ALMA MATER STUDIORUM – UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN

SCIENZE CARDIO NEFRO TORACICHE

Ciclo 35

Settore Concorsuale: 06/D2 - ENDOCRINOLOGIA, NEFROLOGIA E SCIENZE DELLA ALIMENTAZIONE E DEL BENESSERE

Settore Scientifico Disciplinare: MED/14 - NEFROLOGIA

Urine-derived Renal Epithelial Cells from kidney transplanted patients: phenotype, immunomodulatory properties, and effect of the exposition to NGAL.

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Esame finale anno 2023

Summary

Abstract

During kidney transplant procedure transplanted organs can undergo ischaemia reperfusion phenomena, often associated with the onset of acute kidney damage, loss of kidney function and rejection. These events promote cell turnover to replace damaged cells and preserve kidney function, thus cells deriving from nephrons structures are highly voided in urine. Urine derived cells represents a promising cell source since they can be easily isolated and cultured. The aim of this project was to characterise Urine-derived Renal Epithelial Cells (URECs) from transplanted kidney and to evaluate how these cells react to the co-culture with immune cells. URECs expressed typical markers of kidney tubule epithelial cells (Cytokeratin and CD13), and a subpopulation of these cells expressed CD24 and CD133, which are markers of kidney epithelial progenitor cells. The expression of immunosuppressive molecules as HLA-G and CD73 was also observed. As matter of fact, during the co-culture with PBMCs, UREC suppressed the proliferation of CD4 and CD8 Lymphocytes and reduce the T helper 1 subset, while increasing the T regulatory counterpart. Also, preliminary data observed in this study indicated that the exposition to kidney damage associated molecule, such as NGAL, could significantly affect UREC viability and immunomodulatory capacity. These results add new information about the phenotype of urine cells obtained after kidney transplant and reveal that these cells show promising immunomodulatory properties, suggesting their potential application in personalized cell therapy approaches.

Introduction

1. Background

Kidneys are vital life-sustaining organs located below the ribcage, one on each side of the spine. Together with the bladder and the ureter, they constitute the urinary tract. The main function of the kidneys is to filter the blood deriving from other organs, removing waste products and extra water to make urine, while returning amino acids, glucose, hormones, and other substances into the bloodstream. Kidneys also contribute to the acid-base balance of the body and in the regulation of the balance between water, salts, and minerals. In addition, they produce essential hormones, involved in the control of blood pressure and in the production of red blood cells, among many other relevant functions [1].

Nephrons constitute the functional unit of the kidney, processing blood through filtration, reabsorption, and secretion phenomena. The site of blood filtration is the glomerulus, a network of capillaries surrounded by a cuplike structure, known as Bowman's capsule. The filtrate obtained from the glomerulus passes through the tubular structures of the nephron, undergoing different processes of secretion and reabsorption, from which urine is obtained as final product [2,3].

There are several pathological conditions that can irreversibly impair kidney function, including diabetes, autoimmune diseases, drug toxicity, infections, and inherited diseases. The progressive and permanent loss of kidney function caused by a variety of conditions is known as chronic kidney disease (CKD). It is estimated that about 10% of the world's population is affected by CKD and this percentage is expected to significantly increase [4].

CKD identification is based on the evidence of a reduced kidney function for three or more months, with an estimated glomerular filtration rate (eGFR) of less than 60 mL/min/1.73 m^2 and albuminuria higher than 30 mg for 24 hours, as well as the evidence of structural and functional abnormalities [5]. Depending on the eGFR value and albuminuria, CKD can be divided into 5 stages; while in the early stages primary prevention strategies are still possible, stages three to five represent the terminal phase of the disease, with a progressive and irreversible reduction of eGFR, associated with the onset of several symptoms, which are often nonspecific thus they can be

associated with a high number of pathologies. As matter of fact, the lack of clinical evidence in the first stages complicates and delays the diagnosis, thus reducing the efficacy of therapeutic strategies [6].

In the final stage of renal failure, also known as end-stage kidney disease (ESKD), the GFR drops below 15 ml/min, and renal function is irreversibly impaired. At this stage of the disease, the start of the dialysis protocol is needed, to partially replace the functions of the damaged organ, removing waste products, while maintaining the fluid and salts balance [7]. Despite the dialytic treatment represent a fundamental step to take over the functions of the failing organs, both haemodialysis and peritoneal dialysis are associated with several long-term side effects, including cardiovascular complications, chronic inflammation and high risk of infections [8]. Kidney transplantation is the treatment of choice for the resolution of CKD and the ESKD, conferring a significant improvement in the quality of life, and an increase in survival rate compared with dialysis, with 68% lower risk of death. However, because of the increasing demand for kidney transplantation, and the high need of compatible donors, many patients continue to wait prolonged periods before being subjected to transplantation. To overcome these limitations, the inclusion criteria regarding kidney donors have been extended, including donors after cardiac death (DCD), along with living candidates and donors after brain death (DBD). Moreover, the setup of specific therapeutic protocols based on the use of immunosuppressive drugs, and the development of individualized treatment strategies based on immunological and genetic heterogeneity is required to reduce the risk of rejection and irreversible damage of transplanted kidney [9,10].

In addition to the side effects caused by the immune response against the transplanted kidney, we must consider other phenomena that can arise because of the transplant procedure, including nephrotoxicity and ischaemia-reperfusion damage [11]. The ischaemia and reperfusion events occurring during the explant and reimplantation phases of organ transplantation are linked to the onset Acute Kidney Injury (AKI) and are often one of the leading causes of reduced kidney function, increasing the risk of graft rejection. In fact, AKI following kidney transplant represents a common issue in organ recipients, with a particular involvement of the glomerulus and the kidney tubules, resulting in the impairment of tubular cells morphology and functionality [12].

Acute graft damage following kidney transplant also results in an increased cell turnover, as a response to ischaemia and reperfusion injury. Mechanisms that allow the repair of tissue damage

are activated and involve the recruitment of renal progenitors cell, which contribute to the restoration of damaged tubular structures, replacing damaged tubular cells [13]. The result of the cell turnover is an increased exfoliation of epithelial cells lining tubules, which are highly voided in the urine. The study of cell populations isolated from urine of transplanted patients could add more information about tissue regeneration processes occurring right after kidney transplant procedure. In addition, the isolation and culture of kidney derived cells, along with the analysis of their features and properties, could reveal novel potential applications in cell based therapeutic strategies.

Over the years, several markers for the early diagnosis of acute or chronic kidney failure have been discovered, ameliorating the diagnostic procedure. Despite their increasing use in the diagnosis of kidney disease, the interaction between these markers and renal cells is still not completely understood. The analysis of how these markers interact with cells derived from kidneys and the study of their effect on these cell populations during in vitro culture could be a promising tool for the understanding of the mechanism occurring in vivo in both physiological and pathological conditions [14].

2. Urine as a high yield and non-invasive cell source

The kidney consists of various cellular populations, which differ in location, morphology, and function. The study and characterization of different cytotypes has implications in the field of diagnostics, drug screening, basic research and in the development of regenerative medicine and cell therapy approaches [15]. Isolation, in vitro expansion, and cell characterization are crucial steps for the previously listed applications. Biopsies obtained from renal tissue samples represent an invasive procedure and a limited-yield cell source, with several post-biopsy risks, including bleeding, pain and occasionally nephrectomy. Additionally, the isolation process from solid tissues requires the use of both mechanic and enzymatic digestions processes, which can decrease the viability and the proliferation potential of isolated cells. Therefore, non-invasive procedures are highly desirable to increase the viability and growth of primary cells. In this context, urine meets these needs, since it is an easily obtainable cell source, from which it is possible to isolate cells without ethical repercussions and risk for the patients [16].

Urine is about 95% water and 5% waste products, such as urea, creatinine, ammonia, and uric acid, as well as dissolved salts and ions [17]. The analysis of urine samples it's used to detect and manage a wide range of disorders, such as urinary tract infections, kidney diseases and diabetes [18].

In 1972, Sutherland and Bain were the first to successfully describe the isolation and culture of exfoliated kidney cells from urine of newborn children [19].

Through the physiological and daily exfoliation, in fact, it is possible to obtain in the urinary sediment several viable cellular populations, coming from different functional units of the kidney and the lower urinary tract. The exfoliation contributes to the epithelia homeostasis and integrity, with approximately 2.000 to 7.000 cells released in urine daily [20]. As previously reported, the exfoliation rate in healthy subjects is lower that unhealthy patients, and exfoliated cells obtained are usually senescent and unable to proliferate during in vitro expansion [21].

Numerous techniques of cell isolation and characterization have been innovated in recent years, allowing exfoliated cells from the urine to be used as surrogate markers for solid biopsies in predicting changes relating to gene expression, deoxyribonucleic acid (DNA) methylation, DNA damage and protein expression in the kidney [22]. In addition to their application in diagnostics, urine cells are increasingly studied for cell therapy applications in different pathologies, as well as in drug testing procedures [23]. Since the applications of exfoliated kidney cells into clinical practice has increased, the development of protocols to properly isolate and expand the target cell type, and the set-up of proper culture conditions are required [24].

3. Cells in urinary sediment

Urine-derived cells display a certain degree of morphological heterogeneity with cells deriving from the bladder, the urethra, and the renal pelvis, along with squamous epithelia cells from the anterior urethra [25]. Most of the cells isolated and cultured from urine samples derived from kidney tubules and correspond to the two main morphologies that have been previously described as type 1 and type 2 shapes. Type 1 cells derive from the lower urinary tract and display a spindle-like morphology, appearing less elongated than fibroblast or mesenchymal stromal cell populations. In vitro these cells proliferate for up to six passages forming colonies with irregular contours and with relatively low cell-to-cell contact. Type 2 cells are less common and less

proliferating and appear slightly smaller than type 1 cells. This cell type derives from the upper urinary tract, especially from kidney tubules. These cells have a cobblestone-like shape, and usually form compact colonies with smooth-edged contours while proliferating [26,27].

3.1 Podocytes

Podocytes are highly differentiated epithelial cells located on the outer part of the glomerular basement membrane, consisting of three distinct morphological zones: a cell body, major processes, and foot processes. The high number of foot processes in mature podocytes contributes to the selective permeability of the glomerular filtration barrier. The presence of podocytes in urine is a relevant hallmark of kidney damage, since a large amount of these cells is lost in urine in patients with glomerulopathies [28]. Nevertheless, a small percentage of viable cells were found also in healthy subjects. It is possible to easily identify podocytes in urinary sediment targeting specific markers such as nephrin, podocin and podocalyxin, but the in vitro expansion of these cells is limited, due to their low proliferative capacity and the onset of senescence in the early stages of the culture, especially in cells derived from healthy subjects [29]. To overcome these limitations, an increasing number of immortalized podocyte cell lines have been developed over the years [30,31]. The possibility to culture immortalized podocytes for long term period make them an interesting source for the study and the diagnosis of glomerular and genetic diseases, for the study of new therapeutical options and for drug discovery applications [32].

3.2 Proximal and Distal Tubular epithelial cells

Terminally differentiated epithelial cells in the kidney tubules are classified into two main subtypes, Proximal Tubular Epithelial Cells (PTEC) and Distal Tubular Epithelial Cells (DTEC), depending on their localization in the nephron. PTEC are cuboidal and polarized epithelial cells, located in the proximal tube, between the Bowman's capsule and the loop of Henle [33]. PTECs are involved in acid-base control, water homeostasis, reabsorption of compounds deriving from the glomerular filtrate and secretion of metabolites. These cells may undergo alterations because of several inherited and acquired conditions, contributing to kidney failure [34]. Moreover, the presence of high number of tubular cells in urine may correlate to allograft rejection and may be a relevant hallmark of the degree of several kidney disfunctions [35]. A recent study demonstrated the diagnostic potential of urine derived PTEC obtained from transplanted kidneys; donor derived

PTEC where co-cultured with Peripheral Blood Mononuclear Cells (PBMCs) of the graft recipient to analyse the activation of immune population, as markers of graft rejection [36]. In contrast to podocytes, PTECs isolated from the urine retain a slight proliferative activity, that allows them to grow in vitro as a monolayer for up to twelve doublings. As regards the application of PTEC in cell therapy strategies, it was demonstrated that when cultured between two layers of collagen they exhibit the potential to proliferate. Moreover, the formation of organized structures, mimicking in vivo tubules, with the development of numerous microvilli on the apical cell surface indicate that PTEC respond to proper stimuli in certain culture conditions [37].

However, since PTECs quickly lose their phenotypical feature when cultured, most of the studies based on this cell type require the immortalization of the cells for ensure their long term in vitro expansion [38]. The markers for the definition and identification of PTEC population are pan-Cytokeratin, typical epithelial marker, and CD13, which is exclusively expressed on the proximal brush border membrane, along with the expression of CD10, megalin, (LRP2) and cubulin (CUBN) [36,39,40].

Distal Tubular Epithelial cells are located in the ascending limb of Henle's loop and the early distal convoluted tubule [41]. These cells are responsible for the regulation of sodium, chloride, and potassium homeostasis [42]. Compared to cells from proximal tubules, DTEC present a similar morphology, but with a different embryological derivation. Moreover, DTEC are less proliferating and more prone to transdifferentiate during in vitro culture. In urine samples, the expression of the epithelial cell adhesion molecule (EpCAM) allows to distinguish the DTEC subtypes from the proximal counterpart [43].

3.3 Urine Stem Cells (USCs)

Like other organs, adult human kidneys have tissue-specific progenitor cells, located in specific anatomic niches, that contribute to the maintenance of proper renal function, replacing damaged tubular cells with functional ones [44]. Most of progenitor cells of the kidney are in niches located within the Bowman Capsule and in the proximal tubules; these cells are characterized by a spindle-shaped morphology and a hight proliferation rate in vitro. Gupta and colleagues well characterized multipotent renal progenitor cells, demonstrating the expression of typical Embryonic Stem Cell (ESC) markers such as Pax-2 and Oct-4, along with the expression of CD90 and vimentin, while resulting negative for Cytokeratin [45].

The co-expression of CD133 and CD24 surface molecules, which are shared by different types of adult stem cells, along with the lack of CD106, allows to identify a group of progenitor cells mainly located at the urinary pole of Bowman's capsule. Compared to USCs, these cells are more committed toward the epithelial lineage [46] and are able to differentiate into mature podocytes and proximal tubular epithelial cells [47]. It has been proposed that the $CD133^+$ cell subset from kidney retains stemness properties that may contribute to the repair of renal injury [48].

Despite most of the protocols isolate renal progenitor cells from biopsies, using proper culture conditions, it is possible to isolate and expand subpopulation of stem/progenitor cells also from urine samples. The presence of stem cell population in urine was demonstrated by Zhang et al [49], and likely originate from the parietal cell interface of the renal glomerulus. Like kidney tissues, two significant types of undifferentiated donor-derived cells can be detected in human urine. The first subset is constituted by Urine Stem Cells (USCs); compared to stem cells from other sources, USCs are characterized by a higher proliferative activity, with about 70 population doublings, while maintaining their multipotent nature in vitro. Although their phenotype is not an object of consensus in the literature, several studies showed that USCs have a spindle shaped morphology and are able to differentiate into several cell populations, including endothelial, urothelial and smooth muscle cells [50,51]. For what concern their characterization, USC express the typical renal cortex markers *Sine Oculis Homeobox Homologue 2* (SIX2), *Neural Cell Adhesion Molecule* (NCAM), EpCAM, and *Frizzled class receptor* (FZD), suggesting their origin from the kidney. Moreover, mesenchymal stromal cells markers such as CD44, CD73, CD90, and CD105 are highly expressed in USC, with the absence of hematopoietic cell markers (CD45, CD34, and HLA-DR) [52,53]. USCs show immunomodulatory and anti-inflammatory properties, participate in angiogenesis and secrete a panel of growth factors and cytokines involved in the previously listed functions [54]. These cells have been isolated from patients with several disease conditions, including Duchenne Muscular Dystrophy, Systemic Lupus Erythematosus, Epidermolysis bullosa and others. The potential of USCs has been demonstrated also in animal models; the administration of USCs in rats with ischemia reperfusion-induced AKI improved renal function while reducing inflammation, fibrosis and tubular injury [55].

3.4 Urine Renal Epithelial/Progenitor Cells (URECs)

Urine Renal Epithelial Cells (URECs) are another population of progenitor cells founded in urine, considered more differentiated and less proliferating than USCs. URECs can be easily isolated by centrifugation of urine samples and the seeding of cell suspension under proper condition allows to subculture cells for 4 to 10 passages, before they visibly transform, acquiring a more flattened and elongated morphology, suggesting the onset of senescence [56]. The optimization of URECs culture was firstly described in newborn urine samples, the culture medium consists of a 1:1 mixture of Dulbecco's modified Eagles' medium (DMEM) and Ham's F-12 medium supplemented with insulin, transferrin, selenium, and hydrocortisone, URECs were seeded on plates covered by collagen-I matrix in serum free condition [57]. These cells share most of their markers with USCs, differing for their morphology and differentiation potential. In fact, UREC phenotype is intermediate between PTEC and fibroblasts, with the expression of Cytokeratin and other markers typically associated to proximal tubules, along with the expression of fibroblastic-like markers as CD90 and Vimentin. Despite their high heterogeneity, Lazzeri and co-workers showed the presence in urine of a $CD24^+/CD133^+$ subpopulation of renal progenitor cells having epithelial features, with percentages between 28%–70% [58]. The higher expression of CD133 allows to distinguish URECs from USC, that slightly express this marker. There are certain pathological conditions in which URECs are highly voided in urine, but a small amount of progenitor epithelial cells were also founded in urine samples from preterm neonates [59]. For their association with kidney diseases, URECs have already been used in diagnostic procedures as markers of glomerular diseases [60] and in functional and genotypic studies [58]. Recently, Ziegler and colleagues cultured URECs derived from children with hereditary cystic kidney diseases in 3D culture condition to evaluate the epithelial disfunction and to test cells' capacity to perform epithelial morphogenesis in culture [61]. In the same study, they also demonstrated a reduced yield in the establishment of UREC culture from urine of healthy children, confirming the increase in the release of kidney derived cells from stressed and altered tissues.

A loss of renal tubular epithelial cells was also observed in the urinary sediments of renal transplant recipients especially in the first 2 weeks post-kidney transplant, with an increase in their number during acute graft rejection, as demonstrated in a pivotal study published in 1977 [62]. Given the increase in the exfoliation rate occurring after kidney transplant procedure, the present study focuses on the study of URECs derived from patients undergoing kidney transplant, focusing on

their interaction with immune cells and on the effect of their exposition to markers of kidney damage during in vitro culture.

4. Immune cells

Starting from peripheral blood samples it is possible to separate the fraction of Peripheral Blood Mononuclear Cells (PBMCs) from red blood cells and granulocytes. PBMC fraction is an easily isolable source of immune cells and includes lymphocytes, monocytes, and dendritic cells (DCs); it is widely used for in vitro cultures studies regarding peripheral immune cells [63] and for the assessing of immunomodulatory properties of different cell populations [64].

The frequencies of cell subsets in PBMC pool vary across individuals, with lymphocytes being the main component of PBMCs with percentages in the range of 70 and 90% and they mediate the adaptative immune response. Natural killer (NK) cells are effector lymphocytes of the innate immune system, while in the adaptative immune response Thymus-derived lymphocytes (T-lymphocytes), mediate cellular immunity and Bone-marrow-derived (B-lymphocytes) are involved in humoral immune response. The percentage of monocytes is between 10 to 20 %, while DCs founded in peripheral blood samples are rare, in a range between 1-2% [65].

4.1 T Lymphocytes

As regards T cells, they represent the 70 to 85% of lymphocytes in PBMCs, expressing the typical T cell marker CD3. T cells are implicated in the establishment and maintenance of immune response, homeostasis, memory, and tolerance. T lymphocytes originate from bone marrow progenitors that migrate to the thymus for maturation. T cells migrate to peripheral blood and differentiate into several subsets including naïve T cells, which have the capacity to recognise and respond to new antigens, memory T cells that derive from previous antigen recognition and contribute to long-term immunity, and regulatory T (Treg) cells which regulate immune response and self-tolerance. Immune responses commence when naïve T cells encounter antigen and costimulatory ligands presented by dendritic cells (DC), resulting in interleukin 2 (IL-2) production, proliferation, and differentiation to effector cells that migrate to diverse sites to promote pathogen clearance [66].

Since T cells are limited in their natural life span, the in vitro culture of primary T cells is challenging and requires an appropriate stimulation and the set-up of proper culture condition. The polyclonal activation of T cells can be induced by different proliferating stimuli, including antibodies that specifically bind T cell receptors CD3 and to CD28, or with mitogenic agents such as phytohemagglutinin (PHA) [67].

The main components of T cell subset are the T helper $CD4^+$ and the T cytotoxic $CD8^+$, that are characterized by several subtypes, each one with specific cytokine profile and roles in the immune and inflammatory response.

4.1.1 $CD4^+$ T helper cells (Th)

T helper (Th) cells strongly proliferate after the recognition of antigenic peptides associated to MHC-II molecules. Activated Th cells contribute to the maintenance of cytotoxic T cell response while contributing to the activation of B lymphocytes. The synergic effect of circulating cytokine, transcription factors and epigenetic modifications induces the differentiation of activated cells into specific CD4+ subsets that participate in regulation of homeostasis and inflammatory processes.

Th1 cells differentiate in response to IFN-γ and IL-2 stimuli and are involved in the response to intracellular pathogens. Th1 strongly secreted proinflammatory cytokines such as IL-2, IFN-γ, TNF-β [68]. The high secretion of IFN-γ promotes the activation of phagocytes and activates pathways that promote the anti-viral and bactericidal activity of immune cells. Moreover, IFN-γ plays a relevant role in antitumor immunity since it stimulates the response of cytotoxic cells against cancer cells [69]. In addition, the production of IL-2 driven by Th1 cells contributes to the enhancement of phagocytic activity and in the stimulation of T $CD8^+$ proliferation.

Th2 cells are involved in the response against extracellular pathogens and participate in allergic response. This T cell subset is characterized by the secretion of several cytokines, including IL4, IL5, IL9, IL13, IL10, IL25 which play an important role also in the modulation of Th1 activity. As regards humoral response, Th2 cells provide stimuli for B cells to produce IgM, IgA, IgE and IgG immunoglobulins[70].

Th17 cells were first described in 2005, by Harrington and colleagues. These cells share their developmental pathway with Th1 and Th2 cells and are characterized by the release of cytokines involved in the triggering of inflammatory and immune response [71,72]. In particular, the

secretion of IL17A is the hallmark for the identification of Th17 cells, but they also produce IL-21 and IL-22. The binding of IL17 with its receptors initiates several signalling pathways that stimulate the production of proinflammatory cytokines and chemokines, the production and migration of neutrophils and the secretion of antimicrobial peptides. Th17 cells strongly contribute to the response against extracellular agents but also participate in the onset of autoimmune response [73].

T CD4 regulatory cells (Treg) are an heterogenous population that contributes to the modulation of immune response and in the maintenance of tolerance against self-antigen, thus preventing the onset of autoimmune response. $CD4^+$ Treg cells represent about 5–10% of human $CD4^+$ T cells and can be distinguished in thymus-derived (t) and inducible (i) Treg cells, that are stimulated in the peripheral blood. Most Treg cells are characterized by the expression of CD25 surface markers, but the expression of transcription factor FOXP3 is crucial for the identification of functional Treg cells [74]. The high expression of IL-10 and TGF-β by Treg cells, along with cell-cell signals, exert an important role in the control of the immune response [75,76] inhibiting the activation of pro-inflammatory pathways and modulating the activation of immune cells subset including B-cells and NK cells [77,78]. Despite their relevant role in the control of immune-mediated disorders, an excessive induction of Treg could reduce immune response against cancer cells.

4.1.2 $CD8^+$ cytotoxic T cells

CD8 cytotoxic T (T $CD8^+$) cells are stimulated by the recognition of antigens carried by MHC-I molecules on target cells and are important effectors of adaptive immunity and long-term protection against pathogens [79]. Most of $CD8^+$ T cells exert a cytotoxic function, killing cancerous or infected cells by cytolytic action of perforins and granzymes. $CD8^+$ cells also act by releasing a large amount of different cytokines such as IFN-γ and TNF-α, as well as many chemokines, which promote the recruitment of other immune cells that contribute to the elimination of pathogens [80]. The exposition of $CD8^+$ T lymphocytes to stimulating molecules as IL-12 augments the perforin and granzyme production, thus enhancing their cytotoxicity.

In addition to killing infected cells and releasing cytokines at the site of infection, $CD8^+$ T cells have a regulatory role in preventing excessive tissue injury and inflammation, by secretion of the pleiotropic cytokine IL-10 [81].

As for Th cells, several studies demonstrated the presence of different CD8 subsets with different function. $CD8^+$ $IL17^+$ cells are defined by their ability to produce the pro-inflammatory cytokine IL-17A and stimulate the production of many inflammatory mediators by monocytes and fibroblasts, as well as the release of matrix metalloproteinase [82,83]. $CD8^+$ cells expressing IFN-γ, showed an enhanced cytotoxic function, improving their anti-cancer and anti-viral activity [84]. As the $CD4^+$ counterpart, cells with regulatory and tolerogenic functions were also found within CD8 cells. CD8 regulatory T cell strongly express CD122, Ly49 and FOXP3 markers among others, and their differentiation is regulated by transcription factor Helios, homeostatic cytokine IL-15 and TGF-β. The contribution of CD8 regulatory T cells in the downregulation of the immune response is currently the subject of an increasing number of studies [85].

4.2 B Lymphocytes

B-cells represent 5-10% of within the lymphocyte population and are the main mediators of humoral immune response. B-cells mature in response to several signals including CD40 ligand (CD40L), which induce proliferation, isotype switching, Ig secretion, and memory generation. B Lymphocytes differentiate in memory B cells or in antibody secreting plasma cells [86], that recognize and bind specific antigens toward the production of high affinity immunoglobulins [87,88].

B-cells play a pivotal role in the activation of T cell response and can release a panel of cytokines involved in the modulation of inflammation, in the differentiation of specific T cell subsets and in tolerance maintenance, while responding to pro (IL-6, IL-17, IFN-ɣ, PGE2) or anti-inflammatory (IL-4) stimuli deriving from surrounding microenvironment.

Several changes occur in the phenotypic markers of B cell during their development, nevertheless the presence of CD19 surface molecule allows the identification of B cell lineage, while the expression of CD20 is a typical hallmark of mature B lymphocytes [89].

Despite their low proliferative activity, over the years, different culture methods have been developed to support the activation and proliferation of human B cells, that become a useful tool for studying human immunity also in coculture assays [90,91].

4.3 Natural Killer

NK cells where first discovered in 1960 and represent a “naturally” cytotoxic cell population that do not require prior antigen exposure to mediate their cytolytic function [92]. NK cells have innate anti-cancer and antiviral functions, in response to cells that have undergone a malignant transformation or have become infected. In addition, as CD8 or Th1 cells, NK cells release a panel of inflammatory cytokines (IFN-γ, TNF-α, and GM-CSF) that contributes to the induction of adaptative response against intracellular pathogens [93].

4.4 Monocytes

Monocytes are a leucocyte subpopulation developed in bone marrow and released into the peripheral circulation. Together with macrophages and conventional dendritic, monocytes constitute the “mononuclear phagocyte system” (MPS) [94], playing a relevant role in tissue homeostasis and in the resolution of inflammation. The exposition to bacterial signal molecules as Lipopolysaccharide (LPS) and to other stimulatory molecules (CD40L, IFN-ɣ, TNF-α, IL-8, MIP-1α, and MCP-1) provides a strong stimulatory signal for monocytes activation. Monocytes play an essential role in the response to infections and in the regulation of inflammation by producing several mediators. The most known marker for monocyte identification is CD14, but over the years several monocyte subsets with different marker profile and function were discovered [95]. Circulating monocytes have the potential to differentiate into various cell types including macrophages, dendritic cells, liver Kupffer cells or even microglia in the central nervous system [96]. The development of specific culture condition favour the proliferation of human monocytes in culture, allowing their usage in several basic research applications [97].

5. Interaction between urine-derived cells and immune cells

There are several pathological conditions in which the immune system exerts an unregulated response that leads to chronic inflammation and organ damage. Cells from different tissues interact with immune system modulating the immune response, and their application in immuno-related disorders has been widely studied.

Various studies have demonstrated the interaction between renal cells and immune cells in both physiological and pathological condition. Regulatory components of the immune system play a relevant role in promoting kidney tissue regeneration and limit renal inflammation and damage [98,99]. Alteration of the local immune response in renal tissue critically contributes to initiation and progression of acute and chronic kidney damage, with a key role in the mechanisms governing both renal injury and renal repair. Following injury to renal tubular epithelial cells (TECs) or podocytes, resident immune cells recruit lymphocytes, monocytes and DCs from the blood stream to the damaged organ [100]. The unregulated activation of immune cells causes loss of immune homeostasis, inhibits TEC proliferation and induces programmed cell death [101]. Moreover, the inflammatory microenvironment promotes the epithelial mesenchymal transition (EMT) of renal tubular cells, associated with the onset of tubulointerstitial fibrosis. For all these evidences, immune cells are a potential therapeutic targets in the attempt to promote tissue regeneration in AKI and slow the progression to CKD [102]. As other Stem Cell populations, USCs display immunosuppressive abilities, via cell-cell interaction and by the release of paracrine factors. It was demonstrated that USCs strongly suppress PBMCs proliferation, inhibit B-cell and T-cell activation and mediate NK cytotoxicity, by the secretion of immunoregulatory cytokines and chemokines [103].

As regards tubular epithelial cells, they constitute 75% of the kidney parenchymal cells, thus they are particularly exposed to inflammation stimuli and immune response mediators. It was reported that TECs can act as Antigen Presenting Cells (APCs) via the expression of the Major histocompatibility complex class II (MHC-II) molecule [104]. The recognition of antigen on MHC-II by T cells induces their activation in the tubulointerstitial compartment, promoting significative damages in proximal and distal TECs, with alterations in their structure and loss of their function. Moreover, the activation of immune cells against TECs promotes tubular atrophy and fibrosis and plays a pivotal role in the pathogenesis of acute rejection of graft [105,106].

Therefore, understanding the multi-directional interaction between tubular cells and the immune system is crucial for the outcome of kidney transplantation.

Demmers and colleagues previously analysed the immunosuppressive capacity of primary TECs obtained from human kidney nephrectomies due to tumours. They demonstrated that the activation of TEC with proinflammatory cytokines inhibited the proliferation and induce apoptosis of $CD4^+$

and $CD8^+$ T Cells deriving from healthy donors, while increasing the percentage of $CD4^+FOXP3^+$ regulatory cells [107].

Curci and colleagues demonstrated the immunomodulatory potential of Adult Kidney Stem/Progenitor cells (ARPC) isolated from biopsies by coculturing with PBMCs. Their results showed how ARPC interact with immune system by the release of specific chemokines, modulating Treg proliferation [108].

Since the release of cells in urine is often associated with a response to kidney damage, understanding the interactions between URECs isolated from patients undergoing kidney transplant and immune cell using proper in vitro assays could provide new information about the characteristics of urine cells obtained after kidney transplant.

6. Lipocalin-2 (NGAL)

Lipocalin 2 (LCN-2), also known as NGAL, uterocalin or siderocalin is a glycoprotein first discovered and characterized in a G0-arrested mouse kidney cell cultures, then isolated in neutrophil granules released at sites of infection [109]. As its name suggests, NGAL is a member of the lipocalin superfamily, a group of circulatory proteins with shared motifs but varying sequence homology. Lipocalins are involved in the transport of small and hydrophobic molecules, including steroids, fatty acids, retinoids, prostaglandins, and hormones [110].

Lipocalin-2 has a basal expression in bone marrow and mucosal and epithelial barriers, but several conditions can lead to an increase in its production in nearly all tissues. NGAL can be secreted in three different forms: as a monomer, a homodimer, or as a heterodimer binding Matrix Metallopeptidase 9 (MMP-9) depending on the cell type secreting the molecule. Two cell surface receptors have been proposed for this protein, namely megalin/glycoprotein GP330 and Solute carrier family 22 member 17 (SLC22A17), also known as 24p3R [111].

NGAL is well known as a pleiotropic mediator of inflammatory pathways for its bacteriostatic properties. During the antibacterial innate immune response it plays a key role in the inhibition of bacterial growth acting as a shuttle for iron and bacterial siderophores [112,113]. Iron-associated NGAL (holo-NGAL) traffics to endosomes and releases iron from the complex, which results in regulation of iron-responsive genes, such as ferritin and transferrin receptor, the endosomal NGAL protein core is either degraded in lysosomes or recycled to the extracellular space as the

unconjugated apo-NGAL. Some of the biologic effects of this lipocalin may depend markedly on its association with the siderophore:iron complex, but NGAL is well known as a growth factor acting in a high number of different pathways [114].

The involvement of NGAL as a mediator of biological processes, including cell differentiation, apoptosis and organogenesis has been widely demonstrated. Moreover, NGAL plays a pivotal role also in cancer, as well as in kidneys, lung, and heart injury [115].

6.1 NGAL as a player of innate and acquired immune response

Regarding the effect of NGAL on immune cell populations, most of the studies are focused on the pivotal role of NGAL in modulating the innate response against several pathogens. In the urinary tract, the production of NGAL by α–intercalated cells allow the clearance of infecting bacteria [116]. Moreover, NGAL enhances and sustains the inflammatory response by serving as a chemoattracting factor for the recruitment of neutrophils and for Th1 stimulation [117] and by inducing the synthesis of the proinflammatory cytokines as IL-1α, IL-6, IL-8, and TNFα.

In the lungs, the secretion of NGAL by immune cells, such as neutrophils and macrophages, and by airway epithelial cells is induced in both acute and chronic inflammation, and its upregulation also occurs in response to oxidative stress [118]. Current literature suggests that NGAL could play a paradoxical role in the regulation of inflammation, recruiting neutrophils and inducing proinflammatory cytokine signaling, while also promoting "M2-macrophage polarization and IL-10 production, as demonstrated in pneumococcal infections of respiratory tract [119]. Regarding the adaptive immune response, it was demonstrated an increase in T CD4 regulatory cells derived from PBMCs treated with NGAL, suggesting a possible role of NGAL in the establishment and sustainment of immune tolerance [120].

6.2 NGAL as a marker of kidney damage

As regards the kidneys, LCN-2 has been demonstrated as a potential early biomarker for kidney injuries since its amount in both plasma and urine rapidly increase in several pathological conditions and the increase in its expression is often associated with nephron damage in both acute and chronic kidney injuries [121,122]. In addition to its application in diagnostic, in recent years

an increasing number of studies focused on the effects of NGAL exposure in both in vitro and in vivo models [123]. Although the mechanism of action is not yet fully understood, several studies hypnotize that the upregulation of Lcn-2 expression may positively correlates with protection against renal ischemia/reperfusion damage [124]. One of the mechanisms that could be affected by NGAL is apoptosis [125]; the link between NGAL and apoptosis was already investigated on hematopoietic cells [126], while Han and colleagues showed an interaction between NGAL and caspase 3 in proximal tubular epithelial cells in animal models of AKI. In 2020, Anja Urbschat and coworkers showed that the exposition of Cisplatin-injured tubular epithelial cells to NGAL resulted in less damage and induced cellular proliferation, promoting damage repair [127].

The effects of NGAL on some cell features, including the immunomodulatory potential of renal cells is not fully understood. Since kidney derived cells are highly exposed to the increase of NGAL occurring in several pathological conditions, the analysis of how this lipocalin influences cell features and properties of urine derived kidney cells could add new information on the mechanisms occurring in vivo between kidney and immune cells during the exposition to pro-inflammatory and damage associated molecules.

AIM

During the surgical procedure of kidney transplant, received organ can undergo ischaemia reperfusion phenomena, often associated with the onset of acute kidney damage and loss of kidney function. These events promote an increase in tubular cell turnover, removing damaged cells and replacing them with functional ones, to preserve the vital functions exerted by kidneys. During the tissue regeneration process, cells deriving from nephrons structures, particularly from glomerulus and proximal tubules are voided in urine, together with other cell populations of the urinary tract. Urine derived cells represents a promising cell source, since they can be easily isolated and cultured, without ethical concern. The first aim of this project was to isolate Urine-derived Renal Epithelial Cells (URECs), from urine of patients undergoing kidney transplantation, to better characterize the phenotype of urine cells derived from donor kidney after transplant. Moreover,

the analysis of UREC release at different time point after transplant could help in the diagnosis of kidney damage and graft failure. URECs are a heterogeneous cell population that can be easily maintained in vitro under proper culture condition, but their features are less known if compared with other cell types. As matter of fact, data regarding the immunomodulatory potential of these cells are still lacking. The second purpose of the study was to analyse how URECs react to immune cells deriving from different donors during in vitro culture, to improve our knowledge on the interaction occurring between renal cells derived from transplanted patients and the immune system. During the onset of acute or chronic kidney damage, there is the upregulation of several biomarkers. Lipocalin-2 (NGAL) is a well-known marker of kidney damage, which increase in both acute and chronic kidney injury, targeting tubular cells among many others cell populations. NGAL is also involved in inflammatory processes and in the regulation of immune response. The last purpose of the study was to evaluate the effect of NGAL on UREC viability and immunomodulatory characteristics. All together the characterization of URECs, their interaction with immune cell, and the effect of damage-associated molecule, as NGAL, on cell properties could add more information about the events occurring in the early stages after kidney transplant. Moreover, the ability of URECs in regulating the immune response could be a useful tool in cell therapy applications.

Material and Methods

Patients

Urine samples collected for this study derived from patients undergone kidney transplantation in University Hospital of Bologna IRCCS, Sant'Orsola Polyclinic, after approval of ethic commission (protocol number: 312/2021/Oss/AOUBO). The transplanted kidneys derived from circulatory death (DCD) donors and from living donors. Transplanted patients recruited for the study had no residual diuresis, to ensure the donor origin of kidney cells. Urine of four healthy volunteers was collected as control. Data regarding the characteristics of donors and recipients are summarized in table 1 and table 2 respectively.

Table 1 - Characterististics of donors	
Age (years)	57.5 (48-65.25))
Ischaemia time (min)	12.25 (7.54-13.2)
Living donors (%)	10
DCD (%)	80
DBD (%)	0
Hypertension (%)	40
Diabetes (%)	30

Table 2 - Characterististics of recipients	
Age (years)	46 (38-58)
Haemodialisis (%)	100
Residual diuresis (mL/die)	< 50
Creatinin (mg/dl)	7 (6.03-9.4)
Urea (mg/dl)	65 (59-112)
LDH (mg/dl)	201 (157-225)
PCR (mg/dl)	0.3 (0.16-0.5)

Table 1 and 2: Characteristic of kidney transplanted donors and recipients recruited for the study.

Isolation of Urine Renal Epithelial Cells (URECs)

The urine of transplant patients was collected within the first week after transplantation (T0), when patients resume autonomous diuresis. Urine samples were also collected after one (T1) and six (T6) months from kidney transplant. Samples were processed right after the collection and the range of urine volume was 100-300mL. Urine was transferred from the catheter to sterile 50 mL tubes and then centrifuged at 400g for 10 minutes. The pellet was resuspended in phosphate-buffered saline (PBS, Corning, NY, USA) with 1% penicillin-streptomycin (P/S) solution (10,000 U/mL penicillin, 10,000 U/mL Streptomycin, Corning, NY, USA) and centrifuged at 400g for 10 minutes. After the supernatant was removed, the urine sediment was resuspended in the culture medium for renal cell isolation, consisting of Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM F12, Gibco, Life Technologies, CA, USA) with 10% of fetal bovine serum (FBS, Gibco, Life Technologies, CA, USA), Renal Epithelial Growth Medium (REGM)

SingleQuot kit (Lonza Lonza Bioscience, Basel, Swizerland), 1% P/S solution (Corning, NY, USA), 2.5µg/ml amphotericin B (Biochrom), 100µg/ml normocin (InvivoGen, CA, USA) and 10µg/ml ciprofloxacin (Fresenius Kabi, Graz, Austria). The cell suspension was seeded in flasks and incubated at 37°C and 5% CO_2. After 72 hours, the isolation medium was replaced with the proliferation medium, consisting of Renal Epithelial Basal Medium (REBM™, Lonza Bioscience, Basel, Swizerland) at 10% of FBS and supplemented with REGM ™ SingleQuot kit, 2.5mM GlutaMAX (Gibco, Life Technologies, CA, USA), 1% non-essential amino acids (Termo Fisher Scientific, MA, USA) and 1%P/S. The proliferation medium allows the survival and proliferation of epithelial cells, while death cells and cellular debris will be removed with subsequent medium exchange. Cultures were monitored for the presence of growth foci, and when confluence was reached, the cells were detached from the growth surface by incubation with 0,25% Trypsin/EDTA solution (Corning, NY, USA). The freshly isolated cells were counted and tested for viability using Erythrosine B (Sigma-Aldrich, St. Louis, MO, USA). Only samples with > 90% viability were used for further assays. All the experiments were performed on cells within the third culture passage.

Immunofluorescence analysis

For analysis of Ki-67 by immunofluorescence, URECs were seeded onto glass coverslips and cultured in proliferation medium. Cells were fixed with 10% formalin for ten minutes at room temperature, washed with PBS, and permeabilized by adding PBS 0.1% Triton (Triton X-100, Sigma-Aldrich, Co., St. Louis, MO, USA) for 10 min. URECs were incubated for 30 min with blocking solution containing PBS 1% Bovine Serum Albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) and then incubated overnight at 4°C with primary antibodies rabbit anti-Vimentin (1:200,) and mouse anti-Ki67 (1:250) diluted in blocking solution. Secondary antibodies anti-rabbit Alexa Fluor 594 (1:250), was added and incubated 1 hour at room temperature. After three washes with PBS, coverslips were mounted using the Prolong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, Monza, Italy). Stained cells were observed using Nikon Inverted Microscope (Nikon Instruments, Tokyo, Japan), and images were acquired with a Digital Sight camera DS-03 using the imaging software NIS-Elements (Nikon Corporation, Tokyo, Japan). To quantify the expression of Ki67, ten different fields were acquired, and the number of green-stained nuclei was counted and normalized to total number of cells (100%). Data are expressed as mean ± standard deviation (SD).

Flow cytometry analysis for UREC characterization

After isolation, URECs were seeded in culture flasks and cultured until confluence. Cells were harvested by trypsin digestion and fixed with 10% formalin (Sigma Aldrich St. Louis, MO, USA) for ten minutes. Fixed cells were stained with conjugated antibodies and analysed by flow cytometry. For the surface staining, fixed cells were incubated for 30 minutes at 4°C in staining buffer, made of PBS containing 0.1% BSA (Sigma Aldrich St. Louis, MO, USA) avoiding light exposure. After labelling the cells were washed twice and resuspended in PBS 0.1% BSA.

For the intracellular staining, cells were permeabilized using Fixation/Permeabilization Kit, (BD Bioscience, Cat No 554714) according to manufacturer instruction. After permeabilization, cells were incubated for 30 minutes at 4°C in permeabilization buffer with anti-Cytokeratin (Ck) antibody or anti Ki-67 antibody, washed twice in the same buffer and then in PBS 0.1% BSA. The antibodies and the concentration used for surface and intracellular markers are listed in the table below (Table 3). After the surface and intracellular staining cells were resuspended in PBS 0.1% BSA and analysed by flow cytometry using CytoFLEX S (Beckman Counter, CA, USA) instrument. Unstained samples were used as negative controls and the data were analysed using FlowJo X software (Tree Star, Ashland, OR, USA). Characterization of URECs was performed on five independent samples and for each marker results are expressed as percentage (%) of positive cells.

Antibody	**Diluition**	**Company**	**Cat. No**
CD13 APC	1:100	Biolegend	301706
CD326 (EpCam) FITC	1:100	Biolegend	324204
CD24 PE	1:100	Biolegend	311106
CD44 FITC	1:100	Biolegend	560977
CD73 PE	1:100	Biolegend	34004
CD90 PerCP/Cy5.5	1:100	Biolegend	328118
CD133 APC	1:100	Biolegend	397906
HLA-G PerCp-eFluor™ 710	1:100	eBioscience	46-9957-42
Cytokeratin BV421	1:100	BD Bioscience	564709
Ki-67	1:100	Miltenyi-Biotech	130-120-421

Table 3: Conjugated antibodies used for flow cytometry analysis on PBMCs and URECs.

Addition of NGAL to UREC culture

To evaluate the effect of NGAL on UREC characteristics, cells were cultured in standard proliferation medium or in medium supplemented with NGAL (Recombinant Human Lipocalin-2/NGAL Protein, R&D system, MN, USA) at a concentration of 320 ng/mL. The tested concentration was chosen among three different doses already used in previous analysis. The evaluation of proliferation, apoptosis, viability, and immunomodulatory capacity of URECs was performed for both untreated and NGAL-treated cells.

CFSE assay for UREC proliferation

For the analysis of UREC proliferation with or without NGAL, cells were incubated with Carboxyfluorescein Diacetate Succinimidyl Ester (BD Horizon™ CFSE, Becton, Dickinson, NJ, USA). CFSE covalently bound to DNA is divided equally between daughter cells, allowing discrimination of successive rounds of cell division. For the CFSE staining the cells were resuspended in PBS and labelled with 2µM CFSE for five minutes at 37°C. The reaction was stopped using medium with 50% of FBS. Cells were then centrifuged and resuspended in culture medium with or without NGAL treatment and seeded in 6 well plate at a density of 30.000/cm^2. After four days, cells were detached, and proliferation rate was analysed by flow cytometry evaluating the decrease in cell fluorescence, corresponding to dilution of CFSE during cell division. Proliferation of URECs treated with NGAL was normalized to cells cultured in standard medium (100%).

MTT assay for UREC viability

For the analysis of UREC viability cells were seeded on a 96 well-plate in proliferation medium with or without NGAL and incubated for four days at 37°C and 5% CO_2. After four days of culture cells were incubated with 100uL/well of proliferation medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent at a final concentration 0.5 mg/ml and incubated for 2 hours. The MTT colorimetric assay is based on the reduction of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. MTT solution was

removed and 100uL of DMSO were added to each well to dissolve the purple formazan crystals. The absorbance at 500-600 nm of formazan solution was measured using a microplate reader. Results were normalized setting the viability of URECs cultured without NGAL as 100%.

Annexin V PE/ 7-AAD assay for UREC apoptosis

For the detection of apoptotic cells, URECs were seeded in 6 well plate 30.000/cm^2 and cultured for four days with or without NGAL 320 ng/mL. At the end of the incubation cells were collected and stained with Annexin V PE/7-AAD apoptosis detection kit (Biolegend, Cat. No 640934). Annexin V has a high affinity for phosphatidylserine (PS) that is commonly exposed in the inner leaflet of the plasma membrane. During early-stage cell apoptosis, PS is translocated from the inner to the outer leaflet of the cell membrane, where it is detected by Annexin V. As cells progress through apoptosis and towards necrosis, the cell membrane is compromised and consequently the 7-AAD viability dye passes into the cell. For the detection of early and late apoptotic cells, URECs were collected, washed with PBS, and labelled for 15 minutes at room temperature with anti-Annexin V PE and 7-AAD, both used 2:100 in binding buffer. After the staining, 400 µL of binding buffer were added to each tube and samples were analysed by flow cytometry. Unstained samples were used as negative controls and results were represented as percentage of Annexin V PE^+/ 7-AAD^- (early apoptosis) and Annexin V PE^+/7-AAD^+ (late apoptosis) cells in both culture conditions.

Isolation of PBMCs

PBMCs were obtained from the blood of healthy donors according to the protocol approved by the Ethics Committee. PBMCs were isolated by density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA). Then the blood sample was centrifuged at 1500 rpm for 30 minutes without brake. After centrifuge, the ring containing PBMC population was collected and resuspended in PBS. The cell suspension was centrifuged at 1500 rpm for 10 minutes and the washing step was repeated twice. PBMCs were counted with methyl violet (Sigma-Aldrich, St. Louis, MO, USA) to exclude red blood cells and with Erythrosine B to evaluate viability; cells were frozen at –80 °C in FBS with 10% of dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA).

Co-culture of URECs and PBMCs with or without NGAL

URECs were seeded at a density of 40.000 cells/cm^2 in 96 well plates in proliferation medium. After 24h PBMCs were thawed and activated by stimulation with anti-CD3 (CD3 Monoclonal Antibody HIT3a, Functional Grade, eBioscience™, Invitrogen, MA, USA) and anti-CD28 (CD28 Monoclonal Antibody (CD28.2), Functional Grade, eBioscience™, Invitrogen, MA, USA) antibodies. Stimulated PBMCs were seeded at a concentration of 200.000 cells/well above the UREC monolayer (PBMCs + URECs), activated PBMCs seeded in the absence of URECs were set as positive control, while non-activated PBMCs represent the negative control. Cells were incubated for 72 hours and the medium for the co-culture experiments was RPMI 10% FBS, 2.5mM GlutaMAX (Gibco), 1% non-essential amino acids (Termo Fisher Scientific, MA, USA) and 1% P/S. To analyse the effect of NGAL on immunomodulatory properties of URECs, coculture (PBMCs + URECs + NGAL) and positive controls (PBMCs + NGAL) were cultured also with NGAL 320 ng/mL for 72 hours. Proliferation, apoptosis, and analysis of lymphocyte subpopulation were assessed by flow cytometry.

CFSE assay for CD4$^+$ and CD8$^+$ cell proliferation

To analyse the proliferation of CD4$^+$ and CD8$^+$ lymphocytes with or without coculture with URECs and in presence of NGAL, PBMCs were labelled with BD Horizon™ CFSE according to manufacturer protocol. Briefly, thawed PBMCs were resuspended in PBS and labelled with CFSE 2uM for five minutes at 37°C. The reaction was stopped using RPMI with 50% of FBS, cells were then centrifuged and resuspended in culture medium. CFSE-labelled PBMCs were activated with anti-CD3 and anti- CD28 antibodies and seeded at concentration of 200.000 cells/well in 96-well plate for both positive controls and coculture condition, as previously described. After 72h PBMCs were collected, washed with PBS, and stained with anti-CD4 APC (Biolegend, Cat. No 300514) and anti-CD8 Pecy7 (Biolegend Cat. No 344712) antibody for 30 minutes at 4°C in PBS 0,1% BSA, both diluted 1:100. The proliferation rate of CD4 and CD8 cells was evaluated by flow cytometry using Cytoflex S instrument. Results were compared to positive control set as 100%. A total of 20.000 events among both CD4 and CD8 cell subsets were acquired for each sample.

Analysis of PBMCs apoptosis with Annexin V/7-AAD assay

The apoptosis of PBMCs with or without coculture and in presence of NGAL was assessed using Annexin V/7-AAD kit (Biolegend, Cat. No 640934). Cells were stained according to manufacturer

instructions. Briefly, PBMCs were collected, washed with PBS, and then labelled for 15 minutes at room temperature with anti-Annexin V PE and 7-AAD in binding buffer, both used 2:100. After the staining, 400 µL of binding buffer were added to each tube and sample were analysed by flow cytometry. Unstained PBMCs were used as negative controls and results were represented as percentage of Annexin V PE^+/7-AAD^- (early apoptosis) and Annexin V PE^+/7-AAD^+ (late apoptosis) cells among PBMCs.

Flow cytometry analysis of lymphocytes subpopulations

PBMCs from healthy donors were cultured with URECs as described before. After 72h of incubation PBMCs were collected and the characterization of T cell subset was performed. For the analysis of Treg population, anti-CD4 APC (Biolegend, Cat. No 300514) and anti-CD25 FITC (Biolegend, 302604) were used as surface markers, by incubation of 30 minutes in PBS 0.1% BSA. For intracellular staining PBMCs were fixed and permeabilized with eBioscience™ Foxp3/ Transcription Factor Staining Buffer Set (Invitrogen, Cat. No 00-5523-00) according to manufacturer instructions. Briefly, cells were fixed for 30 minutes at 4° with Fixation/Permeabilization solution, previously diluted 1:4 with Foxp3 Fixation/Permeabilization Diluent, and then permeabilized for 15 minutes at room temperature with 1X Foxp3 Permeabilization Buffer. Cells were labelled with anti-FoxP3 PE (Biolegend, Cat. No 320108) antibody for 30 minutes at 4° in permeabilization buffer, then washed twice and resuspended in PBS 0.1% BSA.

For the quantification of intracellular cytokines in Lymphocyte subsets, a fraction of PBMCs of both control and coculture conditions was treated with a mixture of 1nM Phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich, St. Louis, MO, USA), Ionomycin 3mg/ml (Sigma Aldrich, St. Louis, MO, USA) and 1ul/ml Golgi Plug™ (BD, Becton, Dickinson, NJ, USA) and incubated at 37 ºC to 5% CO2 for four hours before staining. For IFNɣ-producing populations, cells were stained for surface markers with anti-CD4 APC (Biolegend, Cat No. 300514) and anti-CD8 Pecy5.5 antibody, while the intracellular staining was performed, after fixation and permeabilization steps, for 30 minutes at 4°C with anti-IFNɣ-PeCy7. For IL17 producing cells, anti-CD4 APC (Biolegend, Cat No. 300514) and anti-CD8 Pecy7 (Biolegend, Cat No. Cat. No 344712) were used for surface markers, while intracellular staining was performed with anti-

IL17A-PE (Biolegend, Cat No. 512306). For the flow cytometry analysis 20.000 events among CD4 and CD8 cells were acquired for each sample.

Luminex® xMAP® Technology for cytokine release quantification

The analysis of cytokine release was performed in cell culture supernatant of PBMCs and PBMCs + UREC conditions. After 72h of culture conditioned media were centrifuged and collected into 1.5 mL tubes; samples were stored at −80°C until subsequent analysis. Samples were thawed at room temperature and cytokines were quantified using the human custom procartaplex-19 (Cat. No. PPX-19-MXRWE2G, Invitrogen) according to manufacturer protocol and the concentration of cytokines was measured by MAGPIX ™ (Luminex® xMAP® Technology, Austin, TX, USA). All samples were analysed in technical triplicates. The amount of each cytokine in the coculture condition was normalized to the control condition (PBMCs) set as 100%. The cytokine analysed are listed in the table below (Table 4).

Cytokines	**Abbreviations**
Interleukin 1 beta	IL-1β
Interleukin 2	IL-2
Interleukin 4	IL-4
Interleukin-5	IL-5
Interleukin-6	IL-6
Interleukin-7	IL-7
Interleukin-8	IL-8
Interleukin-10	IL-10
Interleukin-12p70	IL-12p70
Interleukin-13	IL-13
Interleukin-17	IL-17
Interferon gamma	IFN-ɣ
Tumour necrosis factor-alpha	TNF-α
Tumour necrosis factor-beta	TNF-β
CD40 ligand	CD40-L
Granulocyte-macrophage colony-stimulating factor	GM-CSF

Granulocyte Colony Stimulating Factor	G-CSF
Monocyte Chemoattractant Protein-1 (MCP-1)	MCP-1
Macrophage inflammatory protein 1-β	MIP-1β

Table 4. List of cytokines analysed with MAGPIX ™ Luminex® xMAP® Technology

RNA extraction from URECs and PBMCs

URECs were seeded in T25 flasks at the density of 30.000 cells/cm^2 and cultured in proliferation medium with or without NGAL supplementation. Untreated and NGAL-treated cells were collected after four days. As regards PBMCs, 7*10^^{6} cells were thawed and resuspended in culture medium prior to RNA extraction. For the extraction of RNA, from URECs and PBMCs samples, the RNeasy mini kit (QIAGEN, Valencia, CA, USA) was used following the manufacturer's instructions. The genomic DNA contamination was removed by digestion with RNase-free deoxyribonuclease I (RNase-free DNase set, QIAGEN, Valencia, CA, USA). The evaluation of RNA quality and concentration was assessed using the NanoDrop® 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to reverse transcribe the RNA according to the manufacturer's instructions.

Real-time PCR for gene expression evaluation

Real-time PCR (qPCR) was performed in a Bio-Rad CFX96 real-time thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). For each condition, 25 ng of cDNA were amplified using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in technical triplicate. Data were analysed using the software CFX Manager (Bio-Rad Laboratories, Hercules, CA, USA) and the $2^{-\Delta\Delta Ct}$ method. The TATA box binding protein (TBP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as reference genes. For the analysis of LCN-2 and SLC-22A17 expression in URECs and PBMCs, the normalized expression value of URECs was set to 1, and the other gene expression data were compared with that sample. Data are expressed as fold change ± SD. The primer used are listed in the table below (Table 5).

Primer	Company	Cat. No	ID
GAPDH	Origene	HP205798	NM_002046
TBP	Origene	HP220445	NM_001172085
LNC-2	Origene	HP208681	NM_005564
SLC-22A17	Origene	HP233381	NM_020372
CD24	Origene	HP210404	NM_013230
CD133	Origene	HP209042	NM_006017

Table 5: Primer for real time PCR gene expression analysis on URECs and PBMCs.

Statistical analysis

All the experiments were performed at least on five donors. Data are expressed as mean ± standard deviation (SD) and were analysed with *t*-test using Graph Pad Prism 7.04 software (San Diego, CA, USA). The significance threshold was $p < 0.05$.

Results

Isolation and culture of URECs

Urine was firstly collected between 5-10 days after kidney transplant (T0), sample collection was also performed after one (T1) and after six months (T6), as described in material and method section. Urine sediment was seeded in culture flasks with the isolation medium. After 48 hours, it was possible to observe adherent cells with a cobblestone shaped morphology. The addition of the proliferation medium promoted cells growth, with the formation of numerous colonies; cells reached confluence within 10-12 days from isolation (Fig.1A).

As shown in the graph of figure 1B (Fig. 1B), in all patients recruited for the study the isolation of URECs at T0 was successful. At T1 the possibility to successfully isolate and culture URECs significantly decreased, with only 30% of positive isolation obtained. At time point T6 not successful isolations were observed, and the same result was obtained in healthy subjects (healthy control), used as control.

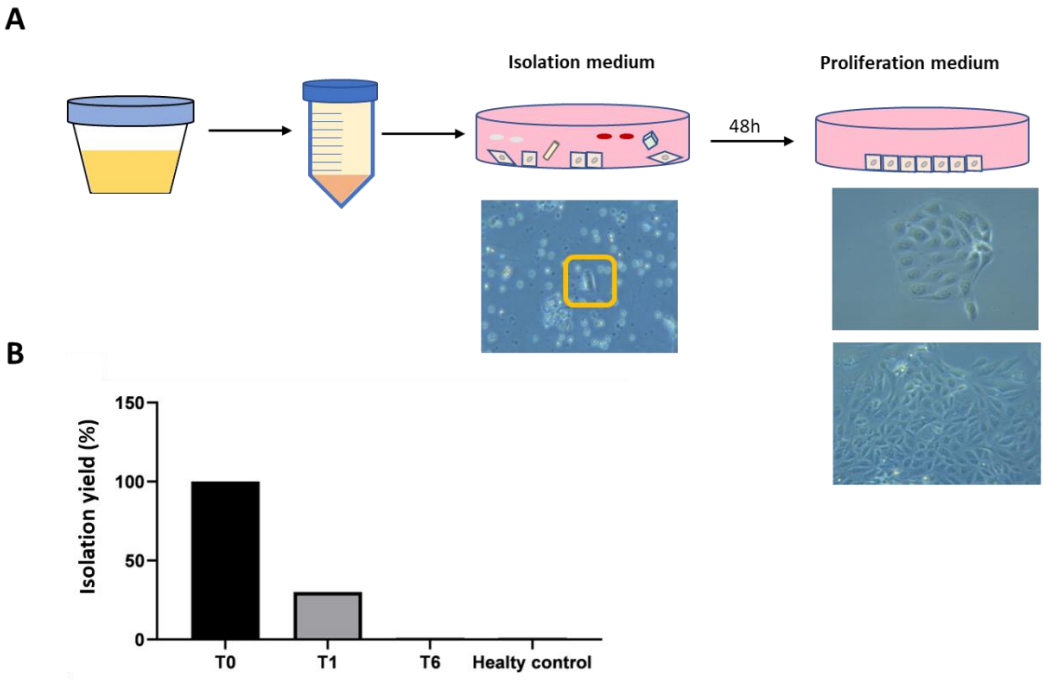

Fig. 1: A) schematic representation of UREC isolation protocol; B) percentage of UREC isolation yield after transplant (T0), and at one (T1) and six (T6) months after surgery. Transplanted patients (n=10), healthy controls (n=5).

Analysis of UREC proliferative activity

The immunofluorescence analysis of Ki67 nuclear expression on isolated URECs revealed the presence of two populations, allowing to distinguish cells in active proliferation ($Ki67^+$) from less proliferating cells ($Ki67^-$). As shown in the representative immunofluorescence images and in the related graph (Fig. 2A), the percentage of $Ki67^+$ cells (78.6 ± 12.53 %) was significantly higher than the $Ki67^-$ negative subset of cells (21.40 ± 12.53 %). Results obtained by immunofluorescence were also confirmed by flow cytometry, where the percentage of $Ki67^+$ cells was assessed at 87% (Fig. 2B).

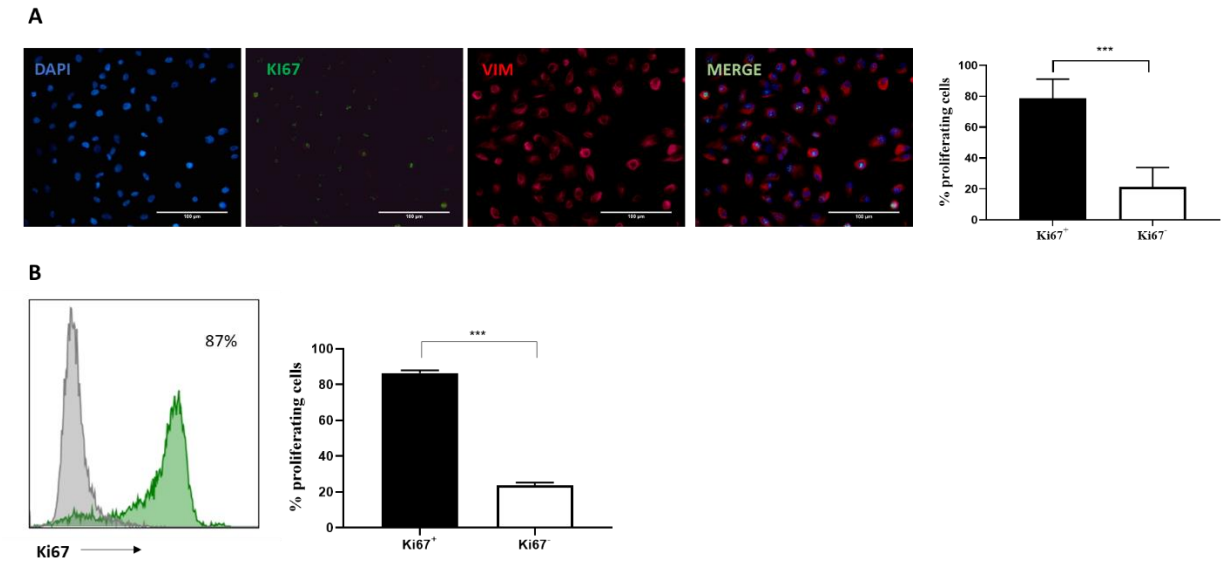

*Fig. 2: A) immunofluorescence analysis of UREC proliferative activity. Images represents cells stained with Dapi (blue), Ki67 (green) and vimentin (red), the related graph shows the percentage of Ki67$^+$ and Ki67$^-$ cells. B) Flow cytometry analysis for Ki67 expression in URECs. Results are expressed as mean ± SD, *** p< 0.001*

Immunophenotype characterization of URECs

To better characterize the isolated cells, the expression of a panel of markers was performed. As shown in the representative panel and in the graph of figure 3, URECs were highly positive for the epithelial marker Cytokeratin (96.8 ± 7.04%). Among Cytokeratin positive cells (Ck$^+$), a high expression of proximal tubules marker CD13 was observed (89.53 ± 12.48%), while the expression of distal tubules marker epithelial cell adhesion molecule (EpCAM) was lower, with percentages of positive cells of 30 ± 12.8 %. Moreover, as shown in the flow cytometry plot, the expression of EpCam majorly co-expresses with CD13 marker, indeed the percentage of CD13$^-$EpCAM$^+$ cells was lower than 4%.

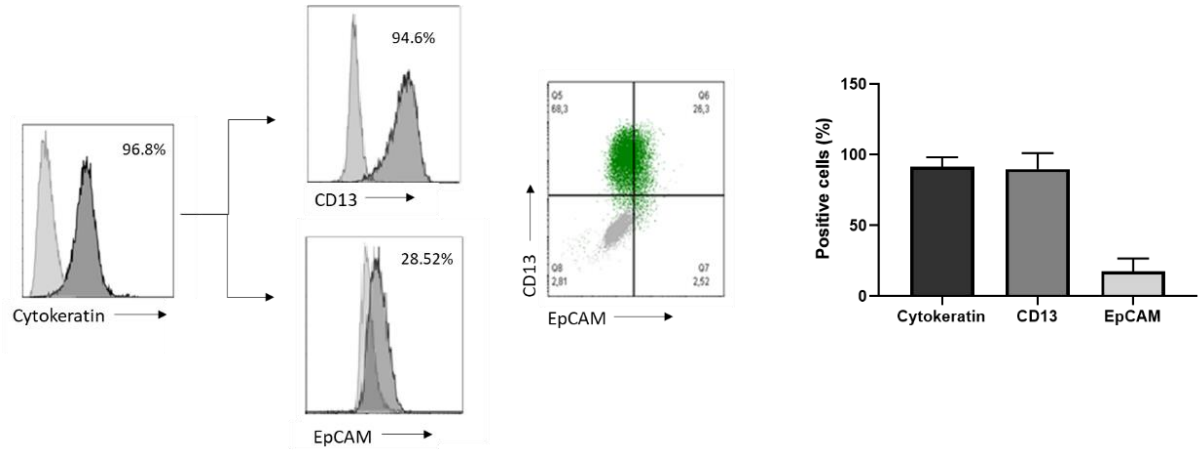

Fig. 3: Representative flow cytometry plots and related graph of Cytokeratin, CD13 and EpCAM expression in URECs. The graph shows the percentage of positive cells for each marker. Results are expressed as mean ± SD, n=5 independent experiments.

The study of UREC phenotype was improved thanks to the analysis of CD24 and CD133 markers, which are both known as typical surface molecules of kidney epithelial progenitor cells. As shown by flow cytometry results in figure 4 A (Fig. 4A), UREC population resulted highly positive for CD24 expression (99.8 ± 5.6 %). The analysis of CD133 expression revealed the presence of two subpopulations, with 67.05 ± 14.3 % of $CD24^{+}CD133^{+}$ double positive cells and 30.2 ± 14 % represented by $CD24^{+}CD133^{-}$ subset. The percentage of both $CD24^{-}CD133^{+}$ and $CD24^{-}CD133^{-}$ population was under 5%. The higher expression of CD24 compared to CD133 was also confirmed by real time PCR analysis (Fig. 4B).

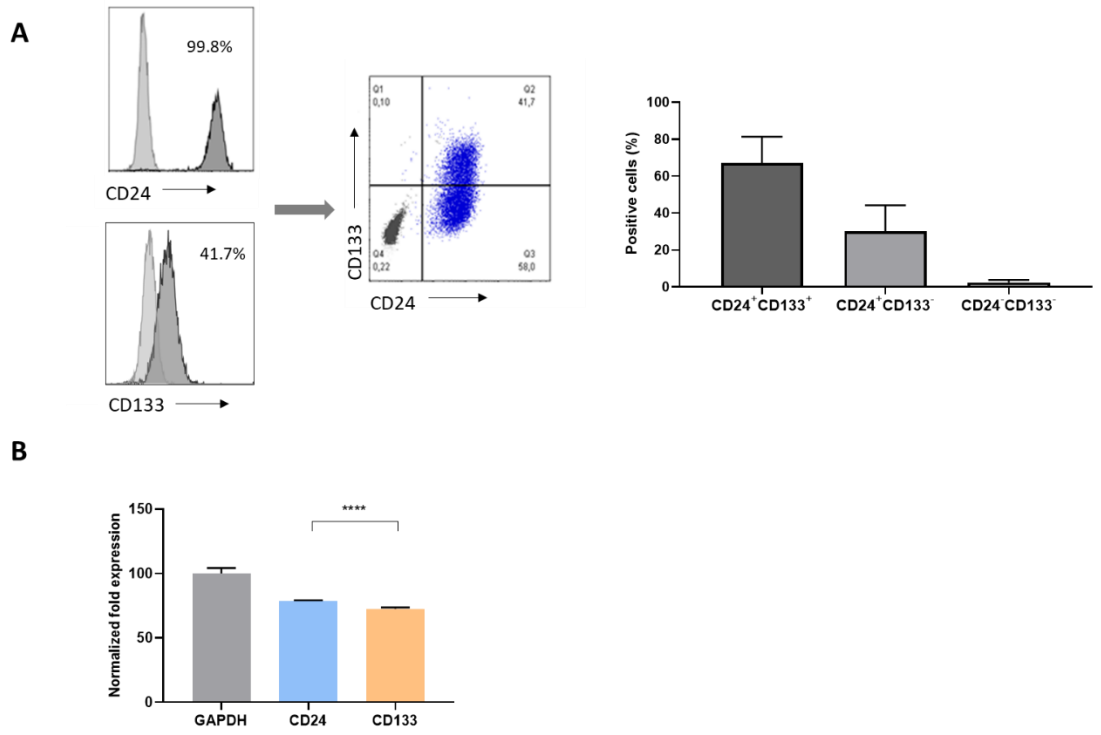

Fig. 4: Analysis of CD24 and CD133 expression in URECs by both flow cytometry (A) and real time PCR (B) Results are expressed as mean ± SD, n=5 independent experiment.

Further characterization of URECs was performed with the analysis of mesenchymal stromal cells markers. As shown in figure 5A, cells resulted highly positive for CD44, CD73, CD90 and CD105 surface molecules. Interestingly, URECs also expressed tolerogenic molecule HLA-G (54.8 ± 16.06%), while the expression of HLA-DR was detected in 5.84 % of cells (Fig. 5B).

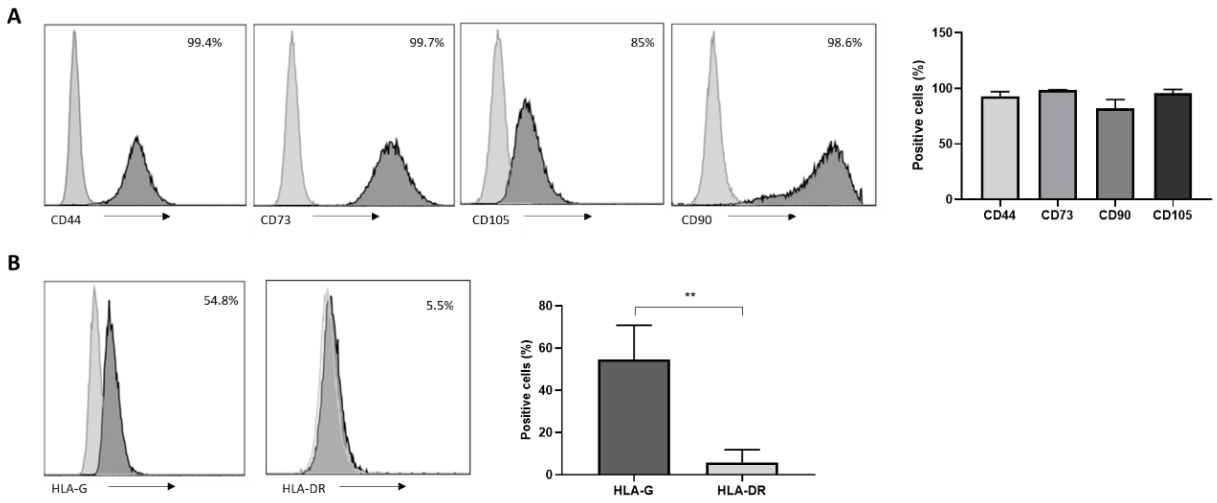

*Fig. 5: Flow cytometry analysis of the expression of mesenchymal stromal cell markers (A) and expression of HLA-G and HLA-DR (B) in URECs. Labels show the percentage of positive cells. Results are expressed as mean ± SD unstained cells were used as control, N= 5 independent experiments, ** p<0.01*

URECs reduced proliferation of CD4 and CD8 T Lymphocytes

The co-culture of URECs with anti-CD3 and anti-CD28 activated PBMCs was set-up to analyse the interaction between these cell populations during in vitro culture and to evaluate the immunomodulatory capacity of kidney derived cells. As shown in pictures of figure 6A (Fig. 6A), the coculture (PBMCs + URECs) significantly reduced the activation of PBMCs compared to positive control (PBMCs). Particularly, as shown in the graphs (Fig. 6B), the proliferation of both $CD4^+$ (49.55 ± 25.8%) and $CD8^+$ (45.08% ± 10.72%) cells was significantly impaired in the co-culture condition if compared with activated PBMCs without URECs, set as control (100%).

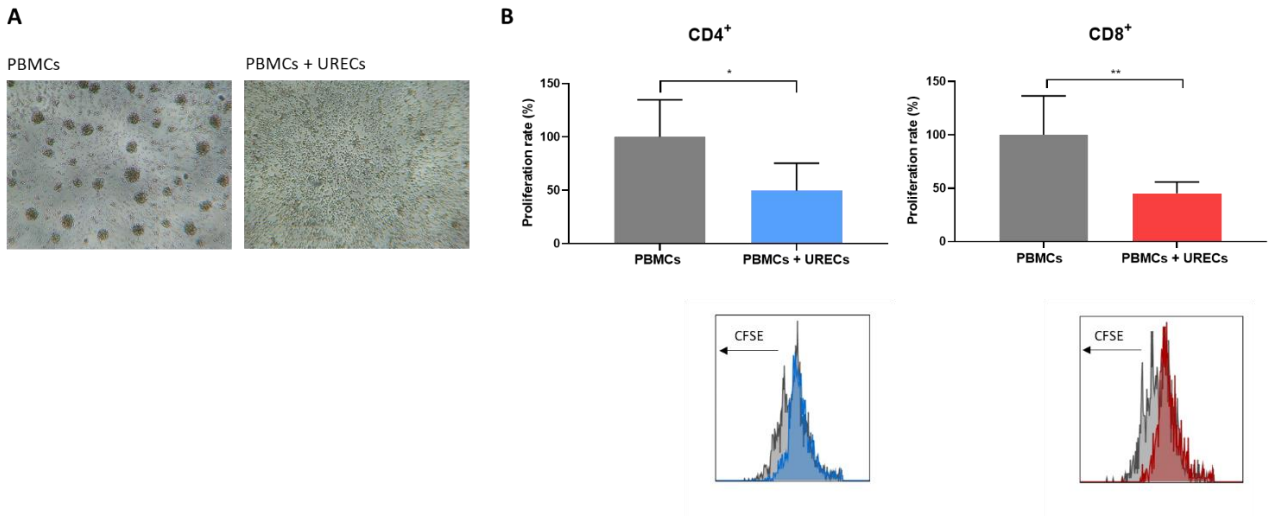

*Fig.6: A) representative images of PBMCs and PBMCs co-cultured with URECs. B) CFSE assay analysis of CD4 (blue) and CD8 (red) proliferation rate in coculture compared with control, set as 100%. Results are expressed as mean ± SD, * p <0.05, ** p <0.01, n=5 independent experiments.*

URECs reduced the percentage of apoptotic PBMCs

To explain the reduction of PBMC proliferation when cultured with URECs, the analysis of early and late apoptosis was performed on PBMCs with or without co-culture and results are represented in figure 7 (Fig. 7). While the number of cells in early apoptosis (Annexin $V^+/7'AAD^-$) remained stable, the percentage of cells undergoing late apoptosis (Annexin $V^+/7'AAD^+$) was significantly lower in co-culture conditions (25.05 ± 1.977 %), compared to controls (37.28 ± 4.0%). These results suggest that the anti-proliferative effect of URECs is not mediated by the activation of apoptotic stimuli acting on PBMCs, surprisingly showing a protective role of urine derived cells against the onset of late apoptosis.

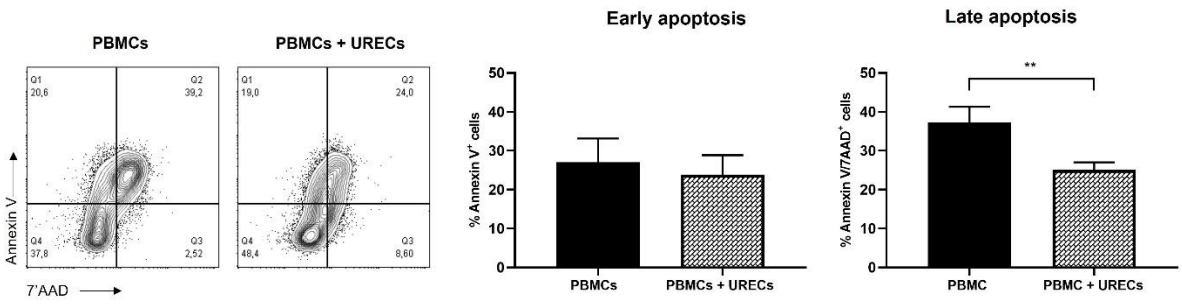

*Fig. 7: Annexin V/7AAD assay on PBMCs and PBMCs co-cultured with URECs. Histograms show the percentage of PBMCs in early (Annexin V^+/7-AAD^-) and late (Annexin V^+/7-AAD^+) apoptosis in the two culture conditions. Results are expressed as mean ± SD, **p < 0.01, n=5 independent experiments.*

URECs increased the percentage of Treg cells, while reducing the Th1 subset

Assessed the suppression of CD4 and CD8 proliferation exerted by URECs, the following analysis focused on the effect of the co-culture on T Lymphocytes subpopulations. Figure 8 shows the effect of URECs on T $CD4^+$ cell subsets during co-culture with PBMCs. Among $CD4^+$ cells an increase in the percentage of Treg cells ($CD4^+CD25^+Foxp3^+$) was observed in co-culture condition (12.85 ± 4.05 %) compared to control (6.93 ± 1.45%) (Fig 8A). A significant decrease in Th1 cells producing IFN-ɣ ($CD4^+$IFN-$ɣ^+$) was observed in co-culture (4.62 ± 2.1%) compared to activated PBMCs used as control (9.73 ± 4.5 %) (Fig. 8B).

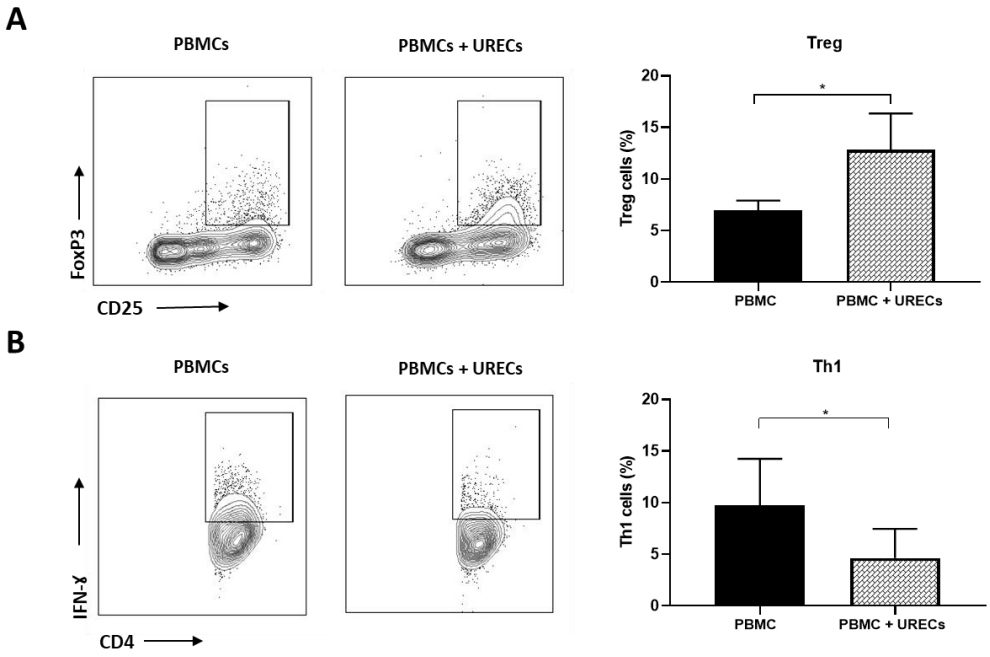

*Fig. 8. Flow cytometry analysis of Treg (A) and Th1 (B) lymphocyte subsets in PBMCs and PBMCs co-cultured with URECs. Results are expressed as mean ± SD, * p<0.05 n= 5 independent experiments.*

For what concern other CD4 and CD8 cell subsets, there were no significant alterations in Th17 and in CD8-producing IL17 percentage, while a slight decrease in CD8 cells producing IFN-ɣ was observed in the co-culture condition (Fig. 9).

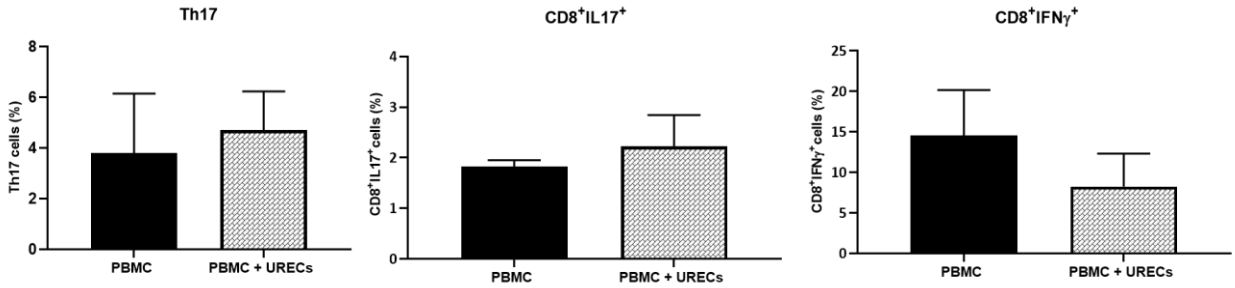

Fig. 9: Analysis of Th17, CD8$^+$IL-17$^+$ and CD8$^+$ IFN-ɣ$^+$ percentage in PBMCs and PBMCs co-cultured with URECs. Results are expressed as mean ± SD, n=5 independent experiments.

URECs influences the secretion profile of PBMCs

The analysis of the supernatants collected after the in vitro culture of activated PBMCs with or without URECs revealed that the coculture with urine derived cells widely influenced the secretion profile of PBMCs. Figure 10 shows the panel of Cytokines measured in the supernatants of the previously described culture conditions. For each cytokine results obtained from the PBMCs + URECs were normalized on activated PBMCs, set as 100. It was observed a significant increase in cytokines and chemokines involved in the recruitment and stimulation of monocytes and macrophages (IL-1β, MCP-1, GM-CSF, G-CSF) along with an increase of IL-2 release, well known for its anti-inflammatory and tolerogenic properties. A decrease of cytokines involved in the stimulation of immune response and inflammation (IL-8, IFN-ɣ, TNF-β, MIP-1β, CD40-L) was observed, along with IL-5 and IL10.

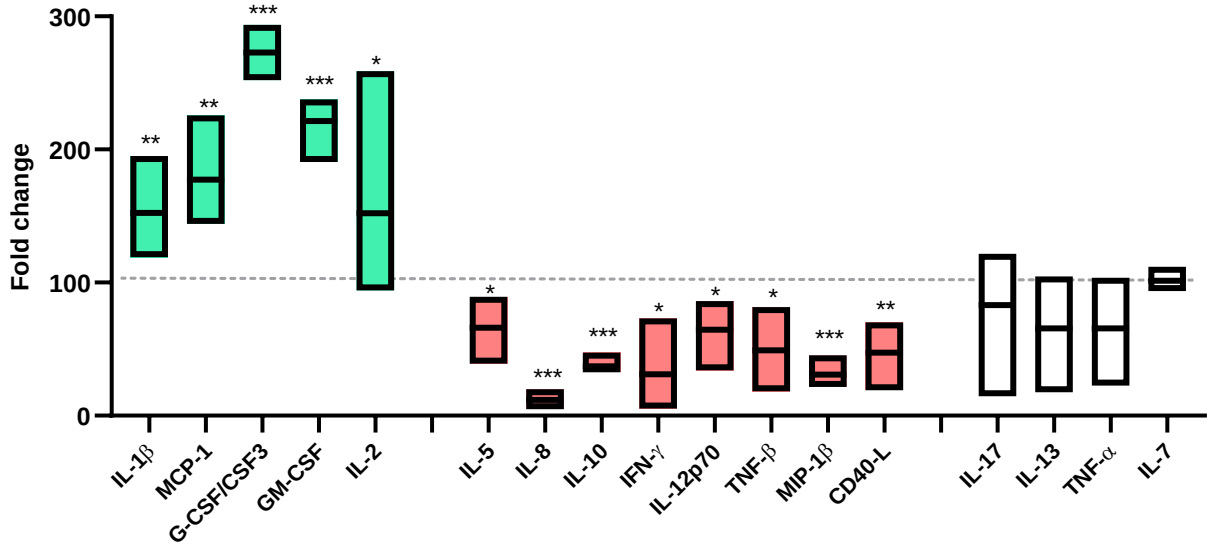

*Fig 10: Secretion profile of PBMCs after coculture with URECs. Results are normalized on PBMCs alone (100). Data are expressed as mean ± SD, * p<0.05, ** p<0.01, *** p<0.001.*

Expression of NGAL (LCN-2) and its receptor (SLC-22A17) in URECs and PBMCs

To evaluate the effect of NGAL on UREC characteristics and immunomodulatory capacity, the expression of NGAL and its receptor SLC22A17 were evaluated via real time PCR in both URECs and PBMCs samples. Results were normalized on UREC cell population set as 1. The higher expression of SLC22A17 in URECs compared to PBMCs, suggests that URECs may respond more to the treatment with NGAL during in vitro culture; on the contrary, NGAL expression (LCN-2) was significantly higher in PBMCs (Fig. 10).

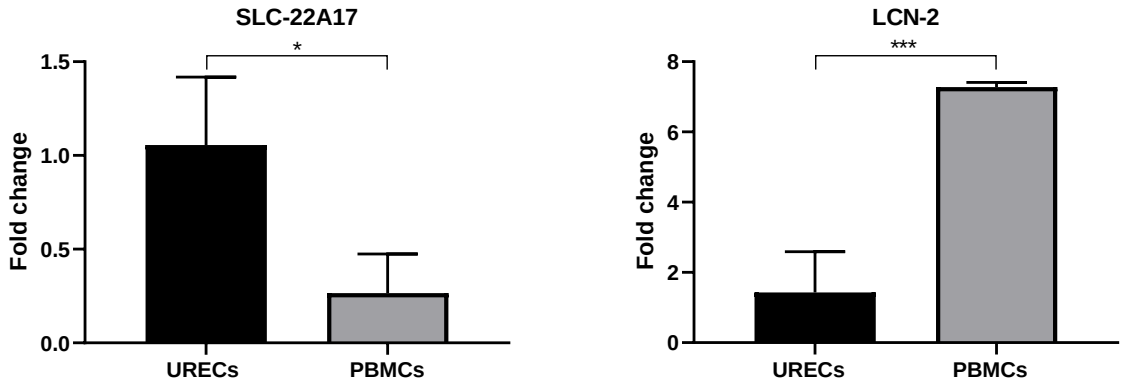

*Fig.11: gene expression analysis via real time PCR of NGAL (LCN-2) and its receptor (SLC-22A17) in URECs and PBMCs. Data were normalized to two reference genes, the normalized expression value of URECs was set to 1 and the PBMC gene expression data were reported to that sample. Data are expressed as fold change ± SD. * p<0.05, ** p<0.001*

NGAL reduced UREC immunomodulatory and anti-apoptotic properties.

After evaluating how NGAL affected UREC viability, the immunomodulatory properties of urine cells cultured with NGAL were also evaluated (Fig. 12). In all the experiments, no significant differences were observed between the two positive controls, represented by PBMCs treated or not with NGAL (PBMCs + NGAL), while in the coculture condition (URECs + PBMCs + NGAL) several changes in UREC immunomodulatory capacity were observed compared to the coculture without NGAL, suggesting that the addition of this molecule specifically affected URECs, but not PBMCs.

As regards proliferation of CD4 and CD8 T cells, the addition of NGAL significantly reduced the immunosuppressive capacity of URECs, with a return of the proliferation rate to values similar to positive controls. For what concern $CD4^+$ subset, the exposure to NGAL increased the proliferation rate of cocultured PBMCs to 94.7 ± 10.62 %. Also, in $CD8^+$ the percentage of proliferation rate was increased to 81.08 ± 18.07 % compared to coculture without NGAL.

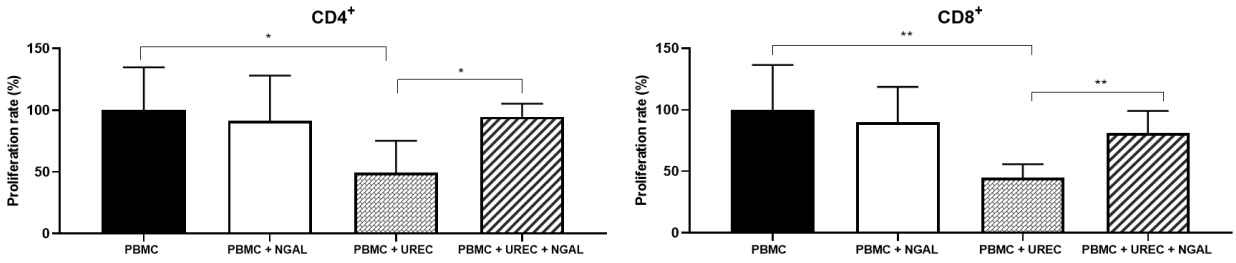

*Fig. 12. CFSE assay analysis of CD4 and CD8 proliferation rate in PBMCs and PBMCs co-cultured with URECs, both treated or not treated with NGAL. Results are expressed as mean ± SD, * p <0.05; ** p < 0.01, n = 5 independent experiments.*

The presence of NGAL in the coculture affected also the antiapoptotic activity of URECs in both early and late apoptosis (Fig.13). In early apoptosis, the percentage of Annexin $V^+/7'AAD^-$ significantly increase in coculture with NGAL (31.2 ± 1.9 %), compared with coculture without NGAL (23.8 ± 5 %). The increase was maintained also in late apoptosis, with percentage that increase from 25.05 ± 1.2 % in standard coculture, to 32.10 ± 1.63 % in NGAL treated condition, reaching the percentage of positive controls. No significant changes in the number of apoptotic cells were observed on control PBMCs in presence of NGAL.

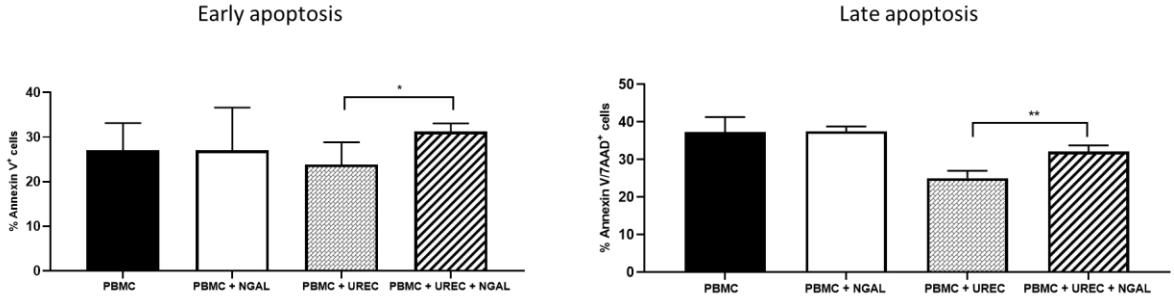

*Fig. 13: Annexin V/7'AAD assay on PBMCs and PBMCs co-cultured with URECs, both treated or not treated with NGAL. Histograms show the percentage of PBMCs in early (Annexin $V^+/7\text{-}AAD^-$) and late (Annexin $V^+/7\text{-}AAD^+$). Results are expressed as mean ± SD, * p< 0.05, **p < 0.01, n=5 independent experiments.*

T reg increase during PBMCs-URECs co-culture was not altered by NGAL

The percentage of CD4 and CD8 cell subsets with or without URECs was analysed in presence of NGAL (Fig. 14). Interestingly, the increase in Treg percentage observed during the co-culture in basal medium (PBMCs + UREC) was not altered by NGAL treatment (PBMCs + UREC + NGAL), with a percentage of Treg cells of 12.11 ± 4.4 %. The significant decrease in Th1 observed during the co-culture in basal medium was not maintained in presence of NGAL, with a percentage of Th1 cells of 5.2 ± 4.6 %.

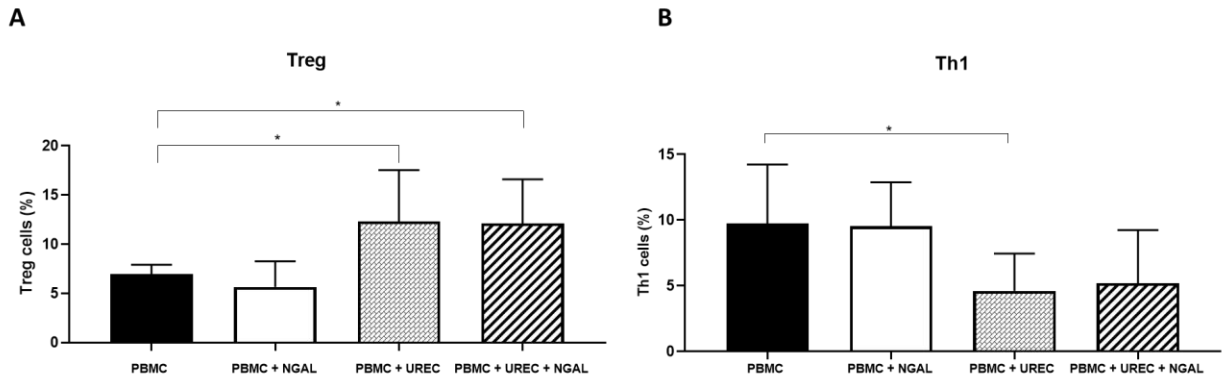

*Fig 14: T reg (A) and Th1(B) percentage in presence or not of NGAL in PBMCs or PMBCs cocultured with URECs. Results are expressed as mean ± SD, * $p < 0.05$, n= 4 independent experiments*.

For what concern other CD4 and CD8 cell population, the exposure of URECs and PBMCs to NGAL during co-culture did not induce significant changes in Th17 cell subsets, nor in CD8 cells producing IL17 and IFN-ɣ (Fig. 15).

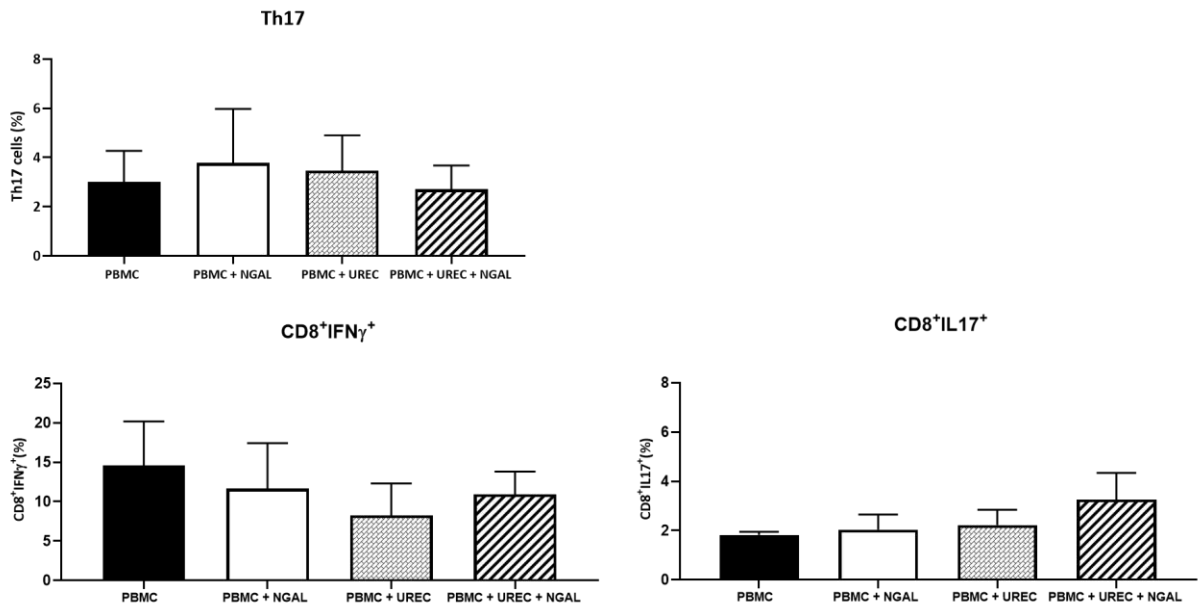

*Fig. 15: percentage of Th17, $CD8^+IL17^+$ and $CD8^+IFN\text{-}\gamma^+$ cells in PBMCs or PMBCs cocultured with URECs in presence or not of NGAL. Results are expressed as mean ± SD, * $p < 0.05$, n= 4 independent experiments.*

In all the experiments there were no significant differences in the comparison of T CD4 and CD8 cell subsets among control PBMCs treated or not with NGAL. This confirmed a specific effect of this molecule on UREC cell type, as observed in the proliferation and apoptosis results.

NGAL reduced UREC viability and proliferation, while increasing apoptosis

Given the inhibitory role of NGAL on UREC immunosuppressive and anti-apoptotic properties, and the high expression of NGAL receptor in urine cells, the effect of NGAL supplementation on UREC viability, proliferation, apoptosis was analysed (Fig. 16). URECs treated with NGAL (UREC + NGAL) presented a significant decrease in viability (84 ± 12.2%) and in their proliferation capacity (48.5 ± 3.04 %), compared with untreated cells. For what concern the effect of NGAL on UREC apoptosis, the percentage of early apoptotic cells (Annexin $V^+/7AAD^-$) was significantly increased in NGAL-treated cells (10.7 ± 0.28 %) compared to untreated ones (7.2 ± 0.14 %), while the percentage of cells in late apoptosis was very low in both culture conditions.

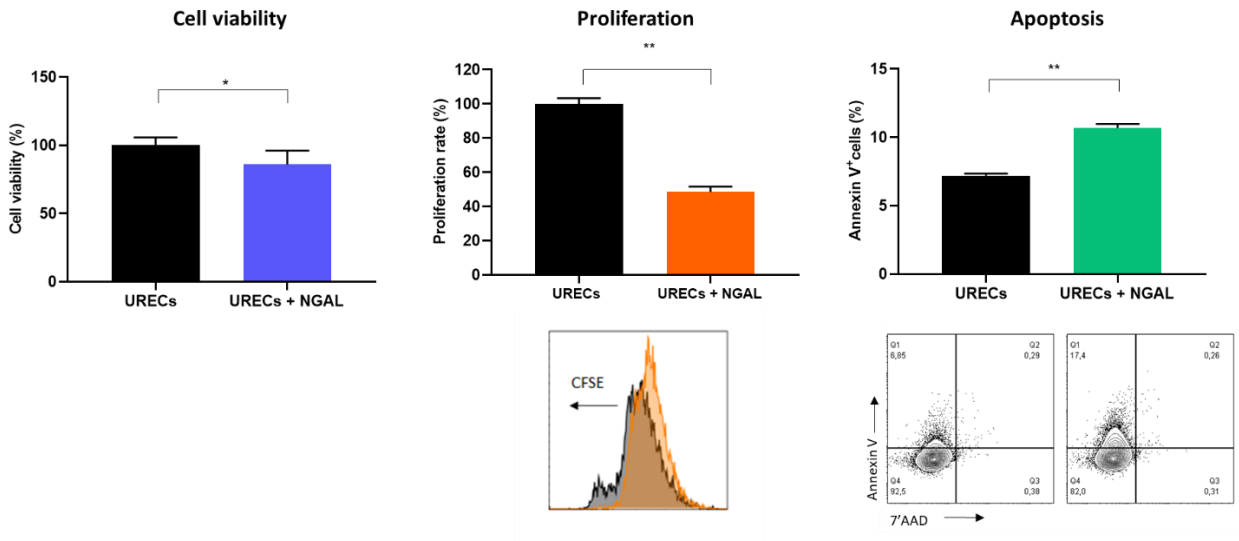

*Fig. 16: Evaluation of URECs viability (MTT assay), proliferation (CFSE assay) and early apoptosis (Annexin V/7-AAD assay) with or without NGAL treatment. Results are expressed as mean ± SD, ** p<0.01*

Discussion

Kidney transplantation is the treatment of choice for the resolution of chronic kidney disease and end stage kidney disease. Despite organs from living donors have shown a superior graft survival compared with deceased donors, the increasing demand of kidneys over the years has resulted in expansion of the donor pool, including the donation after brain death (DBD) and donation after circulatory death (DCD). Kidneys derived from deceased patients, and particularly from DCD donors are highly exposed to ischemia-reperfusion injury, that take place during the transplant procedure, promoting the accumulation of ischaemic metabolites and increasing the risk of acute kidney injury, delayed graft function and rejection [128].

These events result in an increased cell turnover within nephron structures, especially in the glomerulus and in the proximal tubule, to replace the damaged cells with functional ones. Epithelial progenitor cells expressing CD24 and CD133 surface markers are well known for their role in the regeneration of the epithelia lining the functional unit of the kidneys, since they are able to differentiate into epithelial subpopulations, including podocytes and proximal tubule epithelial cells [47]. The result of the tissue regeneration process is an increase in the release of kidney derived cells into the urine, together with other cell types deriving from the urinary tract. Cells released in urine are an easily obtainable and heterogeneous cell population and their use in diagnostic, disease modelling and regenerative medicine applications has obtained increasing interest in recent years [129].

Urine derived renal epithelial cells (URECs) are highly voided in urine under different pathological conditions or in response to stress factors, including post-transplant ischemia and reperfusion events, while a small percentage of these cells is detected in the urine of healthy patients. The characterization of URECs obtained from kidney transplanted patients recruited for this study confirmed the epithelial phenotype of this population, with the high expression of Cytokeratin epithelial marker. According to previous studies, the progressive reduction of successful isolations observed after one and six months from transplant, and the lack of positive isolations from healthy donors confirmed that the release of URECs is higher in the earlier stages after transplant, when the regenerative events are more involved [12]. The difference in kidney derived cell excretion observed among the two groups are in accordance with data already reported for several kidney

disorders [58]. As a results of the increase in cell turnover, significant amount of $CD24^+CD133^+$ kidney progenitor cells were detected during the first passages of the culture. The presence of $CD24^+CD133^+$ progenitor cells in urine suggests their use for modelling genetic disorders and for the development of personalized therapeutic approaches, avoiding both the risk associated with isolation from biopsies and the alteration in cell features caused by the immortalization process [58]. Moreover, the isolated cells resulted highly positive for CD73 surface molecule, which is known to be involved in the regulation of the immune response by converting pro-inflammatory AMP to anti-inflammatory adenosine, reducing myocardial ischemia/reperfusion injury in animal models [130]. A percentage of URECs also expresses the tolerogenic molecule HLA-G, while the level of HLA-DR was very low. The HLA-G belong to the nonclassical HLA-1b family and it is involved in the inhibition of CD4 and CD8 T cell proliferation and in the promotion of Treg subset [131], playing a relevant role in the development of foeto-maternal tolerance and in the regulation of the inflammatory response. HLA-G has also been detected in glomerular and tubular cells of patients with Lupus nephritis [132]. This characteristic HLA profile is in line with well-known immunomodulatory cell populations, including mesenchymal stromal cells. However, the lack of information regarding the role of HLA-G in cells obtained after kidney transplant has aroused the interest in deepening the interaction between URECs and the immune cells in vitro, setting up a co-coculture with Peripheral Blood Mononuclear Cells (PBMCs) from healthy donors. Like other more characterized cell populations, URECs significantly reduce the proliferation of CD4 and CD8 T Lymphocyte. Interestingly, this inhibition of lympho-proliferation was not associated with the promotion of apoptotic mechanisms. On the contrary, URECs protected PBMC against the onset of both early and late apoptosis. The inhibition of the apoptosis on PBMCs exerted by URECs has not been documented to date. Anti-apoptotic mechanisms have been already described in other cell populations, including mesenchymal stem cells, and they are mostly associated with the secretion of factors as TGF-β and BMP15 that inhibit the activation of pro-apoptotic signals [133]. Analysis regarding the paracrine pathway involved in UREC anti-apoptotic function, and the evaluation of PBMC subpopulations mainly targeted by this effect will be required.

To better understand the immunomodulatory activity of URECs, their influence on T CD4 and CD8 cell subsets was analysed. T CD4 cell population producing IFN-ɣ (Th1), commonly associated with inflammation and immune response triggering, was significantly reduced in the coculture, and the percentage of Treg cells was increased. The increase in Treg subset could be

associated with the expression of HLA-G by URECs, given its role in activating the regulatory T component and in the downregulation of the inflammatory response [134]. In addition, URECs significantly influenced the secretory profile of PBMCs during in vitro culture.

Cytokines and chemokines play pleiotropic roles, and their action is strongly influenced by the surrounding microenvironment and by the target cell. For these reasons the analysis of the mechanisms involved in the modulation of such molecules is challenging and requires a deepen investigation. Also, the analysis of the secretory profile of URECs could add more information about their immunomodulatory abilities since cytokines play a pivotal role in the immunomodulatory and tolerogenic properties exerted by most characterized cell types.

In presence of acute and chronic damage, kidney tubular epithelial cells produce several molecules that act as early markers of acute and chronic kidney disease. These markers are widely used in diagnostics, due to their easily measurement in urine or blood samples. In vitro culture of urine derived cells in the presence of such molecules could add new information on the effects of prolonged exposure of kidney cells to damage markers. Lipocalin-2, commonly known as NGAL is well described for its role as biomarker for the diagnosis of acute and chronic kidney damage, but also for its involvement in inflammation and immune response [120].

From the results of gene expression analysis, high expression of NGAL receptor SLC-22A17 in URECs has been observed. Cell exposure to NGAL treatment results in reduced proliferation and viability, with an increase in the number of apoptotic cells. The action of NGAL on the proliferation and apoptosis of different cell populations, including cancer cells, has been previously described, with conflicting results. The ability of NGAL to bind the EGF receptor is critical for maintaining the proliferative activity of many cells type [135], however a pro-apoptotic role of this molecule was observed in different cancer cell lines, via the interaction with IL-3 and IL-8 [126]. Also, despite the increase in NGAL represents an hallmark of tissue damage, its administration in a murine model of renal ischemia-reperfusion injury was associated with the proliferation of proximal tubule cells and the reduction of apoptosis [136]. Since the functions of NGAL are multiple and not completely understood, it has been suggested that NGAL effects may be influenced by its binding with iron [137]; the binding of iron lacking NGAL to its receptor decreases intracellular iron levels promoting apoptosis, while the iron-loaded molecule do not induce apoptotic signals [111].

As regards the immunomodulatory activity of URECs, the addition of NGAL to the culture causes a reduction of the immunosuppressive ability of urine derived kidney cells. In addition, the protective role of URECs against PBMC apoptosis was inhibited by the presence of NGAL. The changes in CD4 and CD8 proliferation and in PBMCs apoptosis caused by NGAL treatment were observed in UREC-PBMCs coculture but not in PBMCs alone. These results could be associated with a higher expression of SLC22A17 receptor on URECs, which in turn negatively affects their viability and immunomodulatory properties. Interestingly, the percentage of Treg lymphocyte observed in the coculture was not affected by exposition to NGAL. As matter of fact, it has been reported that NGAL promotes the upregulation of Treg cells in cultured PBMCs in a dose dependent manner [120].

In conclusion, urine of transplanted patients represents a promising cell source for the evaluation of the characteristics of kidney derived cells, including the stem/progenitor subset, released after the exposition to ischemia and reperfusion events derived from the transplant procedure. Despite the events occurring after transplant, URECs voided in urine have shown promising immunomodulatory properties. Since studies regarding the co-culture of kidney cells and PBMCs with or without the exposition to kidney damage associated molecules, such as NGAL, are not documented to date, future step will be to deepen the mechanism of action of NGAL in the interaction between URECs and PBMCs.

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