Interleukin-30 expression in prostate cancer and its draining lymph nodes correlates with advanced grade and stage

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Statement of Translational Relevance

Mortality for PCa is related to metastatic disease driven by both genetic and epigenetic alterations and multiple signals delivered within the tumor microenvironment. The specific ways in which the microenvironment regulates PCa progression are still poorly understood. In this article, we provide evidence that a newly discovered cytokine, namely IL-30, displays cancer-promoting effects in vitro, and that endogenous IL-30 expression is tightly linked with advanced PCa grade and stage. Our findings candidate this cytokine as a novel molecule shaping the tumor and lymph node microenvironment, and hence one to be targeted by modern integrated therapeutic approaches to metastatic disease.
Abstract

Purpose: The IL-27 cytokine subunit p28, also called IL-30, has been recognized as a novel immunoregulatory mediator endowed with its own functions. These are currently the subject of discussion in immunology, but completely unexplored in cancer biology. We set out to investigate IL-30's role in prostate carcinogenesis and its effects on human (h) prostate cancer (PCa) cells.

Experimental Design: IL-30 expression, as visualized by immunohistochemistry and real-time RT-PCR on prostate and draining lymph nodes from 125 PCa patients, was correlated with clinicopathological data. IL-30 regulation of hPCa cell viability and expression of selected gene clusters was tested by flow cytometry and PCR Array.

Results: IL-30, absent in normal prostatic epithelia, was expressed by cancerous epithelia with Gleason ≥7 of 21.3% of PCa stage I-III and 40.9% of PCa stage IV. IL-30 expression by Tumor Infiltrating Leukocytes (T-ILK) was higher in stage IV that in stage I-III PCa (P=0.0006) or in control tissue (P=0.0011). IL-30 expression in prostate draining Lymph Nodes (LN)-ILK was higher in stage IV than in stage I-III PCa (P=0.0031) or in control nodes (P=0.0023). The main IL-30 sources were identified as CD68+macrophages, CD33+/CD11b+myeloid cells and CD14+monocytes.

In vitro, IL-30 stimulated proliferation of hPCa cells and also down-regulated CCL16/LEC, TNFSF14/LIGHT, Chemokine-like-factor/CKLF and particularly CKLF-like MARVEL transmembrane-domain-containing-3/CMTM3 and greatly up-regulated ChemR23/CMKLR.

Conclusions: We provide the first evidence that IL-30 is implicated in PCa progression since I) its expression by PCa or T- and LN-ILK correlates with advanced disease grade and stage, and II) IL-30 exerts pro-tumor activity in hPCa cells.
Introduction

Prostate cancer (PCa) is the second most common cause of male cancer-related deaths (1). Mortality for PCa is related to metastatic disease driven by both genetic and epigenetic alterations and multiple signals delivered within the tumor microenvironment which are critical factors in skewing cancer toward dormancy or progression (2). Discrimination of molecular pathways driving tumor growth and progression is thus of crucial importance to identify novel prognostic markers and targets for advanced treatments.

The IL-27 cytokine subunit p28, also known as IL-30, is a 28 kDa protein which may be secreted by activated APCs, such as DCs (3, 4), and is biologically active (5) independent of the other cytokine receptor-like component, namely Epstein-Barr virus Induced gene 3 (EBI3) (6, 7). Thus, it has been recently recognized as a novel cytokine endowed with its own properties (7-10). However, while the immunoregulatory functions of IL-27 are fairly well known (11, 12) and the mechanisms of its anti-tumor effects are becoming progressively clear (13, 14), the involvement of IL-30 in cancer biology has not been explored, and its biological functions are currently a matter of controversy.

IL-30 has so far been shown to act as a natural antagonist of gp130-mediated signaling in response to IL-6 and IL-27 and thus resulting in anti-inflammatory effects (6), while more recently it has been reported to signal via IL-6 α-receptor (IL-6R) by recruiting a gp130 homodimer (15).

IL-6R (gp80) and gp130 are both expressed in human (h) PCa (16) and increase during prostate carcinogenesis (17).

Since we and others have found that PCa usually houses Tumor Infiltrating Leukocytes (T-ILK) in its stromal compartment (18-20), we first asked whether IL-30 is expressed in this context, and then assessed its effects in vitro on hPCa cell viability and expression of selected gene clusters.
Material and Methods

Patients and samples

We collected biological samples (cancer and normal prostate samples, and draining lymph nodes), clinical and pathological data of 125 patients (PCa patients), ages 54 to 73, treated by radical prostatectomy (RP) for PCa between 2009 and 2012 at the “S.S. Annunziata” Hospital, Chieti, Italy. Twenty-two of them were diagnosed with lymph-node metastasis at surgery. Preoperative androgen deprivation had not been used.

PCa samples were graded as Gleason score 5 \((n = 22)\), 6 \((n = 19)\), 7 \((n = 37)\), 8 \((n = 32)\), and 9 \((n = 15)\) and staged as \(\text{pT2, organ-confined cancer, (n = 69)}\) \([15 \text{T2aN0M0, 28 T2bN0M0, 21 T2cN0M0, and 5 T2cN1M0}]\) and \(\text{pT3, capsular penetration, (n = 56)}\) \([22 \text{T3aN0M0, 7 T3aN1M0, 17 T3bN0M0, and 10 T3bN1M0}]\).

The cases were then subdivided into two groups: (a) those with a Gleason score < 7 (41 cases), and (b) those with a Gleason score \(\geq 7\) (84 cases).

The cases were divided into two groups on the bases of pathological TNM classification (21): (a) those without (Stage I-III; 103 cases), and those (b) with metastases to the pelvic lymph nodes (Stage IV; 22 cases) (Table 1).

Normal prostates were obtained from 12 untreated patients ages 54 to 62 following prostatectomy for bladder cancer (control patients). They were histologically negative for PCa or benign prostatic hyperplasia. In addition, we obtained pelvic lymph nodes (control lymph nodes) from autopsies of 15 men, aged 51 to 65, who died for reasons other than cancer and were histologically free from PCa.

Patients entering the study had not received hormone or immunosuppressive treatments or radiotherapy, and were free from immune system diseases. Clinical and pathological stages were determined according to the 7th edition of the TNM Classification of Malignant Tumours (22). Tumor grade was assessed according to the Gleason scoring system from the prostate biopsies (23).
One-half of each normal or neoplastic tissue sample was fixed in 4% formalin and embedded in paraffin. The other was embedded in Killik frozen section medium (Bio-Optica, Milano, Italy), snap frozen in liquid nitrogen, and preserved at -80°C.

For histology, paraffin-embedded samples were sectioned at 4 μm and stained with H&E.

Written informed consent was obtained from patients. The study has been approved by the Ethical Committee for Biomedical Research of the Chieti University and Local Health Authority No. 2 Lanciano-Vasto-Chieti in PROT 1945/09 COET of 14/07/2009, and performed in accordance with the principles outlined in the Declaration of Helsinki.

**Immunohistochemistry**

For immunohistochemistry, formalin-fixed, paraffin-embedded sections were treated with H2O2/3% for 5 min. to inhibit endogenous peroxidase and then washed in H2O. Antigen was unmasked with heat-induced epitope retrieval in ethylenediaminetetraacetic acid (EDTA) buffer at pH 8. The slices were then held for 20 min. at room temperature. After washing in PBS/Tween-20, sections were incubated for 30 min. with the primary Ab (polyclonal rabbit anti-IL-30 [anti-IL-27p28, catalog: ab118910]; Abcam, Cambridge, UK) and immunocomplexes were detected using the Bond Polymer Refine Detection kit (Leica Biosystems, Newcastle Upon Tyne, UK) according to the manufacturer's protocol. Negative controls were carried out by replacing the primary Ab with 10% non-immune serum.

**Double and triple immunohistochemistry**

For double and triple immunohistochemistry, formalin-fixed, paraffin-embedded sections were deparaffinized, treated with H2O2/3% for 5 min. to inhibit endogenous peroxidase, and then washed in H2O.
Double stainings were performed with anti-IL-30 antibody (Ab) in combination with anti-CD11b (clone EP1345Y; Abcam), anti-CD14 (clone 7; Leica Biosystems), anti-CD33 (clone PWS44; Leica Biosystems) or anti-CD68 (clone PG-M1; Dako, Glostrup, DK) Abs and, triple immunostaining was performed with anti-IL-30 Ab in combination with both anti-CD33 and anti-CD11b Abs as we previously reported (24).

**Morphometric analyses**

IL-30 expression by primary tumours or lymph node metastases was evaluated using the following criteria based on 1) the widening of the staining expressed as the percentage of tumor or metastasis stained i.e.: $<50\%, \geq 50\% \leq 70\%$, and $>70\%$, and 2) the strength of the staining: defined as absent (−), slight (±), distinct (+) or strong (++)

Thus, IL-30 immunostaining was defined as:

- **positive**, when a) the widening was $>70\%$ and its strength range slight (±) to strong (++; or b) the widening was $50\% \leq 70\%$ and its strength range distinct (+) to strong (++; or

- **weakly positive**, when a) the widening was $50\% \leq 70\%$ and its strength was slight (±), or b) the widening was $=50\%$ and its strength range slight (±) to strong (++; or

- **negative**, when the widening was $\leq 50\%$ and its strength was slight (±) to absent (−).

T-ILK or Lymph Node (LN)-ILK expression of IL-30 was evaluated using the following score based on 1) the percentage of leukocyte expressing the cytokine, i.e. $<50\%, \geq 50\% \leq 70\%$, and $>70\%$, and 2) the strength of the cytokine staining that was defined as absent (−), scarce (±), distinct (+) or strong (++; or

Thus, IL-30 expression by T-ILK or LN-ILK was defined as:

- **strong**, when a) the staining involved more than 70% of leukocytes and its strength range scarce (±) to strong (++; or b) the percentage of positively stained leukocytes was $50\% \leq 70\%$ and the strength of the staining range distinct (+) to strong (++; or
• **distinct**, when a) the staining involved > 50% ≤ 70% of leukocytes and its strength was scarce (±), or b) the staining involved 50% of leukocytes and its strength range scarce (±) to strong (++);

• **scanty**, when the staining involved ≤ 50% of leukocytes and its strength was scarce (±) to absent (–).

Immunostained sections were examined by two pathologists with very good agreement (κ value = 0.82, 0.75 and 0.79 for evaluations of IL-30 staining in tumors, T-ILK or LN-ILK respectively) (25).

The rate of cancer cell positive for Ki-67, in the primary tumor or in lymph node metastasis, was assessed as reported (26). The proliferation rate was measured by quantifying the fraction of Ki-67 antigen-positive cells in immunostained tissue sections. The mean fraction of positive nuclei was estimated, and when one or more positive nuclei were present, it was estimated at 1%. For the analysis, the Ki-67 was grouped into two categories: 0-5% and >5% (*low* and *high* frequency).

**Laser Capture Microdissection (LCM) and real-time RT-PCR**

For LCM, 10 µm frozen sections from cancer and normal prostate specimens (of both control and PCa patients) were mounted on polyethylene naphthalate membrane-covered slides (P.A.L.M. Microlaser Technologies, Bernried, D), thawed at room temperature, and immersed in cold acetone (5 min.). Immediately after H&E staining, sections were used for LCM. Two sections per sample were analyzed. From 1000 to 1500 epithelial cells were cut and catapulted intact into the cap of an LPC-Microfuge Tube (P.A.L.M.), and RNA was immediately isolated with the RNeasy Plus Micro kit (Qiagen, Hilden, D). Tissue sections for microdissection of the stroma were labeled with an mAb that identifies fibroblasts and myofibroblasts (and excludes leukocytes, endothelial and epithelial cells) (clone TE-7, Millipore, Billerica, MA, USA). The stroma was isolated among the glands of low- and high-grade PCa, or in the histologically normal zones far from the PCa foci.
The real-time RT-PCR was carried out as reported (24). Primers for IL-30 and EBI3 were purchased from Qiagen (product number QT00236250 and QT01014104 respectively), while the primers for the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT) were designed and synthesized by Sigma-Aldrich Corporation (St. Louis, MO, USA): HPRT forward 5’-AGACTTGTGCTTTTCCTTGGTCAGG-3’ and HPRT reverse 5’-GTCTGGCTTATATCCAACACTTCG-3’. The sizes of the amplified cDNA fragments were 148 bp for IL-30, 88 bp for EBI3, and 101 bp for HPRT. The samples were processed in triplicate, and wells without added cDNA served as negative controls.

**Cell culture, antibodies, reagents, flow cytometry and immunocytochemistry**

The human PC3 (Interlab Cell Line Collection, CBA/IST San Martino, Genova Italy), 22Rv1 and LNCaP prostatic carcinoma cell lines (both from the American Type Culture Collection) were cultured in RPMI 1640 with 10% FCS (Seromed-BiochromKG, Berlin, Germany). Cell lines were obtained directly from the above mentioned cell banks that performed cell line characterizations by Short Tandem Repeat (STR) profile analysis. PC3, 22Rv1 and LNCaP were passaged in our laboratory for fewer than 6 months after resuscitation. Human recombinant (hr) IL-30 (IL27p28 Recombinant Protein, catalog: H00246778-P01; Abnova, Taipei City, RC) was used at 100 ng/ml, following titration experiments using 10-200 ng/ml. The expression of gp130 and IL-6Rα were analyzed using PE conjugated specific mAb (R&D Systems, Minneapolis, MN, USA). Isotype-matched Abs of irrelevant specificity (Caltag, Burlingame, CA, USA) were used as controls. Cells were run on Gallios flow cytometer (Beckman Coulter, Brea, CA, USA), acquiring at least 10⁴ events. Data were analyzed using Kaluza analysis software (Beckman Coulter). For immunocytochemical staining on PC3 cells, cytospin slides were fixed in acetone for 10 min. and then incubated for 30 min. with rabbit anti-IL-30 (Abcam) Ab or mouse anti-EBI3 (clone EL8; Leica Biosystems) Ab and immunocomplexes were detected using the Bond
Polymer Refine Detection kit (Leica Biosystems) according to the manufacturer's protocol. Negative controls were carried out by replacing the primary Ab with 10% non-immune serum.

Cell proliferation

The human PC3, LNCaP and 22Rv1 cells were cultured for 24, 48 and 72 hours with or without 10-200 ng/ml hrIL-30. Cells were incubated with 2 µM Carboxy-Fluorescein diacetate Succinimidyl Ester (CFSE) in RPMI 1% FCS for 15 minutes at 37 °C, washed in RPMI 10% FCS, plated and analyzed by flow cytometry at the above mentioned time points.

PCR Array

Total RNA was extracted, using the RNeasy micro kit (Qiagen), from PC3 and 22Rv1 cells cultured overnight with 100 ng/ml hrIL-30 or medium alone. Contaminant genomic DNA was removed by Dnase treatment (Qiagen). RNA was retrotranscribed by the RT2First Strand cDNA Synthesis kit (SABioscience, Frederick, MD, USA). Human Tumor Metastasis (code #PAHS-028Z) and Chemokines and Receptors (code #PAHS-022Z) RT2PCR Arrays and RT2 Real-Time SyBR Green/ROX PCR Mix were from SABioscience. PCR was done on the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Gene expression of hrIL-30 treated and control samples was analyzed separately in different PCR Array plates. For each plate, results were normalized on the median value of a set of housekeeping genes. Then, changes in gene expression between hrIL-30 treated and control samples were calculated using the ΔΔCt formula. Results from hrIL-30-treated and control samples, performed in duplicate, were pooled and analyzed by the software provided by the manufacturer. A significant threshold of 4-fold change in gene expression corresponded to $P < 0.001$. 
Statistical analysis

Differences in IL-30 protein expression between control prostates or lymph nodes and Stage I-III or Stage IV PCa and tumor draining lymph nodes were assessed by Fisher's Exact test. Between-group differences in the relative expression of IL-30 mRNA, by real-time RT-PCR, were assessed by one-way analysis of variance (ANOVA) and the difference between each pair of means was evaluated with the Tukey HSD test. Differences in proliferating cell percentages between primary cancers and correspondent lymph node metastases were assessed by Student’s t-test. The Spearman’s rank correlation coefficient (\(\rho\)) was used to examine the correlation between IL-30 protein expression and immunohistochemical staining for Ki-67 in primary PCa and lymph node metastases. The SPSS software, version 11.0 (IBM, Armonk, NY, USA) was employed, with \(P<0.05\) as the significance cut-off.

Results

IL-30 expression by PCa epithelia correlates with high-grade and advanced-stage PCa

To determine whether IL-30 is expressed in hPCa, we first performed immunohistochemistry with a mAb specific against this subunit of IL-27 in a large sets of prostate samples from patients who underwent RP for PCa, at different stages of disease, and from control patients.

IL-30 expression was absent in normal prostatic epithelia (from both PCa, \(n=125\), and control patients, \(n=12\)) and in high-grade prostatic intraepithelial neoplasia (HGPIN), whereas it was detected, ranging positive to weakly positive, in the cancerous epithelia of 22/103 PCa stage I-III (21.3%) and 9/22 metastatic PCa stage IV (40.9%) (Table 1, Fig. 1A). Additionally, we analyzed IL-30 mRNA expression levels by real-time RT-PCR and confirmed data obtained from tissue section immunostainings. IL-30 expression in normal prostate epithelium from PCa patients was comparable to that found in prostate epithelium from control patients. A significant difference (\(P=0.0132\)) was disclosed by Fisher's Exact test in the expression of IL-30 between control tissues
and PCa stage IV, since the percentages of IL-30 negative cases were 100% and 59% respectively. The strength of IL-30 expression in lymph node metastasis was usually comparable to that observed in the primary tumor (Fig. 1A). All the twenty-nine IL-30 positive PCa were graded as Gleason score ≥ 7.

**IL-30 expression by T-ILK, particularly CD68⁺ macrophages and CD33⁺ myeloid cells, correlates with advanced-stage PCa**

Analyses of the PCa stromal compartment revealed that IL-30 expression was lacking in malignant fibroblasts as in the normal counterpart, as assessed by real-time RT-PCR analyses of microdissected PCa stroma, while immunohistochemistry clearly localized IL-30 in the T-ILK. Its expression was particularly evident in T-ILK of metastatic PCa, as assessed by Fisher's Exact test, since the percentage of cases endowed with a distinct pattern of IL-30 staining was significantly higher in stage IV (63.6%) than in stage I-III PCa (29.1%; \(P=0.0006\)) or in control tissue (16.6%) \(P=0.0011\), while the percentage of cases showing a scanty IL-30 staining significantly prevailed in stage I-III PCa \(P=0.0031\) and control tissue \(P=0.0129\) (83.3% and 64.0%, respectively, versus 22.7% in stage IV PCa) (Table 2). Double immunostainings revealed that IL-30 production was mainly attributable to CD68⁺ macrophages and CD33⁺ myeloid cells infiltrating the prostatic stroma (Fig. 1B).

**Prostate draining lymph nodes express IL-30 in the metastatic stage of PCa progression**

Assessment of IL-30 production, by immunohistochemistry, in lymph nodes draining normal prostate, PCa stage I-III, and metastatic PCa stage IV, revealed that:

- the production of IL-30, particularly localized in the lymphatic sinuses, was wider and stronger in lymph nodes draining metastatic PCa stage IV, both those harboring the metastasis and those simply draining metastatic PCa, than in lymph nodes draining PCa stage I-III or control prostate.
draining lymph nodes, (Fig. 2A), since the percentage of lymph nodes endowed with a strong IL-30 staining was significantly higher in the cohort of PCa stage IV (50%) than in that of PCa stage I-III (18.4%, \( P=0.0031 \)) or the controls (0%, \( P=0.0023 \)). Inversely, the percentage of scanty stained lymph nodes was higher in the cohort of controls (66.6%; \( P=0.0498 \)) or PCa stage I-III (66.9% \( P=0.0034 \)) than in that of PCa stage IV (31.8%) (Table 3);

- in lymph nodes draining PCa stage IV, IL-30 production co-localized with CD68\(^+\)macrophages, CD14\(^+\)monocytes and CD33\(^+\)myeloid cells, part of which were also CD11b\(^+\), as assessed by triple immunostaining (Fig. 2B).

**IL-30 stimulates *in vitro* proliferation of hPCa cells and its expression *in vivo* by primary PCa and lymph node metastasis is associated with higher cancer cell proliferation**

Since IL-30 expression in PCa microenvironment and, particularly, in draining lymph nodes correlates with advanced stages of disease, we next try to clarify the mechanisms involved in the supposed tumor promoting activity of this cytokine, through *in vitro* experiments with hPCa cell lines.

It has been found that in the presence of EBI IL-30 binds to a gp130/WSX-1 heterodimer, otherwise it binds to the receptor complex composed of IL-6R and a gp130 homodimer (15). Therefore we first assessed the expression of gp130 and IL-6R in hPCa cell lines PC3, LNCaP and 22Rv1, by flow cytometry.

As shown in Fig. 3A, PC3 cells (left panels) express both gp130 (upper panels) and IL-6R\(\alpha\) (lower panels) at surface level, and hence may respond to IL-30. By contrast, the LNCaP (middle panels) and 22Rv1 cells (right panels) express IL-6R\(\alpha\) only. Immunocytochemical assessment of IL-30 and EBI3 expression in PC3 cells revealed that they expressed IL-30, but were negative for EBI3 (not shown), which should thus be absent in PC3 cell culture.

We next looked to see whether hrIL-30 regulates PC3 cell proliferation, metastasis-related gene
expression and chemokine/chemokine receptor gene expression.

PC3, LNCaP and 22Rv1 cells were cultured in the presence or absence of hrIL-30 (from 10 to 200 ng/ml) for 72 hours. An aliquot was harvested every 24 hours for CFSE intracellular staining. These experiments revealed that the optimal concentration of hrIL-30 that induced PC3 cell proliferation was 100 ng/ml. This event was clearly detected starting from 48 hours of treatment (Fig. 3B, left panel), as demonstrated by the higher CFSE intensity in untreated PC3 cells compared to hrIL-30 treated cells at this time point. Fifty ng/ml of hrIL-30 induced PC3 cell proliferation although to a lower extent compared to 100 ng/ml (not shown). hrIL-30 did not affect LNCaP and 22Rv1 proliferation at the same time points (Fig. 3B, middle and left panels respectively), as expected in consideration of the lack of the gp130 receptor in these PCa cell lines.

Since in patients' prostate samples the expression of IL-30 was particularly frequent in metastatic PCa, we performed double immunostainings with anti-Ki-67 and anti-IL-30 Abs in IL-30 positive primary tumors and related lymph node metastasis (total n = 9) versus IL-30 negative PCa and related metastasis (total n = 13). Cancer cell proliferation was higher in IL-30 expressing tumors and metastasis (8/9; 89%), than in IL-30-lacking samples (4/13; 31%) (Fig. 3C) (ρ = 0.574, P < 0.005226, by Spearman's rank correlation coefficient). The rate of cancer cell positive for Ki-67 was not significantly different between the primary tumor and related metastasis.

**hrIL-30 regulates the expression of various genes encoding chemokines or their receptors in the PC3 line**

To find out whether IL-30 also regulates cancer cell expression of metastasis-related genes or inflammatory chemokine/chemokine receptor related genes which may drive toward cancer progression, we next performed PCR Arrays, after co-culture with hrIL-30, of PC3 cells responsive to IL-30, and 22Rv1 cells, as negative control.

As shown in Fig. 3D, the chemokine/chemokine receptors PCR Array demonstrated that, in PC3
cells, hrIL-30 down-modulates the expression of C-C chemokine Ligand 16/CCL16, also known as Liver-Expressed Chemokine (LEC), (27) (7 fold down-regulation), Tumor Necrosis Factor Ligand Superfamily member 14 (TNFSF14), also known as LIGHT, (28, 29) (9.7 fold down-regulation), and Chemokine-Like Factor (CKLF) (30) (13.4 fold down-regulation). Other down-regulated genes were those coding for chemokine receptors, C-X-C chemokine Receptor 5 (CXCR5) (30 fold down-regulation), C-X-C chemokine Receptor 3 (CXCR3) (31.5 fold down-regulation) and C-C chemokine Receptor-Like 1 (CCRL1), also known as CCX-CKR, (37 fold down-regulation). The most down-regulated gene was the tumor suppressor and androgen co-repressor CKLF-like MARVEL Transmembrane domain containing 3/CMTM3 (31-33) (134 fold down-regulation).

hrIL-30 also up-regulated two molecules of the CKLF-like MARVEL Transmembrane domain containing 1, CMTM1 (34), and Chemokine-Like Receptor 1 (CMKLR1) (146 and 120 fold increase) the multifunctional receptor, also known as chemerinR23 (35). No significant modulation was observed of the gene expression profile included in the tumor metastasis PCR Array (not shown) in PC3 nor in 22Rv1 cells.

**Discussion**

This study provides the first evidence that the newly identified cytokine IL-30 (4, 6, 7), corresponding to the IL27p28 subunit, may be expressed in both the epithelial and stromal compartments of PCa. In the former, IL-30 expression is a hallmark of poorly differentiated, high-grade PCa and is observed in about 41% of cases that have metastatized to the regional lymph nodes. In the latter, IL-30 is basically lacking in malignant fibroblasts, as revealed by real-time PCR, whereas it is clearly produced by infiltrating leukocytes in ~77% of metastatic PCa. Endogenous IL-30, irrespective of its cellular source, is thus implicated in tumor progression and likely conditions tissue-specific “niche” microenvironment of cancer stem cell subsets and thus their metastatic potential (36). This assumption is corroborated by the frequency of a strong IL-30
expression in the regional lymph nodes from stage IV metastatic PCa when compared to those from stage I-III PCa or control lymph nodes.

This assumption is corroborated by the frequency of a strong IL-30 expression in the regional lymph nodes from stage IV metastatic PCa when compared to those from stage I-III PCa or control lymph nodes.

The intriguing finding that leukocyte expression of IL-30 in metastasis-free lymph nodes draining metastatic PCa is comparable or even stronger than in metastasis homing lymph nodes led us to suppose that locally released IL-30 paves the way for PCa seeding to regional lymph nodes. Indeed, CD68+ macrophages, CD14+ monocyte and CD33+CD11b+ myeloid cell populations, firmly recognized as main actors in tumor promotion (37-39), appear to be the major sources of IL-30 in both the primary tumor and the regional lymph node microenvironment.

The possibility that the availability of EBI3 in the tumor or lymph node microenvironment allows IL-30 to engage IL-27R on locally available leukocytes, and thus act like IL-27, is quite low, since our immunohistochemical and PCR analyses (not shown) have demonstrated that EBI3 is almost absent in the epithelia of both primary and metastatic lesions and barely detected in T- or LN-ILK, but far from IL-30.

Gp130 and IL-6Rα expression has been well documented in PCa epithelia and increases during progression (16, 17), suggesting that endogenous IL-30, via autocrine or paracrine signaling, may directly affect PCa cells. We addressed this issue by assessing the viability and expression profiles of selected genes in hPCa cell lines co-cultured with hrIL-30.

PC3 cells are endowed with gp130 and IL-6R. They alone respond to IL-30 stimuli with a significant increase of their proliferation and a quite distinctive regulation of specific chemokine/chemokine receptor genes. IL-30, in fact, was unable to affect the expression of canonical metastasis-related genes. Furthermore, it down-regulated the expression of the chemokine receptor genes CXCR3, CXCR5 and CCRL1, which may favor cancer cell migration (40-42).
Instead, the main effects of IL-30 on PCa cells are suppression of leukocyte chemoattractant expression and dramatic modulation of the expression of multifunctional molecules of the CKLF-like MARVEL Transmembrane domain-containing family (CMTM).

In particular, IL-30 significantly down-regulated PCa cell expression of immunoregulatory mediators such as CCL16 (27), TNFSF14 (28, 29) and CKLF (30) that may recruit and activate different leukocyte populations at the tumor site. The most down-regulated gene (134 fold) is that coding for CMTM3 which is physiologically highly expressed in the testes and deeply involved in male reproductive system maturation (31), inhibits prostate-specific antigen (PSA) expression and represses androgen receptor (AR) transactivation in LNCaP cells (33). Thus, CMTM3 down-regulation, may result in PSA increase and AR transactivation with related boosting of prostate cell proliferation. Moreover, CMTM3 which function as cancer cell growth inhibitor by inducing apoptosis, has been reported to be silenced by aberrant promoter methylation in many carcinomas (32). This epigenetic phenomenon may constitute the mechanism whereby IL-30 regulates CMTM3 expression and eventually boosts PCa cell proliferation.

Basically two chemokine-related genes were highly up-regulated and greatly susceptible to the effect of IL-30. They code for two molecules of the CKLF-like MARVEL transmembrane domain, namely CMTM1 (34) (146 fold increase), whose role is still unclear, and CMKLR1/chemR23 (120 fold increase). The latter is a multifunctional receptor, usually highly expressed by monocyte-derived human macrophages and immature plasmacytoid DCs (35), leading to their chemerin mediated migration. It has also been observed on acute monocytic leukemia cells and human glioblastoma cells (43) to mediate activation of calcium-triggered downstream signaling after interacting with specific chemerin isoforms. Though its functional role in this context remains to be investigated, it may drive the migration of cancer cells, as they were leukocytes, in response to an inflammatory tumor or lymph node chemerin-rich microenvironment.

Taken as a whole our results, by revealing 1) that IL-30 displays cancer-promoting effects in vitro,
and II) that endogenous IL-30 expression is tightly linked with advanced PCa grade and stage, strongly candidate this cytokine as a novel molecule shaping the tumor and lymph node microenvironment, and hence one to be targeted by modern integrated therapeutic approaches to metastatic disease.

Authors’ Contributions

Conception and design: E. Di Carlo.

Development of methodology: I. Airoldi, A. Zorzoli, S. Di Meo.

Acquisition of data: E. Di Carlo, C. Sorrentino.

Analysis and interpretation of data: E. Di Carlo, C. Sorrentino, S. Di Meo.

Writing, review, and/or revision of the manuscript: E. Di Carlo, I. Airoldi.

Administrative, technical, or material support: S. Esposito.

Study supervision: E. Di Carlo.
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Table 1. IL-30 expression by prostatic epithelia

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<th>PCa</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Stage I-III (n = 103)</td>
<td>Stage IV (n = 22)</td>
</tr>
<tr>
<td></td>
<td>(n = 12)</td>
<td>Negative</td>
<td>Weakly positive</td>
</tr>
<tr>
<td></td>
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<tr>
<td>Negative</td>
<td>12</td>
<td>81</td>
<td>16</td>
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*P < 0.05 Fisher's Exact test for comparisons between two classes, within the same category (negative, weakly positive or positive).

- **Negative:**
  - Stage I-III PCa vs Controls = 0.1188; Stage IV PCa vs Controls = 0.0132*; Stage IV PCa vs Stage I-III PCa = 0.0624.

- **Weakly positive:**
  - Stage I-III PCa vs Controls = 0.2116; Stage IV PCa vs Controls = 0.0691; Stage IV PCa vs Stage I-III PCa = 0.2191.

- **Positive:**
  - Stage I-III PCa vs Controls > 0.999; Stage IV PCa vs Controls = 0.2941; Stage IV PCa vs Stage I-III PCa = 0.3586.
Table 2. IL-30 expression by prostate infiltrating leukocytes

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 12)</th>
<th>Stage I-III (n = 103)</th>
<th>Stage IV (n = 22)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Scanty</td>
<td>Moderate</td>
<td>Strong</td>
</tr>
<tr>
<td>Scanty</td>
<td>10</td>
<td>2</td>
<td>-</td>
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</table>

*P < 0.05 Fisher's Exact test for comparisons between two classes, within the same category (scanty, moderate or strong).

- **Scanty:**
  - Stage I-III PCa vs Controls = 0.2176; Stage IV PCa vs Controls = 0.0011
  - Stage IV PCa vs Stage I-III PCa = 0.0006

- **Moderate:**
  - Stage I-III PCa vs Controls = 0.5056; Stage IV PCa vs Controls = 0.0129
  - Stage IV PCa vs Stage I-III PCa = 0.0031

- **Strong:**
  - Stage I-III PCa vs Controls = 0.6084; Stage IV PCa vs Controls = 0.2941; Stage IV PCa vs Stage I-III PCa = 0.3790
Table 3. IL-30 expression by lymph node infiltrating leukocytes

<table>
<thead>
<tr>
<th>Controls (n = 15)</th>
<th>Stage I-III (n = 103)</th>
<th>Stage IV (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scanty</td>
<td>10</td>
<td>69</td>
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<tr>
<td>Moderate</td>
<td>5</td>
<td>15</td>
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<tr>
<td>Strong</td>
<td>−</td>
<td>19</td>
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</tbody>
</table>

*P < 0.05 Fisher's Exact test for comparisons between two classes, within the same category (scanty, moderate or strong).

- **Scanty:**
  - Stage I-III PCa vs Controls > 0.9999;
  - Stage IV PCa vs Controls = 0.0498a;
  - Stage IV PCa vs Stage I-III PCa = 0.0034a.
- **Moderate:**
  - Stage I-III PCa vs Controls = 0.1313;
  - Stage IV PCa vs Controls = 0.4382;
  - Stage IV PCa vs Stage I-III PCa = 0.7439.
- **Strong:**
  - Stage I-III PCa vs Controls = 0.1253;
  - Stage IV PCa vs Controls = 0.0023a;
  - Stage IV PCa vs Stage I-III PCa = 0.0031a.
Figure Legends

Figure 1. IL-30 expression in the prostate. A, immunohistochemistry showed that expression of IL-30 (brown) was absent in normal epithelia, whereas it was evident in PCa epithelial and stromal compartments and in lymph node metastasis. B, IL-30 expression (brown) co-localized with CD68\(^+\) macrophages (fuchsia) (magnification in the inset), as indicated by arrows in the image to the right of the panel and, in part, (C) with CD33\(^+\) myeloid cells (fuchsia) (magnification in the inset), as indicated by arrows in the image to the right of the panel (A: x400; B and C, images to the left: x400, images to the right: x630, insets: x1000).

Figure 2. IL-30 expression in the prostate draining lymph nodes. A, IL-30 expression (brown) was scanty in lymph nodes draining normal prostate, scanty to distinct in PCa stage I-III draining lymph nodes, whereas it was strong (one half of the cases) in lymph nodes draining metastatic PCa stage IV, both without (second-last image of the panel) or with (last image of the panel) metastatic lesion. B, double immunohistochemistry revealed that expression of IL-30 (brown) in lymph node draining metastatic PCa was mostly attributable to CD68\(^+\) macrophages (fuchsia) and CD14\(^+\) monocytes (fuchsia). CD33\(^+\) myeloid cells (fuchsia) contribute to this IL-30 production, and CD11b\(^+\) cells (fuchsia), to a lesser extent. All insets show, in brick red staining, a magnification of IL-30 expressing immune cells. Triple immunostaining (image at the bottom right of the panel) showed IL-30 (brown) co-localization with CD33 (fuchsia), indicated by the arrowhead (brick red staining), and also with CD11b (blue), indicated by arrows and showed in the inset (dark staining). (A and B: x400; insets in B: x1000).

Figure 3. Expression of gp130 and IL-6R\(\alpha\) and modulation of cell proliferation and chemokine/chemokine receptors expression in hPCa cell lines by IL-30. A, flow cytometry of gp130 (upper panels) and IL-6R\(\alpha\) (lower panels) expression in PC3, LNCaP and 22Rv1 cell lines.
These experiments were performed at least in triplicate. B, flow cytometry assessment of PC3 cell proliferation induced by hrIL-30. CFSE staining in PC3 (left panel), LNCaP (middle panel) and 33Rv1 (right panel) cells cultured for 48 hours in the presence (dark profile) or absence (light gray profile) of 100 ng/ml IL-30. C, cancer cell proliferation, as assessed by Ki-67 staining (fuchsia), was higher in IL-30 positive (brown) PCa (image on the top right) and its lymph node metastasis (image on the bottom right), than in IL-30 negative PCa (image on the top left) and its lymph node metastasis (image on the bottom left). D, gene expression profiling of chemokine/chemokine receptor genes by PCR Array in PC3 cells cultured in the presence or absence of hrIL-30. Pooled results ± SD from two experiments performed in duplicate are shown. Histogram represents fold differences of individual mRNA between PC3 cells cultured in the presence or absence of IL-30. A significant threshold of 4-fold change in gene expression corresponded to $P < 0.001$. (C: x400).
Figure 1

A  Normal Prostate  Epithelial Compartment  Primary PCa  Stromal Compartment  LN Metastasis

B  Primary PCa

C  Primary PCa
Figure 3

A. PC3, LNCaP, 22Rv1

- Anti-gp130
- Isotype

B. gp130
- Anti-IL-6Rα
- Isotype

C. IL-30 Ki-67

Primary PCA

LNA Metastatic

D. Field difference

- CAM153
- CKN15
- C5902
- C5906
- C5910
- C5911
- C5915
- C5916
- C5918
- C5921
# Interleukin-30 expression in prostate cancer and its draining lymph nodes correlates with advanced grade and stage

Serena Di Meo, Irma Airoldi, Carlo Sorrentino, et al.

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