Membrane-Bound Interleukin (IL)-15 on Renal Tumor Cells Rescues Natural Killer Cells from IL-2 Starvation-Induced Apoptosis

Sebastian Wittnebel,1 Sylvie Da Rocha,1 Julien Giron-Michel,1 Abdelali Jalil,1 Paule Opolon,1 Bernard Escudier,1 Pierre Validire,1 Krystel Khawam,2 Salem Chouaib1 Bruno Azzarone,2 and Anne Caignard1

1Institut National de la Santé et de la Recherche Médicale U753; 2Institut National de la Santé et de la Recherche Médicale U542, Hôpital Paul Brousse; 3Centre National de la Recherche Scientifique UMR 8121, Laboratoire de Vectorologie et Transfert de Gènes; Unité des Thérapies Innovantes, Institut Gustave Roussy, Villejuif, France and 4Unite des The rapies Innovantes, Institut Gustave Roussy, Villejuif, France

Abstract
Renal cell carcinoma primary tumors and lung metastases are infiltrated by activated natural killer (NK) cells. Interleukin (IL)-15, a major cytokine involved in cross-talk between accessory cells (dendritic cells and macrophages) and NK cells, is produced by epithelial renal cells. We show that renal cell carcinoma cells and normal renal cells express IL-15 mRNA and membrane-bound IL-15 (MbIL-15). These cells also express IL-15 receptor α (IL-15Rα). Silencing of IL-15Rα by specific small interfering RNA in renal cell carcinoma had no effect on MbIL-15 production, indicating that the cytokine is not cross-presented by IL-15Rα in renal cell carcinoma cells but anchored to the membrane. Furthermore, we show that MbIL-15 from renal cell carcinoma cells is functional and involved in rapid nuclear translocation of phosphorylated signal transducers and activators of transcription 3 in IL-2–starved NK cells. MbIL-15 on the target did not interfere with resting NK cell activation and target cell cytolysis but rescued NK cells from IL-2 starvation-induced apoptosis through contact-dependent interaction. Masking of MbIL-15 with soluble IL-15Rα molecules restored NK cell apoptosis. These findings suggest that IL-15 produced by renal tumor cells is involved in the maintenance of active NK cells at the tumor site. [Cancer Res 2007;67(12):5594–9]

Introduction
Immunotherapy for metastatic renal clear cell cancer (RCC) is a treatment option with unique curative potential (1, 2). Unfortunately, the overall response rate is limited. A better understanding of the mechanisms underlying the striking immunogenicity of RCC may contribute to improved immunotherapeutic strategies.

Natural killer (NK) cells were detected in tumors of various origins (3). NK cells are cytotoxic effectors playing a major role in the first line of defense against pathogens and transformed cells. NK cells can kill tumor cells without prior priming. Activation of endogenous NK cells with interleukin (IL)-2 and adoptive transfer of in vitro–activated autologous NK cells stimulate antitumor activity in experimental and clinical settings (4). NK cell activation and survival depend strongly on the presence of cytokines, including IL-2 and IL-15. Due to the reported expression of IL-15 mRNA by renal cells, we investigated the expression of IL-15 by renal tumor cells and its function in the cross-talk between NK and tumor cells.

IL-15 is a pleiotropic cytokine belonging to the four-helix bundle cytokine family and was first characterized by its ability to substitute IL-2 in supporting growth of the murine IL-2–dependent CTL cell line (5). IL-15 shares the IL-2 receptor (IL-2R) β and γ chains with IL-2 but has a unique high-affinity α chain (IL-15Rα). Due to IL-15Rα, IL-15 and IL-2 have different functional properties in cells of the same type. Additionally, a natural soluble form of IL-15 (sIL-15Rα), generated through proteolytic shedding, behaves as a high-affinity IL-15 antagonist (6). Unlike IL-2, IL-15 is a non–T-cell-derived cytokine. IL-15 mRNA is constitutively expressed by many cell types and tissues, including monocytes, fibroblasts, and kidney epithelial cells. However, most primary cell lines do not release detectable amounts of IL-15 into the culture medium due to the complex control of IL-15 expression.

First identified as a soluble factor, IL-15 also has a biologically active, membrane-bound form expressed by monocytes and macrophages. The membrane-bound IL-15 (MbIL-15) signaling involves various specific pathways. MbIL-15 is believed to act mainly by a cross-presentation mechanism involving production of the IL-15Rα chain and the MbIL-15 by the same cell. IL-15 is loaded onto IL-15Rα and presented to IL-2/IL-15Rβ and IL-15Rγ chains on bystander cells by an undefined mechanism. IL-15/IL-15Rα complexes in antigen-presenting cells (APC) in peripheral tissues are essential for the development of NK cells, NK T cells, and CD8+ memory T cells (7). IL-15 can stimulate growth and IFN-γ production in these effector cells. It is also essential for the induction of NK cell differentiation and for NK cell survival in the bone marrow. In lymph nodes, IL-15 produced by APCs controls NK cell survival and IL-2 is required for activation (8).

We analyzed the local immune response in renal tumors by investigating how IL-15 produced by renal tumor cells interferes with bystander NK cells. We show here that renal primary tumors and lung metastases are infiltrated by NK cells. We also show that MbIL-15 produced by RCC cells can rescue NK cells from apoptosis induced by growth factor deprivation. This ability may explain, in part, the unique immunogenicity of RCC.

Materials and Methods
Cell lines and cultures. Primary tumor and normal renal cells were obtained by enzymatic digestion of renal tumor fragments as described previously (9). NK cells were negatively selected from donor peripheral blood mononuclear cells with NK cell isolation kit II (Miltenyi Biotec). NK
cells were used as resting effectors or activated by incubation with 300 units/mL IL-2 for 4 to 18 days.

**Immunohistochemistry.** After deparaffinization and antigen retrieval, 4 μm sections were incubated with anti-CD3 (1:500) and anti-CD57 (1:500) primary antibodies (NeoMarkers) for 1 h. Labelings were revealed with a mixture of Rabbit Power Vision kit coupled to horseradish peroxidase (Ultra Vision) for CD3 and Mouse Vision kit coupled to alkaline phosphatase for CD57. Anti-CD3 was visualized with 3,3′-diaminobenzidine and anti-CD57 with Fast Blue. Sections were counterstained with Mayer’s hematoxylin and mounted.

**Flow cytometry.** Anti-IL-15 monoclonal antibody (mAb; MAB247, R&D Systems) and IL-15Rs α/β heterodimer (Santa Cruz Biotechnology) were used to label IL-15 and the IL-15Rα chain on renal cells. Early apoptotic events were evaluated by flow cytometry with Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences). Activated NK cells were starved for 6 to 8 h in IL-2–free medium and incubated with tumor cells for 20 h before Annexin V staining. RCC cells were incubated with 1.5 μg/mL recombinant human IL-15Rα/Fc chimera (sIL-15Rα/Fc) during the coculture. NK cells were removed and stained with CD56-allophycocyanin mAb and Annexin V-FITC. Flow cytometry was carried out on a FACSort (BD Biosciences) and 10,000 events were collected and analyzed with CellQuest software (BD Biosciences).

**Tagman real-time quantitative reverse transcription-PCR.** Random hexamers were used to reverse transcribe 1 μg of RNA extracted from renal cells. Taqman (Applied Biosystems) real-time quantitative reverse transcription-PCR (RT-PCR) was carried out with primers and probes for IL-15 designed by Applied Biosystems and used as the manufacturer recommended. Relative quantification of the transcripts was derived by the standard curve method (Applied Biosystems User Bulletin 2, ABI PRISM 7700 Sequence Detection system) and normalized to the 18S mRNA of each cell type analyzed.

**Confocal microscopy.** IL-2–starved NK cells were stimulated with IL-2 (300 units/mL) and IL-15 (20 μg/mL) or incubated with adherent RCC7 and MEL1T cells (E:T ratio of 1:1) for 10 to 30 min. Cells on cover slides were fixed, permeabilized, and stained with anti–phosphorylated signal transducers and activators of transcription 3 (pSTAT3; Tyr705) rabbit antibody (Cell Signaling Technology) for 1 h. They were then incubated for 1 h with biotinylated goat anti-rabbit IgG, and signal was detected with a streptavidin Alexa Fluor 488 conjugate, both from Molecular Probes. Nuclei were stained with TO-PRO-3 (Molecular Probes). Conjugates were examined with an LSM 510 confocal microscope (Zeiss). In various experiments, RCC7 cells were treated with sIL-15Rα (1.5 μg/mL) or anti-IL-15 mAb (20 μg/mL) for 30 min before coculture.

**Small interfering RNAs and electroporation.** IL-15 and IL-15Rα gene expression was silenced with sequence-specific small interfering RNA (siRNA; ref. 10). Control siRNA targeting enhanced green fluorescent protein (EGFP) was purchased from Sigma-Proligo. Briefly, Sigma-NK cells were transfected by electroporation with 0.5 μmol/L siRNA in a Gene Pulser Xcell Electroporation System (300 V, 500 μF; Bio-Rad) and grown for 48 h before testing.

**Results and Discussion**

**Activated NK cells infiltrate lung metastases of renal cell carcinoma.** NK cells are potent cytotoxic effectors for tumor cells of various origins. Better defined mechanisms involved in recognition and killing of targets by NK cells emphasize their role in immunotherapy. NK cells are not typically found in large numbers in advanced human neoplasms, but their numbers increase following activation or adoptive transfer (4). The level of NK infiltration positively correlates with prognosis in squamous cell lung carcinoma (3), gastric carcinoma, and colorectal tumors (11). NK cells have been detected in renal tumors by tumor-infiltrating lymphocyte isolation, but in situ studies are rare (12, 13).

We immunostained serial sections of infiltrating T and NK cells from 19 primary renal tumors and 22 lung metastases derived from RCC patients. There were occasional small foci of lymphocyte-like cells within the tumor or at its periphery as determined by visualizing tumor architecture and inflammatory infiltrate density by HES staining. We immunohistochemically detected tumor-infiltrating NK cells by double staining for CD57 (a glycoprotein present on most activated NK cells) and CD3 to exclude T cells. NK cells were present in most primary tumors and lung metastases, but the density of the staining varied among tumors. Staining of NK cells was stronger in 5 of 17 primary tumors and in 6 of 22 metastases. We frequently found CD3+ T cells and NK cells in peritumoral areas but less frequently inside the tumor. Primary renal tumors contained scattered NK cells (Fig. 1A). Lung metastases contained many CD3+ T cells in the periphery, but few NK cells were present in the tumor (Fig. 1B). NK cells are characterized as CD57+/CD3− cells with large digitated cytoplasm and displaying no histologic sign of apoptosis; some were in close contact with renal tumor cells (Fig. 1A).

Thus, NK cells are present in renal primary tumors and lung metastases. As IL-2 treatment has been shown to increase peripheral NK cell activity, it may also activate NK cells in situ. Tumor-infiltrating CD56+/CD16− NK cells have been shown to regain lytic activity following short-term ex vivo IL-2 activation in

**Figure 1.** Immunohistochemical study of NK cells infiltrating primary tumors and lung metastases from RCC patients. Double staining with anti-CD3 (brown) and anti-CD57 (red) was carried out to detect NK and T cells in primary renal tumor (A) and lung metastases (B). CD57+/CD3− NK cells are in red circles. Arrows, tumor cells. Magnification, ×200.
RCC (12). Furthermore, although RCC cells express HLA-I molecules, these cells are susceptible to NK cell-mediated lysis (14). Cellular therapy trials using NK cell adoptive transfer further show that NK cells control the growth of RCC (15). These findings highlight the importance of investigating cross-talk between RCC cells and NK cells.

Renal carcinoma cell lines express MbIL-15. Tumor environment greatly influences the local immune response through RCC (12). Renal tumor cell lines (three of six tested are shown: RCC5, RCC6, and RCC7) produce IL-15 (bold line), but breast cancer MCF7 and melanoma MELT1 cells do not. Paired tumor (TC) and normal (NC) cells produce similar levels of IL-15 (two of five pairs are shown: RCC5 and PIL). B, quantitative analysis of IL-15 mRNA transcripts in primary tumor and normal renal cell pairs derived from eight RCC patients. Results are expressed as IL-15 mRNA relative value (normalized to 18S mRNA of each cell line). P values between normal and tumor cells are indicated (Student’s t test). C, staining of MbIL-15 and IL-15Rα (thin lines) in tumor cells treated with IFN-α (gray) and IFN-γ (black). Dashed lines, isotypic controls. D, RCC7 cells transfected with IL-15 siRNA, IL-15Rα siRNA, or both and labeled with anti-IL-15 and anti-IL-15Rα antibodies 24 h later.
secretion of various immune factors. RCC tumors produce immunosuppressive factors, including cytokines (transforming growth factor-β and IL-10) and gangliosides. The presence of NK cells in RCCs indicates that various factors may maintain their activation and survival in situ. We show that five renal tumor cell lines established from primary tumors and metastases produce MbIL-15. This cytokine was not detected in breast cancer (MCF7) and melanoma (MELT1) cell lines (Fig. 2A). We observed similar production of MbIL-15 in paired, normal renal epithelial cells and tumor cells (Fig. 2A) derived from five patients. We also detected IL-15 mRNA transcripts in eight pairs of normal and tumoral renal cells but not in MCF7 and MELT1 cell lines by quantitative analysis using (real time) RT-PCR (Fig. 2B). Soluble IL-15 was not detected in renal cell supernatants (ELISA test threshold, 4 pg/mL), indicating that the active form is mainly membrane bound. It was previously reported that IL-15 transcripts are present in normal kidney and in renal epithelial cells and that IL-15 acts as a survival factor for normal tubular epithelial cells (16). Furthermore, it was shown that IL-15 mediates many specific responses as a cell membrane–associated molecule and that this may represent its functional form (17). MbIL-15 on monocytes and dendritic cells is cross-presented by IL-15Rα to responder cells expressing the specific IL-15/II-2Rβ and II-2Rγ chains. We show the presence of the private II-15Rα and the common II-2/II-15Rβ in a RCC cell line but the absence of II-2/II-15Rγ mRNA transcripts and membrane protein (data not shown). Clearly, more IL-15 and IL-15Rα were produced in response to IFN-γ and IFN-α than in controls (Fig. 2C). IL-15 and IL-15Rα production in terms of mean fluorescence and intensity and percentage values were decreased by specific siRNAs in eight independent experiments: there were a

**Figure 3.** MbIL-15 on RCC cells induces IL-15 signaling in NK cells.
A, NK cells were IL-2 starved for 6 to 8 h in cytokine-free medium (med) and stimulated in vitro with 300 units/mL IL-2 or 20 ng/mL IL-15 for 10 min. Cells were fixed, permeabilized, stained with anti-pSTAT3 or control mAbs, and analyzed by confocal microscopy. Green nuclear staining, pSTAT3 translocation. Nuclei are stained in red with TO-PRO-3.
B, IL-2–starved NK cells (arrows) were incubated with tumor cells (arrowheads) for 30 min and processed as above.
C, treatment of RCC7 cells with sIL-15Rα, anti-IL-15 mAb, or control IgG1 before their coculture with NK cells abrogated pSTAT3 nuclear translocation in starved NK cells.
D, RCC7 (TC) cells transfected with IL-15 siRNA inhibited pSTAT3 nuclear translocation in IL-2–starved NK cells. IL-15Rα siRNA and EGFP siRNA had no effect.
65% inhibition of MbIL-15 level on RCC7 and a 50% inhibition of IL-15Rα level on RCC7. However, inhibition of IL-15Rα production did not reduce IL-15 production and combined siRNAs did not have additional or synergistic effects, indicating that IL-15 is not cross-presented (Fig. 2D). These data provide the first evidence of a nonsecreted membrane-anchored IL-15 in RCC cells. Consistent with these findings, a MbIL-15 with dual ligand receptor qualities and potential for bidirectional signaling was described in human prostate carcinoma cells (18). This MbIL-15 acts as a ligand to immune cells expressing the IL-15Rαβγ receptor.

MbIL-15 engaged with soluble specific ligands has been shown to stimulate production of various proinflammatory cytokines (IL-6, IL-8, and tumor necrosis factor-α) in cancer cells by reverse signaling. The form of IL-15 described in prostate cancer cells also exerts protumoral activity through reverse signaling, inducing the extracellular signal-regulated kinase pathway (19). It would therefore be interesting to compare the mechanism of IL-15anchoring on tumor cells and normal renal cells and the signaling induced in these two cell types.

**MbIL-15 on RCC cells induces nuclear translocation of pSTAT3 in NK cells.** NK cells produce IL-2 and IL-15Rαβγ and up-regulate the private IL-2Rα and IL-15Rα following stimulation with IL-2 and IL-15, respectively. IL-15 signaling involves Janus-activated kinase (JAK) 1 and JAK3 followed by nuclear translocation of pSTAT3. We studied nuclear translocation of pSTAT3 by confocal microscopy to establish whether tumor MbIL-15 is functional and transduces signal in NK cells. We observed rapid pSTAT3 nuclear translocation, detected as intense green labeling, in >90% of IL-2–starved NK cells (Fig. 3A) incubated for 10 to 30 min with IL-2 (300 units/mL) or IL-15 (20 ng/mL). Stimulation of IL-2–starved NK cells with MbIL-15–positive RCC7 cells induced nuclear translocation of pSTAT3 in >90% of NK cells. In contrast, pSTAT3 staining was faint, diffuse, and cytoplasmic in response to MELT1 cells as in IL-2–starved NK cells (Fig. 3B). Masking of MbIL-15 on RCC7 cells with anti-IL-15 mAb before their incubation with starved NK cells abrogated the nuclear translocation of pSTAT3 in NK cells; control Ig had no effect (Fig. 3C). The nuclear translocation of pSTAT3 in NK cells was also inhibited in RCC7 cells incubated with sIL-15Rα or transfected with IL-15 siRNA before being cocultured with starved NK cells (Fig. 3C and D). This finding confirms that MbIL-15 presented by RCC cells induces signaling in NK cells. In contrast, there was no effect on nuclear

---

**Figure 4.** MbIL-15 on RCC cells rescues NK cells from apoptosis induced by IL-2 starvation. A, percentage lysis of tumor targets (K562, RCC5, RCC7, and MELT1 cells) by resting NK (left) and IL-2–activated NK cells (right). MbIL-15 in RCC cells did not interfere with resting NK cell–mediated lysis. B, IL-2–starved NK cells (med) were layered on tumor cells (RCC7, MCF7, and MELT1) for 20 h before Annexin V/propidium iodide (PI) labeling in CD56–gated NK cells. Results are expressed as mean percentages of apoptotic NK cells. C, addition of a Transwell between RCC7 and NK cells abrogated survival of NK cells. Masking of MbIL-15 with saturating concentrations of sIL-15Rα (1.5 μg/mL) partially restored apoptosis in IL-2–starved NK cells. One of five experiments.
translocation of pSTAT3 in MELT1 cells treated in the same manner (data not shown). These findings show that MbIL-15 on RCC cells is functional and induces signaling in bystander cells expressing IL-15Rα or IL-15Rβγ receptors.

**MbIL-15 on RCC cells does not influence resting NK cell activation.** In the absence of exogenous cytokines, NK cell activation relies on cell/cell interactions. Stimulation of resting NK cells with RCC7, RCC5, and MELT1 for 20 h induced expression of CD69, but the expression levels of NCR1 and NCR3 (NKP46 and NKP30) were unchanged (data not shown). Resting NK cells efficiently lysed K562 cells, sparing RCC7, RCC5, and MELT1 cells that were efficiently killed by IL-2–activated NK cells independently of IL-15 expression (Fig. 4A). Resting NK cells secreted low levels of IFN-γ (<10 pg/ml, except for K562 that secreted >100 pg/ml; data not shown) after incubation for 24 h with tumor cells. Thus, expression of MbIL-15 by tumor target does not contribute to induction of cytokysis and IFN-γ secretion by resting NK cells. However, MbIL-15 may indirectly control NK cell lytic potential, as IL-15 was shown to induce the expression of inhibitory receptors (i.e., CD94/NKG2A) on alloantigen-activated NK cells. Furthermore, IL-15 may be involved in polarization of the tumor immune response by favoring survival of immune NK and antigen-experienced T cells in the absence of IL-2 secreted by CD4+ T cells. This may be important for epithelial tumors that are characterized by a low Th1 response. For example, in renal cell carcinomas, tumor-derived products have been shown to induce a Th2-type response by inducing apoptosis in IFN-γ antigen-specific T cells.

**MbIL-15 on RCC cells rescues NK cells from IL-2 starvation-induced apoptosis.** It was recently reported that tumor cells induce NK cell apoptosis through NCR engagement and subsequent up-regulation of FasL, resulting in Fas-mediated NK cell death (20). We asked whether IL-15 produced by tumor cells interferes with NK cell death. We addressed this question by studying the role of MbIL-15 expressed by tumor cells in the control of NK cell death in 24-h coculture experiments. In these experiments, IL-2–starved NK cells were incubated with adherent tumor cells presenting or not presenting MbIL-15. We measured NK cell apoptosis by Annexin V staining. Deprivation of IL-2 induced apoptosis in NK cells. Overnight incubation with RCC cells (RCC7 and RCC5) rescued NK cells from IL-2 deprivation-induced apoptosis in 10 independent experiments. In contrast, MbIL-15–negative MELT1 and MCF7 cells did not protect against NK cell death (Fig. 4B). Addition of a Transwell between tumor and NK cells abolished survival of NK cells cocultured with RCC7 cells, indicating that the NK cell apoptosis rescued required contact with RCC7 (Fig. 4B). Addition of si-L-15Rα blocked IL-15–induced protection from apoptosis (Fig. 4C), confirming the role of MbIL-15 in rescue of NK cells from apoptosis. The si-L-15Rα chain may mask a functional epitope of MbIL-15 on RCC7 cells and/or modify its function, leading to modified signaling.

Our findings suggest that IL-15 produced by renal tumor cells maintains activated NK cells in the tumors. The production of stress-induced molecules (MICA/MICB molecules, UL-binding proteins, and heat shock proteins) changes in chemokine expression and hypoxia in renal tumors may induce a local inflammation and a local immune response maintained by MbIL-15. The MbIL-15 may mediate activation of involved immune cells and protect them from apoptosis.

The role of IL-15 in renal tumor biology is consistent with this emerging concept. This molecule enables cells to integrate various stimuli under physiologic or pathologic conditions by diversifying downstream signaling. Thus, our findings stress that it is a crucial factor for the local immune response and suggest it would be a good candidate for manipulation by immunotherapeutic strategies for cancer and allograft reactions. Better understanding of cross-communication between diverse signaling networks should provide insight into how complex and diverse environmental stimuli are translated into appropriate cellular reactions and adaptive responses.

**Acknowledgments**

Received 12/4/2006; revised 4/7/2007; accepted 5/7/2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

---

**References**


---


Downloaded from cancerres.aacrjournals.org on July 21, 2017. © 2007 American Association for Cancer Research.
Membrane-Bound Interleukin (IL)-15 on Renal Tumor Cells Rescues Natural Killer Cells from IL-2 Starvation-Induced Apoptosis

Sebastian Wittnebel, Sylvie Da Rocha, Julien Giron-Michel, et al.