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Urine-derived renal epithelial cells isolated after kidney transplant are sensitive to neutrophil gelatinase-associated lipocalin exposure during in vitro culture

Valeria Pizzuti [a], Emma Balducelli [a], Miriam Di Nunzio [b], Diletta Conte [a], Elisa Gessaroli [b], Marcello Demetri [a], Pasquale Marrazzo [a], Francesco Alviano [c], Valeria Corradetti [b], Federica Maritati [b], Gaetano La Manna [a,b,*], Giorgia Comai [a,b]

[a] Department of Medical and Surgical Sciences (DIMEC), University of Bologna, Bologna 40126, Italy
[b] Nephrology, Dialysis and Renal Transplant Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna 40139, Italy
[c] Department of Biomedical and Neuromotor Sciences (DIBINEM), University of Bologna, Bologna 40126, Italy

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ABSTRACT

Urine-derived renal epithelial cells (URECs) are highly voided after kidney transplant and express typical kidney markers, including markers of kidney epithelial progenitor cells. Recently URECs have shown promising immunomodulatory properties when cultured with Peripheral Blood Mononuclear Cells (PBMCs), promoting an increase in the T regulatory cells. In vivo, kidney cells are highly exposed to damage associated molecules during both acute and chronic kidney injury. Neutrophil gelatinase-associated lipocalin (NGAL) is one of the most -known early marker of acute and chronic kidney damage. However, its role on the evolution of renal damage has not yet been fully described, nor has its impact on the characteristics of renal-derived cells during in vitro culture. The aim of this study is to investigate the effect of NGAL on the characteristics of URECs isolated after kidney transplant, by exposing these cells to the treatment with NGAL during in vitro culture and evaluating its effect on UREC viability, proliferation, and immunomodulatory potential. The exposure of URECs to NGAL reduced their viability and proliferative capacity, promoting the onset of apoptosis. The immunomodulatory properties of URECs were partially inhibited by NGAL, without affecting the increase of Treg cells observed during UREC-PBMCs coculture. These results suggest that the exposure to NGAL may compromise some features of kidney stem and specialized cell types, reducing their viability, increasing apoptosis, and partially altering their immunomodulatory properties. Thus, NGAL could represent a target for approaches acting on its inhibition or reduction to improve functional recovery.

1. Introduction

Neutrophil gelatinase-associated lipocalin (NGAL), also known as Lipocalin-2 (LCN-2), is a glycoprotein discovered and characterized in G0-arrested mouse kidney cells, then isolated in activated neutrophil granules released at sites of infection (Kjeldsen et al., 1993). The expression of NGAL was observed in the heart, lung, liver among many other organs and tissues (Ding et al., 2010) as a 25 kDa monomer, a 45 kDa homodimer, or as a heterodimer binding Matrix Metallopeptidase 9, depending on the cell type secreting the molecule (Romejko et al., 2023). NGAL is a player in the antibacterial immune response, since it contributes to the inhibition of bacterial growth, acting as a shuttle for iron and siderophores highly needed for bacterial proliferation and metabolism (Parrow et al., 2013, Bachman et al., 2009). Indeed, iron-associated NGAL (holo-NGAL) traffics to endosomes and releases iron from the complex, regulating iron-responsive genes, such as ferritin and transferrin receptor. The endosomal NGAL protein core is either degraded in lysosomes or recycled to the extra-cellular space as unconjugated apo-NGAL. NGAL has implications also in cancer, obesity, diabetes, and metabolic disorders (Jang et al., 2012). Some of its the biologic effects may depend on its association with siderophore: iron complexes, however NGAL acts in a high number of different pathways, from cell proliferation and apoptosis to inflammatory and immune response (Flo et al., 2004, Schmidt-Ott et al., 2007).

* Corresponding author at: Department of Medical and Surgical Sciences (DIMEC), University of Bologna, Bologna 40126, Italy.
E-mail address: gaetano.lamanna@unibo.it (G.L. Manna).

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In kidneys, NGAL is mostly synthesized by distal tubule epithelial cells, and it increases after kidney injuries caused by ischemia, nephrotoxins exposure, infections, and inflammation (Kuwabara et al., 2009). Given the rapid increase of NGAL in plasma and urine, it is used in diagnostic procedures as an early biomarker of acute kidney injury (AKI) and chronic kidney disease (CKD) (Romejko et al., 2023). Several studies focused on the effects of NGAL exposure in both in vitro and in vivo models (Du et al., 2015, Deis et al., 2019), and two cell surface receptors have been identified: the megalin/glycoprotein GP330 and the Solute carrier family 22 member 17 (SLC22A17), also known as 24p3R (Devireddy et al., 2005).

Kidney-derived cells are exposed to the increase of NGAL occurring during AKI and Chronic Kidney Disease (CKD) progression (Viau et al., 2010), as well as during kidney transplant procedure (Ramirez-Sandoval et al., 2015), thus the analysis of how this lipocalin influences features of kidney cells could add new information on the mechanisms occurring during the exposure to damage-associated molecules. Urine derived kidney cells are an easily obtainable cell model to investigate the role of NGAL during in vitro culture. These cells are highly voided during kidney repair and regeneration processes and represent an important cell source for diagnostic approaches, as documented by several studies, reporting the advantages of using urine derived cells instead of solid biopsies (Bach et al., 2021). Nowadays, the application of these cells for in vitro studies focusing on their use in cell therapy and personalized medicine approaches is increasing, due to the easy collection and the lack of ethical concerns of urine manipulation (Bondue et al., 2021). Recently, we observed that Urine-derived Renal Epithelial Cells (URECs), are highly voided after kidney transplant from Donation after Circulatory Death (DCD) donors, and the possibility to successfully isolate these cells reduced after one and six months from surgery. These results indicated that URECs were highly voided after ischemia and reperfusion injury events occurring during transplant procedure, which are often associated with an increased risk of delayed graft function (DGF). URECs rapidly proliferate during in vitro and expressed typical marker of kidney epithelial cells. Interestingly, URECs showed promising immunomodulatory properties, when cultured with Peripheral Blood Mononuclear Cells (PBMCs) reducing CD4 and CD8 T lymphocytes proliferation, while increasing the T regulatory (Treg) cell subset. URECs inhibited PBMCs apoptosis and influenced the cytokine microenvironment (Pizzuti et al., 2023). The effect of NGAL on in vitro cultured UREC has not been characterized. In this study we aimed to add new information about the role of NGAL in the pathogenesis of DGF, analyzing how it affects UREC viability, proliferation, and immunomodulatory potential. As matter of fact, an excessive production of NGAL by damaged tubular cells, may compromise the immunomodulatory and regenerative potential of renal progenitor cells voided in urine, thus contributing to the worsening of kidney damage. Also, NGAL and its receptor could be used as a therapeutic target for the treatment of kidney disorders in which they are known to play a pivotal role.

2. Materials and methods

2.1. Patients recruitment

Urine samples were collected from patients undergone kidney transplantation in University Hospital of Bologna IRCCS, Sant'Orsola Polyclinic, after the approval of the Ethics Committee (protocol number: 312/2021/Oss/AOUBO). Transplanted organs derived from Donation after Circulatory Death (DCD) donors, and recipients had no residual diuresis, to ensure the donor origin of kidney cells.

2.2. Isolation of Urine Renal Epithelial Cells (URECs)

Urine samples were collected within the first week after the transplant after resuming of autonomous diuresis and centrifuged at 400 g for 10 minutes. Urine sediment was resuspended and seeded in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM F12, Gibco, Life Technologies, CA, USA) with 10 % of foetal bovine serum (FBS, Gibco, Life Technologies, CA, USA), Renal Epithelial Growth Medium (REGMTM) Single Quot kit (Lonza Bioscience, Basel, Swizerland), 1 % P/S solution (Corning, NY, USA), 2.5 μg/ml amphotericin B (Biochrom, Waterbeach Cambridge, UK). Cells were incubated at 37°C and 5 % CO_2. After 72 hours, medium was replaced with the proliferation medium, consisting of Renal Epithelial Basal Medium (REBMTM, Lonza Bioscience, Basel, Swizerland) at 10 % of FBS, with REGM TM SingleQuot kit, 2.5 mM GlutaMAX (Gibco, Life Technologies, CA, USA) and 1 %P/S.

2.3. RNA extraction and real-time PCR

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 qualitative and quantitative evaluation of RNA 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. Real-time PCR (qPCR) was performed in a Bio-Rad CFX96 real-time thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). 25 ng of cDNA were amplified using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The data obtained were analysed using the software CFX Manager (Bio-Rad Laboratories, Hercules, CA, USA) and the $2-\Delta\Delta Ct$ method. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Origene, Cat. No HP205798, ID: NM_002046) was used as reference gene and the expression of NGAL receptor (SLC-22A17) (Origene Cat.no HP233381, ID: NM_020372), and NGAL (LCN-2) (Origene, Cat.no HP208681, ID: NM_005564) was analysed.

2.4. Addition of NGAL to URECs cultures

To evaluate the influence of NGAL on UREC characteristics, cells were cultured in standard proliferation medium or in proliferation medium supplemented with NGAL (Recombinant Human Lipocalin-2/NGAL Protein, R&D system, MN, USA) at a concentration of 320 ng/ml for 72 hours. The assessment of proliferation, apoptosis, viability, and immunomodulatory capacity of URECs was performed for both untreated and NGAL-treated cells.

2.5. Analysis of URECs viability with MTT assay

To analyse cell viability, URECs were seeded on a 96 well-plate in proliferation medium with or without NGAL and incubated for 4 days at 37°C and 5 % CO2. Cells were then incubated for 2 hours with 100uL/well of proliferation medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT) reagent at a final concentration 0.5 mg/ml. 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 %.

2.6. Analysis of URECs proliferation by Carboxyfluorescein Diacetate Succinimidyl Ester assay

For the analysis of URECs proliferation with or without the addition of NGAL, cells were incubated with Carboxyfluorescein Diacetate Succinimidyl Ester (BD Horizon™ CFSE, Becton, Dickinson, NJ, USA). CFSE covalently bounds to DNA and is divided equally between daughter cells, allowing to discriminate successive series of cell divisions. For the CFSE staining, URECs were resuspended in PBS and labelled with 2 μM CFSE for 5 minutes at 37°C. Cells were then centrifuged and resuspended in culture medium with or without NGAL addition and seeded in 6 well plates at a density of 30.000/cm2. The proliferation rate was evaluated

after four days analyzing the dilution of CFSE during subsequent cell divisions. The proliferation of URECs treated with NGAL was normalized to cells cultured in standard medium (100 %).

2.7. Analysis of URECs apoptosis with Annexin V/7-AAD assay

URECs were seeded in 6 well plates at a concentration of 30.000/cm2 and cultured for 4 days with or without the addition of NGAL 320 ng/ml. The cells were then collected and stained with Annexin V PE/7-Aminoactinomycin D (7-AAD) apoptosis detection kit (Biolegend, Cat. No 640934), following kit instructions, for the detection of cells in early and late apoptosis. URECs were labelled for 15 minutes with anti-Annexin V PE and 7-AAD, both used 2:100. After the staining, 400 μL of binding buffer were added to each tube and samples were analyzed by flow cytometry. Unstained samples were used as negative controls and results were represented as percentage of Annexin V PE^+/ $7\text{-}AAD^-$ (early apoptosis) and Annexin V PE^+/$7\text{-}AAD^+$ (late apoptosis) cells in both culture conditions.

2.8. Isolation of PBMCs

PBMCs were isolated from the blood of healthy volunteers and separated from the other blood components by density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA). After isolation PBMCs were counted with Methyl Violet (Sigma-Aldrich, St. Louis, MO, USA) and frozen at –80 °C in FBS with 10 % of Dimethyl Sulfoxide (Sigma-Aldrich, St. Louis, MO, USA).

2.9. Co-culture of URECs and PBMCs

URECs were seeded at a density of 40.000 cells/cm^2 in 96-well plates in proliferation medium. After 24 hours, PBMCs were thawed and stimulated 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 above the URECs monolayer at a concentration of 200.000 cells/well; activated PBMCs cultured without URECs and NGAL represented positive control, and non-activated PBMCs represented the negative control. Cells were incubated for 72 hours, in RPMI 10 % FBS, 2.5 mM GlutaMAX (Gibco), 1 % non-essential amino acids (Termo Fisher Scientific, MA, USA) and 1 % P/S. The analysis of cell proliferation, apoptosis, and lymphocyte subpopulations was performed by flow cytometry.

2.10. Analysis of PBMCs proliferation by Carboxyfluorescein Diacetate Succinimidyl Ester

The analysis of $CD4^+$ and $CD8^+$ Lymphocytes proliferation with or without co-culture with URECs was executed by labelling with CFSE. PBMCs were thawed and labelled with BD Horizon™ CFSE according to manufacturer protocol, as previously described for URECs. 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 co-culture condition. After 72 hours, PBMCs were collected and stained with anti-CD4 APC (Biolegend, San Diego, CA, USA) and anti-CD8 Pecy7 (Biolegend, San Diego, CA, USA) antibodies 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 the CytoFLEX S instrument. Results were normalized on PBMCs not treated with NGAL.

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

The apoptosis of PBMCs with or without co-culture was assessed using Annexin V/7-AAD kit (Biolegend, San Diego, CA, USA). Cells were stained according to manufacturer instructions. Briefly, PBMCs were collected in tubes, washed with PBS, and then labelled for 15 minutes at room temperature with anti-Annexin V PE and 7-AAD iboth used 2:100, as previously described for UREC staining. 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.

2.12. Flow cytometry analysis of lymphocytes subpopulations

After 72 hours of incubation, PBMCs were collected, and the characterization of T cells subpopulations was performed by flow cytometry. For the analysis of the Treg population, anti-CD4 APC (Biolegend, San Diego, CA, USA) and anti-CD25 FITC (Biolegend, San Diego, CA, USA) antibodies were used as surface markers. For intracellular staining, PBMCs were fixed and permeabilized with eBioscience™ Foxp3/ Transcription Factor Staining Buffer Set (Invitrogen, MA, USA) according to manufacturer instructions. Briefly, cells were fixed with Fixation/Permeabilization solution, and permeabilized with 1X Foxp3 Permeabilization Buffer. Cells were then labelled with anti-FoxP3 PE (Biolegend, San Diego, CA, USA) in Permeabilization Buffer, washed twice and resuspended in PBS 0.1 % BSA. For the quantification of intracellular cytokines, PBMCs of both control and co-culture conditions were treated with a mixture of 1 nM Phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich, St. Louis, MO, USA), Ionomycin 3 mg/ml (Sigma Aldrich, St. Louis, MO, USA) and 1ul/ml Golgi Plug™ (BD, Becton, Dickinson, NJ, USA), and incubated at 37°C to 5 % CO2 for 4 hours before staining. For IFN-γ-producing populations, cells were stained for surface markers with anti-CD4 APC (Biolegend, San Diego, CA, USA) antibody, while the intracellular staining was performed, after the fixation and permeabilization steps, for 30 minutes at 4°C with anti-IFN-γ PeCy7 (Biolegend, San Diego, CA, USA).

2.13. Luminex xMAP technology for cytokine quantification

The analysis of cytokine release was performed in supernatant of PBMCs and PBMCs + URECs culture, with and without NGAL after 72 h of culture. Cytokines were quantified using the Human Custom Procartaplex-19 (Cat. No. PPX-19-MXRWE2G, Invitrogen, Waltham, MA, USA) according to manufacturer protocol, and measured with MAGPIX TM (Luminex® xMAP® Technology, Austin, TX, USA). The amount of each cytokine in was normalized to the control condition (PBMCs), set as 100 %. The cytokine analysed were Interleukin 2 (IL-2), Interleukin-5, (IL-5) Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), Interleukin-17 (IL-17), Interferon gamma (IFN-ɣ), Tumour necrosis factor-alpha (TNF-α), Tumour necrosis factor-beta (TNF-β).

2.14. Statistical analysis

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

3. Results

3.1. NGAL treatment reduce UREC viability and proliferation, while increasing apoptosis

The exposure of UREC to NGAL resulted in a reduction of cell viability (85.9 ± 10.2 %) compared to control conditions set as 100 (Fig. 1a). Moreover, the proliferation of NGAL-treated UREC was significantly impaired, resulting in a reduction of the proliferation rate (48.58 ± 3.43), compared to untreated cells (Fig. 1b). The Annexin V/7-AAD assay revealed a significant increase in the percentage of cells

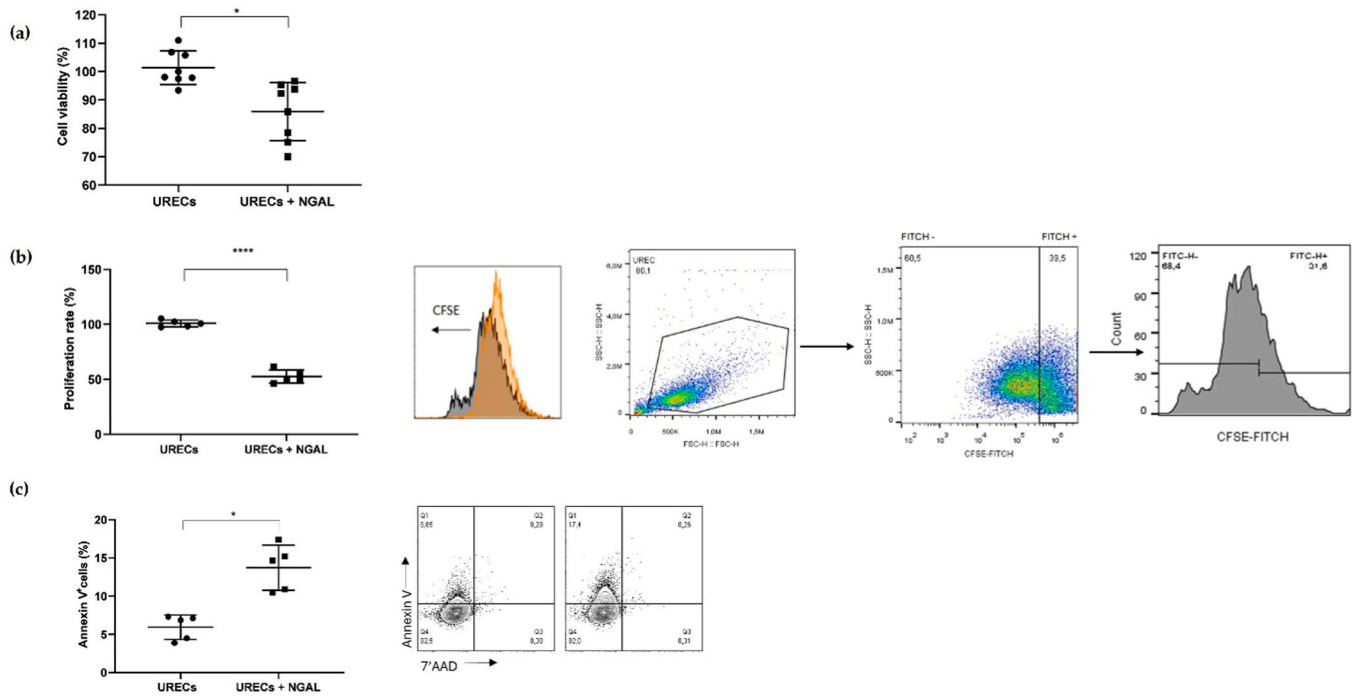

Fig. 1. Effect of Neutrophil gelatinase-associated lipocalin (NGAL) on Urine Renal Epithelial Cells (URECs) viability apoptosis and proliferation. a) evaluation of cell viability with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT) assay in URECs treated (URECs + NGAL) or not treated (URECs), results were normalized setting viability of untreated UREC, set as 100 %. b) histograms and representative results of Carboxyfluorescein Succinimidyl Ester (CFSE) assay for the evaluation of proliferation in URECs treated (URECs + NGAL) or not treated (URECs), results were normalized setting viability of untreated UREC, set as 100 %. c) flow cytometry assessment and representative plots of URECs apoptosis via Annexin V/7-Aminoactinomycin D (7-AAD) in URECs treated (URECs + NGAL) or not treated (URECs). Results are expressed as mean ± SD, * $p<0.05$.

undergoing early apoptosis (Annexin $V^+/7AAD^-$) in the presence of NGAL (12.93 ± 3.93 %), compared to control (7.09 ± 0.24 %) as showed in the graph and in the representative flow cytometry plot in Fig. 1c.

3.2. Expression of NGAL and its receptor (SLC22A17) in URECs and PBMCs

In order to set up a coculture among UREC and PBMCs in presence of NGAL, the expression of both NGAL and its receptor was evaluated in both cell populations. Results indicated that the NGAL receptor (SLC22-A17) was strongly expressed by UREC compared to PBMCs (0.26 ± 0.2). The expression of NGAL gene (LCN-2) was higher in PBMCs with a fold change of 7.28 ± 1.43 as observed in Fig. 2.

3.3. NGAL exposure negatively affected UREC immunomodulatory properties

NGAL was tested during the PBMCs + UREC coculture, to evaluate whether it could influence UREC immunomodulatory properties, previously observed. [15] The addition of NGAL in the coculture medium (PBMCs + UREC + NGAL) reduced the ability to inhibit CD4 and CD8 T cell proliferation, compared to untreated coculture (PBMCs + UREC). The percentage of CD4 T cells increased from 45.56 ± 25.81 % to 94.7 ± 10.62 %, while the CD8 T cell proliferation rate changed from 45.08 ± 10.72 % to 81.10 ± 18.07 % in PBMCs + UREC + NGAL. The presence of NGAL did not affect the proliferation of PBMCs alone (PBMCs + NGAL, 91.51 ± 36.5 %) (Fig. 3a). NGAL also inhibits the antiapoptotic effect of UREC during the PBMCs+UREC coculture. PBMCs undergoing early apoptosis (Annexin $V^+/7AAD^-$ cells) increased in PBMCs + UREC + NGAL condition (31.23 ± 1.88 %), compared to PBMCs + UREC

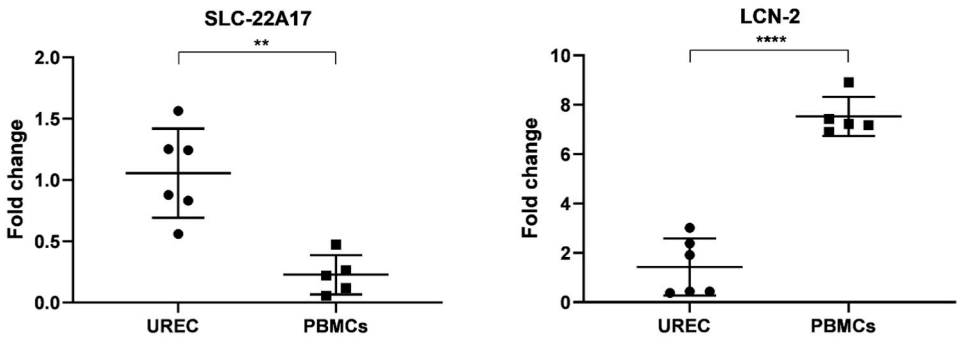

Fig. 2. Expression of NGAL (LCN-2 and its receptor (SLC-22A17) in Urine Renal Epithelial Cells (URECs) and in Peripheral Blood Mononuclear Cells (PBMCs). Real time PCR for the gene expression analysis of NGAL and its receptor in UREC and PBMCs, values were normalized on UREC values and expressed as fold change. Results are expressed as mean ± SD, * $p<0.05$, *** $p<0.001$.

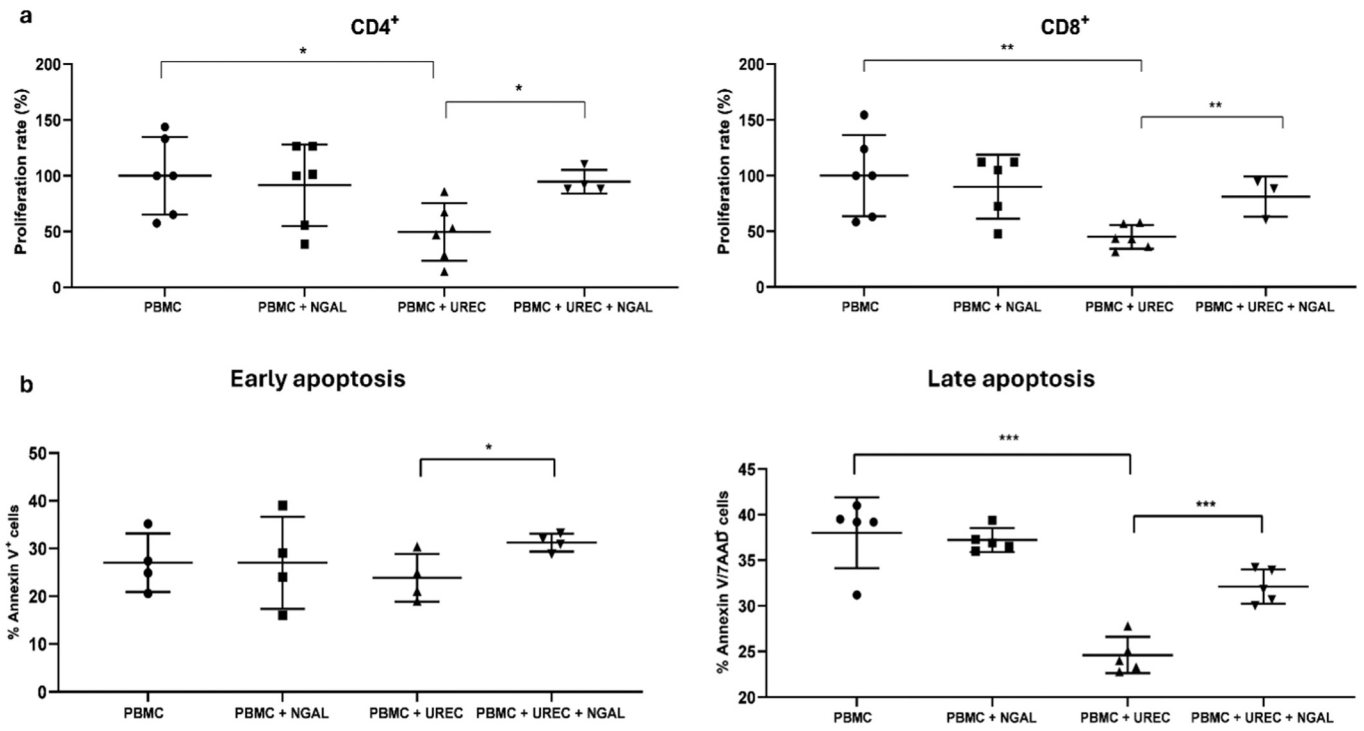

Fig. 3. Evaluation of in Urine Renal Epithelial Cells (URECs) immunomodulatory potential during coculture with Peripheral Blood Mononuclear Cells (PBMCs), in presence or not of NGAL. **a)** Carboxyfluorescein Succinimidyl Ester (CFSE) assay for the evaluation of CD4 and CD8 T Lymphocyte proliferation in control PBMCs (PBMCs), NGAL-treated PBMCs (PBMCs + NGAL) and in PBMCs cocultured with UREC in presence (PBMCs + UREC + NGAL) or not of NGAL (PBMCs + UREC). Results were normalized on control PBMCs set as 100 %. **b)** Annexin V/ 7-Aminoactinomycin D (7-AAD) assay for the analysis of PBMCs in early (Annexin V^+) and late (Annexin $V^+/7AAD^+$) apoptosis in PBMCs and PBMCs + UREC coculture, without or with (PBMCs + NGAL; PBMCs + UREC + NGAL) treatment. Results are expressed as mean ± SD, * $p<0.05$, ** $p<0.01$.

(23.85 ± 5.00 %). PBMCs in late apoptosis (Annexin $V^+/7AAD^+$) significantly reduced in URECs + PBMC condition (25.05 ± 1.98 %) compared to control PBMCs (37.28 ± 4.1 %). However, when NGAL was added to the coculture (PBMCs + UREC + NGAL) PBMCs in late apoptosis increased to 32.13 ± 1.6 % (Fig. 3b).

3.4. NGAL treatment did not counteract the increase of Treg cells during UREC+PBMCs coculture

Considering the previously observed increase in Treg percentage during UREC-PBMCs coculture, we assessed the effect of NGAL treatment on this outcome. The NGAL supplementation did not affect the increase in the Treg ($CD4^+CD25^+FoxP3^+$) promoted by UREC, which resulted significantly higher in the coculture with (PBMCs + UREC + NGAL; 12.11 ± 4.48 %) or without NGAL (PBMCs + URECs; 12.31 ± 5.22 %), compared to control (PBMCs; 6.94 ± 0.96 %). The addition of NGAL on PBMCs alone (PBMCs + NGAL; 5.65 ± 2.61 %) did not influence the Treg percentage (Fig. 4a). Contrarily, the significant decrease of Th1 cells observed in the UREC-PBMCs coculture was not reported in the coculture in presence of NGAL, which was associated with a new increase in Th1 percentage, with values closer to control PBMCs (Fig. 4b).

3.5. NGAL treatment did not affect the cytokine microenvironment in PBMC+UREC coculture

The analysis of different cytokines was performed in both PBMCs alone with and without NGAL treatment, as well as in the standard (PBMCs + URECs) and treated coculture. We confirmed that the coculture modified the amount of several cytokines in the culture medium, without any influence due to the NGAL supplementation, suggesting that URECs exposed to NGAL may have a partial alteration of immunomodulatory properties, which are not completely inhibited (Fig. 5).

4. Discussion

Many molecules that have revealed their positive or negative association with pathological processes have assumed the role of potential biomarkers. Early biomarkers represent a crucial need for diagnostic procedures; thus, several molecules have been studied over the years for their usage as diagnostic and prognostic markers in a wide range of disorders, including kidney diseases (Rysz et al., 2017). NGAL is one of the most prominent biomarkers, since its variation in blood, urine and fecal samples has been associated with AKI and CKD, among other disorders (Izadi et al., 2016, Fassett et al., 2011). The main function of NGAL is linked to its ability to transport siderophore-iron complexes across cell membranes, removing iron and thus counteracting bacterial proliferation and the inflammatory response (Flo et al., 2004).

In the kidney, NGAL has been proposed as a biomarker and a player of AKI onset and its turning into CKD, as well as a possible biomarker for the early prediction of kidney transplant outcome, although in this and other settings it revealed high sensitivity, but not equal specificity (Devarajan, 2008). Regardless of its role as a biomarker, its biological activityin various pathological processes is still less known, especially by observing the numerous and selective binding sites that this molecule has (Bao et al., 2015). The mechanisms by which NGAL contributes to kidney lesions are not completely understood, and in vitro studies focusing on the effect of the NGAL exposure on cell features are still lacking. Ischemia and reperfusion events commonly occur during kidney transplant from deceased donors, promoting the onset of AKI and DGF (Ponticelli, 2014). Several studies showed that NGAL increases in the acellular perfusate used for the preservation of donated kidney, and its increase was directly related to the extent of graft ischemic damage, suggesting its use as a biomarker of delayed graft function and for the assessment of organ quality (Cappuccilli et al., 2018).

This study aims to contribute new insights into the role of NGAL in

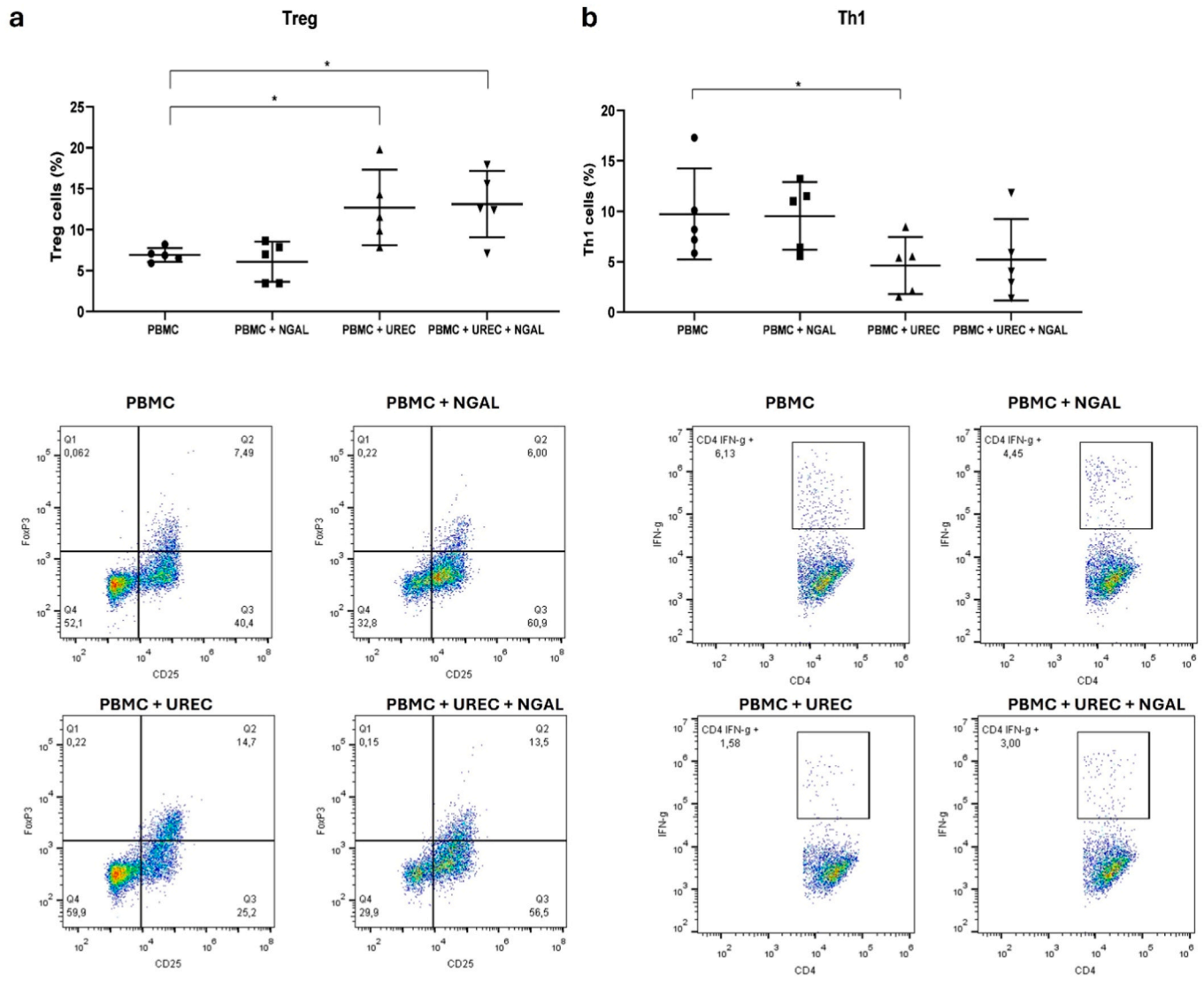

Fig. 4. Treg and Th1 percentage in PBMCs cultured with or without URECs, exposed or not to NGAL treatment. **a)** Percentage of Treg cells ($CD4^+CD25^+Foxp3^+$) in control PBMCs (PBMCs), NGAL-treated PBMCs (PBMCs + NGAL) and in PBMCs cocultured with UREC in presence (PBMCs + UREC + NGAL) or not of NGAL (PBMCs + UREC). **b)** Percentage of Th1 cells ($CD4^+IFN\text{-}\gamma^+$) in control PBMCs (PBMCs), NGAL-treated PBMCs (PBMCs + NGAL) and in PBMCs cocultured with UREC in presence (PBMCs + UREC + NGAL) or not of NGAL (PBMCs + UREC). Results are expressed as mean ± SD, * $p<0.05$, ** $p<0.01$.

kidney transplant, with a focus on the analysis of its effect on kidney cells released in recipient's urine after transplant. We evaluated the effect of NGAL during in vitro culture of Urine Renal Epithelial Cells (URECs) obtained from urine samples of kidney transplanted patients. Our previous studies have shown that URECs are highly released in recipient's urine after transplantation of kidneys deriving from DCD donors; these cells strongly proliferate in vitro and express several kidney markers, including CD24 and CD133, which are typical of kidney epithelial progenitor cells. A characteristic of this cell population is that they are highly voided after kidney transplant in urine of organ recipients, but rapidly disappear after a mean of 6–10 days (Pizzuti et al., 2023). Several groups focused on the characteristics of kidney cells, with a particular interest in tubular epithelial cells (TEC) and in the progenitor subset. Lazzeri and colleagues highlighted the importance of the regenerative potential and proliferative ability of PAX-2^+ TEC in mice model of AKI. Their study also demonstrated that the loss of TEC occurring after kidney damage is partially balanced by the endocycle-mediated hypertrophy of surviving TECs that contributes to the recovery of renal function (Lazzeri et al., 2018). In a recent study involving patients with AKI, tubular epithelial cells were highly detected in urine, confirming the importance of cellular repair and turnover to avoid the onset of CKD. Among different cell populations observed in the urine sediment, a subset of cells expressing CD133 stem cell marker was observed (Gerges et al., 2022). Interestingly, these cells also retained their differentiative ability, giving rise to several tubular cell types.

Since little is known about the effect of damage associated molecule on urine derived cells, in the present study we first analyzed the effect of NGAL treatment on cultured UREC. The first observation is that URECs express the NGAL receptor, which is less expressed in PBMCs. Going further, from the results obtained the exposure to NGAL was associated with a significant increase in the apoptotic rate of UREC, along with a marked reduction of their viability and proliferative activity, compared to UREC not treated with NGAL. Similar observations were previously obtained in mice model of CKD, the apoptotic rate in glomerular and tubular structures was significantly reduced after NGAL inhibition, and both the disease progression and the severity of renal lesions were dramatically reduced in Lcn2-/- mice (Viau et al., 2010). Marques and colleagues demonstrated that NGAL promoted mitochondrial dysfunction in renal tubules of mice, with an activation of mTOR signalling which negatively affects tubular cell viability and proliferation

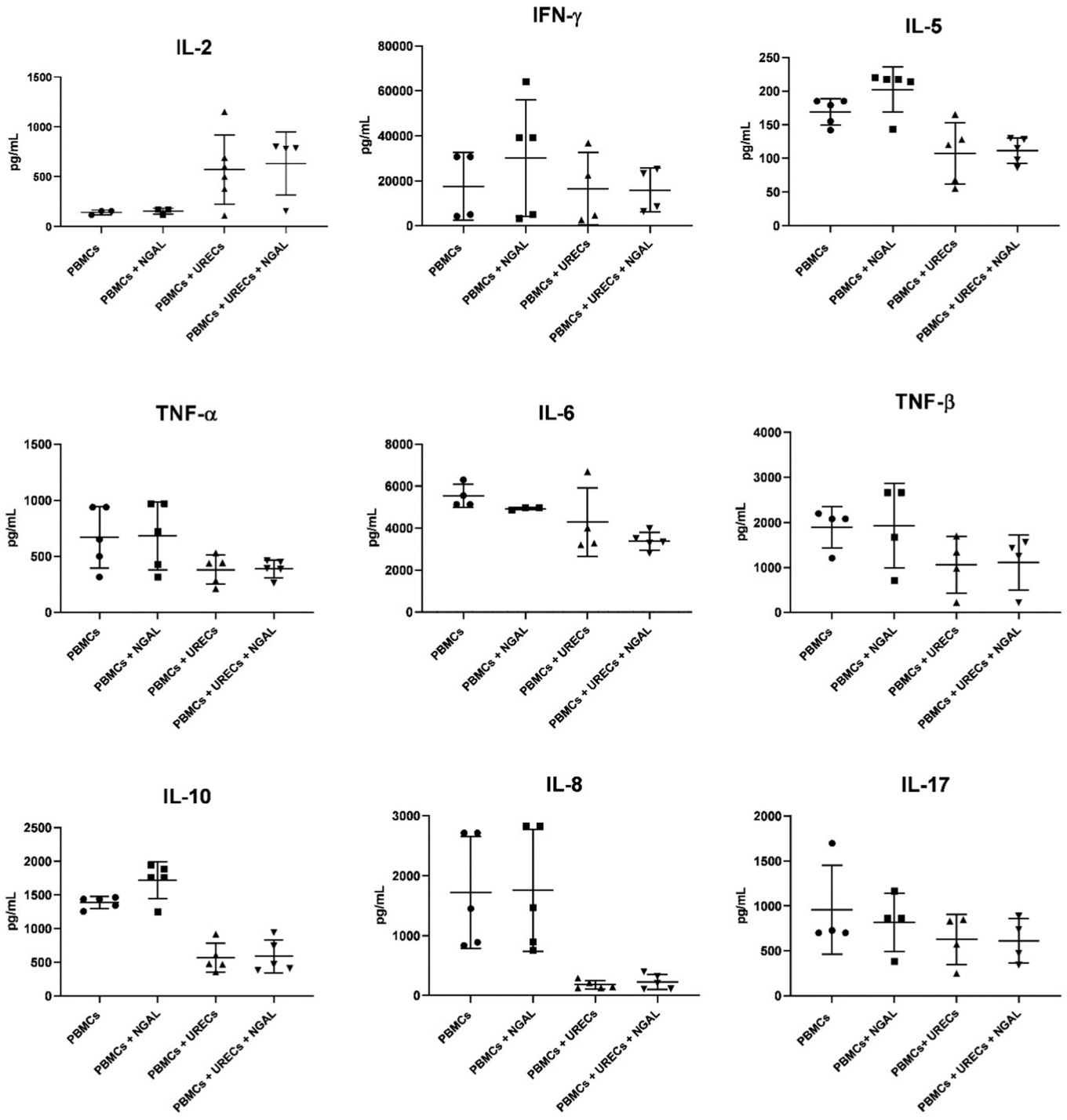

Fig. 5. Analysis of cytokines released in the supernatant of PBMCs and PBMCs + URECs, with or without NGAL. Quantification of Cytokines (pg/ml) in supernatant of control PBMCs (PBMCs), NGAL-treated PBMCs (PBMCs + NGAL) and in PBMCs cocultured with UREC in presence (PBMCs + UREC + NGAL) or not of NGAL (PBMCs + UREC). The cytokines analysed were the following: Interleukin-2 (IL-2), Interferon-gamma (IFN-γ), Interleukin-5 (IL-5), Interleukin-6 (IL-6), Tumour Necrosis Factor alpha and beta (TNF- α/β), Interleukin-10 (IL-10), Interleukin-8 (IL-8), Interleukin-17 (IL-17). Results are expressed as mean ± SD, * $p<0.05$, ** $p<0.01$.

(Marques et al., 2023). Also, NGAL strongly interacts with *Epidermal Growth Factor Receptor* (EGFR) regulating its activation and maintenance on cell surface, thus affecting proliferation and migration kidney cells. The binding of NGAL to EGFR significantly increased kidney lesions in mice models of CKD (Yammine et al., 2019). Since little is known about the effect of NGAL on primary cell derived from urine samples, the results obtained on URECs viability and proliferation need to be further elucidated by analysing the pathways involved in these mechanisms. A hypothesis on which it is possible to speculate is represented by the role of NGAL in negatively conditioning kidney regenerative mechanisms, affecting the turnover of kidney cells and the release of UREC after transplantation. This study also aimed to evaluate whether NGAL can alter the immunomodulatory functions of URECs previously observed. Basing on our previous results, URECs were able to strongly reduce the proliferation of CD4 and CD8 T cells during the coculture with activated PBMCs from healthy donors, without inducing immune cell apoptosis.

Interestingly, URECs promoted a significant increase in the Treg subset and modulated the cytokine microenvironment, reducing several proinflammatory molecules (Pizzuti et al., 2023). We hypothesized that the reduction of the proliferation and vitality of URECs and the increase of apoptosis after exposure to NGAL, could compromise their immunomodulatory capacities. Our results confirmed this hypothesis; NGAL partially suppressed the immunomodulatory properties of URECs during coculture with PBMCs, reducing their anti-lympho proliferative ability.

In detail, NGAL promotes an increase in CD4 and CD8 T cell proliferation during the PBMCs-UREC coculture and the ability of URECs to protect PBMCs from early and late apoptosis was less observed in presence of NGAL. Interestingly, NGAL did not affect the increase in Treg cells promoted by URECs during the coculture and did not alter the panel of inflammatory cytokines. The effect of NGAL in weakening the immunomodulatory capacity was limited to the PBMCs-URECs condition, since not significant results were obtained in PBMCs treated with NGAL and not cultured with URECs. These results may be linked to the higher expression of the gene encoding the NGAL receptor 24p3R in UREC compared to PBMCs. As matter of fact, the NGAL receptor is widely expressed in kidney tubular structures and in vitro study using a tubular epithelial cell line showed that its expression increased after hypoxia-reoxygenation events (Arakawa et al., 2019).

The higher expression of NGAL receptor could made URECs more susceptible to the effect of NGAL during in vitro culture compared to PBMCs. The present study highlights that URECs isolated after kidney transplant are highly sensitive to NGAL exposure during in vitro culture, with consequences on their proliferation and viability. This could indicate that NGAL may negatively contribute to kidney regeneration, which is highly needed after events as kidney transplant, thus contributing to the onset of cell damage and DGF, by reducing viability and proliferation of target cells. This behaviour could also affect the in vitro maintenance of URECs and their expansion for therapeutic purposes which are mainly based on the application of their immunomodulatory properties.

However, the immunomodulatory properties of URECs are not completely suppressed by NGAL, with a preservation of their ability to increase the Treg subset and no significative alteration of the cytokine microenvironment. Despite the role of NGAL in the immune response is well described (Flo et al., 2004), studies focusing on its effect on immunomodulatory properties of cells are still missing. Understanding the mechanisms underlying the effect of NGAL exposure on different kidney-derived cell types and on their features, could improve our knowledge about the effect of this molecule in the pathogenesis of several kidney disorders. Although the pathways in which NGAL could be involved still need to be clarified, NGAL and its receptor could be used as therapeutic targets to reduce kidney cell damage caused by events such as AKI, CKD, and kidney transplantation. Moreover, the understanding of the role of NGAL on target cells may have useful implication for the maintenance of cell features for therapeutic purposes.

Ethics declarations

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of University Hospital of Bologna IRCCS, Sant'Orsola Polyclinic (protocol code 312/2021/Oss/AOUBO, date of approval: 18 May 2021). Informed consent was obtained from all subjects involved in the study.

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CRediT authorship contribution statement

Francesco Alviano: Visualization, Validation. **Valeria Corradetti:** Visualization, Validation, Resources. **Federica Maritati:** Visualization, Validation. **Gaetano La Manna:** Visualization, Validation, Supervision. **Diletta Conte:** Writing – review & editing, Formal analysis. **Elisa Gessaroli:** Resources, Data curation. **Marcello Demetri:** Project administration, Data curation. **Pasquale Marrazzo:** Resources, Data curation. **Miriam Di Nunzio:** Writing – review & editing, Data curation. **Giorgia Comai:** Visualization, Validation, Project administration. **Valeria Pizzuti:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Emma Balducelli:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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