Introduction

Prostate cancer is the second most common cause of male cancer-related deaths (1). Mortality for prostate cancer 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 that may be secreted by activated antigen-presenting cells, such as dendritic cells (DCs; refs. 3, 4), and is biologically active (5), independent of the other cytokine receptor–like component, namely Epstein–Barr virus-induced gene 3 (EBI3; refs. 6, 7). Thus, it has been recently recognized as a novel cytokine endowed with its own properties (7–10). However, although the immunoregulatory functions of IL-27 are fairly well known (11, 12) and the mechanisms of its antitumor effects are becoming progressively clear (13, 14), the involvement of IL-30 in prostate carcinogenesis (17).

As we and others have found that prostate cancer 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.
Translational Relevance

Mortality for prostate cancer 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 prostate cancer progression are still poorly understood. In this article, we provide evidence that a newly discovered cytokine, namely interleukin (IL)-30, displays cancer-promoting effects in vitro, and that endogenous IL-30 expression is tightly linked with advanced prostate cancer grade and stage. Our findings reveal 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.

Materials and Methods

Patients and samples

We collected biologic samples (cancer and normal prostate samples, and draining lymph nodes), clinicopathologic data of 125 patients with prostate cancer, ages 54 to 73, treated by radical prostatectomy for prostate cancer 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.

Prostate cancer 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 $pT2$, organ-confined cancer [$n = 69$ ($15 T2aN0M0, 28 T2bN0M0, 21 T2cN0M0, and 5 T2cN1M0$)], and $pT3$, capsular penetration [$n = 56$ ($22 T3aN0M0, 7 T3aN1M0, 17 T3bN0M0, and 10 T3bN1M0$)].

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

Table 1. IL-30 expression by prostatic epithelia

<table>
<thead>
<tr>
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<th>Controls ($n = 12$)</th>
<th>Stage I–III ($n = 103$)</th>
<th>Stage IV ($n = 22$)</th>
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<tbody>
<tr>
<td></td>
<td>Negative&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Weakened positive&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Positive&lt;sup&gt;c&lt;/sup&gt;</td>
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<td></td>
<td>12</td>
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NOTE: $P < 0.05$ Fisher exact test for comparisons between two classes, within the same category (negative, weakly positive, or positive).

<sup>a</sup>Stage I to III prostate cancer versus controls $= 0.1188$; stage IV prostate cancer versus controls $= 0.0132$; stage IV prostate cancer versus stage I to III prostate cancer $= 0.0624$.

<sup>b</sup>Stage I to III prostate cancer versus controls $= 0.2116$; stage IV prostate cancer versus controls $= 0.0691$; stage IV prostate cancer versus stage I to III prostate cancer $= 0.2191$.

<sup>c</sup>Stage I to III prostate cancer versus controls $> 0.999$; stage IV prostate cancer versus controls $= 0.2941$; stage IV prostate cancer versus stage I to III prostate cancer $= 0.3586$.
in H2O. Antigen was unmasked with heat-induced epitope retrieval in EDTA buffer at pH 8. The slides were then held for 20 minutes at room temperature. After washing in PBS/Tween-20, sections were incubated for 30 minutes with the primary antibody [polyclonal rabbit anti-IL-30 (anti-IL-27p28, catalog: ab118910); Abcam] 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 antibody with 10% nonimmune serum.

**Double and triple IHC**

For double and triple IHC, formalin-fixed, paraffin-embedded sections were deparaffinized, treated with H2O2/3% for 5 minutes to inhibit endogenous peroxidase, and then washed in H2O. Double staining was performed with anti-IL-30 antibody in combination with anti-CD11b (clone EP1345Y; Abcam), anti-CD14 (clone 7; Leica Biosystems), anti-CD33 (clone PW544; Leica Biosystems), or anti-CD68 (clone PG-M1; Dako) antibodies and, triple immunostaining was performed with anti-IL-30 antibody in combination with both anti-CD33 and anti-CD11b antibodies as we previously reported (24).

**Morphometric analyses**

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

Thus, IL-30 immunostaining was defined as

- positive, when (i) the widening was >70% and its strength range slight (±) to strong (++), or (ii) the widening was >50% ≤70% and its strength range distinct (+) to strong (++);
- weakly positive, when (i) the widening was >50% ≤70% and its strength was slight (±), or (ii) the widening was = 50% and its strength range slight (±) to strong (++);
- negative, when the widening was ≤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 (i) the percentage of leukocytes expressing the cytokine, i.e., <50%, ≥50% ≤70%, and >70%, and (ii) the strength of the cytokine staining that was defined as absent (−), scarce (±), distinct (+), or strong (++).

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

- strong, when (i) the staining involved more than 70% of leukocytes and its strength range scarce (±) to strong (++), or (ii) the percentage of positively stained leukocytes was >50% ≤70% and the strength of the staining range distinct (+) to strong (++);
- distinct, when (i) the staining involved >50% ≤70% of leukocytes and its strength was scarce (±), or (ii) 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 (k value = 0.82, 0.75, and 0.79 for evaluations of IL-30 staining in tumors, T-ILK, or LN-ILK, respectively; ref. 25).**

The rate of cancer cell positive for Ki-67, in the primary tumor or in lymph node metastasis, was assessed as reported previously (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% to 5% and >5% (low and high frequency).

** Laser-capture microdissection and real-time reverse transcriptase PCR**

For laser-capture microdissection (LCM), 10-μm frozen sections from cancer and normal prostate specimens (of both control and prostate cancer patients) were mounted on polyethylene naphthalate membrane–covered slides (P.A.L.M. Microlaser Technologies), thawed at room temperature, and immersed in cold acetone (5 minutes). Immediately after H&E staining, sections were used for LCM. Two sections per sample were analyzed. From 1,000 to 1,500 epithelial cells were cut and catapulted intact into the cap of a laser pressure catapulting (LPC) microfuge tube (P.A.L.M. Microlaser Technologies), and RNA was immediately isolated with the RNeasy Plus Micro Kit (Qiagen). Tissue sections for microdissection of the stroma were labeled with a monoclonal antibody (mAb) that identifies fibroblasts and myofibroblasts (and excludes leukocytes, endothelial, and epithelial cells; clone TE-7; Millipore). The stroma was isolated among the glands of low- and high-grade prostate cancer, or in the histologically normal zones far from the prostate cancer foci.

The real-time reverse transcriptase PCR (RT-PCR) was carried out as reported previously (24). Primers for IL-30 and EB13 were purchased from Qiagen (product number QT00236250 and QT01014104, respectively), whereas the primers for the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1) were designed and synthesized by Sigma-Aldrich Corporation: HPRT forward 5’-AGACTTGCTTATATCCAACACTTCG-3’ and HPRT reverse 5’-GTCTGGCTTATATCCAACACTTCG-3’. The sizes of the amplified cDNA fragments were 148 bp for IL-30, 88 bp for EB13, 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), 22Rv1, and LNCaP prostatic carcinoma cell
lines (both from the American Type Culture Collection) were cultured in RPMI-1640 with 10% fetal calf serum (FCS; Seromed-BiochromKG). Cell lines were obtained directly from the above-mentioned cell banks that performed cell line characterizations by short tandem repeat profile analysis. PC3, 22Rv1, and LNCaP were passaged in our laboratory for fewer than 6 months after resuscitation.

Human recombinant (hr) IL-30 (IL-27p28 Recombinant Protein, catalog: H00246778-P01; Abnova) was used at 100 ng/mL, following titration experiments using 10 to 200 ng/mL. The expression of gp130 and IL-6Rα were analyzed using phycocerythrin-conjugated specific mAb (R&D Systems). Isotype-matched antibodies of irrelevant specificity (Caltag) were used as controls. Cells were run on Gallios flow cytometer (Beckman Coulter), acquiring at least 10^6 events. Data were analyzed using Kaluza analysis software (Beckman Coulter). For immunocytochemical staining on PC3 cells, cyto spun slides were fixed in acetone for 10 minutes and then incubated for 30 minutes with rabbit anti-IL-30 (Abcam) antibody or mouse anti-EBI3 (clone EL8; Leica Biosystems) antibody 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 antibody with 10% nonimmune serum.

Cell proliferation

The human PC3, LNCaP, and 22Rv1 cells were cultured for 24, 48, and 72 hours with or without 10 to 200 ng/mL hrIL-30. Cells were incubated with 2 μmol/L carboxyfluorescein 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 RNasy 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 RT2 First Strand cDNA Synthesis Kit (SABioscience). Human tumor metastasis (code #PAHS-028Z) and chemokines and receptors (code #PAHS-022Z) RT2 PCR 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). 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 to III or stage IV prostate cancer and tumor draining lymph nodes were assessed by the Fisher exact test. Between-group differences in the relative expression of IL-30 mRNA, by real-time RT-PCR, were assessed by one-way ANOVA and the difference between each pair of means was evaluated with the Tukey honestly significant difference (HSD) test. Differences in proliferating cell percentages between primary cancers and correspondent lymph node metastases were assessed by the Student t test. The Spearman rank correlation coefficient (p) was used to examine the correlation between IL-30 protein expression and immunohistochemical staining for Ki-67 in primary prostate cancer and lymph node metastases. The SPSS software, version 11.0 (IBM), was used, with P < 0.05 as the significance cutoff.

Results

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

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

IL-30 expression was absent in normal prostatic epithelia (from both prostate cancer, n = 125; and control patients, n = 12) and in high-grade prostatic intraepithelial neoplasia, whereas it was detected, ranging positive to weakly positive, in the cancerous epithelium of 22 of 103 prostate cancer stage I to III (21.3%) and 9 of 22 metastatic prostate cancer stage IV (40.9%; Table 1 and Fig. 1A). All the 29 IL-30–positive prostate cancers were staged as the significance cutoff.

IL-30 expression in lymph node metastasis was usually comparable with that observed in the primary tumor (Fig. 1A). All the 29 IL-30–positive prostate cancers were graded as Gleason score ≥7.

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

Analyses of the prostate cancer 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 prostate cancer stroma, whereas IHC clearly localized IL-30 in the T-ILK. Its expression was particularly evident in T-ILK of metastatic prostate cancer.
prostate cancer, as assessed by the Fisher exact test because 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 to III prostate cancer (29.1%; \( P = 0.0006 \)) or in control tissue (16.6%; \( P = 0.0011 \)), whereas the percentage of cases showing a scanty IL-30 staining significantly prevailed in stage I to III prostate cancer (\( P = 0.0031 \)) and control tissue (\( P = 0.0129 \); 83.3% and 64.0%, respectively, versus 22.7% in stage IV prostate cancer; Table 2). Double immunostainings revealed that IL-30 production was mainly attributable to 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, \( \times 400 \); B and C, images to the left, \( \times 400 \); images to the right, \( \times 630 \); insets, \( \times 1,000 \)).

Figure 1. IL-30 expression in the prostate. A, IHC showed that expression of IL-30 (brown) was absent in normal epithelia, whereas it was evident in prostate cancer epithelial and stromal compartments and in lymph node metastasis. B, IL-30 expression (brown) colocalized 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, \( \times 400 \); B and C, images to the left, \( \times 400 \); images to the right, \( \times 630 \); insets, \( \times 1,000 \)).

Table 2. IL-30 expression by prostate infiltrating leukocytes

<table>
<thead>
<tr>
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<th>Scanty (^a)</th>
<th>Moderate (^b)</th>
<th>Strong (^c)</th>
<th>Scanty (^a)</th>
<th>Moderate (^b)</th>
<th>Strong (^c)</th>
<th>Scanty (^a)</th>
<th>Moderate (^b)</th>
<th>Strong (^c)</th>
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<tbody>
<tr>
<td>Controls (( n = 12 ))</td>
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<td>2</td>
<td>~</td>
<td>66</td>
<td>30</td>
<td>7</td>
<td>5</td>
<td>14</td>
<td>3</td>
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<tr>
<td>Stage I–III (( n = 103 ))</td>
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<td>Stage IV (( n = 22 ))</td>
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</table>

NOTE: \( P < 0.05 \) Fisher exact test for comparisons between two classes, within the same category (scanty, moderate, or strong).

\(^a\)Stage I to III PCa versus controls = 0.2176; stage IV PCa versus controls = 0.0011; stage IV PCa versus stage I to III PCa = 0.0006.

\(^b\)Stage I to III prostate cancer versus controls = 0.5056; stage IV prostate cancer versus controls = 0.0129; stage IV prostate cancer versus stage I to III prostate cancer = 0.0031.

\(^c\)Stage I to III prostate cancer versus controls = 0.6084; stage IV prostate cancer versus controls = 0.2941; stage IV prostate cancer versus stage I to III prostate cancer = 0.3790.
Prostate draining lymph nodes express IL-30 in the metastatic stage of prostate cancer progression

Assessment of IL-30 production, by IHC, in lymph nodes draining normal prostate, prostate cancer stage I to III, and metastatic prostate cancer stage IV, revealed as follows:

- The production of IL-30, particularly localized in the lymphatic sinuses, was wider and stronger in lymph nodes draining metastatic prostate cancer stage IV, both without (second-last image of the panel) or with (last image of the panel) metastatic lesion. B, double IHC revealed that expression of IL-30 (brown) in lymph node draining metastatic prostate cancer 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) colocalization 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, ×400; insets in B, ×1,000.

Prostate draining lymph nodes express IL-30 in the metastatic stage of prostate cancer progression

- In lymph nodes draining prostate cancer stage IV, IL-30 production colocalized 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 prostate cancer and lymph node metastasis is associated with higher cancer cell proliferation

Because IL-30 expression in prostate cancer 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 EB13 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) express both gp130 (top) and IL-6Rα (bottom) at surface level, and hence may respond to IL-30. In contrast, the LNCaP (middle) and 22Rv1 cells (right) express IL-6Rα 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.

Table 3. IL-30 expression by lymph node infiltrating leukocytes

<table>
<thead>
<tr>
<th>Prostate cancer</th>
<th>Controls (n = 15)</th>
<th>Stage I–III (n = 103)</th>
<th>Stage IV (n = 22)</th>
</tr>
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<tr>
<td>Scanty</td>
<td>Moderate</td>
<td>Strong</td>
<td>Scanty</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>69</td>
<td>15</td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

NOTE: *P < 0.05* Fisher exact test for comparisons between two classes, within the same category (scanty, moderate, or strong).

aStage I to III prostate cancer versus controls > 0.9999; stage IV prostate cancer versus controls = 0.0498; stage IV prostate cancer versus stage I to III prostate cancer = 0.0034.

bStage I to III prostate cancer versus controls = 0.1313; stage IV prostate cancer versus controls = 0.4382; stage IV prostate cancer versus stage I to III prostate cancer = 0.7439.

cStage I to III prostate cancer versus controls = 0.1253; stage IV prostate cancer versus controls = 0.0023; stage IV prostate cancer versus stage I to III prostate cancer = 0.0031.

As shown in Fig. 3A, PC3 cells (left) express both gp130 (top) and IL-6Rα (bottom) at surface level, and hence may respond to IL-30. In contrast, the LNCaP (middle) and 22Rv1 cells (right) express IL-6Rα 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.

Figure 3. Expression of gp130 and IL-6Rα and modulation of cell proliferation and chemokine/chemokine receptor expression in hPCa cell lines by IL-30. A, flow cytometry of gp130 (top) and IL-6Rα (bottom) 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), LNCaP (middle), and 22Rv1 (right) 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) prostate cancer (image on the top right) and its lymph node metastasis (image on the bottom right), than in IL-30-negative prostate cancer (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, ×400.
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), as demonstrated by the higher CFSE intensity in untreated PC3 cells compared with hrIL-30–treated cells at this time point. About 50 ng/mL of hrIL-30 induced PC3 cell proliferation although to a lower extent compared with 100 ng/mL (not shown). hrIL-30 did not affect LNCaP and 22Rv1 proliferation at the same time points (Fig. 3B, middle and left, respectively), as expected considering the lack of the gp130 receptor in these prostate cancer cell lines.

Because the expression of IL-30 in patents’ prostate samples was particularly frequent in metastatic prostate cancer, we performed double immunostainings with anti-Ki-67 and anti-IL-30 antibodies in IL-30–positive primary tumors and related lymph node metastasis (total n = 9) versus IL-30–negative prostate cancer and related metastasis (total n = 13). Cancer cell proliferation was higher in IL-30–expressing tumors and metastasis (8 of 9; 89%) than in IL-30–lacking samples (4 of 13; 31%; ρ = 0.574, P < 0.005226, by the Spearman rank correlation coefficient; Fig. 3C). 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 that may drive toward cancer progression, we next performed PCR arrays, after coculture 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 downmodulates the expression of C–C chemokine ligand 16 (CCL16), also known as liver-expressed chemokine (7-fold downregulation; ref. 27), tumor necrosis factor ligand superfamily member 14 (TNFSF14), also known as LIGHT (9.7-fold downregulation; refs. 28, 29), and chemokine-like factor (CKLF; 13.4-fold downregulation; ref. 30). Other downregulated genes were those coding for chemokine receptors, C–X–C chemokine receptor 5 (CXCR5; 30-fold downregulation), C–X–C chemokine receptor 3 (CXCR3; 31.5-fold downregulation), and C–C chemokine receptor–like 1 (CCRL1), also known as CCX–CKR (37-fold downregulation). The most downregulated gene was the tumor suppressor and androgen corepressor Ckfl–like MARVEL transmembrane domain containing 3 (CMTM3; 134-fold downregulation; refs. 31–33).

hrIL-30 also upregulated two molecules, 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 IL-27p28 subunit, may be expressed in both the epithelial and stromal compartments of prostate cancer. In the former, IL-30 expression is a hallmark of poorly differentiated, high-grade prostate cancer 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 approximately 77% of metastatic prostate cancer. 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 prostate cancer when compared with those from stage I to III prostate cancer or control lymph nodes.

The intriguing finding that leukocyte expression of IL-30 in metastasis-free lymph nodes draining metastatic prostate cancer is comparable or even stronger than in metastasis homing lymph nodes led us to suppose that locally released IL-30 shields the way for prostate cancer seeding to regional lymph nodes. Indeed, CD68⁺ macrophages, CD14⁺ monocytes, and CD33⁺CD11b⁺ myeloid cell populations, firmly recognized as main actors in tumor promotion (37–39), seem 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 because 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 prostate cancer epithelia and increases during progression (16, 17), suggesting that endogenous IL-30, via autocrine or paracrine signaling, may directly affect prostate cancer cells. We addressed this issue by assessing the viability and expression profiles of selected genes in hPca cell lines cocultured with hrIL-30.

PC3 cells are endowed with gp130 and IL-6Rα. 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 downregulated 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 prostate cancer cells are...
suppression of leukocyte chemoattractant expression and dramatic modulation of the expression of multifunctional molecules of the CMTM family.

In particular, IL-30 significantly downregulated prostate cancer 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 downregulated 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 downregulation may result in PSA increase and AR transactivation with related boosting of prostate cell proliferation. Moreover, CMTM3, which functions 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 by which IL-30 regulates CMTM3 expression and eventually boosts prostate cancer cell proliferation.

Basically, two chemokine/chemokine receptor-relat ed genes were highly upregulated and greatly susceptible to the effect of IL-30. They code for CMTM1 (146-fold increase; ref. 34), 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 (i) that IL-30 displays cancer-promoting effects in vitro and (ii) that endogenous IL-30 expression is tightly linked with advanced prostate cancer grade and stage, strongly nominate 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Interleukin-30 Expression in Prostate Cancer and Its Draining Lymph Nodes Correlates with Advanced Grade and Stage

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