

Human Renal Normal, Tumoral, and Cancer Stem Cells Express Membrane-Bound Interleukin-15 Isoforms Displaying Different Functions¹

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Abstract

Intrarenal interleukin-15 (IL-15) participates to renal pathophysiology, but the role of its different membrane-bound isoforms remains to be elucidated. In this study, we reassess the biology of membrane-bound IL-15 (mb-IL-15) isoforms by comparing primary cultures of human renal proximal tubular epithelial cells (RPTEC) to peritumoral (ptumTEC), tumoral (RCC), and cancer stem cells (CSC/CD105⁺). RPTEC express a 14 to 16 kDa mb-IL-15, whose existence has been assumed but never formally demonstrated and likely represents the isoform anchored at the cell membrane through the IL-15 receptor α (IL-15R α) chain, because it is sensitive to acidic treatment and is not competent to deliver a reverse signal. By contrast, ptumTEC, RCC, and CSC express a novel N-hyperglycosylated, short-lived transmembrane mb-IL-15 (tmb-IL-15) isoform around 27 kDa, resistant to acidic shock, delivering a reverse signal in response to its soluble receptor (sIL-15R α). This reverse signal triggers the down-regulation of the tumor suppressor gene *E-cadherin* in ptumTEC and RCC but not in CSC/CD105⁺, where it promotes survival. Indeed, through the AKT pathway, tmb-IL-15 protects CSC/CD105⁺ from non-programmed cell death induced by serum starvation. Finally, both mb-IL-15 and tmb-IL-15 are sensitive to metalloproteases, and the cleaved tmb-IL-15 (25 kDa) displays a powerful anti-apoptotic effect on human hematopoietic cells. Overall, our data indicate that both mb-IL-15 and tmb-IL-15 isoforms play a complex role in renal pathophysiology downregulating E-cadherin and favoring cell survival. Moreover, “apparently normal” ptumTEC cells, sharing different properties with RCC, could contribute to organize an enlarged peritumoral “preneoplastic” environment committed to favor tumor progression.

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Introduction

Interleukin-15 (IL-15) is a pleiotropic cytokine that links *in vivo* innate and adaptative immune responses and is characterized by the complexity of its biology [1]. Indeed, IL-15 receptor (IL-15R) consists of a private α -chain and shared IL-2 receptor β - and γ -chains that form various functional receptors with different affinities and signaling capabilities [2–7]. Moreover, several functional forms of IL-15 exist: 1) the soluble monomeric form that is secreted at very low concentrations by accessory cells and activates cells expressing the high affinity receptor [8], 2) the soluble complex IL-15/IL-15R α (sIL-15/IL-15R α) that displays greater half-life and bioavailability in comparison with monomeric IL-15 (>20 hours *vs* <40 minutes, respectively) and essentially interacts with cells expressing low affinity IL-15R [4–7], 3) the membrane-bound form (mb-IL-15), anchored at the cell membrane through the IL-15R α chain [5,9], and 4) the transmembrane form (tmb-IL-15) that is anchored through an IL-15R-independent mechanism [1,10–12]. The sIL-15/IL-15R α [4–7] and the mb-IL-15 [8,13,14] represent the dominant physiological forms of the cytokine. Although the mb-IL-15 and tmb-IL-15 forms could deliver signal *in trans* to surrounding cells expressing low affinity IL-15R [1,5,10–12], only the tmb-IL-15 delivers a reverse signaling to presenting cells on stimulation with a recombinant soluble IL-15R α chain (sIL-15R α) or anti-IL-15 antibodies [1,10–12]. Concerning non-lymphoid cells, human epithelial cells from different tissues produce IL-15, involved not only in potential interactions with immune cells but also in the biologic characteristics of epithelial cells [15–23]. For instance, experiments in IL-15(-/-) and IL-15R α (-/-) mice show that intrarenal IL-15, through autocrine-paracrine and/or juxtacrine loops, behaves as an epithelial survival factor through the IL-15R α chain [24,25]. In addition, recent data highlight new functions of IL-15. Indeed, the cytokine elicits, through the IL-15R γ /JAK3 pathway, a signaling cascade that preserves epithelial phenotype and functions in normal human renal proximal tubular epithelial cells (RPTEC) [26]. Moreover, IL-15 triggers epithelial differentiation of renal cancer stem cells (CSC/CD105⁺) [27]. By contrast, human renal cancer cells (RCC) do not secrete the cytokine and neither express—both *in vivo* and *in vitro*—the IL-15R γ chain (CD132) and JAK3 [28]. In these cells, IL-15 drives the epithelial-mesenchymal transition (EMT) [26,28]. In addition RCC express a tmb-IL-15 isoform, whose stimulation with the sIL-15R α chain triggers a reverse signal that induces E-cadherin down-regulation, triggering the EMT process and thus promoting tumor progression [11].

In the present study, we reassess the biology of mb-IL-15 isoforms by comparing primary cultures of human normal RPTEC, apparently normal peritumoral cells (ptumTEC), and tumor-derived cells (RCC and CSC/CD105⁺). Overall, our data indicate that mb-IL-15 and tmb-IL-15 isoforms play a complex role in renal cancer homeostasis, acting on cell phenotype, tumor microenvironment, and cell survival.

Materials and Methods

Antibodies, Cytokines, and Reagents

Antibodies against IL-15 (L-20), TWIST (sc-15393), and β -actin (sc-47778) were from Santa Cruz Biotechnology (Paso Robles, CA). Antibodies against phospho Extracellular signal-Related Kinase pERK1/2 (9101S), ERK1/2 (4695), pAKT (2967), and SNAIL (3895S) were from Cell Signaling Technology (Danvers, MA). Antibody against SLUG (MAB4371) was from Millipore (Billerica, MA). The phycoerythrin (PE)-conjugated antibodies against IL-15 (IC2471P) and E-cadherin (FAB18381P), the recombinant human

IL-15R α /Fc chimera soluble (sIL-15R α) chain and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) were from R&D Systems Europe Ltd (Abingdon, United Kingdom). The PE-conjugated anti-APO2.7 monoclonal antibody (mAb) was from Beckman Coulter (Roissy, France). 1,10-Phenanthroline (Phen) and crystal violet were from Sigma-Aldrich (St Louis, MO). HRP-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA), and the recombinant human non-glycosylated IL-15 (rhIL-15) was from ImmunoTools (Friesoythe, Germany). The neutralizing anti-IL-15 M111 antibody was provided by Amgen (Thousand Oaks, CA). The RAL 555 kit was from RAL Diagnostics (Martillac, France).

Primary Cells and Cell Lines

Primary normal RPTEC derived from a non-cancerous kidney were purchased from Lonza Technologies (Verviers, Belgium) and expanded *in vitro* following strictly manufacturer's instructions. The REGM medium culture of RPTEC was changed every day to maintain epithelial characteristics. In the absence of culture medium renewal, RPTEC lose E-cadherin expression after 5 days, likely for the exhaustion of the corticosteroids present in the REGM medium that are powerful inducers of E-cadherin [26]. Primary renal cultures were obtained by enzymatic digestion of renal biopsies as described previously [29]. Primary tumoral (RCC) and peritumoral (ptumTEC) cells were derived from the same patient. Peritumoral cells were isolated from "apparently normal" renal fragments surrounding the neoplastic lesion and display *in vitro* normal morphology, contact inhibition of proliferation, and limited life span, whereas primary RCC derived from tumor biopsies generate permanent cell lines that at confluence continue to proliferate. PtumTEC also lack both *in vivo* and *in vitro* the expression of CD132 that differentiates RCC from normal tubular ones and exhibit IL-15R assembly, response to soluble IL-15, and immunomodulatory properties, similar to those detected in RCC [26,28].

RCC primary cultures, ptumTEC, established RCC cell lines ACHN, HIEG, RCC5, and RCC7, and U937 (human leukemic monocyte lymphoma cell line) were grown in RPMI 1640 supplemented with 10% fetal calf serum, 1% minimum essential medium sodium pyruvate, and 1% penicillin/streptomycin (all products from Life Technologies, Carlsbad, CA). The erythroleukemia cell line TF-1 β was maintained in complete RPMI 1640 supplemented with 5 ng/ml GM-CSF and 250 μ g/ml geneticin G418. Renal cancer stem cells CSC/CD105⁺ were identified and isolated from human renal carcinoma as previously described [30]. Monocytes were purified from peripheral blood mononuclear cells by using the CD14-Ademabeads (Ademtech, Pessac, France).

Stimulations and Treatments

Acidic shock was performed by incubating cells in ice-cold 0.1 M sodium acetate (pH 3.5) for 15 minutes at 4°C. Incubation in phosphate-buffered saline was used as a control. Metalloprotease sensitivity was evaluated by incubating epithelial cells with ortho-pervanadate (PVN, 200 μ M) or with phorbol ester phorbol 12-myristate 13-acetate (PMA, 200 ng/ml) in fetal calf serum-free medium at 37°C for 1 and 3 hours, respectively. Competition experiments were performed by incubating the cells with a broad-spectrum metalloprotease inhibitor Phen (2 mM) [21]. For biochemical analysis, culture supernatants (SNT) were collected after treatments, filtrated (0.22 μ m), and concentrated 20-fold using Amicon Ultra-4, Ultracel-10K (Millipore). Soluble IL-15 in the SNT was analyzed by Western blot, and bioactivity assays were performed on TF-1 β cells.

Flow Cytometry

For all assays described below, fluorescence data for 10,000 to 50,000 events were acquired on a FACSCalibur flow cytometer (BD Biosciences, Oxford, United Kingdom), and data were analyzed using version vX.0.7 of FLOWJO software (Treestar, Ashland, OR). Each experiment was repeated at least three times.

Immunoblot Analysis

All Western blot analyses were performed as previously described [11]. For *N*-glycanase treatment, phosphate-buffered saline-washed RCC were lysed for 15 minutes at 4°C in 1% Triton X-100, 50 mM sodium phosphate buffer (pH 7.8), 150 mM NaCl, and protease inhibitor cocktail (complete tablets; Roche). After 30-minute centrifugation (14,000 rpm, 4°C), the supernatants were incubated in the presence or absence of 6 µl/100 µl (6 U) of *N*-glycanase (Roche, Indianapolis, IN) at 37°C for 18 hours. Proteins were separated on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and IL-15-immunoblotted as described above.

In Vitro Scratch Assay

In vitro scratch assay was performed as previously described [31]. Briefly, cells were pretreated or not (control condition) with sIL-15Rα (100 ng/ml) for 5 days and seeded in a six-well plate (400,000 cells per well) and allowed to reach confluence (24 hours). Cells were then scratched and shifted to a serum-free medium in the presence or absence of sIL-15Rα (100 ng/ml). Analysis was performed 24 hours later. Cells were fixed and stained using the RAL 555 kit, and images were acquired using fluorescence microscopy (Leica, Wetzlar, Germany).

Transwell Invasion Assay

Invasion assay was performed in Boyden chambers (polycarbonate filters of 8 mm pore size, precoated with Matrigel matrix). Cells (3×10^5) in 300 µl of medium (containing 0.1% FBS) with or without 100 ng/ml sIL-15Rα chain were seeded in the upper chamber. Six hundred microliters of medium with 10% FBS were added to the lower chamber and served as a chemotactic agent. After 4-day incubation, Matrigel was mechanically removed from the filter with a cotton swab. The cells adhering to the underside of the filter were fixed and stained with crystal violet and analyzed under an upright microscope (five fields per chamber).

Bioactivity Assays

GM-CSF-starved TF-1β cells were cultured at a concentration of 5×10^3 cells per well in microtiter plates in 50 µl of culture medium containing increasing concentrations of rhIL-15 or supernatant of PMA-treated RCC (10% final). To evaluate IL-15 activity, SNT were preincubated for 30 minutes with 10 µg/ml neutralizing anti-IL-15 mAb M111. Early-stage apoptosis induction was determined by measuring the expression of the mitochondrial membrane protein APO2.7 (7A6 antigen) using a PE-labeled antibody as well as the respective isotype control by flow cytometry.

Serum Starvation and Cell Death Detection

CSC/CD105⁺ were seeded in T25 culture flasks in complete medium. The day after, the medium was replaced with fresh medium without fetal calf serum for starvation. Controls included untreated cells or cells treated with DMSO (1/1000); sister cultures were treated with sIL-15Rα (100 ng/ml) or with sIL-15Rα plus 1 µM MK-2206 (1 mM stock solution in DMSO) or with 1 µM MK-2206 alone (not shown). All treatments were performed in

duplicate, and the experiment was repeated three times. After 48 hours of culture, cells were collected, and programmed (apoptosis) and non-programmed (necrosis) cell death were determined using Annexin V-FITC Apoptosis Detection Kit (e-Bioscience, San Diego, CA) according to the manufacturer's protocol. Analysis was performed by flow cytometry using Cell Quest software (FACScan; Becton Dickinson, Franklin Lakes, NJ).

Results

Renal Normal and Tumor-Derived Cells Express Different mb-IL-15 Isoforms

To characterize the type of IL-15 isoforms expressed in the different components of renal clear cell carcinoma, we took advantage of normal and tumoral epithelial cells, cancer stem cells from renal origin, and also peritumoral renal cells (ptumTEC). Indeed, the road to cancer begins long before the growth of a clinically detectable lesion, with the replacement of the normal cells by histologically non-dysplastic but pro-tumorigenic cells (field cancerization) that subsequently coexist and share with the nearby cancer cells several epigenetic alterations [32]. In this context, we integrated herein the analysis of renal ptumTEC that for their properties likely represent cells belonging to a field cancerization that up to now had not yet been identified in renal clear cell carcinoma.

Analyzing by flow cytometry the expression of IL-15 in these different cell subtypes, we show that normal, peritumoral, and tumor-derived cells (RCC and CSC/CD105⁺) constitutively express an mb-IL-15 resistant to acidic shock, with the exception of normal RPTEC, where IL-15 is shed from the cell surface by a pH 3.5 treatment (Figure 1A). These data indicate that RPTEC likely express a mb-IL-15 anchored at the cell membrane through the IL-15Rα chain [5,9], while ptumTEC, RCC, and CSC express a transmembrane IL-15 (tmb-IL-15) [1,10–12]. Western blot analysis of the recombinant non-glycosylated cytokine, used as control, shows the 12.5 kDa canonical monomeric band and a dimeric band around 25 kDa (Figure 1B) likely formed by self-aggregation of the cytokine [33]. Immunoblot analysis of cell lysates with the anti-IL-15 L20 antibody shows that normal RPTEC express two IL-15 isoforms with apparent molecular mass of 14 and 16 kDa, which likely represent two different N-glycosylated patterns of the cytokine [34]. These isoforms were also found in LipoPolySaccharide (LPS)-treated human monocytes and in the Interferon (IFN)-γ-treated macrophage cell line U937. The abovementioned IL-15 isoforms are faintly expressed in peritumoral (RCC5-ptumTEC and Pil-ptumTEC), tumoral (HIEG, RCC7, ACHN, RCC/CD105⁻), and CSC/CD105⁺ that in addition contain, as main form, a band close to 27 kDa, previously observed only in RCC [11].

Mb-IL-15 Is N-glycosylated in RCC

In eukaryotic cells, N-linked glycosylation is necessary for IL-15 complete processing and secretion [34]. Although IL-15 displays N-glycosylation on two of three potential glycosylation sites (Asn-X-Ser/Thr) in the mature protein, there are no available data on the role of N-linked glycosylation in the expression of the tmb-IL-15 isoforms. Overnight exposure of RCC to tunicamycin (1 and 10 µg/ml), an inhibitor of N-linked glycosylation, causes dose-dependent inhibition of the tmb-IL-15 expression on these cells (Figure 2A). Tunicamycin withdrawal (Figure 2B) allows, within 48 hours, the recovery of tmb-IL-15 expression, which is dependent not only on N-linked glycosylation but also on protein neosynthesis because cycloheximide inhibited this effect. These data show that

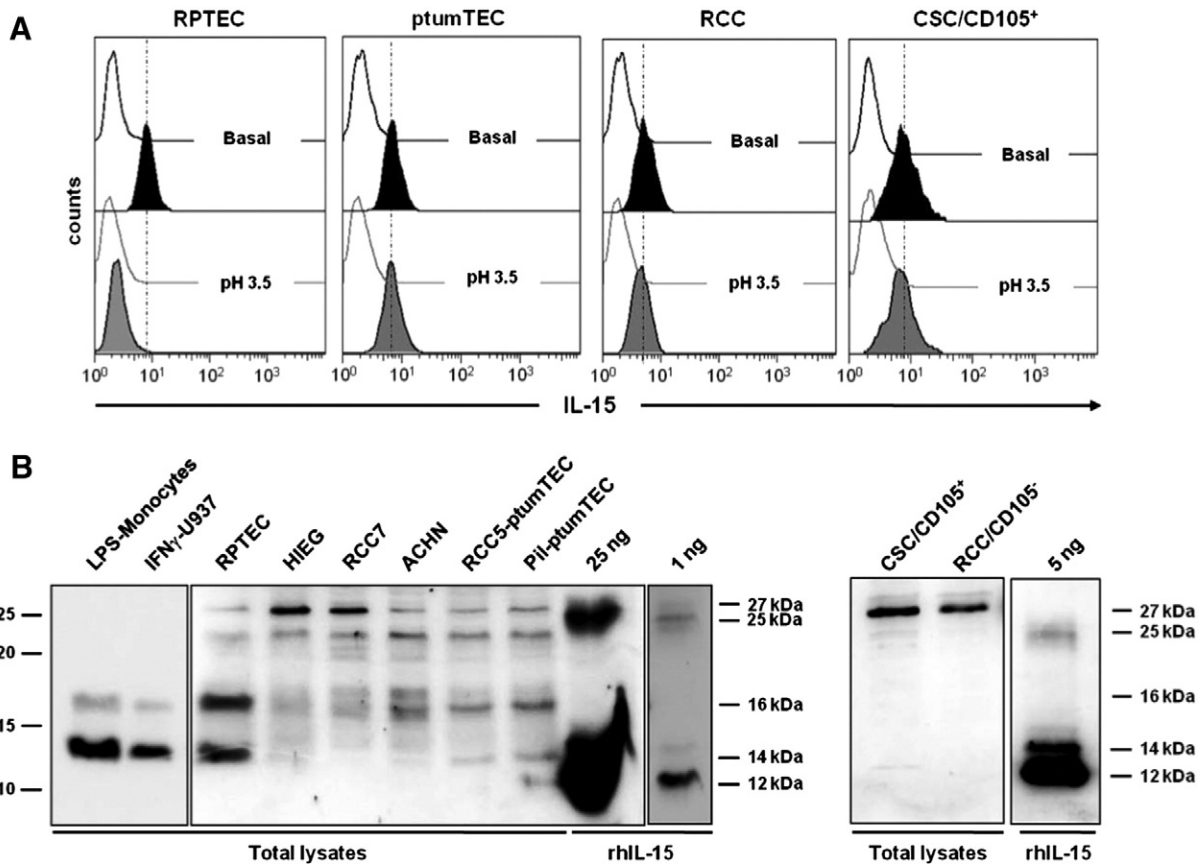


Figure 1. Mb-IL-15 characterization in human renal normal and tumor-derived cells. (A) FACS analysis of membrane IL-15 expression on epithelial cells after acidic shock. Primary normal (RPTEC), peritumoral (ptumTEC), tumoral (RCC) epithelial cells and cancer stem cells (CSC/CD105⁺) were treated or not with acidic buffer (pH 3.5) before staining with anti-IL-15 monoclonal antibody. These data are representative of three independent experiments. (B) Western blot analysis performed with anti-IL-15 L20 antibody detects different IL-15 isoforms in the total lysates from primary normal (RPTEC), peritumoral (RCC5-ptumTEC and Pil-ptumTEC), tumoral RCC cell lines (RCC7, HIEG, ACHN, and RCC/CD105⁺) and CSC/CD105⁺.

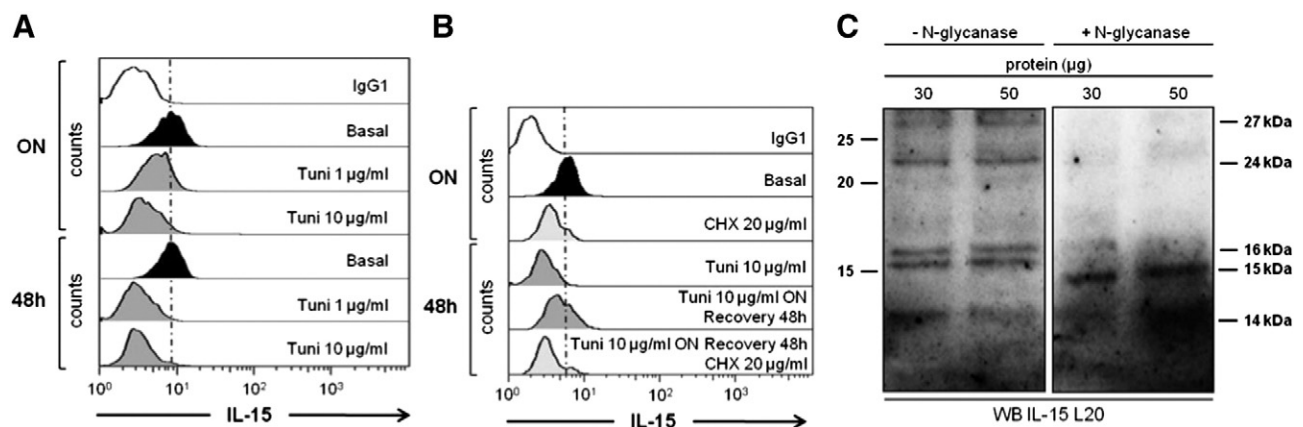


Figure 2. Characterization of mb-IL-15 isoforms in RCC. (A) FACS analysis of membrane IL-15 expression on RCC. Cells were treated overnight with 1 or 10 μ g/ml tunicamycin (Tuni, an inhibitor of N-linked glycosylation) or (B) with 20 μ g/ml cycloheximide (CHX, a protein synthesis inhibitor). In some experiments, overnight-treated tunicamycin cells were washed three times and cultured for an additional 48 hours in fresh medium in the absence or presence of CHX for the last 24 hours. Mb-IL-15 expression in untreated (black) or treated cells (gray) was analyzed using an anti-IL-15 monoclonal antibody. White histograms refer to isotype-matched control. A representative experiment out of three is shown. (C) Immunoblots on total RCC cell lysates of *N*-glycanase digested and mock digested samples. Varied amounts of extracts were applied to a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Release of oligosaccharides after *N*-glycanase treatment (right panel) was monitored by immunodetection of altered protein band migration.

in RCC mb-IL-15 expression is subjected to a rapid turnover that requires optimal N-linked glycosylation and protein synthesis. Western blot analysis in the RCC cell lysate (Figure 2C) shows that *N*-glycanase treatment leads to the loss of the isoforms ranging between 16 and 27 kDa and the acquisition of an under-glycosylated isoform around 13 to 15 kDa. These data strongly suggest that 27 kDa represents a hyper-glycosylated isoform of the cytokine that could be due either to the engagement of the third potential glycosylation site [34] or to an excess of N-glycosylation of the two described sites.

Released mb-IL-15 from Renal Epithelial Cells Is Metalloprotease-Dependent and Biologically Active

To investigate whether mb-IL-15 isoforms are metalloprotease sensitive, primary RPTEC, peritumoral (ptumTEC), tumoral (RCC) epithelial cells, and CSC/CD105⁺ cells were incubated for 1 hour in the presence of PVN or PMA for 3 hours. Both treatments cause the loss of mb-IL-15 expression in all tested cultures, while exposure to Phen (a wide spectrum metalloprotease inhibitor) counteracts the effects of PVN and PMA indicating that the cleavage of the mb-IL-15 is mediated by metalloproteases (Figure 3A). To analyze the released

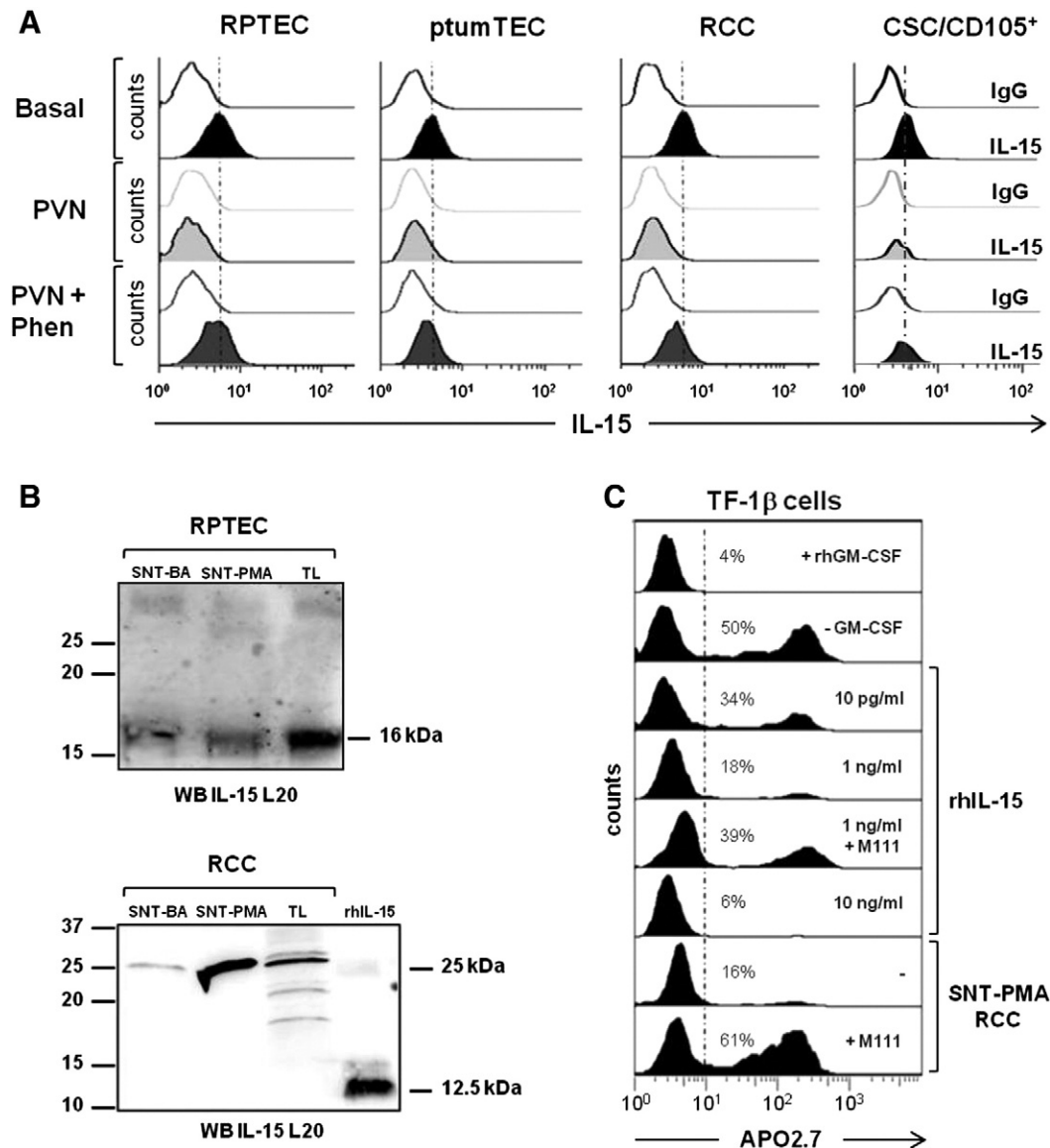


Figure 3. Metalloprotease sensitivity and biologic activity of the released mb-IL-15 from renal epithelial cells. (A) Flow cytometry analysis of mb-IL-15 in RPTEC, ptumTEC, RCC, and CSC/CD105⁺, after 1-hour treatment with ortho-PVN (200 μ M) in the presence or absence of metalloprotease inhibitor Phen (2 mM). These data are representative of three independent experiments. (B) Western blot analysis with anti-IL-15 L20 antibody in total cell lysates (TL, 25 μ g) and 50-fold concentrated supernatant (SNT, 25 μ l) of RPTEC and RCC treated or not with 200 ng/ml PMA for 3 hours. rhIL-15 (5 ng) was used as specific control. (C) Released mb-IL-15 protects TF-1 β cells from apoptosis through IL-15R $\alpha\beta\gamma$ receptors. TF-1 β cells were GM-CSF deprived and cultured with rhGM-CSF (positive control), increasing concentrations of rhIL-15, or supernatant of 3-hour PMA-treated RCC (SNT-PMA-RCC) in the absence or presence of 10 μ g/ml neutralizing anti-IL-15 monoclonal antibody M111. To determine the induction of apoptosis in an early stage, the expression of the mitochondrial membrane protein APO2.7 was measured using a PE-labeled antibody as well as the respective isotype control (mouse IgG1-PE) by flow cytometry. The percentage of APO2.7 positive cells is indicated for each cell treatment. Data are representative of three identical experiments.

mb-IL-15 isoforms, we compared by Western immunoblots the concentrated supernatants from RPTEC and RCC before and after exposure to PMA (Figure 3B). The 16-kDa IL-15 isoform, observed in the RPTEC cell lysate, was barely detectable in the supernatant from untreated cells (SNT-BA) and significantly increased in the supernatant collected after PMA treatment (SNT-PMA). By contrast, in supernatants of RCC, we only detected a 25-kDa band that was strongly enhanced after exposure to PMA. To determine if the hyper-glycosylated IL-15 isoforms released by PMA-treated RCC exerts a biologic activity, the erythroleukemic cell line TF-1 β , whose survival and proliferation are GM-CSF- or IL-15-dependent, was used [35]. The anti-apoptotic activity of IL-15 present in SNT-PMA-RCC on TF-1 β was evaluated by analyzing the surface expression of the apoptotic marker APO2.7 (Figure 3C), whose expression is strongly induced after 72-hour GM-CSF starvation (50%). While monomeric rhIL-15 protects TF-1 β cells from apoptosis in a dose-dependent manner (10 pg/ml, 34% to 10 ng/ml, 6% of apoptotic cells), treatment of TF-1 β cells with SNT-PMA-RCC, containing cleaved tmb-IL-15, causes a powerful anti-apoptotic effect similar to that obtained using 1 ng/ml rhIL-15. Use of the neutralizing anti-IL-15 mAb M111 markedly inhibits, after 72 hours, the anti-apoptotic effect of rhIL-15 (1 ng/ml, 39%), as well as the SNT-PMA-RCC (61%), while added alone it had no effect (data not shown), demonstrating therefore that the anti-apoptotic effect is promoted by the released IL-15.

Tmb-IL-15 Mediates Reverse Signaling in Renal Tumor-Derived Cells but Not in Normal Cells

Tmb-IL-15 displays a potential to induce bidirectional signaling, whereas mb-IL-15 transpresented by the IL-15R α chain only activates bystander cells. For this reason, we investigated the competence of the mb-IL-15 expressed by the different human renal cells to mediate the outside-to-inside signal transduction in response to soluble specific ligands [11]. Stimulation with 100 ng/ml sIL-15R α triggers the phosphorylation of the Mitogen-Activated Protein (MAP) kinase ERK1/2 in all examined tumor-derived cells but not in RPTEC (Figure 4A). These data clearly state that ptumTEC, RCC, and CSC, but not the RPTEC, express an mb-IL-15 able to mediate a reverse signaling after activation by a soluble ligand. Because RCC treated with sIL-15R α underwent epithelial-mesenchymal transdifferentiation [11], we tested the effects of reverse signaling on the abovementioned cells, analyzing the expression of the epithelial marker E-cadherin. Five days of treatment with the sIL-15R α chain (100 ng/ml) causes the loss of this epithelial marker on ptumTEC and RCC, whereas it had no effect on RPTEC and CSC (Figure 4B), suggesting that in the latter ones tmb-IL-15-dependent reverse signal is not competent to modify E-cadherin expression. This event was associated with an enhanced expression of the transcription factors, SNAIL and SLUG in RCC, confirming that these factors are involved in the sIL-15R α -induced EMT process. As expected, both transcription factors were not detected in RPTEC (Figure 4C). We further checked what could be the functional effect of the sIL-15R α -induced reverse signaling. Using an *in vitro* scratch assay, we show that sIL-15R α treatment allowed a nearly complete wound healing in 24 hours only in RCC. We also addressed the invasive properties of tumor cells treated or not with sIL-15R α chain and show that treated cells highly invaded the Matrigel matrix in comparison to untreated ones. Taken together, these data indicate that the sIL-15R α -mediated reverse signaling favors a migratory/invasive phenotype in tumor cells.

Tmb-IL-15 Mediates Reverse Signaling in CSC/CD105⁺ Inducing Protection from Cell Death

It has been reported that tmb-IL-15-dependent reverse signal controls cancer cell secretion of pro-inflammatory cytokines, cell migration [1,12], or EMT induction [11]. Because in CSC/CD105⁺ tmb-IL-15-dependent reverse signal does not modify E-cadherin expression, we explored other biologic parameters usually controlled by IL-15 such as cell survival. Therefore, we tried to induce cell death subjecting CSC to prolonged serum starvation and to study whether a single stimulation of the tmb-IL-15 with 100 ng/ml sIL-15R α chain could assure CSC survival in these conditions. Flow cytometry analysis shows that serum starvation essentially induces cellular necrosis [propidium iodide⁺ (PI⁺)/Annexin V⁻ cells] and very limited apoptosis (PI⁺/Annexin V⁺ cells) and that sIL-15R α chain rescues about 70% of CSC from non-programmed cell death (Figure 5, A and B). We next investigated what signaling pathway might be associated with CSC cell survival. Protection from non-programmed cell death may be induced through the activation of the AKT pathway [36]. In Figure 5C, Western blot analysis shows that in CSC/CD105⁺, reverse signal triggered by the sIL-15R α chain activates the AKT pathway, while the use of the AKT-specific inhibitor MK-2206 [37] counteracts the protective effect induced by the sIL-15R α chain (Figure 5D).

Discussion

Experiments in IL-15(-/-) and IL-15R α (-/-) mice show that the production of intrarenal IL-15 is involved in renal cell survival and homeostasis, and it has been hypothesized that the dominant intrarenal IL-15 isoform is probably represented by the mb-IL-15 anchored at the cell surface through the IL-15R α [24,25]. Subsequently, several papers have reported the *in vitro* production of IL-15 by both murine and human normal renal epithelial cells, but in no instance the expression of mb-IL-15 has been demonstrated [20,24,25,21]. A major distinguishing feature between normal RPTEC and peritumoral (ptumTEC) and renal cancer-derived ones (RCC and CSC) is represented by the expression of different mb-IL-15 isoforms. Indeed, RPTEC express, similarly to monocytic cells of normal or neoplastic origin, a cell surface 16-kDa IL-15 sensitive to acidic buffer treatment (pH 3.5) and not competent for inducing a reverse signaling in response to sIL-15R α . This isoform therefore represents the classic mb-IL-15 anchored through the IL-15R α chain, competent for transactivation of bystander cells through juxtacrine loops [1,8,9,14,38].

In contrast, ptumTEC and CSC/CD105⁺, like RCC [11], constitutively express a functional 27-kDa transmembrane IL-15 isoform (tmb-IL-15), since it is resistant to acidic buffer treatment and delivers a reverse signal following stimulation with its soluble ligand [1,10–12]. We had previously hypothesized that the 27-kDa tmb-IL-15 could represent an IL-15 homodimer [11]. However, further biochemical characterization shows that the 27-kDa tmb-IL-15 is a hyper-N-glycosylated isoform. Indeed, a homodimeric 27-kDa IL-15 should, after deglycosylation, display a molecular weight of 25 kDa similar to that exhibited by the self-aggregated and unglycosylated recombinant IL-15. By contrast, treatment of RCC cell lysates with N-glycanase causes the loss of different IL-15 N-glycosylated isoforms (16 to 27 kDa) and the generation of under-glycosylated isoforms 13 to 15 kDa, rendering unlike the homodimeric hypothesis. In addition, IL-15 homodimers generated by self-aggregation of recombinant unglycosylated IL-15 do not exhibit biologic activity owing to the self-aggregation process [33],

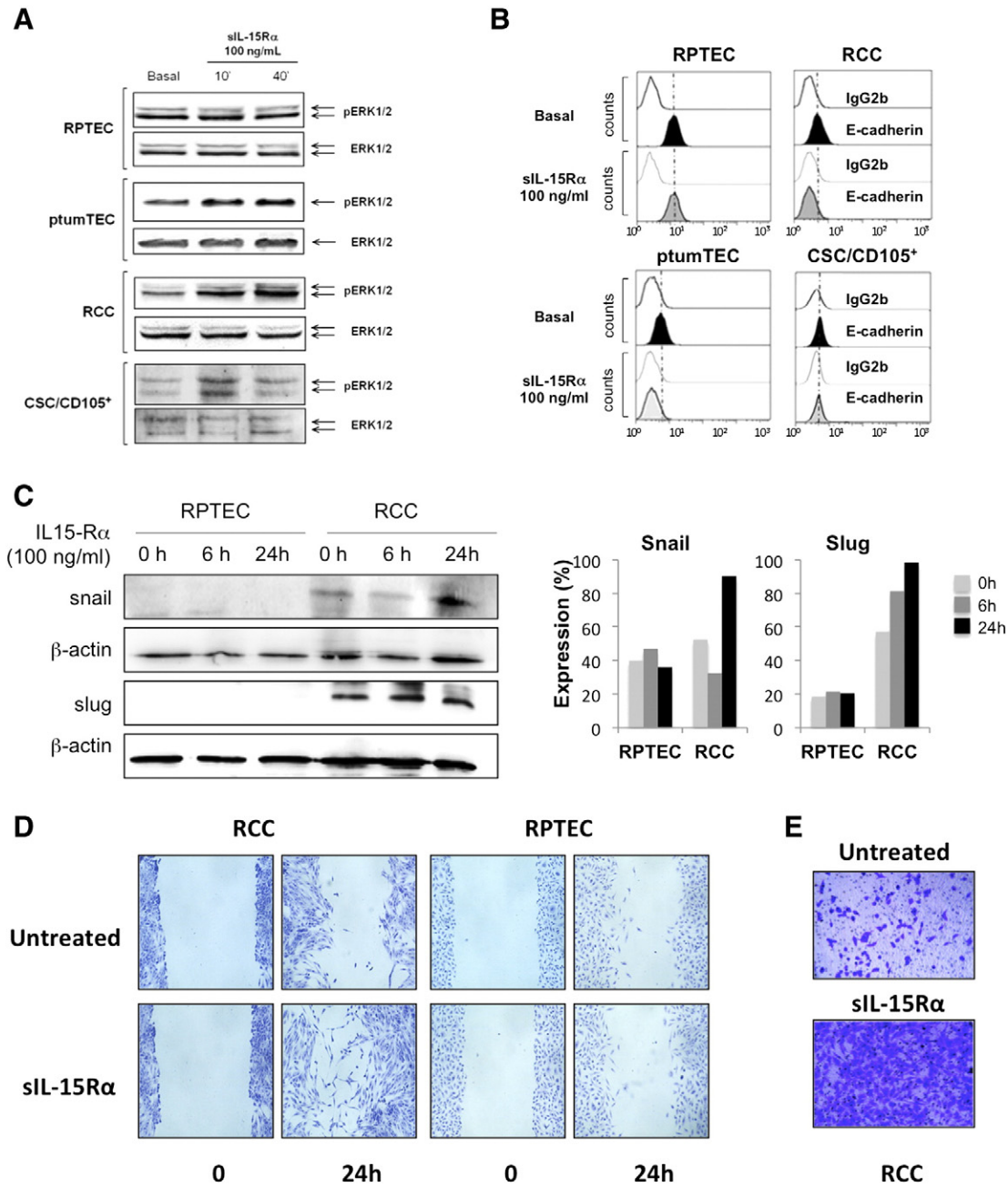


Figure 4. Mb-IL-15 mediates reverse signaling in RCC, ptumTEC, and CSC/CD105⁺ but not in RPTEC. (A) Time course Western blot analysis of MAP kinase ERK1/2 activation in RPTEC, ptumTEC, RCC, and CSC/CD105⁺ stimulated with 100 ng/ml sIL-15R α chain. Membranes were reblotted with an anti-ERK1/2 antibody used as loading control. A representative experiment out of three is shown. (B) FACS analysis of E-cadherin surface expression in RPTEC, ptumTEC, RCC, and CSC/CD105⁺, after 5 days of stimulation with sIL-15R α chain (100 ng/ml). RPTEC were cultured in fresh medium every day to maintain epithelial morphology. In these cells, treatment with sIL-15R α chain was also renewed every day. Gray and black histograms represent E-cadherin expression in treated or untreated cells, respectively. White histograms refer to isotype-matched control. The data are representative of four separate experiments. (C) Time course Western blot analysis of SNAIL and SLUG activation in RPTEC and RCC stimulated with 100 ng/ml sIL-15R α chain. β -Actin was used as loading control. Images were quantified using ImageJ software. (D) *In vitro* scratch assay in RPTEC and RCC after stimulation with sIL-15R α chain (100 ng/ml). Cells were treated with sIL-15R α for 5 days and scratched, and images were acquired 24 hours later. (E) Transwell invasion assay in RCC. After 4 days of sIL-15R α chain (100 ng/ml) treatment, cells adhering to the underside of the filter were fixed and stained with crystal violet and analyzed under an upright microscope (five fields per chamber).

while in RCC and ptumTEC, tmb-IL-15 released in the supernatant following PMA treatment (25 kDa) displays a powerful biologic activity, because it protects hematopoietic cells from apoptosis, thus excluding the homodimer hypothesis. Moreover, the treatment with

tunicamycin and cycloheximide shows that on RCC tmb-IL-15 appears to undergo a rapid turnover that strictly depends on an optimal N-glycosylation and protein neosynthesis. These latter parameters further distinguish the RCC tmb-IL-15 isoform from

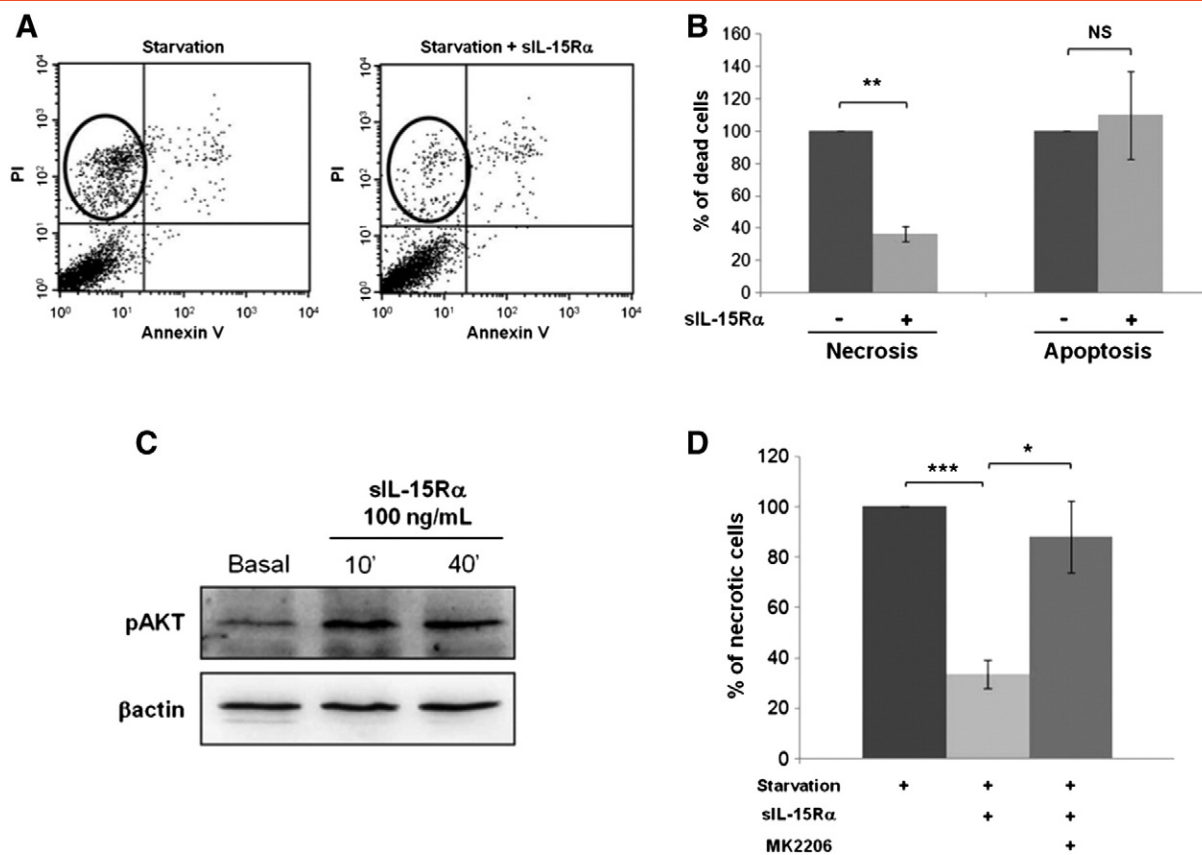


Figure 5. Reverse signaling through transmembrane IL-15 in CSC/CD105⁺. (A) Dot plot analysis and (B) statistical analysis of cell death triggered by serum starvation and protective effect induced by sIL-15Rα. Black circles identify cells undergoing non-programmed cell death (PI⁺/Annexin V⁻ cells). (C) Western blot analysis of AKT activation induced by sIL-15Rα. (D) Statistical analysis of non-programmed cell death triggered by serum starvation, protective effect induced by sIL-15Rα, and inhibition of survival effect by the MK-2206 AKT inhibitor. Starvation-induced cell death was set at 100%. Each figure is representative of at least three independent experiments. * $P < .05$, ** $P < .01$, and *** $P < .005$ compared to control by paired Student's t test.

the IL-15Rα-dependent one that after *trans*-endosomal recycling of the complex is expressed and persists at the cell membrane up to 72 hours [9].

Interestingly, activation of a reverse signal through stimulation of the tmb-IL-15 with sIL-15Rα causes the down-regulation of E-cadherin in RCC and ptumTEC but not in CSC. In the latter ones, serum starvation induces the non-programmed death, which is inhibited by the tmb-IL-15-induced reverse signal through the activation of the survival AKT pathway [36]. The protection from cellular necrosis, through the tmb-IL-15-induced reverse signal, is a novel observation that sheds a new light on the multiple biologic properties of tmb-IL-15 and could represent an additional mechanism functionally defining the CSC niche [39].

The present data and previous ones [26,28] indicate that peritumoral epithelial renal cells express an IL-15 system similar to the one observed in RCC [11,26,28]. Thus, activation of the IL-15 system in ptumTEC, through the loss of E-cadherin, could contribute to organize around the tumor a crown of “pre-neoplastic” cells that, losing their polarization, would become more prone to neoplastic transformation favoring tumor progression [40]. In this context, the depletion of E-cadherin is *per se* sufficient to promote the migratory and invasive potential of embryonic kidney epithelial cells [41,42]. In addition, the loss of E-cadherin was observed in premalignant foci of Von Hippel-Lindau patients, which concomitantly showed increased

CAIX expression, suggesting that E-cadherin loss is an early tumorigenic event [43]. Finally the loss of E-cadherin could drastically modify the repertoire of tumor infiltrating regulatory T lymphocytes whose intratumor homing is strictly dependent on the E-cadherin/CD103 interaction [44,45].

In conclusion, these data show that IL-15 acts not only as sensor of the normal epithelial homeostasis but also plays a major role in the pathophysiology of human renal epithelial cells modifying both epithelial cells and their microenvironment.

References

- [1] Bulfone-Paus S, Bulanova E, Budagian V, and Paus R (2006). The interleukin-15/interleukin-15 receptor system as a model for juxtacrine and reverse signaling. *Bioessays* **28**, 362–377.
- [2] Anderson DM, Kumaki S, Ahdieh M, Bertles J, Tometsko M, Loomis A, Giri J, Copeland NG, Gilbert DJ, and Jenkins NA, et al (1995). Functional characterization of the human interleukin-15 receptor alpha chain and close linkage of *IL15RA* and *IL2RA* genes. *J Biol Chem* **270**, 29862–29869.
- [3] Giri JG, Kumaki S, Ahdieh M, Friend DJ, Loomis A, Shanebeck K, DuBose R, Cosman D, Park LS, and Anderson DM (1995). Identification and cloning of a novel IL-15 binding protein that is structurally related to the alpha chain of the IL-2 receptor. *EMBO J* **14**, 3654–3663.
- [4] Bergamaschi C, Bear J, Rosati M, Beach RK, Alicea C, Sowder R, Chertova E, Rosenberg SA, Felber BK, and Pavlakis GN (2012). Circulating IL-15 exists as heterodimeric complex with soluble IL-15Rα in human and mouse serum. *Blood* **120**, e1–e8.

- [5] Giron-Michel J, Giuliani M, Fogli M, Brouty-Boye D, Ferrini S, Baychelier F, Eid P, Lebousse-Kerdiles C, Durali D, and Biassoni R, et al (2005). Membrane-bound and soluble IL-15/IL-15R α complexes display differential signaling and functions on human hematopoietic progenitors. *Blood* **106**, 2302–2310.
- [6] Kroemer A, Xiao X, Degauque N, Edtinger K, Wei H, Demirci G, and Li XC (2008). The innate NK cells, allograft rejection, and a key role for IL-15. *J Immunol* **180**, 7818–7826.
- [7] Stoklasek TA, Schluns KS, and Lefrancois L (2006). Combined IL-15/IL-15R α immunotherapy maximizes IL-15 activity in vivo. *J Immunol* **177**, 6072–6080.
- [8] Waldmann TA (2006). The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol* **6**, 595–601.
- [9] Dubois S, Mariner J, Waldmann TA, and Tagaya Y (2002). IL-15R α recycles and presents IL-15 in trans to neighboring cells. *Immunity* **17**, 537–547.
- [10] Bo H, Wei XQ, Dong H, Zhang Y, Lv P, Liu W, Koutoulaki A, and Gao XM (2009). Elevated expression of transmembrane IL-15 in immune cells correlates with the development of murine lupus: a potential target for immunotherapy against SLE. *Scand J Immunol* **69**, 119–129.
- [11] Khawam K, Giron-Michel J, Gu Y, Perier A, Giuliani M, Caignard A, Devocelle A, Ferrini S, Fabbi M, and Charpentier B, et al (2009). Human renal cancer cells express a novel membrane-bound interleukin-15 that induces, in response to the soluble interleukin-15 receptor alpha chain, epithelial-to-mesenchymal transition. *Cancer Res* **69**, 1561–1569.
- [12] Neely GG, Epelman S, Ma LL, Colarusso P, Howlett CJ, Amankwah EK, McIntyre AC, Robbins SM, and Mody CH (2004). Monocyte surface-bound IL-15 can function as an activating receptor and participate in reverse signaling. *J Immunol* **172**, 4225–4234.
- [13] Koka R, Burkett P, Chien M, Chai S, Boone DL, and Ma A (2004). Cutting edge: murine dendritic cells require IL-15R α to prime NK cells. *J Immunol* **173**, 3594–3598.
- [14] Lodolce JP, Burkett PR, Boone DL, Chien M, and Ma A (2001). T cell-independent interleukin 15R α signals are required for bystander proliferation. *J Exp Med* **194**, 1187–1194.
- [15] Maiuri L, Ciacci C, Auricchio S, Brown V, Quarantino S, and Londei M (2000). Interleukin 15 mediates epithelial changes in celiac disease. *Gastroenterology* **119**, 996–1006.
- [16] Nishiyama R, Sakaguchi T, Kinugasa T, Gu X, MacDermott RP, Podolsky DK, and Reinecker HC (2001). Interleukin-2 receptor β subunit-dependent and -independent regulation of intestinal epithelial tight junctions. *J Biol Chem* **276**, 35571–35580.
- [17] Obermeier F, Hausmann M, Kellermeier S, Kiessling S, Strauch UG, Duitman E, Bulfone-Paus S, Herfarth H, Bock J, and Dunger N, et al (2006). IL-15 protects intestinal epithelial cells. *Eur J Immunol* **36**, 2691–2699.
- [18] Regamey N, Obregon C, Ferrari-Lacraz S, van Leer C, Chanson M, Nicod LP, and Geiser T (2007). Airway epithelial IL-15 transforms monocytes into dendritic cells. *Am J Respir Cell Mol Biol* **37**, 75–84.
- [19] Sheller JR, Polosukhin VV, Mitchell D, Cheng DS, Peebles RS, and Blackwell TS (2009). Nuclear factor kappa B induction in airway epithelium increases lung inflammation in allergen-challenged mice. *Exp Lung Res* **35**, 883–895.
- [20] Tejman-Yarden N, Zlotnik M, Lewis E, Etzion O, Chaimovitz C, and Douvdevani A (2005). Renal cells express a functional interleukin-15 receptor. *Nephrol Dial Transplant* **20**, 516–523.
- [21] Weiler M, Rogashev B, Einbinder T, Hausmann MJ, Kaneti J, Chaimovitz C, and Douvdevani A (1998). Interleukin-15, a leukocyte activator and growth factor, is produced by cortical tubular epithelial cells. *J Am Soc Nephrol* **9**, 1194–1201.
- [22] Yanagita M, Shimabukuro Y, Nozaki T, Yoshimura N, Watanabe J, Koide H, Terakura M, Saho T, Takedachi M, and Jang MH, et al (2002). IL-15 up-regulates iNOS expression and NO production by gingival epithelial cells. *Biochem Biophys Res Commun* **297**, 329–334.
- [23] Zhang SQ, Luo X, Yang S, Liu JL, Yang CJ, Yin XY, Huang HL, and Zhang XJ (2008). Autoinhibition of IL-15 expression in KC cells is ERK1/2 and PI3K dependent. *Scand J Immunol* **68**, 397–404.
- [24] Eini H, Tejman-Yarden N, Lewis EC, Chaimovitz C, Zlotnik M, and Douvdevani A (2010). Association between renal injury and reduced interleukin-15 and interleukin-15 receptor levels in acute kidney injury. *J Interferon Cytokine Res* **30**, 1–8.
- [25] Shinozaki M, Hirahashi J, Lebedeva T, Liew FY, Salant DJ, Maron R, and Kelley VR (2002). IL-15, a survival factor for kidney epithelial cells, counteracts apoptosis and inflammation during nephritis. *J Clin Invest* **109**, 951–960.
- [26] Giron-Michel J, Azzi S, Khawam K, Mortier E, Caignard A, Devocelle A, Ferrini S, Croce M, Francois H, and Lecru L, et al (2012). Interleukin-15 plays a central role in human kidney physiology and cancer through the γ c signaling pathway. *PLoS One* **7**, e31624.
- [27] Azzi S, Bruno S, Giron-Michel J, Clay D, Devocelle A, Croce M, Ferrini S, Chouaib S, Vazquez A, and Charpentier B, et al (2011). Differentiation therapy: targeting human renal cancer stem cells with interleukin 15. *J Natl Cancer Inst* **103**, 1884–1898.
- [28] Giron-Michel J, Azzi S, Ferrini S, Chouaib S, Camussi G, Eid P, and Azzarone B (2013). Interleukin-15 is a major regulator of the cell-microenvironment interactions in human renal homeostasis. *Cytokine Growth Factor Rev* **24**, 13–22.
- [29] Wittnebel S, Da Rocha S, Giron-Michel J, Jalil A, Opolon P, Escudier B, Validire P, Khawam K, Chouaib S, and Azzarone B, et al (2007). Membrane-bound interleukin (IL)-15 on renal tumor cells rescues natural killer cells from IL-2 starvation-induced apoptosis. *Cancer Res* **67**, 5594–5599.
- [30] Bussolati B, Bruno S, Grange C, Ferrando U, and Camussi G (2008). Identification of a tumor-initiating stem cell population in human renal carcinomas. *FASEB J* **22**, 3696–3705.
- [31] Liang CC, Park AY, and Guan JL (2007). In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* **2**, 329–333.
- [32] Ulrich C (2009). Topical treatment of field cancerization. *Cancer Treat Res* **146**, 439–446.
- [33] Olsen SK, Ota N, Kishishita S, Kukimoto-Niino M, Murayama K, Uchiyama H, Toyama M, Terada T, Shirouzu M, and Kanagawa O, et al (2007). Crystal Structure of the interleukin-15-interleukin-15 receptor α complex: insights into *trans* and *cis* presentation. *J Biol Chem* **282**, 37191–37204.
- [34] Kurys G, Tagaya Y, Bamford R, Hanover JA, and Waldmann TA (2000). The long signal peptide isoform and its alternative processing direct the intracellular trafficking of interleukin-15. *J Biol Chem* **275**, 30653–30659.
- [35] Farner NL, Gan J, de Jong JL, Leary TP, Fenske TS, Buckley P, Dunlap S, and Sondel PM (1997). Alteration of the CD34⁺Tf-1 β cell line profile in response to long-term exposure to IL-15. *Cytokine* **9**, 316–327.
- [36] Morse D, Lin L, Choi AM, and Ryter SW (2009). Heme oxygenase-1, a critical arbitrator of cell death pathways in lung injury and disease. *Free Radic Biol Med* **47**, 1–12.
- [37] Westin JR (2014). Status of PI3K/Akt/mTOR pathway inhibitors in lymphoma. *Clin Lymphoma Myeloma Leuk* **14**, 335–342.
- [38] Koka R, Burkett PR, Chien M, Chai S, Chan F, Lodolce JP, Boone DL, and Ma A (2003). Interleukin (IL)-15R α -deficient natural killer cells survive in normal but not IL-15R α -deficient mice. *J Exp Med* **197**, 977–984.
- [39] Scadden DT (2014). Nice neighborhood: emerging concepts of the stem cell niche. *Cell* **157**, 41–50.
- [40] Evans AJ, Russell RC, Roche O, Burry TN, Fish JE, Chow VW, Kim WY, Saravanan A, Maynard MA, and Gervais ML, et al (2007). VHL promotes E2 box-dependent E-cadherin transcription by HIF-mediated regulation of SIP1 and snail. *Mol Cell Biol* **27**, 157–169.
- [41] Russell RC and Ohh M (2007). The role of VHL in the regulation of E-cadherin: a new connection in an old pathway. *Cell Cycle* **6**, 56–59.
- [42] Esteban MA, Tran MG, Harten SK, Hill P, Castellanos MC, Chandra A, Raval R, O'Brien TS, and Maxwell PH (2006). Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. *Cancer Res* **66**, 3567–3575.
- [43] Webster WS, Thompson RH, Harris KJ, Frigola X, Kuntz S, Inman BA, and Dong H (2007). Targeting molecular and cellular inhibitory mechanisms for improvement of antitumor memory responses reactivated by tumor cell vaccine. *J Immunol* **179**, 2860–2869.
- [44] Foureau DM, Amin A, White RL, Anderson W, Jones CP, Sarantou T, McKillop IH, and Salo JC (2014). Sequential immune monitoring in patients with melanoma and renal cell carcinoma treated with high-dose interleukin-2: immune patterns and correlation with outcome. *Cancer Immunol Immunother* **63**, 1329–1340.
- [45] Le Floch A, Jalil A, Vergnon I, Le Maux Chansac B, Lazar V, Bismuth G, Chouaib S, and Mami-Chouaib F (2007). α E β 7 integrin interaction with E-cadherin promotes antitumor CTL activity by triggering lytic granule polarization and exocytosis. *J Exp Med* **204**, 559–570.