The possible role of ChemR23/chemerin axis in the recruitment of dendritic cells in lupus nephritis

Giuseppe De Palma[1,6], Giuseppe Castellano[1,6], Annalisa Del Prete[2,3], Silvano Sozzani[4], Nicoletta Fiore[1], Antonia Loverre[1], Marc Parmentier[5], Loreto Gesualdo[1], Giuseppe Grandaliano[1] and Francesco P. Schena[1]

[1]Department of Emergency and Organ Transplantation, Nephrology, Dialysis, and Transplantation Unit, University of Bari, Bari, Italy; [2]Department of Medical Biochemistry, Biology, and Physics, University of Bari, Bari, Italy; [3]Istituto Clinico Humanitas IRCCS, Rozzano, Italy; [4]Department of Biomedical Sciences and Biotechnology, Section of General Pathology and Immunology, University of Brescia, Brescia, Italy and [5]Institut de Recherche Interdisciplinaire en Biologie Humaine et Moléculaire, Université Libre de Bruxelles, Brussels, Belgium

Dendritic cells (DCs) have a pivotal role in the autoimmune response of systemic lupus erythematosus. Plasmacytoid DCs infiltrate the kidney of patients with lupus nephritis, but factors regulating their recruitment to the kidney are unknown. Chemerin is the recently identified natural ligand of ChemR23, a receptor highly expressed by plasmacytoid DCs. We performed immunohistochemical and immunofluorescence analysis to study the ChemR23/chemerin axis in renal biopsies from patients with lupus nephritis. We found ChemR23-positive DCs had infiltrated the kidney tubulointerstitium in patients with severe lupus nephritis. Chemerin association with tubular epithelial cells and renal lymphatic endothelial cells was found in patients with lupus nephritis but not in normal kidneys. Proximal tubular epithelial cells produced chemerin *in vitro*, which was significantly down-modulated by added tumor necrosis factor (TNF)-α and interferon-γ as measured by quantitative PCR and enzyme-linked immunosorbent assay. Interestingly, TNF-α was capable of inducing a functionally active form of renal chemerin, resulting in an efficient transendothelial migration of plasmacytoid DCs measured in transwell systems. Thus, the ChemR23/chemerin axis may have a role in the recruitment of DCs within the kidney in patients affected by lupus nephritis.

Kidney International advance online publication, 23 February 2011; doi:10.1038/ki.2011.32

KEYWORDS: chemokine; chemokine receptor; lupus nephritis; systemic lupus erythematosus

Correspondence: *Giuseppe Castellano, Department of Emergency and Organ Transplantation, Nephrology, Dialysis, and Transplantation Unit, University of Bari, Policlinico, Piazza Giulio Cesare 11, Bari 70124, Italy. E-mail: g.castellano@nephro.uniba.it*

[6]These authors equally contributed to the present study.

Received 11 May 2010; revised 1 December 2010; accepted 4 January 2011

Dendritic cells (DCs) are professional antigen-presenting cells that patrol tissues to sense danger signals, activating specific immune responses.[1] Although all DCs are capable of Ag uptake, processing, and presentation to naïve T cells, DC subtypes differ in location, migratory pathways, detailed immunological functions, and differentiation pathways.[2,3] One division is into myeloid DCs, which already have a dendritic form and exhibit DC functions in steady state, and precursors of DCs, which require further development to acquire a dendritic form and full DC function.[2] Plasmacytoid DCs, also known as natural interferon-producing cells, are one of the DC lineages appearing as DC precursors.

A dysregulation in DC biology has been demonstrated in patients affected by systemic lupus erythematosus (SLE).[4–6] In SLE patients, an enhanced production of type I interferons by plasmacytoid DCs promotes the aberrant presentation of auto-antigens to T and B cells by myeloid DCs,[7] which are actively involved in the phagocytosis of apoptotic cells.[5]

SLE is characterized by multi-organ involvement[6] and nephritis, which occurs in up to 60% of patients.[8] SLE patients with nephritis have abundant infiltrate of plasmacytoid DCs mostly localized at tubulointerstitial level.[9] However, the mechanism by which plasmacytoid DCs enter the tubulointerstitium is not fully understood, although it is likely that chemotactic factors are involved.

Recent data on SLE patients with skin involvement have shown an increased number of plasmacytoid DCs expressing the G-protein-coupled receptor ChemR23, at the level of skin lesions. This receptor is able to drive plasmacytoid DC migration in response to chemerin, a non-chemokine chemoattractant polypeptide.[10]

Given the increased presence of plasmacytoid DCs at renal level in SLE patients, aim of the present study has been to investigate the possible role of the ChemR23/chemerin axis in the recruitment of plasmacytoid DCs in kidney of patients with lupus nephritis.

RESULTS

ChemR23 + cells infiltrate kidney of SLE patients with nephritis

Lupus nephritis is characterized by an increased recruitment of plasmacytoid DCs, which express the ChemR23 receptor, whose natural ligand is chemerin.[10] We first investigated the presence of cells expressing the ChemR23 receptor. We did not detect infiltrating cell expressing the chemerin receptor in normal renal tissue (Figure 1a). However, as shown in Figure 1b, we found ChemR23 + cells in kidney from SLE patients with nephritis, mostly localized at tubulointerstitial level. At higher magnifications, ChemR23 + cells clearly surrounded tubular epithelial cells, with a typical DC pattern (Figure 1c). Moreover, ChemR23 + cells frequently localized around the glomeruli (Figure 1d and e thin arrows). In addition, we found ChemR23 + cells associated with renal vessels (Figure 1f, arrow). No ChemR23 + cells were detected in renal biopsies from patients with minimal change disease (Figure 1g). Quantification of percentage of ChemR23 + staining area (Figure 1h) revealed a significant increase of ChemR23 + cells in class III–IV patients ($4.4 \pm 1.1\%$) compared with class I–II patients ($1.1 \pm 2.1\%$).

ChemR23 + cell populations express specific markers of human DCs

We then evaluated the phenotypical characteristics of ChemR23 + infiltrating cells. In humans, markers for plasmacytoid DCs include CD123 and BDCA-2.[11,12] Double-immunofluorescence staining on frozen sections from kidneys of class IV lupus nephritis (Figure 2a–d) revealed that CD123 + cells expressed also ChemR23. In addition, BDCA-2 + plasmacytoid DCs colocalized with ChemR23 (Figure 2e-g). Finally, we found that rare ChemR23 + cells expressed BDCA-1 (Figure 2h-j), a specific marker of myeloid DCs.

Expression of chemerin in renal biopsies

Considering the presence of infiltrating DCs expressing the ChemR23 receptor, we then stained renal tissue with a specific anti-chemerin antibody. Normal subjects, SLE patients with lupus nephritis, and patients with minimal change disease were therefore analyzed (Figure 3). We did not find any sign of chemerin production in normal kidney (Figure 3a). On the contrary, when we analyzed SLE patients with nephritis, chemerin associated to renal tubular epithelial cells was found (Figure 3b–e). The intensity of chemerin staining varied between class II (Figure 3b) and class IV (Figure 3c) lupus nephritis, indicating an increased production in patients with worse kidney involvement. In class V lupus nephritis, chemerin deposits were significantly less present than in class III–IV specimens (Figure 3d). No signal was observed in the minimal change disease (Figure 3f).

Chemerin was also associated to lymphatic endothelium, identified using an antibody recognizing podoplanin, a specific marker for lymphatic endothelial cells. The lymphatic endothelium in lupus nephritis expressed podoplanin along

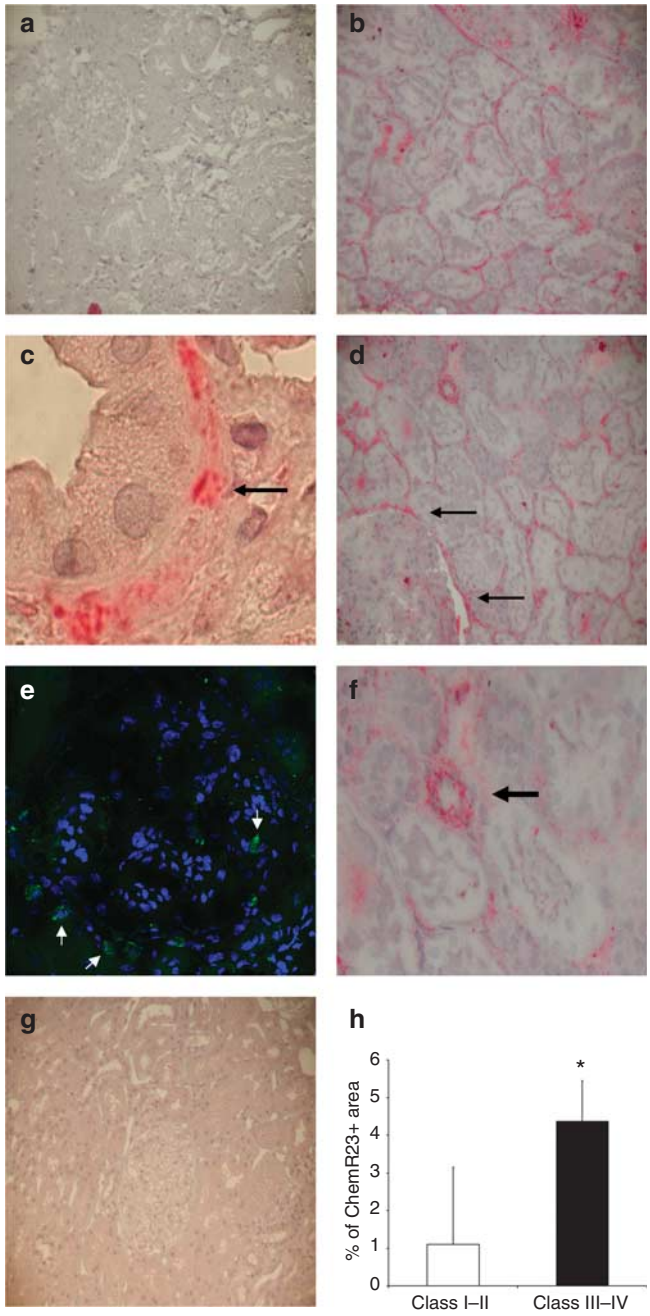

Figure 1 | ChemR23+ cells infiltrate kidney of systemic lupus erythematosus patients with nephritis. Normal kidneys (**a**), kidneys from patients affected by lupus nephritis (class IV; **b**–**f**), from patients with minimal change disease (**g**) were used to investigate the presence of plasmacytoid dendritic cells expressing the ChemR23 receptor. No ChemR23 + cells were observed in normal kidneys (**a**, × 20) and in minimal change disease (**g**, × 20). Several ChemR23 + cells were present in the tubulointerstitium of patients with class IV lupus nephritis (**b**, **d**; × 20). At a higher magnification (**c**, × 100) ChemR23 + cells showed a peritubular location (arrow). ChemR23 + cells frequently localized around the glomeruli (**d**, **e** arrows). ChemR23 + cells were detected in peritubular vessels (**f**, arrow; × 40). (**h**) Quantitative analysis of ChemR23 staining was obtained indicating the percentage (%) of the total area that was positive for the specific signal (Adobe Photoshop software).

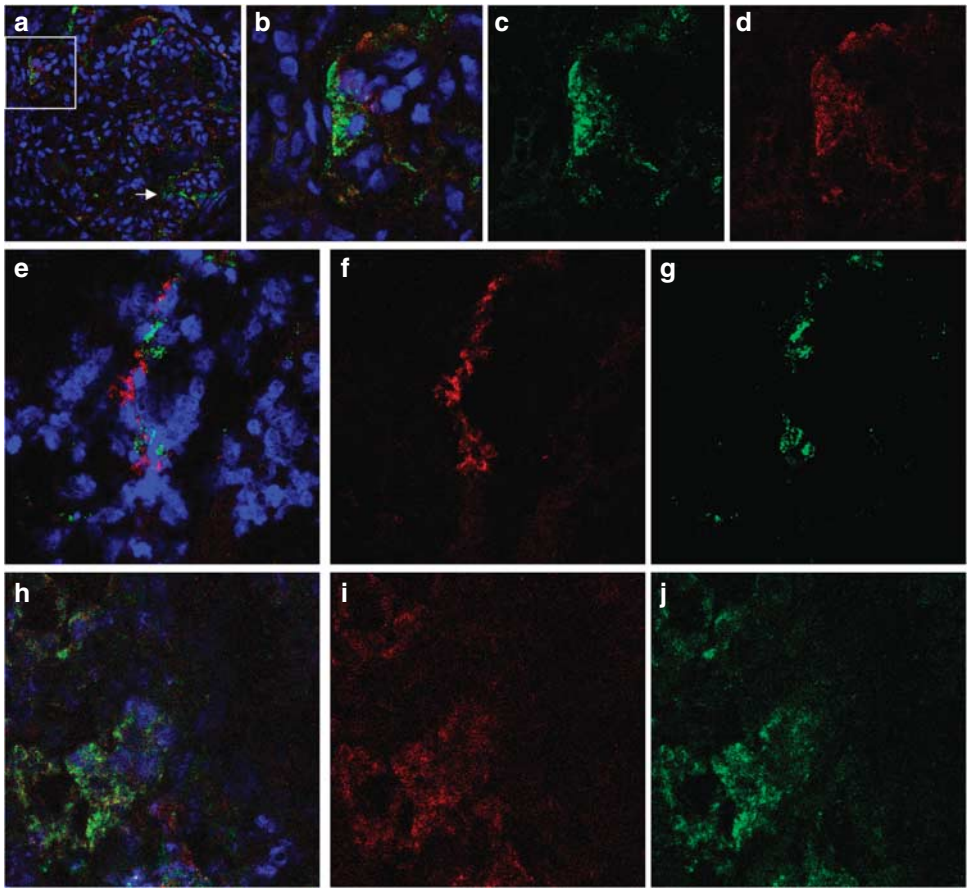

Figure 2 | Characterization of ChemR23+ cells as plasmacytoid dendritic cells (DCs) and myeloid DCs in kidney of patients with lupus nephritis. (**a**–**d**) Overlay double immunofluorescence of ChemR23 (green) and CD123 (red) together with TO-PRO-3 nuclear staining (blue) in renal biopsy of a patient with class IV lupus nephritis (**b**; ×63). The enlargement (**b**), originating from the area indicated by the white box in **a**, shows a double-labeling image illustrating the colocalization between the green (**c**) and red channels (**d**). Merge image (**e**) of red (**f**; ChemR23) and green (**g**; BDCA-2) channels with TO-PRO-3-stained nuclei (blue). Merge image (**h**) of red (**i**; ChemR23) and green (**j**; BDCA-1) channels with TO-PRO-3-stained nuclei (blue).

the vessel walls (Figure 3g), with a significant presence of chemerin (Figure 3h). The yellow staining in Figure 3i indicated that the two signals colocalized on lymphatic vessels.

Chemerin production by renal epithelial and endothelial cells

To test the hypothesis that renal tubular epithelial cells may represent a significant source of chemerin, we performed reverse transcription PCR (RT-PCR) experiments. We found that proximal tubular epithelial cells expressed chemerin mRNA *in vitro* (Figure 4a) and the use of a pro-inflammatory stimulus such as tumor necrosis factor (TNF)-α was able to reduce chemerin mRNA levels. On the contrary, an anti-inflammatory stimulus such as dexamethasone plus 1,25 dihydroxyvitamin D3, did not modify the chemerin mRNA expression (Figure 4a). Next, we performed real-time RT-PCR analysis to quantify chemerin mRNA expression in proximal tubular epithelial cells cultured for 48 h. As shown in Figure 4b, anti-inflammatory stimulus such as transforming growth factor (TGF)-β did not modify the basal level of chemerin mRNA expression in proximal tubular epithelial cells. The same effect was seen after exposure to dexamethasone and 1,25 dihydroxyvitamin D3, that was reported to stimulate chemerin gene in stromal cell line.[13] When we used IL1-β we did not observe any change in mRNA expression (Figure 4b). On the contrary, interferon (IFN)-γ and TNF-α were capable to downregulate significantly chemerin mRNA expression in proximal tubular epithelial cells (Figure 4b).

Considering the presence of ChemR23+ DCs around renal vessels and the colocalization between chemerin and endothelial cells, we also isolated renal CD146+/CD105+ endothelial cells from normal kidneys (Figure 4c) and performed real-time RT-PCR analysis. As shown in Figure 4d, renal endothelial cells expressed the specific mRNA for chemerin as the proximal tubular epithelial cells.

Regulation of chemerin production in tubular epithelial cells

As the major site of chemerin localization in SLE patient was the renal tubules, we then studied the regulation of chemerin production in proximal tubular epithelial cells *in vitro*. Therefore, we stimulated cells with IFN-γ and TNF-α for 72 h and collected culture supernatants to perform sandwich enzyme-linked immunosorbent assay (ELISA). Proximal

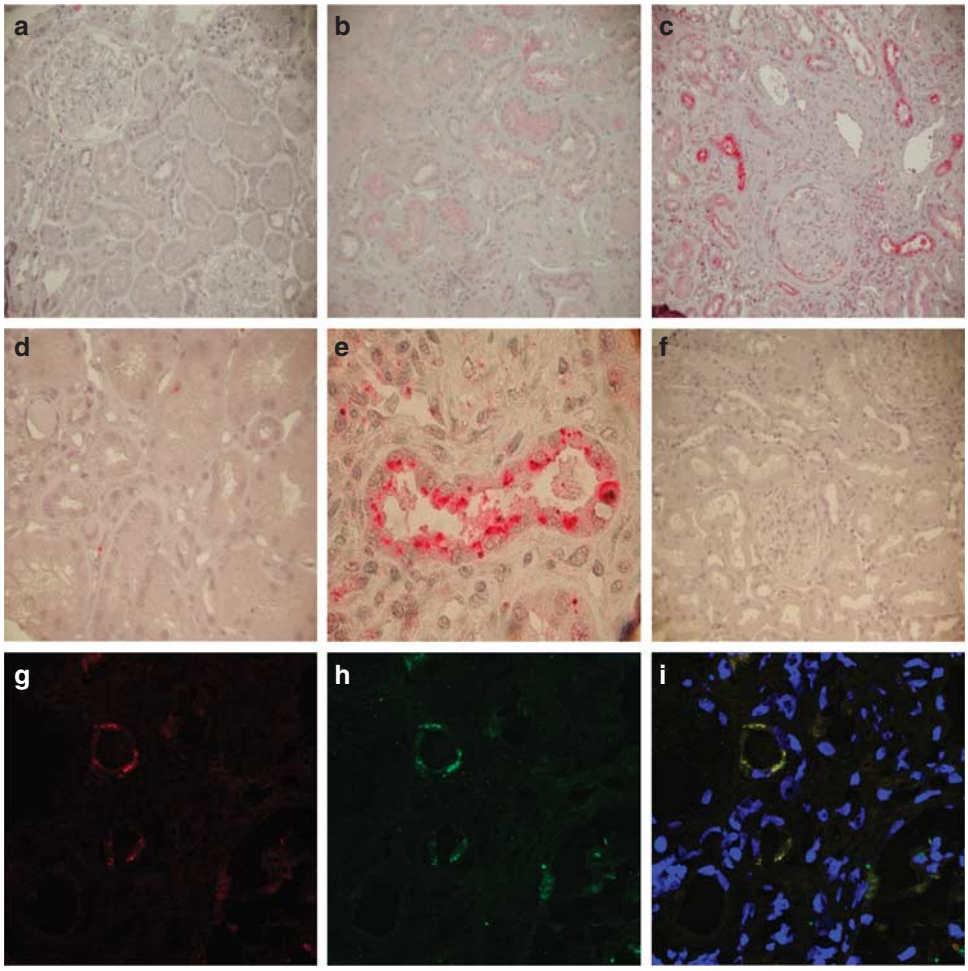

Figure 3 | Expression of chemerin in renal biopsies. A mouse anti-human chemerin antibody was used in immunohistochemistry to stain renal biopsies from normal (**a**; × 20), lupus nephritis (**b**–**e**), and minimal change disease (**f**; × 20). A specific but weak signal was observed in tubular epithelial cells in class II lupus nephritis (**b**; × 20). Chemerin was detected in tubular epithelial cells from class IV lupus nephritis (**c**; × 20). Class V lupus nephritis was characterized by a scarce staining for chemerin (**d**; × 40). At higher magnification (× 100), chemerin was clearly detectable within tubular epithelial cells (**e**). Indirect immunofluorescence-double staining of kidney section from class IV lupus nephritis with anti-podoplanin (**g**; red) and anti-chemerin (**h**; green) antibodies. Yellow colors indicated colocalization of the red and green signal (**i**). TO-PRO-3 staining of DNA is shown in blue.

tubular epithelial cells were capable to produce chemerin in a concentration around 25 ng/ml in presence of culture medium (Figure 5a). TNF-α and IFN-γ significantly down-regulated the release of chemerin after 72 h (Figure 5a). Dose-response experiments on stimulated proximal tubular epithelial cells showed that the production of chemerin progressively increased in culture cells in normal medium (Figure 5b). TNF-α used at the concentration of 5 and 10 ng/ml decreased chemerin production of 40–30%. A stronger effect was induced by 50 ng/ml of TNF-α (70% reduction of protein release). Under stimulation by IFN-γ, we found a decrease in chemerin production by 50 ± 5.8%, with no significant differences between the concentrations used (200, 500, and 1000 U/ml; Figure 5c).

TNF-α activates renal chemerin to attract plasmacytoid DCs

Finally, we tested whether chemerin produced by proximal tubular epithelial cells was functionally active in the recruitment of plasmacytoid DCs using a transendothelial chemotaxis assay in presence of a specific blocking antibody for ChemR23. This method, although indirect, provides a precise indication about the presence of biological active chemerin.[14] Therefore, we isolated plasmacytoid DCs from healthy subjects as previously described.[10] Chemerin-induced transendothelial migration of plasmacytoid DCs through HUVEC monolayer was assessed using supernatants from unstimulated and stimulated proximal tubular epithelial cells as a source of chemoattractants. First, we tested the culture media derived from unstimulated proximal tubular epithelial cells that showed *in vitro* the major production of chemerin (Figure 6a). Surprisingly, despite the high release of chemerin by proximal tubular epithelial cells in basal condition (25 ng/ml), we found a very low capacity to induce plasmacytoid DC migration by those supernatants (Figure 6a). The same effect was seen when we tested the supernatants derived from IFN-γ-activated proximal tubular

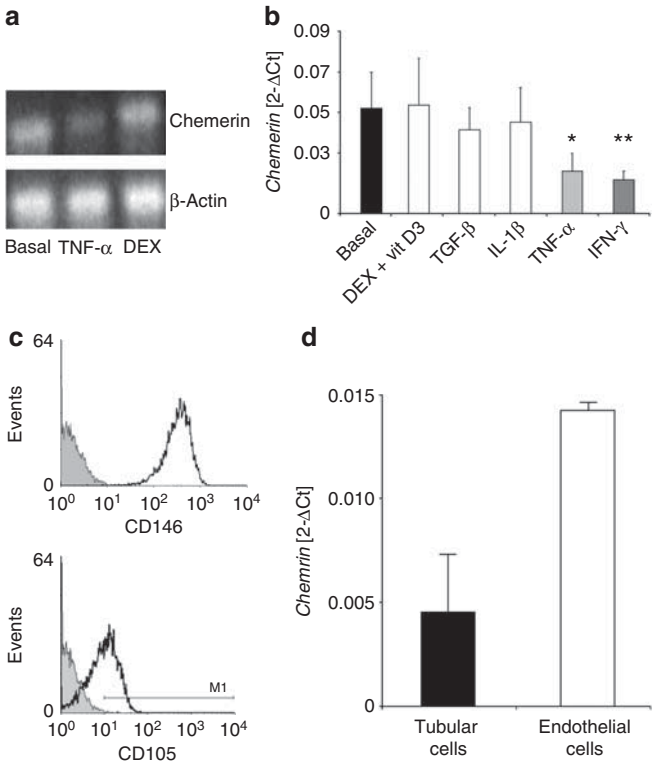

Figure 4 | Analysis of chemerin mRNA expression in renal tubular epithelial and endothelial cells. (**a**) Reverse transcription PCR (RT-PCR) analysis of mRNA isolated from unstimulated proximal tubular epithelial cells (lane 1), and after 48 h of incubation with tumor necrosis factor (TNF)-α (lane 2) and dexamethasone (lane 3). β-Actin was used as housekeeping gene. (**b**) Real-time RT-PCR for chemerin in proximal tubular epithelial cells stimulated for 48 h in presence of different factors. β-Actin mRNA was used as internal control (*$P < 0.05$ compared with basal expression; **$P < 0.03$ compared with basal expression). Bars represent the mean values ± s.d. of three independent experiments. (**c**) Representative histograms of fluorescence-activated cell sorting analysis on isolated renal endothelial cells, showing the expression of CD146 and CD105 marker, confirming their endothelial lineage. (**d**) Real-time RT-PCR analysis for chemerin mRNA expression in renal endothelial cells and proximal tubular epithelial cells under basal conditions. β-Actin mRNA was used as internal control. Bars represent the mean values ± s.d. of three independent experiments. IFN, interferon; IL, interleukin; TGF, transforming growth factor.

epithelial cells that were characterized by low plasmacytoid DCs migration (Figure 6a).

On the contrary, the supernatants derived from proximal tubular epithelial cells stimulated by TNF-α were capable to attract significantly the plasmacytoid DCs through the endothelial cell monolayer (Figure 6a). This effect was significantly inhibited by the use of anti-ChemR23 blocking antibody (Figure 6b). This result showed that the recruitment of plasmacytoid DCs by TNF-α-stimulated proximal tubular epithelial cells supernatants was mediated by chemerin interaction with the ChemR23 receptor and was likely due to the ability of TNF-α to promote pro-chemerin conversion into biologically active chemerin.

DISCUSSION

In this paper, we have demonstrated for the first time the presence of chemerin at renal level in SLE patients affected by nephritis. Chemerin has been detected in tubular epithelial cells and lymphatic endothelial vessels of patients with severe renal damage. In accordance, we have found infiltrating ChemR23 + DCs localized at peritubular and periglomerular levels, at the sites of chemerin production. *In vitro*, chemerin was produced by endothelial and proximal tubular epithelial cells. When they were stimulated by TNF-α and IFN-γ, proximal tubular epithelial cells significantly down-regulated chemerin mRNA expression and protein release. However, only TNF-α was likely capable to induce the biological activation of chemerin, leading to active recruitment of plasmacytoid DCs.

SLE is a multi-organ autoimmune syndrome, wherein patients who develop lupus nephritis are regarded as those with the worst prognosis and the highest morbidity rate.[6] Patients with severe lupus nephritis have massive infiltrates of plasmacytoid DCs at tubulointerstitial level, when compared with patients with mild nephritis.[9] In proliferative and non-proliferative glomerulonephritis, plasmacytoid DCs have also been found in significant numbers, along with myeloid DCs, mainly localized at tubulointerstitial level.[15] So far, the factors regulating the recruitment of plasmacytoid DC at renal level are unknown.

Recent data on SLE patients with skin involvement showed an increased number of plasmacytoid DCs expressing the G-protein-coupled receptor ChemR23 at the level of skin lesions. This receptor has been shown to drive plasmacytoid DC migration in response to chemerin, a non-chemokine chemoattractant polypeptide, that has been found in spleen and lymph nodes.[10] Our data indicate that plamacytoid DCs, and to a lower extent myeloid DCs, express the ChemR23 receptor, that might mediate their recruitment during renal inflammation. In addition, we have shown that chemerin is mainly produced by tubular epithelial cells that have a pivotal role in the recruitment of leukocytes at renal level.[16–20] The local production of chemerin might be crucial to promote the specific chemotaxis of DCs within the kidney at the tubulointerstitial level. The association of chemerin to lymphatic endothelial vessels suggests that this chemotactic factor may also regulate the migration of DC toward draining lymph nodes.[21,22]

The modulation of chemerin expression has been investigated in other cellular systems. Corticosteroid, HGF, and IL1-β have been shown to modulate chemerin mRNA.[23,24] Our analysis in proximal tubular epithelial cells indicated that only TNF-α and IFN-γ are able to modulate chemerin production. A large body of evidence implicates TNF-α in lupus nephritis, but with an ambiguous role.[25] TNF-α is highly expressed at the renal level in this setting[26] and can be detected in high concentration in SLE sera, correlating with disease activity.[27] Accordingly, IFN-γ is also overexpressed in lupus nephritis.[25,28,29] As we have detected chemerin in SLE patients with severe renal involvement, it

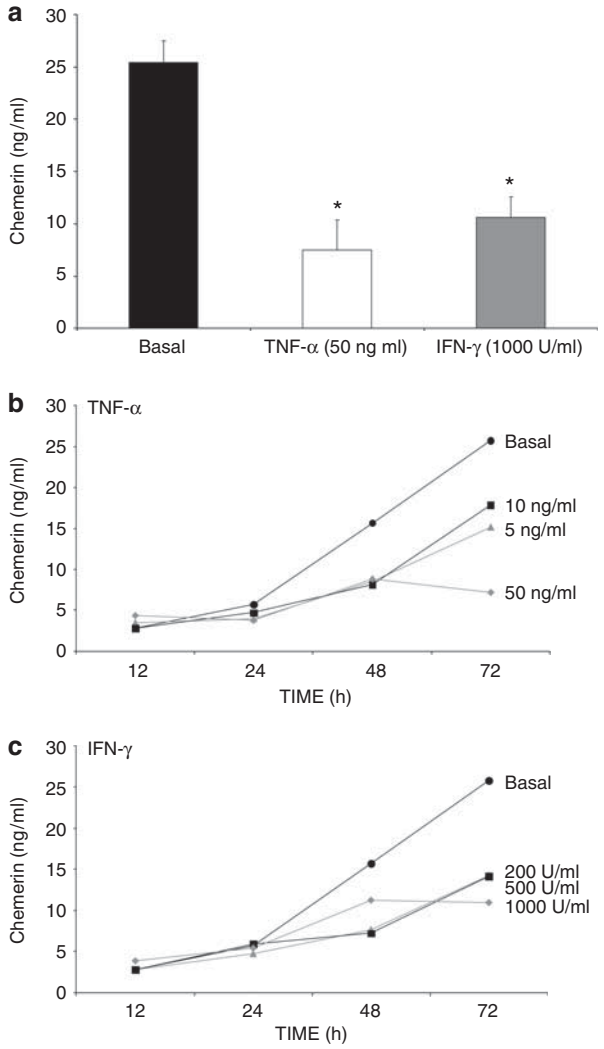

Figure 5 | Chemerin production by tubular epithelial cells *in vitro*. (**a**) Effect of tumor necrosis factor (TNF)-α and interferon (IFN)-γ on the production of chemerin by proximal tubular epithelial cells. After 72 h of stimulation, supernatants were collected and assessed for chemerin levels by enzyme-linked immunosorbent assay (ELISA). Results are expressed as mean ± s.d. of experiments in triplicate culture. (**b**) ELISA experiments on chemerin secretion by proximal tubular epithelial cells exposed to TNF-α at different concentrations. (**c**) ELISA experiments on chemerin secretion by proximal tubular epithelial cells exposed to IFN-γ.

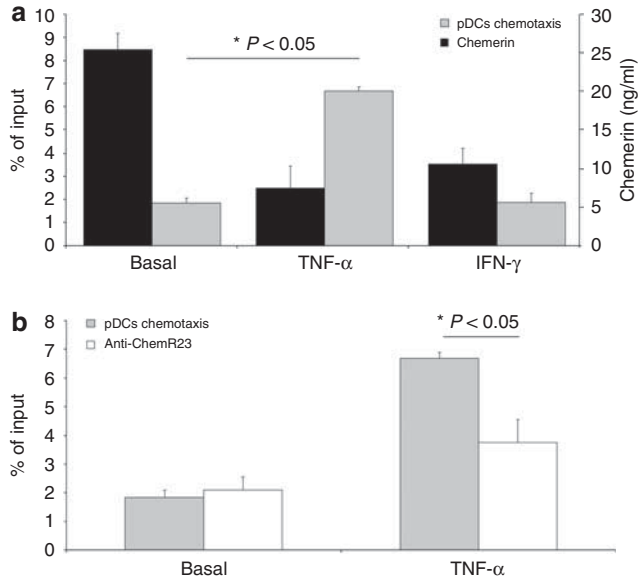

Figure 6 | Renal chemerin induces transendothelial migration of human plasmacytoid dendritic cells (DCs). (**a**) Transendothelial migration experiments were used to test the biological activity of chemerin produced by renal proximal tubular epithelial cells. Black bars indicate the concentration of chemerin in supernatants of proximal tubular epithelial cell cultures (ng/ml). Gray bars indicate the percentage of transmigrating plasmacytoid DCs across the endothelial monolayer (% of plasmacytoid DCs input). Bars represent the mean values ± s.d. of three independent experiments (*$P < 0.05$ by *t*-test). (**b**) Effect of anti-ChemR23 monoclonal antibody on plasmacytoid DC transmigration. *In vitro* transmigration was assessed in the absence (gray bars) or presence (white bars) of anti-ChemR23 blocking antibody. Anti-ChemR23 antibody did not block migration to CXCL12 indicating the specificity of the effect (not shown). Bars represent the mean values ± s.d. of three independent experiments. IFN, interferon; TNF, tumor necrosis factor.

might be possible to hypothesize the existence of some chemerin-inducing factors that can counteract the inhibitory effects of TNF-α and IFN-γ, leading to a balance in protein expression and production.

Chemerin is secreted as pro-chemerin, a poorly active precursor protein that requires to be processed by proteases into a functional form.[30,31] It is important to realize that in our study we could not discriminate the activation state of chemerin on tissue, as the anti-chemerin monoclonal antibody recognize an epitope located on both active chemerin and pro-chemerin. Therefore, we cannot demonstrate whether the chemerin detected in SLE patients with nephritis was the inactive or active form of the protein. We evaluated the levels of active chemerin, using a chemotaxis assay in the presence of a specific blocking antibody for ChemR23. This approach, although indirect, provides a precise indication about the presence of biological active chemerin.[14] Our data *in vitro* clearly show that the presence of TNF-α can induce the activation of chemerin produced by renal epithelial cells, leading to specific plasmacytoid DC recruitment across the endothelial monolayer. The generation of chemerin seems to be regulated first at transcriptional level by TNF-α and IFN-γ, both capable to downregulate protein release. Next, a second level of regulation occurs as the pro-chemerin requires activation at extracellular level to acquire the biological activity. It has been shown that proximal tubular epithelial cells treated with TNF-α have an increase in plasminogen activating ability due to the synthesis of the urokinase-type plasminogen,[32] a serine protease that is capable to activate chemerin.[33] Therefore, it is conceivable that TNF-α might be pivotal in the recruitment of DCs by inducing the cleavage of pro-chemerin via the local production of serine proteases in proximal tubular epithelial cells.

In conclusion, we demonstrated that the production of chemerin occurs at renal level in SLE patients with nephritis.

The local production of chemerin is accompanied by infiltrating ChemR23+ DCs localized at peritubular and periglomerular level. *In vitro*, chemerin is produced by proximal tubular epithelial cells and endothelial cells, with a significant downregulation in protein synthesis and release on TNF-α and IFN-γ activation. Finally, the presence of TNF-α is required to induce the active form of chemerin, resulting in specific chemotactic effect on plasmacytoid DCs.

These findings strongly suggest a role for the ChemR23/chemerin axis in the recruitment of DCs at renal level in lupus nephritis.

MATERIALS AND METHODS

Immunofluorescence and immunohistochemistry

Renal tissue samples were obtained from 13 SLE patients with lupus nephritis who underwent kidney biopsy in the period 2000–2009 (five classes I–II, five classes III–IV, three class V). Five normal controls were obtained from apparently normal portion of kidneys removed for renal cell carcinoma and a second control group of renal tissue samples was also obtained from five minimal change disease patients with proteinuria (more than 5 g per day) who underwent renal biopsy. For all biopsy specimens, standard analysis using immunofluorescence microscopy was performed. Acetone-fixed frozen sections were rehydrated in phosphate-buffered saline, blocked with fetal bovine serum containing 2% of bovine serum albumin, and then were incubated with a mouse monoclonal antibody against ChemR23 (1:15; IgG2b; clone 4C7)[10] or rabbit anti-ChemR23 polyclonal antibody (1:50; IgG; Abcam, Cambridge, UK), mouse anti-chemerin monoclonal antibody (1:30; IgG1; clone 14G10), rabbit anti-CD123 polyclonal antibody (1:100; IgG; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-BDCA-2 monoclonal antibody (1:10; IgG1), mouse anti-BDCA-1 monoclonal antibody (1:10; IgG2a; all Miltenyi Biotec, Bergisch Gladbach, Germany), rabbit anti-podoplanin polyclonal antibody (1:100; IgG; Novus Biologicals, Littleton, CO), a specific marker of lymphatic endothelium. As secondary antibodies we used Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 555 goat anti-rabbit IgG (Molecular Probes, Eugene, OR). The sections were counterstained with TO-PRO-3 (Molecular Probes), coverslipped using Gel Mount mounting medium (Biomeda, Foster City, CA), and sealed with clear nail varnish. Negative controls were performed by using irrelevant primary monoclonal antibodies as well as in double stains secondary antibody specificity was confirmed using unmatched primary and secondary antibodies. Specific fluorescence was acquired by confocal laser scanning microscopy using the Leica TCS SP2 (Leica, Wetzlar, Germany).

For the immunohistochemistry protocol, we used the same anti-chemerin and anti-ChemR23 antibodies. The slides were first deparaffinized in xylene, hydrated in graded ethanol, boiled in Tris-EDTA buffer (pH 9) in the microwave for retrieving antigens and blocked with Protein Block (Dako, Carpinteria, CA). The binding of the secondary antibodies against mouse immunoglobulins was visualized with the EnVision G|2 System/AP (Dako). Visualization of alkaline phosphatase was performed by incubation in Permanent Red Chromogen supplemented with Levamisole (Dako) for blocking endogenous alkaline phosphatase, giving a red precipitate. The sections were counterstained with hematoxylin and mounted in Glycergel (Dako). Negative controls were obtained using irrelevant antibodies.

Cell culture

Human renal proximal tubular epithelial cells obtained from Lonza (Valais, Switzerland) were maintained in REGM medium (Lonza) according to the manufacturer's instructions. The cells were used at passages 3 to 6 and grown on 6-well plates (Corning Life Sciences, Acton, MA). To induce quiescence, we incubated proximal tubular epithelial cells in fetal bovine serum-free media for 24 h. Then, proximal tubular epithelial cells were stimulated with cytokines, such as TNF-α, IFN-γ, IL-1β, and TGF-β (Sigma-Aldrich, St Louis, MO), and with an anti-inflammatory drug, dexamethasone sodium phosphate (Hospira, Napoli, Italy). Proximal tubular epithelial cell culture medium was changed every two days. The medium was collected and analyzed at different time points.

Isolation and culture of renal endothelial cells

To isolate renal endothelial cells, we used the normal part of renal cortex obtained from surgically removed kidneys. After dissection and passage through a graded series of meshes,[34] we used anti-CD146 immunomagnetic microbeads (Miltenyi Biotec), according to the manufacturer's instructions. The phenotype of endothelial cells was assessed by flow cytometric analysis using the following antibodies: anti-human CD146 (Immunotech Beckman Coulter, Marseille, France) and CD105 (R&D Systems, Minneapolis, MN) PE-monoclonal antibody. Nonspecific sites were blocked with the FcR blocking reagent. Data were collected and analyzed using System II software (Beckman Coulter, Fullerton, CA). The isolated renal endothelial cells were cultured in EGM medium (Lonza).

Semiquantitative and quantitative RT-PCR

Total RNA was extracted from the cells using QIA shredder and RNeasy Mini Kit (QIAGEN, Valencia, CA) and 500 ng of RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

Chemerin gene and the endogenous control gene β-actin were included in the same PCR reaction for semiquantitative RT-PCR. All PCR reactions were verified to be in the linear range by testing with different cycle numbers.

Quantitative real-time RT-PCR was performed on an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) using the Hs_RARRES2_1_SG QuantiTect Primer Assay (QIAGEN) in combination with SYBR Green dye. The relative amounts of chemerin mRNA were normalized to β-actin mRNA as housekeeping gene.

ELISA

Chemerin levels in proximal tubular epithelial cell supernatant were measured using the DuoSet ELISA Development kit (R&D Systems), which does not discriminate between pro-chemerin and chemerin. The antibody reagents were titrated and used as follows: mouse anti-chemerin capture antibody used to coat plate at 4 μg/ml in phosphate-buffered saline and biotinylated goat anti-chemerin detection antibody used at 0.2 μg/ml. Bound detection antibody was detected using streptavidin-HRP and the absorbance was measured at 450 nm with a correction wavelength of 550 nm using a microplate reader DV 990 (Gio. De Vita & C., Rome, Italy).

Transendothelial migration assay

Human peripheral blood mononuclear cells were isolated from buffy coats by Ficoll gradient (Amersham Biosciences, Buckinghamshire, UK); peripheral blood plasmacytoid DCs were magnetically sorted with blood DC Ag (BDCA)-4 cell isolation kits (Miltenyi Biotec), as

previously described.[30] Transendothelial migration assay was evaluated using 24-well Costar Transwell chambers (5 μm pore size; Corning Life Sciences). HUVEC (passage <5) were cultured to confluent monolayers on transwell inserts pre-coated with gelatin. Monolayers were rinsed with chemotaxis medium (RPMI 1640 containing 10% fetal calf serum) before use. Plasmacytoid DC suspension (100 μl, $1–2 \times 10^6$ cells/ml) was seeded in the upper well and 600 μl of proximal tubular epithelial cell supernatants, or control medium, was added to the lower well. The chamber was incubated at 37 °C in humidified atmosphere in the presence of 5% CO_2 for 4 h. Migrated cells were recovered from the lower well and counted; results were expressed as the percentage of input cells. In some experiments, plasmacytoid DCs were pre-incubated in the presence of anti-ChemR23 (3 μg/ml) antibody at 37 °C for 30 min before the chemotaxis assay.

Statistical analysis

Data were presented as mean ± s.d. of triplicate analysis. Statistical analysis was performed using paired, unpaired Student's *t*-test or analysis of variance, as appropriate. Differences were considered statistically significant when P values were <0.05. Data were analyzed using GraphPad Prism (GraphPad software, San Diego, CA).

DISCLOSURE

The authors have no financial disclosure to make relevant to the subject matter of this study.

This study was partially supported by MIUR (Ministero dell'Istruzione Università e Ricerca) and Istituto Superiore di Sanità (ISS); GDP was supported by a Ph.D. fellowship from the Doctorate in Biotechnologies applied to organ and tissue transplantation, University of Bari.

ACKNOWLEDGMENTS

We thank Vincenzo Gesualdo, Claudia Curci and Margherita Gigante for thier valuable technical assistance.

REFERENCES

1. Steinman RM, Banchereau J. Taking dendritic cells into medicine. *Nature* 2007; **449**: 419–426.
2. Shortman K, Naik SH. Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* 2007; **7**: 19–30.
3. Sozzani S. Dendritic cell trafficking: more than just chemokines. *Cytokine Growth Factor Rev* 2005; **16**: 581–592.
4. Blanco P, Palucka AK, Gill M *et al.* Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. *Science* 2001; **294**: 1540–1543.
5. Pascual V, Farkas L, Banchereau J. Systemic lupus erythematosus: all roads lead to type I interferons. *Curr Opin Immunol* 2006; **18**: 676–682.
6. D'Cruz DP, Khamashta MA, Hughes GR. Systemic lupus erythematosus. *Lancet* 2007; **369**: 587–596.
7. Sozzani S, Bosisio D, Scarsi M *et al.* Type I interferons in systemic autoimmunity. *Autoimmunity* 2010; **43**: 196–203.
8. Bihl GR, Petri M, Fine DM. Kidney biopsy in lupus nephritis: look before you leap. *Nephrol Dial Transplant* 2006; **21**: 1749–1752.
9. Fiore N, Castellano G, Blasi A *et al.* Immature myeloid and plasmacytoid dendritic cells infiltrate renal tubulointerstitium in patients with lupus nephritis. *Mol Immunol* 2008; **45**: 259–265.
10. Vermi W, Riboldi E, Wittamer V *et al.* Role of ChemR23 in directing the migration of myeloid and plasmacytoid dendritic cells to lymphoid organs and inflamed skin. *J Exp Med* 2005; **201**: 509–515.
11. Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. *Nat Rev Immunol* 2002; **2**: 151–161.
12. Dzionek A, Fuchs A, Schmidt P *et al.* BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J Immunol* 2000; **165**: 6037–6046.
13. Adams AE, bu-Amer Y, Chappel J *et al.* 1,25 Dihydroxyvitamin D3 and dexamethasone induce the cyclooxygenase 1 gene in osteoclast-supporting stromal cells. *J Cell Biochem* 1999; **74**: 587–595.
14. Albanesi C, Scarponi C, Pallotta S *et al.* Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment. *J Exp Med* 2009; **206**: 249–258.
15. Segerer S, Heller F, Lindenmeyer MT *et al.* Compartment specific expression of dendritic cell markers in human glomerulonephritis. *Kidney Int* 2008; **74**: 37–46.
16. van KC, Daha MR. Cytokine cross-talk between tubular epithelial cells and interstitial immunocompetent cells. *Curr Opin Nephrol Hypertens* 2001; **10**: 55–59.
17. Perez de LG, Maier H, Nieto E *et al.* Chemokine expression precedes inflammatory cell infiltration and chemokine receptor and cytokine expression during the initiation of murine lupus nephritis. *J Am Soc Nephrol* 2001; **12**: 1369–1382.
18. Banchereau J, Pascual V, Palucka AK. Autoimmunity through cytokine-induced dendritic cell activation. *Immunity* 2004; **20**: 539–550.
19. Marshak-Rothstein A. Toll-like receptors in systemic autoimmune disease. *Nat Rev Immunol* 2006; **6**: 823–835.
20. Benigni A, Caroli C, Longaretti L *et al.* Involvement of renal tubular Toll-like receptor 9 in the development of tubulointerstitial injury in systemic lupus. *Arthritis Rheum* 2007; **56**: 1569–1578.
21. Kerjaschki D, Regele HM, Moosberger I *et al.* Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. *J Am Soc Nephrol* 2004; **15**: 603–612.
22. Fairhurst AM, Mathian A, Connolly JE *et al.* Systemic IFN-alpha drives kidney nephritis in B6.Sle123 mice. *Eur J Immunol* 2008; **38**: 1948–1960.
23. Yuan R, Fan S, Achary M *et al.* Altered gene expression pattern in cultured human breast cancer cells treated with hepatocyte growth factor/scatter factor in the setting of DNA damage. *Cancer Res* 2001; **61**: 8022–8031.
24. Kralisch S, Weise S, Sommer G *et al.* Interleukin-1beta induces the novel adipokine chemerin in adipocytes *in vitro*. *Regul Pept* 2009; **154**: 102–106.
25. Aringer M, Smolen JS. Cytokine expression in lupus kidneys. *Lupus* 2005; **14**: 13–18.
26. Malide D, Russo P, Bendayan M. Presence of tumor necrosis factor alpha and interleukin-6 in renal mesangial cells of lupus nephritis patients. *Hum Pathol* 1995; **26**: 558–564.
27. Studnicka-Benke A, Steiner G, Petera P *et al.* Tumour necrosis factor alpha and its soluble receptors parallel clinical disease and autoimmune activity in systemic lupus erythematosus. *Br J Rheumatol* 1996; **35**: 1067–1074.
28. Masutani K, Akahoshi M, Tsuruya K *et al.* Predominance of Th1 immune response in diffuse proliferative lupus nephritis. *Arthritis Rheum* 2001; **44**: 2097–2106.
29. Uhm WS, Na K, Song GW *et al.* Cytokine balance in kidney tissue from lupus nephritis patients. *Rheumatology (Oxford)* 2003; **42**: 935–938.
30. Wittamer V, Franssen JD, Vulcano M *et al.* Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. *J Exp Med* 2003; **198**: 977–985.
31. Wittamer V, Gregoire F, Robberecht P *et al.* The C-terminal nonapeptide of mature chemerin activates the chemerin receptor with low nanomolar potency. *J Biol Chem* 2004; **279**: 9956–9962.
32. Kanalas JJ, Hopfer U. Effect of TGF-beta 1 and TNF-alpha on the plasminogen system of rat proximal tubular epithelial cells. *J Am Soc Nephrol* 1997; **8**: 184–192.
33. Zabel BA, Allen SJ, Kulig P *et al.* Chemerin activation by serine proteases of the coagulation, fibrinolytic, and inflammatory cascades. *J Biol Chem* 2005; **280**: 34661–34666.
34. Sallustio F, De Benedictis L, Castellano G *et al.* TLR2 plays a role in the activation of human resident renal stem/progenitor cells. *FASEB J* 2010; **24**: 514–525.