistical analysis and interpretation of molecular profiling studies (www.genedata.com).

Among all the deregulated genes determined by ¨Genedata¨ (7313 de-regulated genes) we focused our comparisons at the set of genes that were deregulated as a function of macrophage presence even in injury or recovery phase. This selection restricted the number of de-regulated genes at 110 in the case of injury phase and 38 in the case of recovery phase. With this preliminary selection, we refined our selection focusing the study in genes that are implicated in the transformation of the macrophage towards its anti-inflammatory phenotype; More specifically we focused on the same genes that there were down-regulated during injury (24 hours) and together were up-regulated during reparation (96 hours) reaching a number of 14 possible proliferative genes.

As a last step, we performed an ¨in vivo¨ assay test to validate the effect of those 14 genes on tissue repair by performing cell therapy, silencing each one of them in

macrophages. After silencing the macrophages we administrate them in control groups as well as in groups that undergo ischemia and reperfusion. More correctly we used the model of 45 min of ischemia and 48 hours of reperfusion, corresponding with the beginning of the reparation phase.

The overall result of the study indicates that all 14 genes are relevant to promote repair after I / R injury and that the absence of each one of those provokes a significant decrease in renal regeneration, indicating their importance in macrophages acquiring a regenerative phenotype.

After establishing the importance of those 14 proliferative genes concerning macrophage capacity of tissue repair, in this study we decided to focus on and continue with the Ivns1abp gene for further experimenting.

Ivns1abp belongs to the kelch family proteins that contain 4-7 kelch motifs that form a propeller-like structure (128, 129). Gene Ivns1abp codes for a novel actin binding protein, Nd1, which belongs to the Kelch family of proteins (137). Is demonstrated that kelch repeats of Nd1-L directly associate with F-actin (147). Thus, Nd1-L is an actin-associated protein that protects against the actin disorganization and play a critical role in fundamental cellular functions such as cell division by coordinating with the dynamics of actin cytoskeleton as a housekeeping gene.

In order to study whether the Ivns1abp gene plays a role in renal regeneration through transformation of macrophage phenotype from M1 to M2, we decided to use a specific tool, which was macrophages over-expressing the Ivns1abp gene.

To achieve such macrophages, we used a tailored specific adenovirus produced by ¨Viraquest¨; a company specialized in this particular field.

After establishing the best conditions for the function of the vector, we proceeded in performing an assay test in order to study possible effects in renal regeneration. Our study groups where mice control and mice submitted in 45 minutes of ischemia and reperfusion of 48 hours. Those mice where untreated or injected with macrophages transfected with a control vector as well as with the vector that contained Ivns1abp.

Results of the study demonstrate the ability of Ivns1abp to enhance macrophage's capacity to regenerate since a significant increment of the regeneration markers Stathmin, Ki-67 and PCNA is observed in mice treated with macrophages over-expressing this particular gene compared to the mice control.

After obtaining the results about macrophage's capacity of tissue repair, our objective was to exam the inflammatory state of the macrophages when they were over-expressing the Ivns1abp gene. Since macrophages secrete a broad number of pro and anti-inflammatory mediators, depending on their state, we decided to examine through RT-PCR the levels of some of those reported mediators that characterize each state. Results indicate that Ivns1abp is connected and can alter the inflammatory stage of macrophages since the expression of pro-inflammatory mediators such as TNF-α and iNOS is considerably decreased in macrophages that over-express Ivns1abp, whereas, the expression of anti-inflammatory mediators such as IL-10 and Mannose Receptor is increased in the same group of macrophages compared to the control groups.

This direct connection of Ivns1abp expression and macrophage state was also observed by phalloidin staining of macrophages control and macrophages over-expressing Ivns1abp. Control macrophages, that are considered as resting macrophages, present a rather round phenotype with the actin found all around the cytoplasm while macrophages over-expressing Ivns1abp adopt a more extended morphology with actin forming filaments being present mostly at the borders of the cytoplasm, a characteristic morphology of the M2 macrophages.

After relating Ivns1abp with tissue regeneration and with the modulation of the macrophage's inflammatory state, we decided to study whether the presence of this particular gene con also affect the resistance of the macrophages against inflammation. It is known that the states that adopt macrophages (M1 or M2) depend on the cytokine environment that they find in every tissue. Based on that, we decided to study whether the presence of Ivns1abp can prevent macrophages from adopting a pro-inflammatory phenotype when they encounter a pro-inflammatory environment. Indeed, after performing RT-PCR to examine the expression levels of certain pro-inflammatory (TNF-α and iNOS) and anti-inflammatory (IL-10 and Mannose Receptor) mediators in macrophages control as well as in macrophages over-expressing Ivns1abp, untreated or treated with cytokines, we observed that the level of pro-inflammatory mediators secreted by macrophages was decreased while the level of anti-inflammatory mediators was increased in macrophages over-expressing Ivns1abp compared to control macrophages treated with cytokines indicating that this particular

gene can increase macrophage resistance against inflammation. Observing the phalloidin staining of macrophages transfected with Ivns1abp and treated with cytokines, we can see that even if the actin starts forming clusters next to the nucleus (characteristic of M1 macrophages), cells are still preserving their elongated form which characterizes the M2 macrophage phenotype.

After establishing the importance of the presence of the Ivns1abp gene in macrophages regarding to their regenerative capacity and phenotype acquisition, we proceeded in studying the effect of the absence of this specific gene.

In order to achieve that, we transfected macrophages with a specific adenovirus that was able to knock out Ivns1abp gene expression using technology based in RNA interference and we performed an ¨in vivo¨ assay test to study its effects during tissue injury. Our study groups where mice control and mice submitted in ischemia and reperfusion of 48 hours. Those mice where untreated or injected with macrophages transfected with a control vector as well as with the vector that silenced Ivns1abp. Results indicate that Ivns1abp absence can lead macrophages in causing greater damage on the tissue compared to the macrophages control as seen from the levels of BUN and creatinine in the plasma. After histological analysis of renal tissue of the same experimental groups we can observe that the repairing capacity of macrophages lacking Ivns1abp gene expression is reduced since they are incapable of reverting tissue damage compared to control macrophages. After measuring the expression of regeneration markers such as Ki-67, PCNA and stathmin by RT-PCR and by Immunofluorescence in the same groups as above we observe a loss in repairing capacity in macrophages that cannot express Ivns1abp. Previous results indicate that lack of Ivns1abp gene expression not only turns macrophages more pro-inflammatory by increasing the damage they cause in the tissue, but also causes a significant loss of their capacity of regeneration, pointing out Ivns1abp gene's importance.

For further studying of the effect of the lack of Ivns1abp on macrophages, we proceeded with experiments ¨in vitro¨. At this end, we used a renal epithelial cell line (NRK-52e) damaged by addition of colchicine in the culture medium or by just provoking mechanical damage. In both models, we then co-cultivated the injured cells with control macrophages (resting) or macrophages silenced for Ivns1abp expression. As expected, silenced macrophages where unable to reverse the damaged provoked to

the renal epithelium cell line compared to control macrophages, indicating again that the proliferative capacity of macrophages is linked to the presence of the Ivns1abp gene transforming this specific gene in a potential target of future macrophage based gene therapy.

After establishing the connection of Ivns1abp to macrophage phenotype and capacity of regeneration, in this study we focused on the mechanism through which Ivns1abp gene expression is regulated. As we already shown in study 1, all the 14 genes selected as candidate proliferative genes, where down-regulated during the injury period, which is a period characterized by the presence of pro-inflammatory cytokines and up-regulated during the period of repair which is characterized by the presence of anti-inflammatory cytokines. Those results indicate that the expression of Ivns1abp, along with the expression of the rest of those 14 genes, could be controlled by cytokines.

Measuring Ivns1abp expression in control macrophages as well as in macrophages treated with various cocktails of pro-inflammatory cytokines, we observed a significant decrease in the expression of this gene by the cytokine cocktails, confirming in one hand the results obtained with the array system in study 1 and in the other hand confirms the control of cytokines over this gene.

Firstly, we decided to focus on micro RNAs since those specific RNAs form an emerging class of regulators of gene expression acting at the post-transcriptional level via an RNA interference mechanism (104, 105). Mature micro RNAs form a 21 to 23 nucleotide double-stranded RNA duplexes (116) and then are loaded into the micro-RNA silencing complex which guides the recognition and translational repression or degradation of target mRNAs (124).

Using a specific micro RNA data web searcher (www.microrna.org/microrna/) we found that one of the micro RNAs highly aligned with Ivns1abp, is the micro RNA mir-9, which is also induced by the presence of LPS (125). Mir-9 expression in macrophages treated with pro-inflammatory and anti-inflammatory cytokines, confirmed that the expression of mir-9 was related to the presence of certain types of cytokines, converting it in a good candidate regarding Ivns1abp gene regulation. But measuring the Ivns1abp expression in macrophages transformed to over-express mir-9, we didn't observe any difference, indicating that, contrary to the expected results provided by

the RNA data web-search, mir-9 is not an important factor for the regulation of the expression of the Ivns1abp gene.

After obtaining those results we decided to focus on other possible cytokine regulated transcription factors that have as a target the Ivns1abp promoter. To this end we used ¨Genecard¨ database tool (www.genecard.com) which provide full gene information by cross-linking selected information extracted from ¨SABiosciences Text Mining Application¨ with the information provided by ¨UCSC Genome Bioinformatics Site¨. Using those tools we founded a set of transcription factors that bind to the promoter of Ivns1abp, being c-myc one of the most relevant.

c-Myc expression is tightly regulated in response to growth signals under non-pathological situations. It is ubiquitously expressed during embryogenesis and in tissue compartments of the adult possessing high proliferative capacity (152). Non-proliferating or quiescent cells generally express non-detectable levels of c-Myc, but the gene is rapidly induced following mitogenic stimulation and, thereafter, continues to be expressed in proliferating cells. C-myc is a gene regulated by cytokines and transcription factors (153, 154, 155) such as PDGF family, EGF, bFGF, etc.

In our experimental, after treating resting macrophages with cytokine cocktail or LPS we observed a significant decrease in the expression of the c-myc transcription factor, confirming its regulation by cytokines.

In order to observe the connection of c-myc transcription factor and Ivns1abp gene expression, we used macrophages over-expressing Ivns1abp and we treated those macrophages with an inhibitor named 10058-F4 that specifically inhibits the c-Myc-Max interaction and prevents transactivation of c-Myc target gene expression. Results indicate a significant decrease in Ivns1abp expression when the c-myc function was blocked both in control macrophages and macrophages overexpressing Ivns1abp. After performing phalloidin staining in resting macrophages and in macrophages treated with 10058-F4, phenotype of treated macrophages changes into a typical M1 phenotype, with acting forming clusters around the nucleus, same as the result obtained after silencing the Ivns1abp expression in macrophages in study 2, indicating that c-myc transcription factor is implicated into Ivns1abp gene regulation.

Phagocytocis is one of the main functions of alternatively activated or M2 macrophages, while in M1 or classically activated macrophages the level of

phagocytosis is decreased. After performing phagocytosis assay with macrophages control as well as with macrophages treated either with cytokines or with 10058-F4, we observe that this particular activity is decreased in both treated groups indicating that in both cases macrophages are acquiring the M1 phenotype.

In order to study the protein degradation profile of macrophages we measured proteasome activity in macrophages control as well as in macrophages treated either with cytokines or with 10058-F4. The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds. The proteasomal degradation pathway is essential for many cellular processes, including the cell cycle, the regulation of gene expression, and responses to oxidative stress. Results indicate an increased proteasome activity in macrophages treated with cytokines as well as in macrophages treated with 10058 F4 indicating the importance of Ivns1abp function in preventing cell protein degradation and apoptosis.

With the present work we have demonstrated the central role of Ivns1abp gene in promoting regenerative macrophages and how the expression of this gene may be regulated by the presence of cytokines and c-myc transcription factor. This study may contribute to a better understanding of renal regeneration and to the control of acute renal failure.

6. CONCLUSIONS

1. We have identified by functional genomics, the expression of the 14 genes crucial for the induction of the endogenous reparative capacity of the kidney that are modulated by the presence of macrophages.

2. Over-expression of Ivns1abp gene in macrophages enhances their regenerative capacity and switches their phenotype towards M2, provoking modulation of their inflammatory state and increasing its resistance against inflammation inputs.

3. Silencing of the Ivns1abp gene to macrophages leads to acquire a M1 phenotype and decrease their reparative and fagocytosis capacity.

4. Ivns1ab gene expression in macrophages is regulated by the c-myc transcription factor and modulated by the presence of cytokines determining cell fate.

7. RESUMEN

El aparato urinario desempeña una labor vital de gran relevancia, ya que no sólo se encarga del mantenimiento y la homeostasis del líquido corporal, sino que también es responsable de la eliminación de desechos metabólicos del organismo, potencialmente tóxicos, que pueden perjudicar el funcionamiento normal del sistema. El aparato urinario de vertebrados se compone de dos riñones (productores de la orina), dos uréteres (conductores de la orina hacia un emplazamiento de reserva o almacén), la vejiga (reservorio temporal de la orina) y la uretra (conducto que comunica la vejiga con el exterior para evacuar su contenido).

Las funciones básicas del riñón pueden ser; excreción de productos de desecho del metabolismo, regulación del medio interno a través del mantenimiento del equilibrio hidroelectrolítico y ácido-base y función endocrina, mediante la síntesis de metabolitos activos de la vitamina D y del sistema renina-angiotensina (1).

Estructura y organización del riñón

Desde el punto de vista anatómico el riñón de mamíferos es un órgano rojizo con forma de habichuela, localizado en el retroperitoneo a ambos lados de la columna vertebral Desde el punto de vista morfológico, se pueden distinguir desde el exterior hacia el interior tres estructuras: la cápsula, la corteza y la médula renal.

La corteza renal está constituida esencialmente por corpúsculos renales y una compleja red de túbulos asociados que forman parte de la nefrona (1).

La nefrona

La nefrona es la unidad estructural y funcional básica del riñón. Está constituida por un corpúsculo renal y un sistema de túbulos asociados.

El corpúsculo renal es esferoidal, comprende el glomérulo y la cápsula de Bowman circundante. El glomérulo es un ovillo capilar que contiene entre 10 y 20 asas capilares y se encuentra englobado por una estructura bilaminar con aspecto caliciforme que se denomina cápsula de Bowman o cápsula renal.

Desde el corpúsculo renal arrancan los segmentos tubulares de la nefrona, que se designan según el trayecto que adoptan (contorneado o recto), según la ubicación (proximal o distal) y según el espesor de la pared (delgado o grueso) (2).

Fallo renal agudo

El Fallo Renal Agudo (FRA) se define como una disminución rápida de la función renal, que ocurre en horas o algunos días. Este deterioro provoca una incapacidad de los riñones para excretar los productos nitrogenados derivados del metabolismo proteico y posteriormente para mantener la homeostasis hidroelectrolítica y el equilibrio ácido-base. Los electrolitos, como potasio, sodio y calcio, que se necesitan para el funcionamiento normal del organismo, llegan a ser dañinos, y a veces venenosos, cuando alcanzan valores anormalmente altos o bajos. En todos los casos existe un descenso de la tasa de filtración glomerular, que en la clínica se mide por medio del aclaramiento de creatinina.

El daño renal puede dividirse desde el punto de vista etiológico en tres categorías(3) :

1) Prerrenal (60-70% de los casos): FRA caracterizado por la disminución del flujo sanguíneo, supone una respuesta fisiológica a la hipoperfusión renal en la cual está preservada la integridad de los riñones.

2) Renal intrínseca: FRA debido al tejido renal dañado (causas glomerulares, tubulointersticiales, necrosis tubular aguda, necrosis cortical aguda). Afecta directamente al parénquima renal Constituyen el 20-40% de casos.

3) Postrenal (5-10% casos): FRA debido a la obstrucción del tracto urinario (uréteres/uretra).

Entre los factores que incrementan el riesgo de FRA, se incluyen la edad avanzada, insuficiencia renal crónica, enfermedad renal, diabetes, presión sanguínea elevada, fallo cardiaco y obesidad.

Aunque se considera un proceso multifactorial, con varias condiciones solapadas (incluyendo isquemia, sepsis, medicación y nefrotoxicidad, y en etapas posteriores efectos retrógrados de obstrucción renal o uretral por piedras), se conoce que el grado agudo o crónico de isquemia es la causa principal de fallo renal (4).

Isquemia / Repercusión

La isquemia se define como la parada del flujo sanguíneo, que se traduce en una disminución de la presión parcial de oxígeno, privando a los tejidos isquémicos de oxígeno y de nutrientes y provocando una alteración del metabolismo celular. A escala celular, si la disminución de la presión de oxígeno es parcial, se conoce como hipoxia, y si es total, como anoxia. Cuando se restablece de nuevo el riego sanguíneo se produce

el fenómeno de reperfusión. Cabría pensar que al volver a circular la sangre y restaurar el aporte de oxígeno y nutrientes al órgano, éste se recuperaría, quedando sólo la lesión isquémica, sin embargo, y paradójicamente, la reperfusión da lugar a una lesión más grave que la lesión isquémica, denominada lesión por reperfusión. En conjunto, los acontecimientos fisiopatológicos que acontecen como consecuencia del restablecimiento del flujo sanguíneo tras un período de isquemia se denomina síndrome de isquemia-reperfusión (I/R)(8). Se trata de un proceso complejo en el cual la lesión se inicia durante la isquemia y aumenta durante la reperfusión.

La isquemia es crítica para múltiples condiciones de enfermedad afectando al riñón, incluyendo enfermedades renales agudas y crónicas en las cuales un insulto inicial lleva a daño tisular y consecuentemente al fallo del órgano(10).

Fases de la I/R

El periodo de tiempo que media desde la exposición al agente etiológico y el comienzo del daño parenquimatoso se denomina fase de inicio. Esta fase se caracteriza por la pérdida de la polaridad celular, hinchazón, pérdida de adherencia de la matriz extracelular, y muerte celular. La tasa de filtración glomerular desciende debido a la caída del flujo sanguíneo renal, a la caída de la presión de ultrafiltración glomerular y a la pérdida de integridad del epitelio tubular. La porción terminal del túbulo proximal y la porción medular ascendente del asa de Henle, son los segmentos de la nefrona más vulnerables a la isquemia debido a que ambos tienen una alta tasa de transporte activo de solutos, ATP dependiente, y por tanto también un elevado consumo de oxígeno.

La siguiente fase se denomina fase de mantenimiento. Durante esta fase el daño parenquimatoso está consolidado. Se produce cuando aparecen las complicaciones urémicas. No se conoce con exactitud porqué la tasa de filtrado glomerular permanece baja a pesar de que se corrija la causa del fracaso renal (por ejemplo, se estabilice la situación hemodinámica).

La fase de recuperación es el periodo durante el cual ocurren los fenómenos de reparación y regeneración del tejido renal y es absolutamente dependiente del grado de daño inicial. Esto depende tanto de la capacidad de recuperación de las células dañadas subletalmente, como de la eliminación de restos necróticos y cilindros

intratubulares y de la capacidad de regeneración de las células tubulares para restaurar la continuidad y función normal del epitelio tubular. Su presencia señala un proceso regenerativo tras la I/R renal (12, 13).

Macrófagos

Los macrófagos son células fagocíticas muy diversas que participan en un gran número de funciones, tanto en el desarrollo de patologías como en el mantenimiento de la integridad del tejido. Son fundamentales tanto para el mantenimiento de una buena defensa contra los patógenos invasores como para la regulación de la homeostasis, promoviendo la angiogénesis, remodelación y reparación de tejidos.

Los macrófagos forman parte del sistema fagocítico mononuclear, el cual engloba a un conjunto de células derivadas de un precursor común y con una función principal: la fagocitosis. Junto con los neutrófilos, el sistema fagocítico mononuclear constituye el principal mecanismo de defensa del organismo frente a agentes extraños, como los microorganismos. A diferencia de otras células del sistema inmunológico, los macrófagos, se encuentran en estado proliferativo o se activan y pasan a ejercer sus funciones específicas. En ausencia de estímulos adecuados, los macrófagos sólo pueden permanecer en estado de reposo durante un periodo corto y posteriormente mueren por apoptosis.

Los macrófagos infiltrados son heterógenos, se adaptan al microambiente local y desarrollan una serie de atributos que les permiten realizar una función particular. En los tejidos, los macrófagos se diferencian bajo la influencia de citocinas y, dependiendo del tipo de tejido, se convertirán en células de microglía del sistema nervioso central, en osteoclastos del hueso, en histiocitos del tejido conectivo, en células de Kupffer del hígado, en células de Langerhans de la dermis o en células dendríticas. Las propiedades de los macrófagos en el tejido y lugar dañados varían según la naturaleza y la evolución del daño o de la inflamación (14).

Una vez en los tejidos, los macrófagos juegan un papel crítico en el desarrollo de la respuesta inmunológica. Estas células actúan como mediadoras de innumerables mecanismos diferentes dentro de la respuesta inmunológica; no sólo participan en la inmunidad natural, sino que además, están adaptados a desempeñar funciones esenciales en la inmunidad específica adquirida, tanto humoral como celular. De forma

basal, los macrófagos se encuentran en disposición de realizar una serie de funciones sin necesidad de ningún tipo de estímulo, aunque muchas de estas funciones pueden realizarse con mayor eficiencia su activación (17).

Fenotipos de los macrófagos

Debido a la plasticidad en sus respuestas funcionales, los macrófagos participan en muchas situaciones patofisiológicas. La activación clásica inducida por componentes de membranas celulares de bacterias como LPS (Lipopolisacaridos) o IFN-γ promueven la formación de mediadores como óxido nítrico (NO), superóxido (O2-), factor de necrosis tumoral (TNF)- α, Interleucina (IL)- 1β o IL-6. Este perfil de mediadores se considera pro-inflamatorio y generalmente está asociado al fenotipo M1 del macrófago. Este fenotipo ayuda a eliminar patógenos invadidos, pero, si se produce de manera incontrolada, contribuye a la destrucción del tejido .

Por el contrario, la activación del macrófago hacía un fenotipo anti-inflamatorio o M2 puede ser provocada por estímulos como glucocorticoides, y por citocinas de tipo Th2, como son IL-4, IL-13 o IL-10 , o también por la fagocitosis de cuerpos apoptóticos. Las consecuencias funcionales de la activación M2 están relacionadas directamente con la protección celular, la regeneración y la angiogénesis.

Los fenotipos M1 y M2 son específicamente distinguidos por las citocinas que producen y el modo en que procesan la arginina. A diferencia de los macrófagos M1, el fenotipo M2 no es capaz de producir NO a partir de la L-Arginina y tampoco consigue controlar el crecimiento de patógenos intracelulares. Por otra parte, los macrófagos M2 muestran una elevada capacidad endocítica y fagocítica, y son capaces de producir una elevada cantidad de L-Arginina obteniendo como producto final la prolina, el glutamato y las poliaminas. Estos metabolitos favorecen la reparación tisular; la prolina actúa como precursora del colágeno y las poliaminas han sido relacionadas con procesos de proliferación y diferenciación celular, e incluso con la inhibición de la producción de NO por macrófagosactivados con LPS. Así, estos macrófagos alternativamente activados son capaces de proteger los tejidos circundantes de procesos inflamatorios prolongados o de respuestas inmunes agresivas, induciendo la reparación y/o remodelación del tejido (26, 31).

Ivns1abp

Ivns1abp pertenece a una familia de proteínas denominada como proteínas Kelch porque contiene 4-7 de los unos motivos denominados motivos Kelch. Muchas de las proteínas que contienen motivos Kelch interactúan con la actina y son importantes mediadores de funciones celulares fundamentales, tales como la regulación de la architectura celular, la organización celular y la migración celular. El gen Ivns1abp (Nd1) expresa dos variantes de proteínas. La forma larga de Nd1 (Nd1-L) que contiene 6 motivos Kelch en su terminal C y la forma corta (Nd1-S) que carece de estos motivos. La dinámica del citoesqueleto de actina regula una amplia gama de estructuras y funciones de las células eucariotas. El citoesqueleto de actina es responsable de una amplia gama de funciones celulares, tales como la regeneración y el mantenimiento de la polaridad celular y la motilidad celular. Estas propiedades de los filamentos de actina dependen de un gran sequito de proteínas que se unan a los filamentos de actina y modulan sus propiedades y funciones. Está demostrado que los motivos Kelch de la proteína Nd1-L se asocian directamente con F-actina indicando que Nd1-L es una proteína asociada a la actina (137, 141).

Con estos antecedentes, el objetivo general de esta tesis es: encontrar genes que su modulacion podria controlar el fenotipo y las funciones de los macrófagos con el fin de encontrar nuevos enfoques para la resolución del FRA.

Con este fin, se han desarrollado los siguientes objetivos:

1. La identificacion de un conjunto de genes reparativos que estan relacionados con la presencia de macrofagos M2, que intervienen en la reparacion tisular.
2. Demonstrar que la modulación del gen Ivns1abp, la inflamación y la reparación renal podría ser modulada.
3. Definir las vías que regulan la transcripción del gen Ivns1abp

Resultados y conclusiones

El primer lugar de la lesión tisular después de una lesión isquémica, que se produce en las primeras 24 horas de reperfusión, ha sido bien caracterizado en varios órganos por estudios anteriores e incluye principalmente un aumento de la turgencia, perdida de adhesión y polaridad y muerte celular. Estos cambios se acompañan de un aumento del estrés oxidativo y los mediadores inflamatorios. Pero con el tiempo, la fase de lesión es seguida por una fase de recuperación, en la cual se observa un aumento de células mitóticas y células de-diferenciadas en las áreas dañadas. También, nuestro grupo ha demostrado que la fase temprana de la lesión se caracteriza por reclutamiento masivo de macrófagos, mientras en la fase de recuperación el reclutamiento disminuye.

Después de someter ratones a 45 minutos de isquemia y a 24 y 96 horas de reperfusión, se midió BUN, un marcador de la función renal, en el plasma y expresión de statmina que es un marcador de la regeneración de tejidos. Los resultados nos indicaron que los niveles de BUN son muy superiores en el punto de tiempo de 24 horas de reperfusión en comparación con los niveles de control y los niveles de los grupos de 96 horas, lo que indica una función renal alterada y confirma que las 24 horas de reperfusión pueden ser consideradas como la fase de lesión. Por otro lado, la expresión de statmina sigue siendo baja en los grupos control y las primeras horas después de la reperfusión y muestra su expresión más alta en el punto de 96 horas de reperfusión, indicando las 96 horas como fase de recuperación.

A continuación, se confirmo que los macrófagos están implicados y juegan un papel muy crítico en la determinación de esas dos fases mediante el uso de la técnica de la transferencia adoptiva de macrófagos tanto durante el daño como la fase de regeneración, después de haber eliminado los macrófagos endógenos de los animales usando liposomas de clodronato. En cuanto la fase de lesión, los resultados indican que los niveles de BUN en el plasma son muy reducidos después de la eliminación de los macrófagos. Después de la adición de macrófagos exógenos, los niveles de BUN están aumentanto de nuevo lo que indica la importancia de la presencia de los macrófagos en el establecimiento de la fase de la lesión. Centrantose en la fase de recuperación, midiendo expresión de statmina, también podemos observar una conexión entre este marcador de regeneración y la presencia o la ausencia de los

macrófagos. Estos resultados confirman la doble función de los macrófagos dependiendo de la naturaleza del ambiente que se encuentran (pro-inflamatorio o anti-inflamatorio).

Después de confirmar las dos fases diferentes de la lesión y la regeneración en el riñón isquémico, nos centramos en la identificación de los genes que están implicados en la regeneración renal y que también están relacionados con macrófagos. Para este fin y con el marco del proyecto europeo PROLIGEN, procedemos en establecer el perfil de la expresión génica de cada uno de los grupos experimentales por análisis de micro-array a través del sistema "Applied Biosystems 1700 Chemiluminescent Microarray Analyzer" realizado en el CNIC (Centro Nacional de Investigaciones Cardiovasculares, Carlos III, Madrid, Spain). Los datos obtenidos fueron procesados y analizados por "Genedata", una empresa bioinformática con gran experiencia en el análisis estadístico y la interpretación de estudios de perfiles moleculares.

Entre todos los genes desregulados que alcanzaron el número 7313, nos centramos en el conjunto de genes que fueron desregulados como una función de la presencia de macrófagos en lesión y en la fase de recuperación. Esta selección limitó el número a 110 genes desregulados en la fase de la lesión y 38 genes desregulados en la fase de la recuperación. Con el fin de centrar el estudio en los genes que están implicados en la transformación de los macrófagos hacia su fenotipo anti-inflamatorio, nos centramos en genes que estaban sub-regulados durante la lesión y a la vez sobre-regulados durante la fase de la regeneración llegando a un numero de 14 posibles genes regenerativos.

Como último, se realizó un ensayo "in vivo" para validar el efecto de estos 14 genes en la reparación tisular, silenciando a cada uno de ellos en los macrófagos. Después, estos macrófagos silenciados se administraron en grupos control así como en grupos sometidos en isquemia y reperfusión. Más correctamente, se utilizó un modelo de 45 minutos de isquemia y 48 horas de reperfusión que corresponde con el comienzo de la fase de reparación.

El resultado general del estudio ha indicado que todos los 14 genes son relevantes para promover la reparación después de la lesión por I/R y que la ausencia de cada uno de ellos provoca una disminución significativa en la regeneración renal, indicando su relación con el fenotipo regenerativo de los macrófagos.

Después de establecer la importancia de los 14 genes proliferativos sobre la capacidad de los macrófagos de regenerar, en este estudio nos hemos decidido a enfocar y seguir con el gen Ivns1abp para posterior experimentación. Para estudiar si el gen Ivns1abp juega un papel regenerativo mediante la transformación del fenotipo de macrófagos de M1 a M2, decidimos utilizar como herramienta macrófagos que sobre-expresan el dicho gen. Para lograr estos macrófagos se utilizó un adenovirus especifico producido por "Viraquest" una empresa especializada en este ámbito particular.

Después de establecer las mejores condiciones para la función del vector, procedimos en un "assay test" con el fin de estudiar los posibles efectos en la regeneración renal. Por eso usamos ratones control y ratones sometidos a 45 minutos de isquemia y 48 horas de reperfusión. En una parte de estos grupos de administraron macrófagos transfectados con un vector control asi como con el vector que contenía Ivns1abp.

Los resultados del estudio han demostrado que Ivns1abp ha mejorado la capacidad de los macrófagos para regenerar ya que se ha observado un incremento significativo de marcadores de regeneración en ratones tratados con macrófagos que sobre-expresan Ivns1abp en comparación con los ratones control.

A continuación, hemos examinado el estado inflamatorio de los macrófagos y su relación con la expresión del gen Ivns1abp. Dado que los macrófagos secretan un amplio número de mediadores pro y anti-inflamatorios, en función de su estado, decidimos examinar los niveles de la expresión de algunos de estos mediadores que caracterizan cada estado. Los resultados han indicado que Ivns1abp está conectado y puede alterar la fase inflamatoria de los macrófagos, ya que se reduce la expresión de mediadores pro-inflamatorios y se aumenta la expresión de mediadores anti-inflamatorios en los macrófagos que sobre-expresan Ivns1abp en comparación con los macrófagos control.

La conexión entre Ivns1abp y estado de macrófagos también ha sido observada por tinción de faloidina de macrófagos control y macrófagos que sobre-expresan Ivns1abp. Los macrófagos control presentan un fenotipo redondo y con la actina ubicada en todo el citoplasma, mientas los macrófagos que sobre-expresan Ivns1abp adoptan una morfología más extendida con los filamentos de actina mas presentes en los bordes del citoplasma, una morfología característica de los macrófagos M2.

A continuación se decidió estudiar si la presencia de este gen puede también afectar la resistencia de los macrófagos contra la inflamación. Se sabe que los fenotipos que adoptan los macrófagos (M1 o M2) dependen del entorno de citoquinas que encuentran en los tejidos. En base a esto, se decidió estudiar si la presencia del Ivns1abp puede impedir que los macrófagos adopten un fenotipo proinflamatorio cuando se encuentran en un entorno pro-inflamatorio.De hecho, después de realizar RT-PCR para examinar los niveles de expresión de ciertos marcadores pro y anti-inflamatorios en macrófagos control, así como en macrófagos que sobre-expresan Ivns1abp, sin tratar o tratados con citoquinas, se observó que el nivel de mediadores pro-inflamatorios secretados por los macrófagos se disminuyó mientras que el nivel de mediadores anti-inflamatorios se aumento en macrófagos que sobre-expresaban Ivns1abp en comparación con los macrófagos control tratados con citoquinas, indicando la capacidad del Ivns1abp en aumentar la resistencia de los macrófagos frente la inflamación. Observando la tinción de faloidina de los macrófagos transfectados con Ivns1abp y tratados con citoquinas, podemos ver que aunque la actina empieza a formar “clusters” próximos al nucleo (fenotipo M1), las células aun están preservando su forma alargada que caracteriza el fenotipo M2.

Después de establecer la importancia de la presencia del gen Ivns1abp en los macrófagos con respecto a su capacidad de regeneración y la adquisición de su fenotipo, procedimos en el estudio del efecto de la ausencia de este gen de los macrófagos.

Con el fin de lograrlo, usamos macrófagos transfectados con un adenovirus capaz de derrotar la expresión del gen Ivns1abp usando tecnología que se basa en RNA interference y realizamos un “assay test” para estudiar sus efectos en la lesión tisular. Nuestros grupos de estudio han sido ratones control y ratones sometidos a 45 minutos de isquemia y 48 horas de reperfusión. Parte de los ratones ha sido inyectado con macrófagos transfectados con un vector control o transfectados con el vector que silencia el Ivns1abp. Los resultados indicaron que la ausencia del Ivns1abp puede conducir los macrófagos en causar un mayor daño en el tejido en comparación con los macrófagos control como se puede observar por los niveles del BUN y creatinina en plasma. Tras el análisis histológico del tejido renal de los mismos grupos experimentales, pudimos observar que la capacidad de la reparación de los

macrófagos que carecen de la expresión del gen Ivns1abp se reduce, ya que son incapaces de revertir el daño tisular en comparación con los macrófagos control. Después de medir marcadores de regeneración por inmunofluorescencia en los grupos anteriores se observa una perdida en la capacidad de reparación en los macrófagos silenciados por Ivns1abp. Estos resultados indican que la falta de expresión del gen Ivns1abp no solo vuelve los macrófagos más pro-inflamatorios mediante el aumento del daño que causan en el tejido, pero también causa una pérdida significativa de su capacidad de regeneración, señalando la importancia del gen Ivns1abp.

Como estudio adicional de los efectos de la falta de la expresión del gen Ivns1abp en los macrófagos, procedimos en experimentos “in vitro” A este fin, se utilizó una línea de células epiteliales renales (NRK-52e) dañadas o por adición de colchicina en el medio de cultivo o por simplemente provocar daño mecánico. A continuación en ambos modelos co-cultivamos las células dañadas con macrófagos control o con macrófagos que no expresaban Ivns1abp. Como era de esperar, los macrófagos silenciados han sido incapaces a revertir el daño provocado a las células NRK-52e comparados con los macrófagos control, lo que indica una vez más que la capacidad proliferativa de los macrófagos está vinculada a la presencia del gen Ivns1abp transformando este gen en una diana potencial de alguna futura terapia génica basada en macrófagos.

Después de establecer la conexión del Ivns1abp con el fenotipo de los macrófagos y su capacidad de regeneración, como siguiente estudio nos centramos en el mecanismo a través de cual se regula la expresión génica del Ivns1abp. Como ya hemos demostrado en el estudio 1, todos los 14 genes seleccionados como genes candidatos de proliferación, han sido sub-regulados durante el periodo de la lesión, que es un periodo caracterizado por la presencia de citoquinas pro-inflamatorias y sobre-regulados durante el periodo de la regeneración que se caracteriza por un ambiente anti-inflamatorio. Esos resultados indican que la expresión del Ivns1abp, junto con la expresión del resto de los 14 genes, podría ser controlado por citoquinas.

Midiendo la expresión del Ivns1abp en los macrófagos control así como en macrófagos tratados con diversos cocteles de citoquinas pro-inflamatorias, se observó una disminución significativa en la expresión de este gen por los cocteles de citoquinas, lo

que confirma por un lado los resultados obtenidos con el micro-array en el estudio 1 y por otro lado confirma el control de las citoquinas en este gen.

En primer lugar, hemos decidido centrarnos en microRNAs, ya que estos específicos RNAs forman una clase emergente de reguladores de expresión génica, actuando a nivel post-transcripcional a través de un mecanismo de inteferencia de RNA. Usando un buscador del web especifico para micro RNAs, encontramos un micro-RNA altamente alineado con Ivns1abp, el micro-RNA mir-9, que también esta inducido por la presencia de LPS. La expresión del mir-9 en macrófagos tratados con citoquinas pro y anti-inflamatorias, confirmo que la expresión de mir-9 se relaciona con la presencia de ciertos tipos de citoquinas, convirtiéndolo en un buen candidato con respecto a la regulación del gen Ivns1abp. Sin embargo, cuando se midió la expresión de Ivns1abp en macrófagos transformados para sobre-expresar mir-9, no se observo ninguna diferencia, indicando que, contrariamente a los resultados esperados, mir-9 no es un factor importante para la regulación de la expresión del gen Ivns1abp.

A continuación, hemos decidido a centrarnos en otros posibles factores de transcripción regulados por citoquinas y que tengan como diana el promotor del gen Ivns1abp. Para ello utilizamos como herramienta la base de datos " Genecard" que nos proporcionó información completa sobre el gen por entrecruzamiento de información seleccionada y extraída por ¨SABiosciences Text Mining Application¨ con información proporcionada por ¨UCSC Genome Bioinformatics Site¨. Usando estas herramientas, se encontraron una serie de factores de transcripción que se unen al promotor del Ivns1abp, siento el c-myc uno de los más relevantes.

En nuestro experimental, después de tratamiento de macrófagos en reposo con coctel de citoquinas o LPS se ha observado una disminución significativa en la expresión del factor de transcripción c-myc, lo que confirma su regulación por citoquinas. Con el fin de observar la conexión del c-myc con la expresión del Ivns1abp, hemos utilizado macrófagos que sobre-expresan Ivns1abp y los tratamos con un inhibidor especifico del c-myc llamado 10058-F4 que inhibe especialmente la interacción de c-Myc-Max previniendo la expresión de los genes diana del c-myc. Los resultados indican una disminución significativa en la expresión del Ivns1abp cuando la función de c-myc está bloqueado tanto en macrófagos control como en macrófagos que sobre-expresan Ivns1abp. Después de realizar tinción con faloidina de macrófagos control y

macrófagos tratados con el inhibidor de c-myc hemos observado que los macrófagos tratados obtenían un fenotipo M1, igual que el resultado obtenido después de silenciar la expresión del Ivns1abp en el estudio 2, lo que indica que el factor de transcripción c-myc está implicado en la regulación del gen Ivns1abp.

Fagocitosis es una de las principales funciones de los macrófagos alternativamente activados o M2, mientras que en los macrófagos M1 el nivel de fagocitosis se disminuye. Después de realizar el ensayo de fagocitosis en macrófagos control, así como en macrófagos tratados con citoquinas o con el inhibidor del c-myc, se observa que esta actividad se reduce en ambos grupos tratados, indicando que en ambos casos los macrófagos adquieren el fenotipo M1.

Con el fin de estudiar el perfil de degradación de las proteínas de los macrófagos se midió la actividad de proteasoma en macrófagos control y macrófagos tratados con citoquinas o con inhibidor del c-myc. La función principal del proteasoma es la degradación de proteínas innecesarias o dañadas, por proteólisis. La vía de la degradación proteasomal es esencial para muchos procesos celulares, incluyendo el ciclo celular, la regulación de la expresión génica y las respuestas al estrés oxidativo. Los resultados indican un aumento de la actividad del proteasoma en los macrófagos tratados con 10058-F4 algo que indica la importancia de la función del Ivns1abp en la prevención de la degradación de las proteínas celulares y la apoptosis.

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