REVERSE SIGNALING THROUGH MEMBRANE-BOUND INTERLEUKIN-15

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Running title: Reverse signaling through membrane-bound IL-15

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Abbreviations: IL-15, interleukin-15; IL-15Rα, IL-15 receptor α; sIL-15Rα, soluble IL-15Rα; Abs, antibodies; IFNγ, interferon γ; ER, endoplasmic reticulum; L, ligand; hMo, human monocytes; LSP, long signal peptide; SSP, short signal peptide; LPS, lipopolysaccharide; MFI, median fluorescence intensity; TACE, TNFα-converting enzyme; FAK, focal adhesion kinase; WGA, wheat germ agglutinin.
SUMMARY

This study demonstrates that membrane-anchored interleukin-15 (IL-15) constitutively expressed on the cell surface of PC-3 human prostate carcinoma cells and IFNγ-activated human monocytes is implicated in reverse signaling upon stimulation with sIL-15Rα or anti-IL-15 antibodies, mediating the outside-to-inside signal transduction that involves the activation of members of MAP kinase family ERK and p38, and focal adhesion kinase. The presence of membrane-bound IL-15 is not dependent on the expression of the trimeric IL-15 receptor complex by these cells and resists the treatment with acidic buffer or trypsin. The reverse signaling through membrane-bound IL-15 considerably increases the production of several pro-inflammatory cytokines by monocytes such as IL-6, IL-8, and TNFα, indicating thereby the relevance of this process to the complex immunomodulatory function of these cells. Furthermore, the stimulation of transmembrane IL-15 also enhances the transcription of IL-6 and IL-8 in PC-3 cell line, and promotes migration of PC-3 cells as well as LnCap human prostate carcinoma cells stably expressing IL-15 upon the cell surface. Thus, IL-15 can exist as a biologically active transmembrane molecule that possesses dual ligand/receptor qualities with potentiality to induce bi-directional signaling. This fact highlights a new level of complexity in the biology of IL-15 and offers novel important insights to our understanding of the cellular responses modulated by this pleiotropic cytokine.
INTRODUCTION

Interleukin-15 (IL-15) is a pleiotropic cytokine which belongs to the four-helix bundle cytokine family and was first identified because of its ability to substitute IL-2 in supporting the growth of murine IL-2-dependent CTLL cell line (1). IL-15 shares with IL-2 the IL-2 receptor beta (IL-2Rβ) and IL-2 receptor gamma (IL-2Rγ/γc) chains (2), but has a unique high affinity α chain (IL-15Rα) responsible for the differential functional effects of IL-15 versus IL-2 on cells of the same type (3). IL-15 can replace IL-2 in most of its activities in the immune system, including induction of T-cell proliferation and chemotaxis, stimulation of natural killer (NK) cell growth and interferon γ (IFNγ) production, generation of cytotoxic effector cells, and co-stimulation of B-cell growth and immunoglobulin synthesis (4-6). IL-15 and IL-15Rα knock-out mice display a marked reduction in numbers of NK cells, memory phenotype CD8+ T lymphocytes, and a distinct population of intestine intraepithelial lymphocytes, suggesting an important role for IL-15 in the development and/or survival of these cells (7, 8). In addition, IL-15 has a potent anti-apoptotic function, inhibiting apoptosis of activated T and B cells, keratinocytes and melanoma cells in vitro, and protecting mice from Fas-induced hepatic failure and multi-system apoptosis in vivo (9-11).

IL-15 mRNA is constitutively expressed by a large variety of cell types and tissues, including monocytes/macrophages, fibroblasts, keratinocytes, kidney epithelial cells, nerve cells, placenta, heart, and skeletal muscle (4, 7, 12-15). Interestingly, most primary cells and cell lines which express IL-15 mRNA do not release detectable amounts of this cytokine into the culture medium. This discrepancy is explained by the fact that IL-15 has a complex, multifaceted control of expression with regulation at the
levels of transcription, translation, and intracellular trafficking. The existence of two IL-15 isoforms has been reported which differ in the length of the signal peptide (16). These isoforms exhibit a differential intracellular trafficking, secretion, and endosomal localization, indicating an important role for signal peptide in multiple mechanisms controlling IL-15 production (17, 18). IL-15 associated with the short signal peptide (IL-15SSP) is not secreted but rather stored intracellularly in the nucleus and cytoplasm, whereas the alternative isoform characterized by the longer signal peptide (IL-15LSP) is located in the Golgi, early endosomes, and the endoplasmic reticulum (ER), and has been suggested to follow a pathway that may result in the cytokine secretion (17, 18).

In monocytes/macrophages, IL-15 is expressed in a biologically active membrane-bound form, and its mRNA expression can be upregulated by exogenous stimuli, such as IFNγ and lipopolysaccharide (LPS) (12). Furthermore, TNFα-stimulated dermal fibroblasts were able to sustain proliferation of activated T cells through expression of membrane-bound IL-15 (13). The presence of biologically active membrane-anchored IL-15 upon the cell surface of normal human monocytes, several monocytic cell lines, and TNFα-stimulated dermal fibroblasts suggests that, under physiological conditions, IL-15 may mainly be present in a membrane-bound rather than secreted form. This report highlights the ability of membrane-anchored IL-15 to mediate outside-to-inside (reverse) signaling that activates focal adhesion kinase (FAK) and MAP kinases in the human prostate carcinoma PC-3 cell line and IFNγ-activated human monocytes. Furthermore, reverse signaling through membrane IL-15 promotes migration of prostate cancer cells, and induces production of pro-inflammatory cytokines both in PC-3 cells and monocytes. Thus, our findings attribute yet another function to this pluripotent
cytokine by demonstrating the capacity of IL-15 to function both as a ligand and a receptor molecule.
MATERIALS AND METHODS

*Cytokines, antibodies, recombinant proteins and ELISA kits* – Recombinant IL-15 and IFNγ were purchased from TEBU (London, UK). Monoclonal anti-CD122/IL-2Rβ PE (TMβ1), anti-CD132/γc PE (4G2) antibodies (Abs) were from BD PharMingen (Heidelberg, Germany). A panel of anti-IL-15 Abs was used: L-20 from Santa Cruz Biotechnology (Santa Cruz, CA), MAB647 from R&D Systems (Wiesbaden, Germany), MAK-hIL-15 from Strathmann Biotec (Hamburg, Germany). Abs against ERK (C-16), pERK (E-4), pJNK (G-7), pp38 (D-8), FAK (C-20), and IL-15Rα (N-19) were purchased from Santa Cruz. Mouse anti-phosphotyrosine (anti-pTyr) Abs (RC20) were from BD Transduction Laboratories (Heidelberg, Germany) and anti-phosphoserine (anti-pSer) Abs were from Sigma-Aldrich (St. Louis, MO). Rabbit anti-goat, rabbit anti-mouse and goat anti-rabbit horseradish peroxidase conjugates (Amersham Pharmacia Biotech, Freiburg, Germany) were used as secondary Abs. IL-15-IgG2b fusion protein and recombinant soluble IL-15Rα (sIL-15Rα) were produced as previously described (9, 19). Briefly, the histidine-tagged sIL-15Rα recombinant protein was expressed in *E.coli* (strain BL21), extracted from bacteria under denaturing conditions and purified using a nickel agarose purification system (Qiagen, Dorking, UK) according to the manufacturer’s recommendations. The purity of recombinant sIL-15Rα was analyzed by SDS-PAGE followed by Coomassie blue staining, and Western blotting with specific anti-IL-15Rα Abs. The purified sIL-15Rα inhibited IL-15- but not IL-2-mediated proliferation of CTLL cells. Endotoxin from sIL-15Rα preparations was removed using Detoxi-Gel endotoxin removing kit (Pierce, Rockford, IL). The concentration of
endotoxin in all preparations used for cell activation was below 10 ng per 1 mg of sIL-15Rα.

Concentration of TNFα, IL-6, and IL-8 in cell supernatants was detected by standard ELISA procedure using DuoSet kits from R&D Systems.

**Cell culture, stimulation, acidic treatment, and transfection conditions** – PC-3 (ATCC CRL-1435) and LnCap (ATCC CRL-1740) human prostate carcinoma cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Human monocytes (hMo) were obtained from healthy donors by elutriation procedure. Monocytes were incubated with 10 ng/ml of IFNγ for 24 h prior to stimulation in order to increase the membrane IL-15 expression. Before stimulation, cells were washed twice with Dulbecco’s PBS and serum-starved for 4 h. For each assay, 5 x 10⁶ cells/ml were stimulated with sIL-15Rα (1 ng/ml) or anti-IL-15 (100 ng/ml) Abs for 5 or 15 min at 37°C. Activation was interrupted by adding 8-10 volumes of ice-cold PBS with 10 mM EDTA and 100 mM sodium vanadate. Cells were pelleted and frozen at -80°C before electrophoresis.

Acidic treatment was performed as described earlier (20). Cells were incubated in ice-cold glycine buffer (25 mM glycine, 150 mM NaCl, pH 3.0) for 10 min.

LnCap cells were transfected with IL-15LSP or IL-15SSP cDNA (16) in pcDNA3 mammalian expression vector (Invitrogen, Groningen, Netherlands) by electroporation (960 μF, 350V) using a Gene-Pulser (Bio-Rad, Munich, Germany). Stable transfectants were selected by limiting dilutions in the presence of G418 (1 mg/ml) (PAA Laboratories, Coelbe, Germany) for 4 weeks. Transient transfection was performed by
GenePORTER 2 transfection kit (Gene Therapy Systems, San Diego, CA). Cells were analysed 48 h post-transfection.

**Immunoprecipitation and Western blotting** – Cell pellets were lysed for 15 min on ice in 1% NP-40 cell extraction buffer: 20 mM Tris-HCl, pH 8.0, 15 mM NaCl, 2 mM EDTA, 10 mM sodium fluoride, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 10 mM phenylmethylsulphonylfluoride and 100 µM sodium vanadate (all reagents from Sigma-Aldrich). The detergent-insoluble material was removed by centrifugation at 13,000 rpm for 15 min at 4°C.

For immunoprecipitation studies, lysates containing 500 µg of proteins were immunoprecipitated overnight at 4°C by incubation in 0.5% NP-40 buffer with 2 µg/ml of Abs. Immunocomplexes were captured on protein A/G-agarose. After washing, pellets were re-suspended in SDS-PAGE sample buffer, boiled for 5 min and analysed in 10% SDS-PAGE. The resolved proteins were transferred onto nitrocellulose (Bio-Rad). Blots were blocked for 1 h in PBS with 0.05% Tween-20 and 3% BSA (Sigma-Aldrich). After incubations with first and second Abs and washing with PBS-T, visualization of specific proteins was carried out by an enhanced chemiluminescence (ECL) method using ECL Western blotting detection reagents (Amersham Pharmacia) according to the manufacturer’s instructions.

**RT-PCR** – Total RNA was extracted from cells using TRIZOL reagent (Invitrogen). cDNA was synthesized from 5 µg of total RNA using random oligonucleotides as primers and SuperScriptII™ kit (Invitrogen). cDNA was amplified by
PCR in a reaction mixture (20 µl) containing 2 µl of 10-fold PCR buffer with 1.5 mM 
MgCl₂, 250 µM of each dNTP, 200 nM 5’ and 3’ primers, and 1 U Taq DNA polymerase 
(Peqlab, Erlangen, Germany). The following primers were used: hIL-15 sense 5’-
GGCTTTGAGTAATGAGAATTTCGA-3’, anti-sense 5’-
ATCAGTTGCAATCAAGAATGTTTG-3’; hIL-15Rα sense 5’-
GCCAGCGCCACCCTCCACAGTAA-3’, anti-sense 5’-
GCCAGCGGGGGAGTTTGCCCTTGAC-3’; hIL-2Rβ sense 5’-
GAATTCCCTGGAGAGATGGCCACGGTCCCA-3’, anti-sense 5’-
GAATTCGAGGTTTGGAAATGGATGGACCAAGT-3’, hIL-2Rγ sense 5’-
AGCCCCAGCCTACACCTCCTCAGT-3’, anti-sense 5’-
TTAAAGCGGCTCCGAACACGAA-3’; hIL-6 sense 5’-
CCTTCGGTCCAGTGGCCTTCT-3’, anti-sense 5’-TCCAAAAGACCAGTGATGATT-
3’; hIL-8 sense 5’-GGGCTCTGTTGTAAGGAGTGC-3’, anti-sense 5’-
TGTGGATCTGGCTAGCAGA-3’; hTNFα sense 5’-
GGGCTCCAGGCGGTGCTTTC-3’, anti-sense 5’-
GCGGCTGATGGTGGGTAGGAGG-3’; and β-actin sense 5’-
GTGGGGCGCCCACCGCACA-3’, anti-sense 5’-
CTCCTTAATGTCAAGCAGGATTTC-3’. All primers were purchased from TIB 
MolBiol (Berlin, Germany).

Samples were amplified in a DNA Thermocycler (Eppendorf, Hamburg, 
Germany) for 30 cycles. Each cycle consisted of denaturation at 94°C for 15 sec, 
annealing at 60°C for 30 sec and elongation at 72°C for 30 sec, preceded by initial 
denaturation at 94°C for 5 min and followed by a final extension step at 72°C for 5 min.
To evaluate mRNA expression semi-quantitatively, in addition to the PCR product from 30 cycles, 15 μl of the PCR product from the 26, 28 cycles and the 32 cycles were run simultaneously. Aliquots of PCR products were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining. β-actin message was used to normalize the cDNA amount to be used. A mock PCR (without cDNA) was included to exclude contamination in all experiments.

_Wound healing assay_ – Exponentially growing cells (2 x 10⁶) were plated onto rat tail collagen (Roche, Heidelberg, Germany) coated (10 μg/ml) cell culture plates in complete growth media. After 8 hours, the monolayers of cells were wounded by manual scratching with a pipet tip, washed with PBS, placed into complete growth medium and photographed in phase-contrast with a Nikon microscope (Nikon Diaphot 300, Tokyo, Japan). Matched pair marked wound regions were photographed again after 18 (PC-3 cells) or 8 (LnCap cells) hours.

_Flow cytometric analysis_ – Cells were stained with monoclonal Abs or IL-15-IgG₂b fusion protein as previously described (21), and analyzed on a FACScalibur (Becton Dickinson, San Jose, CA) using CELL Quest software. Negative controls consisted of isotype-matched, directly conjugated, nonspecific Abs (BD PharMingen).

_Confocal Microscopy_ – Cells were seeded in concentration of 5 x 10⁴ cells/well in 12-well plates containing 18 mm glass coverslips. Next day coverslips were fixed with 2% paraformaldehyde for 10 min at room temperature. To stain cell membranes, fixed
cells were incubated with rhodamine-labelled wheat germ agglutinin (WGA, Molecular Probes, Leiden, Netherlands) for 15 min at room temperature, washed and permeabilized with 0.25% Triton X-100 followed by staining with primary (anti-IL-15, 1:100) and secondary (Alexa Fluor-488 donkey anti-goat IgG (H+L), 1:100, Molecular probes) Abs. Nuclei were stained using TOTO-3 dye (Molecular probes). The specimens were mounted in DABKO (1,4-Diazabicyclo [2,2,2]octane) anti-fading solution and analysed by scanning confocal microscopy (Leica TCS SP, Bensheim, Germany).

Data analysis – All experiments were performed in at least three independent assays, which yielded highly comparable results. Protein sequences were analysed using ProteinPredict software for sequence analysis and prediction of protein function and structure (http://www.embl-heidelberg.de/predictprotein/predictprotein.html). Blots were quantitated using ImageQuant TL software (Amersham Pharmacia). Data are summarized as mean ± SD. Statistical analysis of the results was performed by Student’s t test for unpaired samples. A p value of <0.05 was considered as statistically significant.
RESULTS

*PC-3 cells and IFNγ-activated monocytes express membrane-bound IL-15 –*

Biologically active form of membrane-bound IL-15 is constitutively expressed on the cell surface of human monocytes and several monocytic cell lines (12, 22), whereas IFNγ stimulation further upregulates its surface expression (12). Therefore, monocytes were stimulated with IFNγ for 24 h in all experiments described thereafter. Furthermore, we found that PC-3 prostate carcinoma cell line expresses only membrane-bound form of IL-15, whereas the IL-15Rα, IL-2Rβ, IL-2Rγ/γc chains were absent in these cells. To assess the expression of IL-15 in PC-3 cells and monocytes in more detail, we first analyzed its mRNA content by RT-PCR using a pair of primers that recognize two distinct IL-15 isoforms corresponding to the cytokine with the short (21 aa) and long (48 aa) signal peptide (16). As shown at Fig. 1A, PC-3 cells express only IL-15LSP isoform which is 513 bp in length, whereas monocytes express both IL-15LSP and IL-15SSP (494 bp plus additional 119 bp) isoforms. LnCap prostate carcinoma cell line, which does not express IL-15, was used as a control. Next, we analyzed the presence of IL-15 on the cell surface of PC-3 cells and IFNγ-activated monocytes by flow cytometry using anti-IL-15 Abs. These experiments confirmed that both PC-3 cells and monocytes express IL-15 on the cell membrane (Fig. 1B). However, the amount of membrane-bound IL-15 on monocytes was higher than in PC-3 cells. The expression of IL-15 in PC-3 cells was further corroborated by Western blotting experiments using immunoprecipitation with anti-IL-15 Abs (Fig. 1C). Isotype-matched Abs did not precipitate IL-15 and served as a control. The presence of IL-15 on the cell surface of PC-3 cells was also confirmed by confocal
microscopy (Fig. 1D). IL-15LSP associated with the cell membrane and was also found in the nuclei, which is in agreement with recent findings (11). Thus, only IL-15LSP isoform is present in PC-3 cells, whereas both IL-15LSP and IL-15SSP are found in IFNγ-activated monocytes.

*PC-3 cells do not express components of IL-15 receptor complex* – To exclude the presence of the IL-15Rα, IL-2Rβ, IL-2Rγ/γc chains in PC-3 cells, we assessed their expression by RT-PCR and FACS analysis. In parallel, the expression pattern of these receptor subunits in monocytes was evaluated. Indeed, IL-15Rα, IL-2Rβ, and IL-2Rγ/γc mRNA was absent in PC-3 cells (Fig. 2A), and staining with specific Abs did not reveal the presence of these receptor chains on the cell membrane (Fig. 2B). On the other hand, activated monocytes expressed all subunits of the IL-15R complex. The presence of IL-15-binding sites on the cell membrane was convincingly demonstrated by the ability of monocytes to bind IL-15-IgG2b fusion protein, whereas no IL-15-IgG2b-binding activity was detected in PC-3 cells (Fig. 2B).

A recent report by Musso et al. showed the presence of membrane-bound IL-15, which did not elute with the acetate buffer or trypsin treatment, suggesting that it is an integral membrane protein not associated with the IL-15R complex (12). Additionally, another study demonstrated the expression of biologically active IL-15 on the cell surface of TNFα-stimulated dermal fibroblasts (13). Conversely, Dubois et al. reported that IL-15 is non-covalently attached to the cell surface via its interaction with IL-15Rα and is released after acidic treatment (pH 3.0) of monocytes (20). Thus, we tested whether IL-15 upon PC-3 cells and monocytes could be removed from the cell surface by such acidic
treatment. Fig. 2C shows that no changes in the median fluorescence intensity (MFI) was detected in PC-3 cells after the treatment with the acidic buffer and staining with anti-IL-15 Abs as compared with control cells incubated in PBS. Notwithstanding, we observed a decrease in the amount of the cell surface-bound IL-15 on monocytes treated in a similar way. However, activated monocytes clearly retained membrane-bound IL-15 after the acidic treatment as confirmed by staining with specific Abs, whereas no bound IL-15-IgG₂b fusion protein was detected. These results strongly support the idea that the presence of membrane-bound IL-15 is not dependent on the IL-15R complex. The experiments using trypsin further corroborated this concept. Trypsin treatment renders the IL-15R complex to lose its ability to bind IL-15-IgG₂b fusion protein, which is used as receptor detector (12). Thus, it was reasonable to expect that if IL-15 detected on the cell membrane was indeed bound to its receptor, the whole ligand-receptor complex should be disrupted by trypsin, and either IL-15-IgG₂b fusion protein or anti-IL-15 antibody should not be bound to trypsinized cells. Although anti-IL-15 Abs still detected IL-15 on the cell membrane, IL-15-IgG₂b did not bind any longer to the cell surface (data not shown). Thus, these findings suggest that IL-15 likely exists on the cell surface of monocytes both in membrane-anchored and IL-15Rα-bound form, whereas PC-3 cells express only membrane-bound IL-15. While treatment with the acidic buffer or trypsin does not affect the expression of transmembrane IL-15, it elutes non-covalently bound IL-15 from IL-15Rα, resulting in the decrease in MFI.

Membrane-anchored IL-15 mediates reverse signaling that involves protein phosphorylation, and activation of MAP kinases and FAK in PC-3 cells and monocytes –
The phenomenon of reverse signaling or the ability of a membrane-bound ligand to induce activation of intracellular mediators has surfaced recently as an important mechanism to regulate qualitatively distinct cellular responses to specific stimuli (23). Many ligand-receptor pairs have been shown capable of bi-directional signal transduction (24-29). The presence of membrane-bound IL-15 on the cell surface of PC-3 cells and monocytes strongly invited to investigate its biological relevance for host cells. To test whether membrane IL-15 may mediate reverse signaling events, PC-3 and monocytes were treated with recombinant sIL-15Rα for different time intervals, and the pattern of phosphorylation of cellular proteins was assessed. Notably, the concentration of endotoxin in all sIL-15Rα preparations was extremely low and LPS in such concentration was not able to induce any signaling in PC-3 cells and monocytes (data not shown). Nevertheless, in order to verify that the observed effects of membrane IL-15 stimulation were genuine and not due to nonspecific activation through contamination with endotoxin associated with sIL-15Rα preparations, we also stimulated cells with anti-IL-15 Abs, whereas treatment with isotype-matched Abs was used as a control. Fig. 3A illustrates that stimulation with sIL-15Rα or anti-IL-15 Abs clearly induced tyrosine phosphorylation of several proteins with molecular mass ranging from 30 to 120 kDa in PC-3 cells and monocytes within the first minutes of action. Notably, the phosphorylation pattern in the cells stimulated with sIL-15Rα was slightly different than in the cells treated with anti-IL-15 Abs. Pre-incubation of sIL-15Rα by with 200-fold excess of IL-15 to saturate IL-15-binding sites or anti-IL-15Rα Abs to neutralize sIL-15Rα prior to the stimulation of PC-3 cells or monocytes with sIL-15Rα, respectively, was able to abolish the ability of sIL-15Rα to induce protein phosphorylation in both cell types (Fig.
Furthermore, the phosphorylation of these cellular proteins was still observed in the cells treated with the acidic buffer (Fig. 3B) or trypsin (data not shown) prior to incubation with sIL-15Rα (Fig. 3B) or anti-IL-15 Abs (data not shown).

Next, we sought to establish the identity of the phosphorylated molecules. To this end, the membranes were reprobed with Abs directed against several cellular proteins which reportedly play important roles in mediating downstream signaling from a wide range of the membrane-expressed receptor molecules. These experiments revealed that the stimulation with sIL-15Rα induced the phosphorylation of ERK and p38 (Fig. 3C). Activation of ERK was evaluated by employment of phospho-specific Abs directed against C-termini of ERK1 (p44) and ERK2 (p42) (both phosphorylated at Y204) (30). Interestingly, the predominant tyrosine phosphorylation of ERK2 (p42) was observed in human monocytes, and to a lesser extent, in PC-3 cells (Fig. 3C). Moreover, the phosphorylation of FAK was detected. However, the phosphorylation status of JNK (Fig. 3C) and the FAK-related kinase Pyk2 (data not shown) was not altered. Similar results were obtained after the stimulation with anti-IL-15 Abs, whereas treatment with the acidic buffer or trypsin was not able to abolish these effects (data not shown).

Noteworthy, the stimulation with a lower concentration of sIL-15Rα induced a stronger change in the phosphorylation of MAP kinases and FAK as compared with anti-IL-15 Abs (1 ng/ml of sIL-15Rα versus 100 ng/ml of anti-IL-15 Abs, respectively) (data not shown).

Given the ability of membrane-bound IL-15 to mediate the phosphorylation of these intracellular signaling molecules upon stimulation with sIL-15Rα or anti-IL-15 Abs, series of immunoprecipitation experiments were performed to test whether
membrane IL-15 may physically associate with FAK, ERK or p38 to induce their activation. However, no direct interactions between IL-15 and FAK or MAP kinases were detected (data not shown). Notwithstanding, we found that stimulation with sIL-15Rα or anti-IL-15 Abs resulted in the phosphorylation of IL-15 at serine but not tyrosine residues (Fig. 3C and data not shown). Taken together, these experiments demonstrate that membrane IL-15 is phosphorylated upon the stimulation by sIL-15Rα or anti-IL-15 Abs, and mediates tyrosine phosphorylation of MAP kinases and FAK both in PC-3 cells and activated monocytes.

Membrane IL-15 mediates signaling in LnCap prostate cancer cell line – To validate the ability of membrane-anchored IL-15 to mediate activation of signaling molecules, LnCap cell line which does not express endogenous IL-15 as well as components of the IL-15R complex (Fig. 1) was transiently or stably transfected with a vector coding for IL-15LSP or IL-15SSP. Fig. 4A illustrates that transfected cells express mRNA for the respective IL-15 construct. Interestingly, transient transfection with IL-15LSP clearly rendered the cells responsive to sIL-15Rα treatment, resulting in the characteristic pattern of phosphorylation, whereas the cells transfected with IL-15SSP were unresponsive (Fig. 4B). However, when cells were stably transfected with IL-15SSP or IL-15LSP, tyrosine-phosphorylated proteins were observed in cells expressing both constructs, although the phosphorylation was significantly stronger in the IL-15LSP-transfected cells (Fig. 4C). In accordance with these findings, reprobing of the membrane with specific Abs allowed us to identify ERK as one of the phosphorylated substrates (Fig. 4B and C). We did not observe the phosphorylation of JNK or p38 in these cells.
upon sIL-15Rα stimulation (data not shown). The stimulation with anti-IL-15 Abs mimicked action of sIL-15Rα, while isotype-matched Abs were ineffective (data not shown). Notably, the protein tyrosine phosphorylation was most prominent within the first 5-15 min of sIL-15Rα action, although the phosphorylation of ERK1/2 was sustained in both stably transfected cell lines for 30 min. Treatment with the acidic buffer or trypsin was not able to abrogate these effects (data not shown). Confocal analysis demonstrated that, upon stable overexpression in LnCap cells, both IL-15LSP and IL-15SSP were detected on the cell membrane, in the cytoplasm and nuclei (Fig. 4D), although the amount of IL-15LSP protein associated with the cell membrane was higher. The presence of IL-15SSP on the cell membrane provides a plausible explanation for the ability of this isoform to mediate signaling in LnCap cells stably overexpressing IL-15SSP protein.

Reverse signaling through membrane-anchored IL-15 triggers cytokine production — The MAP kinase cascade represents a key signaling pathway critical for the linking membrane receptors to cytoplasmic and nuclear effectors, and regulates a wide range of cellular functions. Given that treatment with sIL-15Rα clearly induced dramatic changes in the level of phosphorylated proteins in PC-3 cells and monocytes, including ERK and p38, we next tested the production of pro-inflammatory cytokines by these cells. The stimulation with sIL-15Rα or anti-IL-15 Abs for 24 h considerably enhanced the expression of TNFα, IL-6, and IL-8 by monocytes, as detected by RT-PCR (Fig. 5A) and ELISA (Fig. 5B), whereas treatment with the acidic buffer was not able to abolish these responses (Fig. 5B). Isotype-matched control Abs were without effect.
In contrast to monocytes, PC-3 cells abundantly release a number of cytokines into the culture medium in the absence of stimulation, and we were not able to detect changes in the cytokine secretion pattern of these cells by ELISA in response to the stimulation of membrane IL-15 (data not shown). However, a semi-quantitative RT-PCR analysis of IL-6, IL-8, and TNFα mRNA expression showed that stimulation of PC-3 cells for 8 h by sIL-15Rα or anti-IL-15 Abs enhanced the transcription level of IL-6 and IL-8 (Fig. 5C), while not affecting TNFα expression (data not shown). Thus, the reverse signaling through membrane-bound IL-15 leads to signal transduction and gene expression, significantly upregulating the production of IL-6, IL-8, and TNFα by human monocytes, and increasing the transcription level of IL-6 and IL-8 in PC-3 cells.

Reverse signaling through membrane-bound IL-15 promotes migration of prostate cancer cells – A number of studies have demonstrated strong correlations between elevated FAK expression and the increased invasive potential of human tumors by providing support for a role of this kinase in cell migration (31, 32). It has been shown that FAK is required for both integrin- and growth factor-stimulated cell motility (32, 33). FAK is highly tyrosine-phosphorylated at a number of different sites in either growing, integrin-stimulated, or in migrating cells (31). Given the observed phosphorylation of FAK upon the stimulation of membrane-bound IL-15, we performed wound healing scratch assay to determine whether reverse signaling through membrane IL-15 would affect cell migration. To this end, equal numbers of growing prostate cancer cells were placed into 6-well plates. After 18 h of culture, cells were cleared within a defined area by scratching with a pipet tip, washed with PBS, placed into complete
growth medium, photographed in phase-contrast (Fig. 6, 0 h), and allowed to migrate into the cleared area in the presence of sIL-15Rα or anti-IL-15 Abs, whereas cells cultured in the medium or treated with isotype-matched Abs were used as controls. In fact, PC-3 cells treated with sIL-15Rα or anti-IL-15 Abs had separated from the monolayer at the wound edges and significant numbers of these cells started to migrate into the cleared area after 18 h as compared with untreated or treated with isotype-matched Abs cells (Fig. 6, left panels). These results were further confirmed by experiments using LnCap cells transfected with IL-15LSP, whereas parental LnCap cells showed reduced migration properties (Fig. 6, right panels). Notably, the migration of LnCap cells expressing IL-15LSP into the scratched area was already detectable after 8 h. The time-course of total wound closure was significantly shorter for PC-3 and IL-15LSP-transfected LnCap cells after the stimulation with sIL-15Rα or anti-IL-15 Abs (nearly 26 or 16 h, respectively), as compared with control cells where wound closure was observed considerably later presumably due to continued cell proliferation. These results show that reverse signaling through membrane-bound IL-15 promotes cell migration and may contribute to the enhanced invasive properties of prostate cancer cells.
DISCUSSION

In this study, we provide experimental evidence that membrane-bound IL-15 is constitutively expressed on the cell surface of human prostate carcinoma PC-3 cell line and IFNγ-activated human monocytes, and mediates reverse signaling events in both cell types upon stimulation with recombinant sIL-15Rα or anti-IL-15 Abs. This signaling scenario involves the phosphorylation of members of the MAP kinase family ERK and p38, and FAK. Furthermore, the reverse signaling through transmembrane IL-15 upregulates the transcription level of IL-6 and IL-8, and dramatically enhances migratory properties of prostate cancer cells. Moreover, the stimulation of membrane-bound IL-15 results in a positive feedback also in monocytes, leading to a significant increase in the production of several pro-inflammatory cytokines, such as IL-6, IL-8, and TNFα. Given that mediators released by these innate immune response cells can profoundly influence adaptive immunity, and the ability of transmembrane IL-15 to activate neighboring cells, a bi-directional signaling through the membrane-bound IL-15 provides another striking example of a unique role of this pleiotropic cytokine in immune homeostasis.

Our results suggest that IL-15 on activated monocytes is directly anchored to the cell membrane rather than binds to the IL-15Rα chain (12, 20). This suggestion is in agreement with two recent studies which also reported the presence of membrane-bound, biologically active IL-15 on monocytes (12, 22). The bioactivity of IL-15 was convincingly demonstrated by the ability of mitomycin-treated or fixed monocytes to support proliferation of concanavalin A-stimulated human T cells or an IL-15-dependent CTLL cell line, respectively (12, 22). The presence of biologically active membrane IL-
15 was also shown upon the cell surface of TNFα-stimulated dermal fibroblasts (13).

Moreover, the stimulation with sIL-15Rα or specific Abs did not induce any detectable changes in the phosphorylation pattern of intracellular molecular targets in experiments using macrophages from IL-15-/- mice (data not shown). However, these results should be interpreted with caution and cannot be directly extended onto human system.

Notwithstanding, a decrease in the amount of membrane-bound IL-15 on activated monocytes observed after the acidic treatment indicates that, in addition to transmembrane IL-15, a certain number of IL-15 molecules are bound to IL-15Rα in the absence of exogenous IL-15. This binding reportedly enables IL-15Rα to recycle between endosomes and the cell membrane, and to present IL-15 in trans to neighboring target cells that express either only βγ or IL-15Rαβγ complex, thus prolonging their survival (20). Given, however, that human IL-15 has at least two binding sites for recombinant sIL-15Rα (34), it is tempting to speculate that the binding of IL-15Rα/IL-15 complex on monocytes to IL-15Rα on cells which express this high affinity chain might result in the formation of a receptor-ligand-receptor structure, where IL-15 is captured by two IL-15Rα molecules expressed on the surface of different cell types. This complex structure may theoretically be able to induce signaling in both directions, triggering activation of IL-15R-mediated signaling events in both cell types. Future studies must address the plausibility of such bi-directional signaling, and the contribution of the IL-2Rβ and IL-2Rγ/γc subunits to this process.

However, PC-3 human prostate cancer cells were clearly devoid of components of the IL-15R complex, and did not exhibit any reduction in the number of membrane-bound IL-15 molecules after the acidic treatment or trypsinization. The highly invasive
human prostate cancer PC-3 cell line was also found to express the α₅β₃ integrin; in contrast, the noninvasive LnCap prostate cancer cell line did not express this adhesion molecule (32) as well as membrane IL-15. Prostate carcinoma has been estimated to be the second leading cause of death due to cancer among men in the United States (35). A strong correlation between elevated FAK expression that controls cell motility and an increased invasive potential of human tumors has already been demonstrated (36). The observed phosphorylation of FAK in PC-3 cells may serve as a hallmark of the oncoming changes in the cellular behavior that promote cancer cell invasion. In fact, the stimulation of PC-3 cells with sIL-15Rα or anti-IL-15 Abs clearly enhanced the migratory properties of these cells, as assessed by their ability to migrate into the scratched area in wound healing assay. Moreover, sIL-15Rα or anti-IL-15 Abs were also able to promote migration of LnCap cells genetically modified to stably express membrane-bound IL-15LSP. In addition, members of MAP kinase family play a role in modulating integrin-mediated cell migration (37, 38). Thus, the activation of FAK and MAP kinase signaling pathways by membrane-anchored IL-15 renders prostate carcinoma cells to acquire a migratory phenotype in vitro. Furthermore, semi-quantitative RT-PCR showed that stimulation of PC-3 cells with sIL-15Rα or anti-IL-15 Abs induced an increase in the transcription level of IL-6 and IL-8, indicating thereby that the activation of the intracellular signaling molecules in PC-3 cells enhances gene expression. It remains to be elucidated whether the reverse signaling through transmembrane IL-15 may also affect the malignant properties of prostate carcinoma cells, such as survival, proliferation, and tumor invasion, leading to an accelerated development of cancer and enhanced metastatic potential in vivo.
Moreover, the existence of a natural soluble form of the high affinity IL-15R\(\alpha\) chain in the mouse serum and cell-conditioned medium (39) strongly suggests that, in addition to the interaction between the soluble ligand and the membrane-bound receptor or the membrane-coupled receptor-ligand pair, the binding of natural sIL-15R\(\alpha\) to IL-15 that is expressed in membrane-bound form might potentially represent a physiologically relevant mechanism to regulate distinct cellular responses. Importantly, the stimulation with sIL-15R\(\alpha\) induced a stronger change in the phosphorylation of MAP kinases and FAK at lower concentrations. A plausible explanation for this difference is provided by recent study (34), which identified four regions in human IL-15: the first one is located in the C-D loop and is recognized by a set of non-inhibitory Abs, whereas the second region is located in helix D and is recognized by two Abs that are inhibitory for IL-15 bioactivity. The two remaining regions are located in B or C helix, respectively, and react with a recombinant soluble form of IL-15R\(\alpha\). Thus, it seems likely that distinct IL-15 binding properties of sIL-15R\(\alpha\) versus anti-IL-15 Abs might account for the ability of sIL-15R\(\alpha\) to induce a stronger change in the phosphorylation of MAP kinases and FAK. Furthermore, the preferential tyrosine phosphorylation of ERK2 (p42) in response to the stimulation with sIL-15R\(\alpha\) or anti-IL-15 Abs is intriguing and additional experiments are required to address its biological significance. Notably, ERK1 and ERK2 have also been shown to be selectively or differentially activated by distinct stimuli, including EGF (40), TNF\(\alpha\) (41), insulin (42), and membrane immunoglobulin cross-linking (43).

However, the molecular mechanisms underlying the ability of transmembrane IL-15 to mediate activation of signaling molecules are unclear and deserve systemic exploration in follow-up experiments. IL-15, by analogy with TNF\(\alpha\), has been predicted
to have several consensus sequences, including potential phosphorylation sites for casein
kinase II and protein kinase C (data not shown). Importantly, we observed the
phosphorylation of transmembrane IL-15 at serine but not tyrosine residues, although no
physical association between IL-15 and MAP kinases or FAK was detected in the
immunoprecipitation experiments upon the stimulation with sIL-15Rα or anti-IL-15 Abs.
Noteworthy, the predicted cytoplasmic portion of IL-15LSP contains several serine
residues (Fig. 7). Current studies in our laboratory are focused on the mutational analysis
of IL-15LSP to identify which regions of this protein might be important for the
downstream signaling. Furthermore, it seems plausible that transmembrane IL-15 may be
internalized upon binding of the recombinant sIL-IL15Rα or anti-IL-15 Abs to mediate
the subsequent signaling events. Internalization from the plasma membrane has been
shown to occur either via clathrin coated pits to sorting endosomes and the recycling
compartment or via caveolae to smooth ER tubules (44). On the other hand, IL-15Rα has
been demonstrated to mediate recycling of IL-15Rα/IL-15 complexes between
endosomes and the cell surface, leading thereby to the persistence of IL-15 after
withdrawal of the cytokine from the culture medium (20). Although the essential role of
the IL-15Rα cytoplasmic domain for the recycling process has been demonstrated (20), it
is not clear yet whether the membrane-bound IL-15 may reappear again on the plasma
membrane after the interaction with its natural soluble receptor, contributing herewith to
the total amount of IL-15 upon the cell surface.

Despite widespread expression of IL-15 mRNA, detection of significant amounts
of IL-15 in cell culture supernatants has proven to be extremely difficult (18). It has been
suggested that the production of IL-15 is regulated at the level of protein translation and
intracellular trafficking rather than transcription. Two distinct isoforms of IL-15 exhibit different patterns of intracellular distribution and trafficking (16-18). Whereas IL-15LSP is found in the Golgi and early endosomes (17), and in the ER (18), IL-15SSP is present in the cytosol but does not co-localize either with the Golgi, early endosomes, or the ER (17, 18). However, both IL-15LSP and IL-15SSP may exhibit nuclear localization (11). Although the stable overexpression of IL-15SSP in LnCap cells resulted in the appearance of IL-15 on the cell membrane, it seems likely that under physiological conditions only IL-15LSP is expressed in a transmembrane form. This notion gains further support from the observation that PC-3 cells expressed only IL-15LSP isoform on the cell surface.

The ability of a membrane protein to induce bi-directional signal transduction was discovered several years ago. Most members of the TNF ligand (L) family exist as transmembrane proteins with relatively long intracellular domains, and a number of them are involved in reverse signaling. This phenomenon has been shown for such members of the TNFL family as TNFα, CD30L, CD40L, OX40L, CD137L, and FasL (24-29, 45). In addition, transmembrane ligands of the EPH family receptor Nuk are implicated in the regulation of axon guidance, fasciculation, and compartment definition (46). Outside-to-inside signal transduction via membrane TNFα induced the expression of E-selectin (CD62) on activated human CD4+ T cells (47). Another member of the TNFL family, CD137L, was shown to induce monocyte activation through bi-directional signaling (28). An implication in the reverse signaling of a casein kinase I motif present in the cytoplasmic domain of members of the TNFL family has been suggested (48). Members of the TNFL family, with the exception of lymphotoxin α, are type II transmembrane
proteins (24-29). They have large and evolutionary highly conserved leader sequence which spans the cell membrane, and a small N-terminal cytoplasmic portion. The presence of the extended signal peptide in IL-15LSP (48 aa in length) is rather unusual for secreted cytokines. Analysis of amino acid sequences of membrane-bound IL-15LSP and TNFα showed striking similarities in the protein structure organization (Fig. 7). Moreover, as mentioned above, IL-15 has been predicted to contain several consensus sequences, including potential phosphorylation sites for casein kinase II and protein kinase C, sharing thereby not only structural similarities with TNFα (data not shown).

Thus, IL-15LSP may naturally exist as a membrane-bound type II protein and, similar to the members of the TNFL family, can mediate biologically relevant reverse signaling events. Despite the structural relationship with IL-2 and the fact that both IL-15 and IL-2 belong to a four-helix bundle cytokine family (1, 2), post-translational modifications and functional features make it tempting to speculate that IL-15 may be more close to TNFα than to other cytokines. Members of the TNFL family are not secreted but rather shed from the cellular membrane by TNFα-converting enzyme (TACE), as was already demonstrated for TNFα (49). Experiments are underway to explore the ability of TACE to cleave membrane-bound IL-15.

Abnormalities of IL-15 expression have been reported in diverse neoplastic and inflammatory diseases, including adult T cell leukemia and certain autoimmune disorders like rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, chronic liver disease, and pulmonary sarcoidosis (4). It has been suggested that IL-15 may be at the apex of a cytokine cascade that includes downstream production of IL-1, IL-6, GM-CSF, and other biologically active substances (50). Our results demonstrate that highly
invasive human prostate cancer cells and IFNγ-activated human monocytes express membrane-anchored IL-15, which is able to activate a reverse signaling cascade that involves the phosphorylation of FAK and MAP kinases. Moreover, agonistic stimulation of membrane-bound IL-15 promotes migration of prostate cancer cells, and increases transcription or release of pro-inflammatory cytokines in PC-3 cells or human monocytes, respectively. Thus, IL-15 acts as a bipolar molecule, inducing bi-directional signaling both as a ligand and as a novel receptor protein that transmits signals to activate the cells expressing it on their surface. Given a pivotal role of IL-15 in many physiological processes or their pathological deviations and the bioavailability of the soluble high affinity IL-15Rα chain, the reverse signaling through membrane-bound IL-15 provides novel insights to our understanding of the pleiotropic properties of IL-15 within and beyond the immune system.
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REFERENCES

1. Burton, J. D., Bamford, R. N., Peters, C., Grant, A. J., Kurys, G., Goldman, C. K., Brennan, J., Roessler, E., and Waldmann, T. A. (1994) Proc. Natl. Acad. Sci. USA. 91, 4935-4939

2. Carson, W. E., Giri, J. G., Lindemann, M. J., Linett, M. L., Ahdieh, M., Paxton, R., Anderson, D. M., Eisenman, J., Grabstein, K. H., and Caligiuri, M. A. (1994) J. Exp. Med. 180, 1395-1403

3. Giri, J. G., Kumaki, S., Ahdieh, M., Friend, D. J., Loomis, A., Shanebek, K., DuBose, R., Cosman, D., Park, L. S., and Anderson, D. M. (1995) EMBO J. 14, 3464-3663

4. Waldmann, T. A., Dubois, S., and Tagaya, Y. (2001) Immunity 14, 105-110

5. Bulfone-Paus, S, Dürkop, H., Paus, R., Krause, H., Pohl, T., and Onu, A. (1997) Cytokine 9, 507-513

6. Armitage, R. J., Macduff, B. M., Eisenmann, J., Paxton, R., Grabstein, K. H. (1995) J. Immunol. 154, 483-490

7. Kennedy, M. K., Glaccum, M., Brown, S. N., Butz, E. A., Viney, J. L., Embers, M., Matsuki, N., Charrier, K., Sedger, L., Willis, C. R., Brasel, K., Morrissey, P. J., Stocking, K., Schuh, J. C., Joyce, S., and Peschon, J. J. (2000) J. Exp. Med. 191, 771-780
8. Lodolce, J. P., Boone, D. L., Chai, S., Swain, R. E., Dassopoulos, T., Trettin, S., and Ma, A. (1998) IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* **9**, 669-676

9. Bulfone-Paus, S., Ungureanu, D., Pohl, T., Lindner, G., Paus, R., Rückert, R., Krause, H., and Kunzendorf, U. (1997) *Nat. Med.* **3**, 1124-1128

10. Rückert, R., Asadullah, K., Seifert, M., Budagian, V., Arnold, R., Trombotto, C., Paus, R., and Bulfone-Paus, S. (2000) *J. Immunol.* **165**, 2240-2250

11. Pereno, R., Giron-Michel, J., Gaggero, A., Cazes, E., Meazza, R., Monetti, M., Monaco, E., Mishal, Z., Jasmin, C., Indiveri, F., Ferrini, S., and Azzarone, B. (2000) *Oncogene* **19**, 5153-5162

12. Musso, T., Calosso, L., Zucca, M., Millesimo, M., Ravarino, D., Giovarelli, M., Malavasi, F., Negro Ponzi, A., Paus, R., and Bulfone-Paus, S. (1999) *Blood* **93**, 3531-3539

13. Rappl, G., Kapsokefalou, A., Heuser, C., Rösler, M., Uğurel, S., Tilgen, W., Reinhold, U., and Abken, H. (2001) *J. Invest. Dermatol.* **116**, 102-109
14. Shinozaki, M., Hirahashi, J., Lebedeva, T., Liew, F. Y., Salant, D. J., Maron, R., and Kelley, V. R. (2002) *J. Clin. Invest.* **109**, 951-960

15. Quinn, L. S., Anderson, B. G., Drivdahl, R. H., Alvarez, B., and Argiles, J. M. (2002) *Exp. Cell. Res.* **280**, 55-63

16. Onu, A., Pohl, T., Krause, H., and Bulfone-Paus, S. (1997) *J. Immunol.* **158**, 255-262

17. Gaggero, A., Azzarone, B., Andrei, C., Mishal, Z., Meazza, R., Zappia, E., Rubartelli, A., and Ferrini, S. (1999) *Eur. J. Immunol.* **29**, 1265-1274

18. Kurys, G., Tagaya, Y., Bamford, R., Hanover, J. A., and Waldmann, T. A. (2000) *J. Biol. Chem.* **275**, 30653-30659

19. Ruchatz, H., Leung, B. P., Wei, X. Q., McInnes, I. B., and Liew, F.Y. (1998) *J. Immunol.* **160**, 5654-5660

20. Dubois, S., Mariner, J., Waldmann, T., and Tagaya, Y. (2002) *Immunity* **17**, 537-547

21. Bulanova, E., Budagian, V., Orinska, Z., Krause, H., Paus, R., and Bulfone-Paus, S. (2003) *J. Immunol.* **170**, 5045-5055
22. Neely, G. G., Robbins, S. M., Amahkwah, E. K., Epelman, S., Wong, H., Spurrell, J. C. L., Jandu, K. K., Zhu, W., Fogg, D. K., Brown, C. B., and Mody, H. (2001) *J. Immunol.* **167**, 5011-5017

23. Newell, M. K. and Desbarats, J. (1999) *Apoptosis* **4**, 311-315

24. Domonkos, A., Udvardy, A., Laszlo L., Nagy T., and Duda, E. (2001) *Eur. Cytokine Netw.* **12**, 411-419

25. Wiley, S. R., Goodwin, R. G., and Smith, C. A. (1996) *J. Immunol.* **157**, 3635-3639

26. Miyashita, T., McIlraith, M. J., Grammer, A. C., Yasushi, M., Attrep, A. F., Shimaoka, Y., and Lipsky, P. E. (1997) *J. Immunol.* **158**, 4620-4633

27. Stuber E., Neurath, M., Calderhead, D., Fell, H. P., and Strober, W. (1995) *Immunity* **2**, 507-521

28. Langstein, J., Michel, J., Fritsche, J., Kreutz, M., Andereesen, R., and Schwarz, H. (1998) *J. Immunol.* **160**, 2488-2494

29. Suzuki, I., and Fink, P.J. (1998) *J. Exp. Med.* **187**, 123-128

30. Crews, C.M., Alessandrin, A., and Erikson, R.L. (1992) *Science.* **258**, 478-480
31. Sieg, D. J., Hauck, C. R., and Schlaepfer, D. D. (1999) *J. Cell. Sci.* **112**, 2677-2691

32. Zheng, D.-Q., Woodard, A. S., Fornaro, M., Tallini, G., and Languino, L.R. (1999) *Cancer Res.* **59**, 1655-164

33. Hauck, C. R., Hsia, A. D., and Schlaepfer, D. D. (2000) *J. Biol. Chem.* **275**, 41092-41099

34. Bernard, J., Harb, C., Mortier, E., Quemener, A., Meloen, R. H., Vermot-Desroches, C., Wijdeness, J., van Dijken, P., Grötzinger, J., Slootstra, J. W., Plet, A., and Jacques, J. (2004) *J. Biol. Chem.* **279**, 24313-24322

35. Silverman, R. H. (2003) *Biochemistry* **42**, 1805-1812

36. Owens, L. V., Xu, L. H., Craven, R. J., Dent, G. A., Weiner, T. M., Kornberg, L., Liu, E. T. and Cance, W. G. (1995) *Cancer Res.* **55**, 2752-2755

37. Wei, J., Shaw, L. M., and Mercurio, A. M. (1998) *J. Biol. Chem.* **273**, 5903–5907

38. Eliceiri, B. P., Klemke, R., Stromblad, S., and Cheresh, D. A. (1998) *J. Cell. Biol.* **140**, 1255-1263
39. Budagian, V., Bulanova, E., Orinska, Z., Ludwig, A., Rose-John, S., Saftig, P., Borden, E. C., and Bulfone-Paus, S. (2004) *J. Biol. Chem.* (in press)

40. Keel, B. A., Hildebrandt, J. M., May, J. V., and Davis, J. S. (1995) *Endocrinology* 136, 1197-1204

41. Winston, B. W., and Riches, D. W. H. (1995) *J. Immunol.* 155, 1525-1533

42. Zhou, G. X., Meier, K. E., and Buse, M. G. (1993) *Biochem. Biophys. Res. Commun.* 197, 578-584

43. Gold, M. R., Sanghera, J. S., Wellstein, A., and Riegel, A. T. (1993) *Cell. Growth. Differ.* 4, 647-656

44. Nabi, R. (1999) *J. Cell. Sci.* 112, 1803-1811

45. Eissner, G., Kirchner, H., Kolch, W., Janosch, P., Grell, M., Scheurich, P., Andreesen, R., and Holler, E. (2000) *J. Immunol.* 164, 6193-6198

46. Holland, S. J., Gale, N. W., Mbamalu, G., Yancopoulos, G. D., Henkemeyer, M., and Pawson, T. (1996) *Nature* 383, 722-725
47. Harashima, S., Horiuchi, T., Hatta, N., Morita, C., Higuchi, M., Sawabe, T.,
Tsukamoto, H., Tahira, T., Hayashi, K., Fujita, S., and Niho, Y. (2001) J. Immunol. **166**, 130-136

48. Watts, A. D., Hunt, N. H., Wanigasekara, Y., Bloomfield, G., Wallash, D.,
Roufogalis, B. D., and Chaudhri, G. (1999) *EMBO J.*, **18**, 2119-2126

49. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M.
F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N.,
Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J.,
March, C. J., and Cerretti, D. P. (1997) *Nature* **385**, 729-733

50. McInnes, I.B., Leung, B.P., Sturrock, R.D., Field, M., and Liew, F.Y. (1997). *Nat.
Med.* **3**, 189–195
LEGENDS TO ILLUSTRATIONS

Fig. 1. **PC-3 cells and IFNγ-activated monocytes express membrane-bound IL-15.**  
**A,** RT-PCR analysis of two IL-15 isoforms expression in PC-3, LnCap cells, and monocytes. The amount of cDNA analysed was similar in different samples, as shown by PCR amplification of β-actin. A mock PCR (without cDNA) was used to exclude contamination.  
**B,** FACS analysis of membrane IL-15 expression in PC-3 cells and monocytes using anti-IL-15 Abs. White histograms refer to the background staining of isotype-matched control Abs.  
**C,** Western blotting analysis of IL-15 protein expression. IL-15 was immunoprecipitated from PC-3 cell lysates, the precipitates were subjected to 10% SDS-PAGE, and the membrane was probed by anti-IL-15 Abs. Precipitation with isotype-matched Abs was used as a negative control.  
**D,** Localization of IL-15 on the cellular membrane of PC-3 cells. Cells were fixed with 2% paraformaldehyde and incubated with WGA (labeled with rodamine, red) for 20 min, washed, permeabilized, and stained with anti-IL-15 Abs (ALEXA-488, green). Yellow color shows the co-localization of IL-15Rα with the cell membrane.

Fig. 2. **Membrane-bound IL-15 is not associated with the IL-15R complex in PC-3 cells and human monocytes.**  
**A,** RT-PCR analysis of IL-15Rα, IL-2Rβ and IL-2Rγ expression. Total RNA extracted from cells was reverse transcribed and subjected to PCR amplification using specific primers for IL-15Rα, IL-2Rβ, IL-2Rγ, and β-actin. The amplified products were electrophoresed on 1.5% agarose gel. cDNA from LnCap prostate cancer cell line was used as a negative control. A mock PCR (without cDNA)
was included to exclude contamination. The amount of cDNA analysed was similar in different samples, as shown by PCR amplification of β-actin. B, FACS analysis of the IL-15R complex expression on PC-3 cells and monocytes. Cells were incubated with IL-15-IgG2b fusion protein, or specific Abs to detect IL-15Rα, IL-2Rβ or IL-2Rγ expression, and analyzed by FACS. White histograms refer to the background staining by isotype-matched control Abs. C, Acidic treatment does not affect the expression of IL-15 on the cell membrane. PC-3 cells and monocytes were treated with the acidic buffer (pH 3.0) as described in Materials and Methods. Incubation in PBS was used as a control. For the detection of IL-15-IgG2b binding, cells were first incubated with the fusion protein and then with the acidic buffer, pH 3.0, stained with secondary Abs and analysed by flow cytometry. To confirm the presence of membrane IL-15 after the acidic treatment, cells were stained with anti-IL-15 or isotype-matched control Abs, followed by FACS analysis.

Fig. 3. Membrane-bound IL-15 mediates reverse signaling in PC-3 cells and monocytes. A, PC-3 cells and monocytes were activated with sIL-15Rα (1 ng/ml) or anti-IL-15 Abs (100 ng/ml) for 5 and 15 min. Then, the cell lysates were subjected to Western blotting and tyrosine-phosphorylated proteins were detected on the membrane using antiphosphotyrosine Abs. Incubation of sIL-15Rα with 200-fold excess of IL-15 or anti-IL-15Rα Abs prior to the stimulation of PC-3 cells or monocytes with sIL-15Rα, respectively, was used to saturate IL-15-binding sites and block sIL-15Rα-induced signaling. B, Acidic treatment does not affect protein phosphorylation mediated by the membrane-bound IL-15. Cells were treated with the acidic buffer or PBS (as a control).
for 10 min prior to treatment with sIL-15Rα. Tyrosine-phosphorylated proteins were subjected to 10% SDS-PAGE, transferred onto nitrocellulose membrane and detected using anti-phosphotyrosine Abs. C, Cells were stimulated with sIL-15Rα and phosphorylation of ERK, JNK and p38 kinases was detected using phospho-specific Abs. Positions of phosphorylated kinases are indicated on the right. Results are mean ± SD (*, \( p<0.05 \) compared with control). The equal amounts of ERK and p38 are shown as controls for loading. FAK or IL-15 were precipitated from cell lysates using specific Abs and phosphorylation of FAK or IL-15 was detected by probing the membrane with anti-phosphotyrosine or anti-phosphoserine Abs, respectively. Blots were re-probed with anti-FAK Abs after stripping to prove equal loading of precipitated FAK protein.

Fig. 4. **Membrane IL-15 mediates signaling in LnCap prostate cancer cells.** LnCap cells were transfected with IL-15LSP or IL-15SSP. Mock- (empty vector) transfected cells served as a control. A, Total RNA from transfected LnCap cells was isolated and analyzed by RT-PCR using primers amplifying two IL-15 isoforms. Bands corresponding to two IL-15 isoforms are indicated on the right. IL-15 expression in monocytes was used as a positive control. The amount of cDNA was equalized by PCR amplification of β-actin. Transiently (B) or stably (C) transfected LnCap cells were treated with 1 ng/ml of sIL-15Rα for the indicated time intervals. Then, the cells were lysed and pattern of tyrosine and ERK phosphorylation was analyzed by Western blotting. Blots were re-probed with anti-ERK Abs as a loading control. Results are mean ± SD (*, \( p<0.05 \) compared with control). D, Cellular localization of IL-15 in mock-, IL-15SSP- and IL-15LSP-transfected cells was analyzed by confocal microscopy. Cells were fixed with 2%
paraformaldehyde and stained with rodamine-labeled WGA (red), permeabilized and stained with anti-IL-15 Abs (green). Yellow color shows the co-localization of IL-15Rα with the cell membrane.

Fig. 5. **Reverse signaling through membrane IL-15 induces production of cytokines in human monocytes and PC-3 cells.**

**A**, Monocytes were stimulated with sIL-15Rα (1 ng/ml) or anti-IL-15 Abs (100 ng/ml) for 24 h. Unstimulated or stimulated with isotype-matched Abs cells were used as controls. Total RNA was extracted from cells, reverse-transcribed and expression of TNFα, IL-6, and IL-8 was detected by PCR using specific primers. The amount of cDNA analysed was similar in different samples, as shown by PCR amplification of β-actin. **B**, Untreated or acidic-buffer treated monocytes were stimulated with sIL-15Rα (1 ng/ml) or anti-IL-15 Abs (100 ng/ml) for 24 h. Supernatants from 24 h cell cultures were analysed for TNFα, IL-6, and IL-8 release by ELISA. Results represent mean ± SD of three independent experiments (*, p < 0.05). **C**, PC-3 cells were cultured for 8 h in medium (control) or in the presence of sIL-15Rα, anti-IL-15 or isotype-matched Abs. Then, total RNA was extracted and reverse-transcribed to cDNA. For semi-quantitative analysis, in addition to 30 cycles, 15 ml aliquots of the PCR product from 26, 28 and 32 cycles were also evaluated. The picture shows the amplified bands after 28 cycles. Beta-actin message was used to equalize the amount of cDNA used (lower panel). A mock PCR (no cDNA) was included as a negative control. The data represent three separate experiments with comparable results.
Fig. 6. **Stimulation of membrane IL-15 promotes migration of prostate carcinoma cells.** Cells were plated onto collagen-coated 6-well plates and allowed to grow in the presence of 10% FCS. After 18 hours, a wound was created by scratching with a pipet tip (0 h). The cells were washed and incubated in the presence of sIL-15Rα (1 ng/ml), isotype-matched Abs, and anti-IL-15 Abs (100 ng/ml) or in the absence of stimuli (medium) to allow migration into the wounded area. Matched pair marked wound regions phase-contrast images were taken 18 (PC-3 cells) or 8 (LnCap cells) hours later to assess cell migration.

Fig. 7. **A comparative analysis of human IL-15LSP and TNFα.** Both cytokines are predicted to exhibit a high degree of structural similarity, having rather short cytoplasmic and transmembrane domains, and relatively long extracellular regions (predicted using ProteinPredict software).
Fig. 1
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hIL-15LSP
NH₂-MRISKPHLSISIQCYLCLLNSH[FLTEAGIHFILGCFSAG]LPKTEAN
  cytoplasmic domain  transmembrane domain
WVNVISDLKKIEDLIQSMHIDATLYTESDVHSCKVTAKLKCFLLELQVISL
  extracellular domain
ESGDASIHTVENLIIANNLSNSNGNVTESGCKECEELEEKNIKEFLQSFV
  extracellular domain
HIVQMFINTS-COOH
  extracellular domain

hTNFα
NH₂-MSTESMIRDVELEEALPKKTGPGQSRRCL[FLSLSFSLIVAGATTLF]
  cytoplasmic domain  transmembrane domain
LLHFGVIGPQREEFPRDLSLISPLAQAVRSSRTPSDKPVAHVANPQAEGQ
  extracellular domain
LQWLNRANALLANGVELNQVLVPSEGLYLISQVLFKGQGCPSTHVL
  extracellular domain
LTHTISRIAVSYQTKVNLSAIKSPCQRETPEGAEAKPYEPIYLGGVQLEK
  extracellular domain
GDRLSAEINRPDYLDFAESGQVYFGIAL-COOH
  extracellular domain

Fig. 7
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Vadim Budagian, Elena Bulanova, Zane Orinska, Thomas Pohl, Ernest C. Borden, Robert Silverman and Silvia Bulfone-Paus

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