The IL-18 Antagonist IL-18-Binding Protein Is Produced in the Human Ovarian Cancer Microenvironment

Grazia Carbotti1, Gaia Barisione1, Anna Maria Orengo1, Antonella Brizzolara1, Irma Airoldi3, Marina Bagnoli2, Patrizia Pincirol1, Delia Mezzanzanica3, Maria Grazia Centurioni3, Marina Fabbi1, and Silvano Ferrini1

Abstract

Purpose: Interleukin (IL)-18 is an immune-enhancing cytokine, which induces IFN-γ production, T-helper 1 responses, and antitumor effects. In turn, IFN-γ stimulates IL-18–binding protein production, which blocks IL-18 activity. In view of the potential use of IL-18 in epithelial ovarian cancer (EOC) immunotherapy, here, we studied IL-18BP expression and its regulation by cytokines in EOC cells in vitro and in vivo.

Experimental Design: Expression and production of IL-18BP in EOC cell lines, primary ovarian carcinomas, and the corresponding normal tissues, patients’ serum, and ascites were investigated by immunochemistry, ELISA, screening of gene expression profiles, and reverse-transcription PCR.

Results: Analysis of gene expression profiles revealed that IL18BP mRNA is increased in EOC tumors compared with normal ovary cells. Release of IL-18BP was detectable in EOC sera and to a greater extent in the ascites, indicating production at the tumor site. Indeed, immunochemical analyses on cells isolated from the ascites and on tumor sections indicated that IL-18BP is expressed in both tumor cells and tumor-associated leukocytes, which displayed a CD3+CD20−NKp46−CD13+CD14low phenotype. EOC cell lines do not constitutively express IL-18BP. However, its release is inducible both by IFN-γ stimulation in vitro and by xenotransplantation of EOC cells in immune-deficient mice, suggesting a role for the microenvironment. In vitro experiments and immunochemistry indicated that IL-27 is also involved in IL-18BP upregulation in EOC cell lines and primary cells through STAT1 activation. Together, these data indicate that IL-18BP, which is produced in EOC in response to microenvironmental factors, may inhibit endogenous or exogenous IL-18 activity. Clin Cancer Res; 19(17); 4611–20. ©2013 AACR.

Introduction

Epithelial ovarian cancer (EOC) represents 80% of all ovarian malignancies, is frequently diagnosed at advanced stages, and has a poor 5-year survival rate (1). Evidence from several studies indicates that T lymphocytes capable of recognizing EOC cells are present at the tumor site (2, 3) and that T-cell–mediated immunity may have an impact on clinical course of EOC (3). However, similar to other tumors, EOC has the ability to escape from immune system control through several mechanisms (4, 5).

Several cytokines and chemokines were found to be elevated in serum and in the ascites of patients with EOC and have been implicated in tumor development, angiogenesis (6, 7), progression (8), drug resistance (9), or immune suppression (10). Some of these cytokines have been considered as serologic biomarkers of EOC (11). Among them, interleukin (IL)-18 was proposed as a potential biomarker by gene expression profiling (12) and, indeed, IL-18 levels were elevated in serum and ascites of patients with EOC (12, 13).

IL-18 is a proinflammatory and immune-enhancing cytokine, which induces IFN-γ production by T cells and natural killer (NK) cells, mediates T-helper 1 cell (Th1) polarization, and is involved in the defense from pathogens (14–16). IL-18 is synthesized as an inactive precursor (pro-IL-18), which is converted to a mature form (mat-IL-18) by caspase-1 (17). IL-18 is released from cells as both pro- and mat-IL-18 forms, but only mat-IL-18 can bind to IL-18R. Moreover, in preclinical tumor models, mat-IL-18 exhibited antitumor properties through the induction of an immune response, whereas pro-IL-18 had no activity (18, 19).

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M. Fabbi and S. Ferrini contributed equally to this work.

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Clinical Cancer Research

Human Cancer Biology

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Several cytokines and chemokines were found to be elevated in serum and in the ascites of patients with EOC and have been implicated in tumor development, angiogenesis (6, 7), progression (8), drug resistance (9), or immune suppression (10). Some of these cytokines have been considered as serologic biomarkers of EOC (11). Among them, interleukin (IL)-18 was proposed as a potential biomarker by gene expression profiling (12) and, indeed, IL-18 levels were elevated in serum and ascites of patients with EOC (12, 13).

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IL-18 present at high levels in EOC ascites is predominantly
defective caspase-1 expression or activation, whereas normal ovarian epithelial cells secrete mat-IL-18 (20). Indeed, IL-18 present at high levels in EOC ascites is predominantly the inactive pro-IL-18 (13), although we cannot exclude that low levels of mat-IL-18, eventually present, may contribute to the immune response.

IL-18 binding protein (IL-18BP) is an inhibitor of IL-18 activity, as it binds the mature form of this cytokine and blocks its interaction with IL-18R (21) and its isoform “a” is the mostly expressed form (22). IL-18BP is produced by monocytes and macrophages (23) and by prostatic (24) and colorectal tumor cells (25) in response to IFN-γ stimulation. IFN-γ is the physiologic inducer of IL-18BP, which in turn inhibits IL-18 biologic activity in a negative feedback loop. IL-18BP accumulates in the serum in chronic renal failure, in which it may contribute to the defective immune response (26).

To further address the biologic role of endogenous IL-18 in human EOC, we conducted an integrated analysis of IL-18 and IL-18BP in patients with EOC. The study of IL-18BP also seemed relevant, as IL-18–based immunotherapy is being evaluated in patients with EOC (refs. 27, 28; NCT00659178) and high levels of IL-18BP blocks its interaction with IL-18R (21) and its isoform “a” is the mostly expressed form (22). IL-18BP is produced by monocytes and macrophages (23) and by prostatic (24) and colorectal tumor cells (25) in response to IFN-γ stimulation. IFN-γ is the physiologic inducer of IL-18BP, which in turn inhibits IL-18 biologic activity in a negative feedback loop. IL-18BP accumulates in the serum in chronic renal failure, in which it may contribute to the defective immune response (26).

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Materials and Methods

Cells and cell treatments

The human EOC cell lines SKOV3 (ATCC), A2780 (ICLC), A2774 (IST, Genoa, Italy), and OVCAR3 (INT, Milan, Italy) were grown in RPMI-1640, with L-glutamine, 10% FCS, and antibiotics (Lonza). NK-92 cells (ATCC) were grown in medium containing 600 IU IL-2 (Novartis). A vial of each cell line master stock was recently genotyped using the Cell ID System (Promega) and GeneMapper software, version 4.0.

Cells (50 × 10³/well) were seeded in 24-well plates in culture medium. The following day, culture medium was replaced with medium with or without human recombinant IFN-γ (PeproTech), human recombinant IL-27 (R&D System) or human recombinant IL-35 (Enzo Life Sciences). For IL-27R blocking experiments, an anti-gp130 antibody was added [monoclonal antibody (mAb) 228, R&D Systems]. Treatment was carried out for 48 hours. Conditioned media were then collected, centrifuged, and used for IL-18BP detection.

RT-PCR analysis of IL18BP mRNA expression

Cells were detached by trypsin and washed, and total RNA was isolated by the NucleoSpin RNA II kit (Macherey-Nagel) and reverse-transcribed using the SuperScript II Reverse Transcriptase (Invitrogen). Quantitative RT-PCR analysis was conducted using the following primers: POLR2A upper primer GACAATGCAAGAAGCTGG, lower primer GGAAGAGACTCATCATCC; GAPDH upper prim-
er GAAGGTGAGCTGAGGT, lower primer CATGGGTGGAATCATITGGAA; IL18BP upper primer GTGTCAGGCG-
ATTCCAGTGGT, lower primer GGAAGGCTCAATGAGGAAC. Amplification was carried out by the Mas-
tercycler ep realplex instrument (Eppendorf International) using the iQ™ SYBR Green Supermix system (Bio-Rad Laboratories). Relative quantification of mRNAs was calculated by the ΔΔCt method. For semiquantitative RT-PCR, 2 µL of cDNA was separately amplified with 0.25 U of Taq DNA Polymerase (Roche) in the presence of 1 µmol/L of the following primers: IL18BP upper primer ACCATG-
AGACACAAGTGCCACAGC, lower primer TTACCCGT-
CTCAGTGACGACTG; housekeeping gene ACTB upper primer GCCAATGTGATGACCCCG, lower primer GCTG-
GAAGGTGAGCTGAGGT, housekeeping gene ACTB upper primer GCCAATGTGATGACCCCG, lower primer GCTG-
GAAGGTGAGCTGAGGT. PCR products were analyzed on 1% agarose gel stained with ethidium bromide.

Patients

Clinical samples were obtained upon written informed consent and previous approval by the Institutional Review Board from patients and tumor-free, age-matched (median = 60 years; range = 43–75) women. All 55 patients showed evidence of disease and untreated or off treatment for at least 2 months (Table 1). Ascitic fluids were collected during surgical procedures. Tumor histopathology, grade, and stage were assigned according to the International Federation of Gynecology and Obstetrics (FIGO) criteria.

EOC xenotransplant model

Female homozygous nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (The Jackson Laboratory) were bred in-house. Nude mice were from Janvier. The experiments were carried out according to the
National Regulation on Animal Research Resources and approved by the Institutional Review Board. Six-week-old NOD/SCID animals were injected intraperitoneally with \(5 \times 10^6\) SKOV3 or A2774 cells. When ascites developed, animals were euthanized and blood and ascites collected. Nu/Nu mice were surgically implanted in the left ovary with \(2 \times 10^6\) SKOV3 cells. Tumor masses were excised and fixed in 10% buffered formalin.

ELISA for IL-18, IL-18BP, and IFN-\(\gamma\)

Samples were tested with commercially available ELISA kits for human IL-18 (MBL), IL-18BPa (DuoSet R&D Systems), and IFN-\(\gamma\) (Quantikine, R&D Systems). Assays were conducted in duplicate and background values were subtracted.

Immunohistochemistry

Immunohistochemistry detection of IL-18BP was conducted on sections of formaldehyde-fixed paraffin-embedded cell pellets or tumors explanted from mice or on commercially available tissue microarrays of patients with EOC (Super Bio Chips). Antigen retrieval was done with high-pH citrate buffer in a microwave oven. The sections were immunostained using rabbit anti-IL-18BP (clone EP10889, Epitomics), anti-EBI3 (Novus Biologicals Europe), or anti-IL27A (LifeSpan Biosciences) overnight at 4°C. The antibody complex was revealed with the EnVision+ System-Peroxidase (Dako) and 3-amino-9-ethylcarbazole (AEC, Calbiochem). The sections were counterstained with modified Mayer hematoxylin and mounted in PermaFluor (Thermo Scientific). The sections were observed with a Nikon Eclipse 80i light microscope equipped with a color camera imaging head, using a \(\times 40\) objective.

Public EOC datasets of gene expression

We explored IL18BP gene expression in our dataset (Iorio, GSE19532) and in public EOC datasets processed through the Affymetrix HG U133 Plus 2.0 arrays (Tothill, GSE9891 and Anglesio GSE12172). Only type II tumors (31) were considered for all three datasets when exploring pattern of correlation among different relevant genes. We considered only the platforms including probes 222868_s_at because this is the only probe that comprises the IL-18BPa isoform. The Tothill dataset consists of 18 low malignant potential (LMP), 10 type I and 210 type II EOC; the Anglesio dataset consists of 30 LMP and 58 type II EOC cases; and the Iorio dataset includes 17 EOC high-grade tumors, two preparations of normal ovarian surface epithelial cells (OSE), and six EOC cell lines. Raw data were downloaded from GEO and normalized through the RMA algorithm (with Expression Console software, Affymetrix) except for the Iorio dataset, for which the processed matrix was downloaded from GEO.

Statistical analysis

The normal distribution of the data was verified before applying parametric tests by transforming data to logarithms. The one-way ANOVA and appropriate multiple comparison tests were used to compare expression levels between patients and control subjects. The paired Student \(t\) test was used when appropriate. Parametric methods were used to examine the correlation among gene or protein expression levels (Pearson correlation coefficient). An \(\alpha\) level of 0.05 was used for all statistical tests. GraphPad Prism 5.0d software and R statistical language (http://www.R-project.org; version 12.2) were used.

Western blot analysis

For the analysis of phosphorylated proteins, \(10^7\) EOC cells were incubated for 10 minutes at 37°C with or without 20 ng/mL of rIL-27 in 0.5 mL of medium. Cells were lysed in 100 \(\mu\)L of buffer containing 1 mmol/L sodium orthovanadate. Lysates were resolved under reducing conditions by 10% SDS-PAGE and analyzed by Western blotting using rabbit anti-phospho-STAT1 (pY701) anti-serum (Cell Signaling Technology) or anti-\(\beta\) actin or tubulin mAb (Sigma) and chemiluminescence detection.

### Table 1. Distribution by tumor characteristics for EOC patients with evidence of disease

<table>
<thead>
<tr>
<th>Cases at diagnosis, (n)</th>
<th>Cases at relapse, (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>48</td>
</tr>
<tr>
<td><strong>Age at diagnosis, y</strong></td>
<td></td>
</tr>
<tr>
<td>&gt;55</td>
<td>35</td>
</tr>
<tr>
<td>≤55</td>
<td>12</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>15</td>
</tr>
<tr>
<td>II</td>
<td>7</td>
</tr>
<tr>
<td>III</td>
<td>22</td>
</tr>
<tr>
<td>IV</td>
<td>3</td>
</tr>
<tr>
<td><strong>Histotype</strong></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>30</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>10</td>
</tr>
<tr>
<td>Mucinous</td>
<td>3</td>
</tr>
<tr>
<td>Others</td>
<td>3</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td><strong>Borderline</strong></td>
<td>1</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.

*Stage at diagnosis.
Results

Gene expression of IL18BP in EOC tumors

IL18BP mRNA was first to be analyzed in our gene expression dataset (Iorio) including primary EOC tumors, tumor cell lines, and normal ovarian surface epithelial (OSE) cells. A significantly higher expression intensity of IL18BP was observed in tumor samples than in EOC cell lines (P < 0.0001) and in OSE cells (P = 0.01; Fig. 1A). In addition, IL18BP expression was significantly higher in type II (high-grade) tumors (31) relative to low malignant potential tumors, in two independent datasets (Tothill and Anglesio, P = 0.0043 and P < 0.0001, respectively), suggesting a possible relationship of high IL18BP expression with malignancy (Fig. 1B).

IL-18BP levels are elevated in EOC sera and ascites

We then tested IL-18BP serum levels in 48 patients with EOC (Table 1), all with evidence of disease and untreated, in 7 patients at relapse, and in 13 age-matched healthy women by ELISA for IL18BPa, the major isoform of IL-18BP (22). IL-18BP serum levels were higher in both untreated (onset, mean ± SD = 11.06 ± 5.7; median = 9.45 ng/mL, P = 0.03) and in patients with relapsing EOC (mean ± SD = 11.76 ± 3.2; median = 11.21 ng/mL, P = 0.01) than in healthy women (mean ± SD = 7.4 ± 3.5; median = 6.86 ng/mL; Fig. 1C). By stratifying patients in stage I/II and stage III/IV, no significant difference between the two groups was observed (P = 0.43), suggesting that IL-18BP is altered at early stages of EOC and that IL-18BP serum levels are independent from tumor burden (Fig. 1C). In addition, when patients were stratified in type I and in type II, according to a recent classification (31), no difference was observed between the two groups (data not shown). Consistently, no differences were observed by stratifying patients in accordance with tumor grading (data not shown). Performance of serum IL-18BP as classifier, evaluated by Receiver Operating Characteristic analysis, yielded an area under the curve of 0.734, suggestive of a poor performance (Supplementary Fig. S1).

To gain information on the possible tumor origin of the high serum IL-18BP levels, we tested 18 EOC serum and ascites pairs, collected at the same time. By paired analysis, IL-18BP levels were significantly higher in the ascites than in serum of the same patients (mean ± SD = 31.9 ± 14.7, median 32.8 vs. mean ± SD = 11.7 ± 5.9, median 10.5 ng/mL, P < 0.0001; Fig. 1D), thus suggesting that IL-18BP derives from the tumor site, where it could limit the IL-18–driven immune response.

Analyses of IL-18BP correlation with IL-18 and IFN-γ

It is known that IL-18BP inhibits IL-18 as the result of a negative feedback loop mediated by IL-18-induced IFN-γ (21, 23). In agreement with our previous data (13), IL-18 ELISA levels were elevated in EOC serum and ascites (Supplementary Fig. S2A and S2B).
We therefore first evaluated whether there was any relationship between IL18 and IL18BP mRNA in two public access datasets of ovarian cancers and found a moderate, yet significant correlation (Anglesio dataset, \(P = 0.0056\); Tothill dataset, \(P < 0.0001\); Fig. 2A). In addition, a correlation was found for IFNG and IL18BP and for IFNG and IL18 in the Tothill dataset (IFNG vs. IL18, \(P < 0.0001\); IL18BP vs. IFNG, \(P < 0.0001\)), although only a minority of patients showed elevated levels of IFNG expression (Fig. 2B).

However, when we investigated possible correlations at the protein level, we found no significant correlation between IL-18 and IL-18BP in the sera (\(r = 0.12\), \(P = \text{ns}\) by Pearson correlation) and in the ascites (\(r = 0.3, P = \text{ns}\)) of patients with EOC (Supplementary Fig. S2A and S2B). Moreover, IFN-\(\gamma\) levels were low or undetectable in ascites and sera from patients with EOC, in agreement with previous reports, and showed no correlation with IL-18 and IL-18BP (Supplementary Fig. S2C).

Although microarray data suggest that the IL-18/IFN-\(\gamma\) axis may be involved in the regulation of IL-18BP expression in the tumor tissue of a few patients, no evidence of this regulation was found in sera and ascites.

**Both EOC cells and reactive cells from the microenvironment express IL-18BP**

To address the possible cellular origin of the elevated IL-18BP in EOC, we conducted immunochemical analyses on cells isolated from the ascites and on tissue arrays. Among cells present in EOC ascites, tumor cell nests showed positivity for IL-18BP, although stronger expression was found in tumor-associated leukocytes (Fig. 3A). Staining appeared specific, as no reactivity was found on a contiguous section stained with secondary antiserum. Immunohistochemical analyses of tissue microarrays revealed that most EOC tumors expressed IL-18BP, irrespective of the tumor histotype, although at variable intensity in different tumors. Both neoplastic cells and infiltrating leukocytes showed expression of IL-18BP (Fig. 3B).

The IL-18BP–expressing leukocytes showed monocyte- or granulocyte-like morphologic features and had a nonlymphoid (CD3\(^{-}\)CD20\(^{-}\)NKP46\(^{-}\)) but myeloid (CD13\(^{+}\)CD14\(^{-}\)/low) surface phenotype (Supplementary Fig. S3). These features may be consistent with...
"myeloid-derived suppressor cells" (32). Together, these data indicate that different cell populations express IL-18BP in the EOC microenvironment. In contrast, normal ovary and Fallopian tube tissue showed no expression of IL-18BP by immunohistochemistry (Fig. 3C).

**IFN-γ and IL-27 upregulate IL-18BP in human EOC cell lines**

Unlike EOC cells present in tumor specimen, four EOC cell lines showed no constitutive expression of IL-18BP mRNA or protein (Fig. 4). However, culture in the presence of IFN-γ increased IL-18BP protein secretion (Fig. 4A) and IL18BP mRNA (Fig. 4B) expression in EOC cell lines. In addition, although human IL-18BP was undetectable in sera, it was found in the ascites of nude mice bearing orthotopic xenotransplants of the human A2774 and SKOV3 cell lines (Fig. 4C), further suggesting that EOC cells can contribute to IL-18BP production in vivo. Indeed, A2774 and SKOV3 cells grown in immune-deficient mice showed IL-18BP expression by immunohistochemistry, whereas IL-18BP was virtually undetectable in the same EOC cell lines in vitro (Supplementary Fig. S4). These findings suggest that factor(s) present in the microenvironment are responsible for production of IL-18BP in vivo. Although IFN-γ mediates IL-18BP expression in EOC cell lines in vitro, it should not be involved in vivo, as it was virtually undetectable in human ascites from patients and, moreover, mouse IFN-γ is inactive on human cells. These considerations prompted us to examine whether other cytokines, known to be elevated in patients with EOC, such as IL-6, TNF-α, VEGF-A, EGF, IL-18, and IL-8, could mediate IL-18BP expression in EOC cell lines, but none proved active (data not shown).

A recent report indicated that the heterodimeric cytokine IL-27, consisting of EBI3 and IL-27 chains, could mediate IL-18BP production by human keratinocytes in an IFN-γ-independent manner (33). We then tested whether IL-27 or the related cytokine IL-35 could mediate IL-18BP expression in EOC cells. Indeed, IL-27 induced IL-18BP secretion (Fig. 5A; S5A and B) in four EOC cell lines in a dose-dependent fashion, whereas IL-35 showed no activity (not shown). IL-27 also increased IL18BP mRNA expression (Supplementary Fig. S5C). Importantly, the IL-18BP-containing supernatant of IL-27–stimulated A2780 cells significantly inhibited IL-18 bioactivity in a concentration-dependent manner, as detected through IFN-γ release by the human NK cell line NK-92. Controls such as IL-27–containing medium or the supernatant from unstimulated EOC cells produced no inhibition (Fig. 5B).

Because IL-18BP expression is activated through the STAT1 pathway, we also analyzed STAT1 activation by IL-27 in EOC cells. Indeed, IL-27 activates STAT1 signaling in EOC cell lines (Fig. 5C and Supplementary Fig. SSD), as reported for other IL-27–sensitive cell types (33, 34). In addition, Western blot analysis showed that STAT1 was constitutively tyrosine-phosphorylated in cells isolated from ascites ex vivo and was further activated by in vitro treatment with IL-27 (Fig. 5C). Confocal microscopy confirmed constitutive STAT1 phosphorylation, which increased with IL-27 stimulation, in both EPCAM-positive EOC and EPCAM-negative inflammatory cells (Supplementary Fig. S5E). Consistently, cells isolated from ascites showed spontaneous IL-18BP secretion in culture, which could be further enhanced by in vitro IL-27 stimulation (Fig. 5A).

Because IL-27 activity is mediated through a heterodimeric receptor consisting of gp-130 and WSX-1 molecules, we asked whether antibodies neutralizing gp-130 could inhibit the effect of IL-27. Anti-gp130 mAb significantly inhibited IL-27–mediated IL-18BP production in two different EOC cell lines, further supporting the involvement of the IL-27/IL-27R pathway in IL-18BP regulation (Fig. 5A).
IL-27A and EBI3 are expressed in EOC tissues

Further analyses of two microarray datasets of EOC indicated a correlation between the expression of EBI3 and IL18BP mRNA in EOC primary tumors (Supplementary Fig. S6A). In addition, although IL18BP mRNA expression showed no significant correlation with outcome (not shown), high levels of EBI3 expression correlated with a shorter relapse-free survival (Supplementary Fig. S6B). The correlation between IL18BP and EBI3 gene expression suggested a possible paracrine loop of IL-18BP induction in vivo. This hypothesis was also suggested by the use of an anti-IL-27A (p28) specific antibody in immunohistochemistry, which revealed IL-27 expression predominantly in a fraction of tumor-associated leukocytes isolated from the ascites and in tissue microarrays, whereas tumor cell nests appeared negative (Fig. 5C and D). Also, EBI3 protein showed a similar distribution both in ascites (Supplementary Fig. S7A) and within tumor tissues (Supplementary...

Figure 5. Involvement of IL-27 in IL-18BP expression in EOC cells. A, IL-18BP secretion is detected by ELISA in culture supernatants following A2780 and A2774 EOC cell stimulation with IL-27 (20 ng/mL for 48 hours, \( P < 1 \times 10^{-5} \) vs. untreated control). Anti-gp130 mAb (from 0.5 to 500 ng/mL) significantly inhibits IL-18BP secretion mediated by IL-27 stimulation (\(*, P < 0.05; **, P < 0.001; *** P < 1 \times 10^{-5}\). Black bars show constitutive and IL-27–induced IL-18BP in vitro secretion by cells isolated from patients’ ascites A98, representative of three cases with similar results. B, the conditioned medium of IL-27–stimulated A2780 cells containing 2 ng/mL IL-18BP (BP \( ^{+} \text{cm} \)) significantly inhibits IL-18–induced IFN-\( \gamma \) release by the NK-92 cells in a concentration (50, 25, or 12\%/v/v)–dependent fashion (\(*, P < 0.05; **, P < 0.001\). As controls, conditioned medium of unstimulated cells (ctrl cm) and IL-27–containing medium (IL-27 \( ^{+} \text{cm} \)) were used. C, Western blot analysis of tyrosine-phosphorylated STAT1 protein in A2780 cells and in cells isolated from three different ascites unstimulated or stimulated for 10 minutes with IL-27 (20 ng/mL); \( \beta \)-actin was used as loading control. D, tumor-associated leukocytes show staining for IL-27A, whereas tumor cell nests show no reactivity (top). Negative control of a contiguous section is shown. Scale bar, 100 \( \mu \)m. Arrows indicate examples of negative tumor cell nests. E, EOC tissue array staining. Different EOC histotypes show expression of IL-27A in infiltrating leukocytes (enlarged in the inset).
Discussion

In this study, we show that IL-18BP levels are elevated in the serum of patients with EOC and are even higher in the ascites, reaching four-fold higher levels than those found in normal serum. This finding suggested a local production of IL-18BP in the microenvironment of EOC. Indeed, immunohistochemical analyses showed that IL-18BP is expressed by neoplastic cells of different EOC histotypes and by tumor-associated leukocytes with myeloid features. Therefore, the high concentration of IL-18BP in the tumor environment of EOC may limit the effect of endogenous or exogenously administered IL-18. On the other hand, IL-18BP also binds the anti-inflammatory cytokine IL-37 (35), which may suppress the host immune response (36), and this may result in a beneficial effect for the host. However, to our knowledge, no evidence for IL-37 expression in ovarian cancer has been provided to date.

Elevated IL-18BP levels were recently described also in serum of patients with prostate (24) and pancreatic cancer (37). In the latter, the concomitantly elevated levels of free-IL-18 in the serum suggested the existence of a biologic paradox, in view of the immune-enhancing properties of IL-18. Indeed, increased levels of both IL-18 and its natural inhibitor IL-18BP were also found in patients with systemic lupus erythematosus, in whom biologically active free IL-18 was still higher than in controls and was a marker of disease activity and a potential contributor to autoimmunity (38).

It was previously shown that high levels of IL-18 are present in serum and ascites of patients with EOC (12) and that pro-IL-18 is largely predominant (13). In fact, although IL-18 ELISA preferentially recognized mat-IL-18 in sera, this assay also detected pro-IL-18, albeit with a reduced sensitivity. Therefore, the presence of "free IL-18" in EOC could be explained by the predominance of pro-IL-18, which is unable to bind IL-18BP and is detected by IL-18 ELISA. It is likely that a similar situation may occur in other tumors in which alterations of IL-18 processing have been reported (39).

Because the presence of mat-IL-18 in the ascites of EOC could not be formally excluded and mat-IL-18 is an inducer of IL-18BP, via IFN-γ production (25, 40), we explored possible correlations between immune-reactive IL-18 and IL-18BP or IFN-γ levels. No significant correlation was found in the ascites and serum of patients with EOC, and IFN-γ levels were very low to undetectable in the ascites. However, a correlation between IL18 and IL18BP mRNA levels was found in two independent datasets of EOC gene expression profiles. Moreover, IFNG mRNA showed a correlation with IL18BP in one dataset, although IFNG mRNA was elevated only in a minority of cases. These data suggest that the IL-18/IFN-γ loop may be active in the tumor tissue microenvironment in some patients and that other factor(s) may participate in the induction of IL-18BP expression.

Evidence exists to suggest that IL-18BP production is a result of the interaction between EOC cells and the microenvironment. Human EOC cell lines do not produce IL-18BP in culture but, once grafted in immune-deficient mice, they display IL-18BP expression, suggesting a role for factor(s), which function across the species. This is not the case for IFN-γ, in view of its species specificity. Because other cytokines, which are elevated in the ascites of EOC failed to induce IL-18BP expression in vitro, we focused on IL-27, which was recently shown to stimulate IL-18BP expression in human keratinocytes (33).

IL-27 is a member of the IL-12 family that may have pro- or anti-inflammatory properties in different systems (29, 30). IL-27 is a heterodimeric cytokine, composed of p28 and EBV-induced gene 3 (EBI3), which upregulates IL-12R expression and is relevant for Th1 polarization (41, 42). However, the precise contribution of IL-27 to immune response, inflammation, and cancer is still poorly understood. On one hand, IL-27 has proinflammatory effects through the induction of CXCL10 in macrophages in inflammatory skin disorders (43). However, IL-27 may limit the proinflammatory and immune-enhancing activities of IL-18 in the skin through IL-18BP induction (33) and may dampen autoimmunity, as Il27−/− mice were more susceptible to experimental autoimmune encephalomyelitis (44).

Here, we show that IL-27 induces the expression of IL-18BP mRNA and protein in human EOC cell lines in culture and activates STAT1 signaling in these cells, whereas IL-35, another EBI3-containing cytokine (30), was inactive. Such activity was specifically induced through the IL-27R complex as indicated by the significant inhibition of IL-18BP induction upon treatment with a neutralizing antibody against the gp130 chain. A potential role of IL-27 in vivo was suggested by the expression of IL-27A and EBI3 found by immunohistochemistry in tumor-associated leukocytes in both ascites and tumor tissues and by the correlation between EBI3 and IL18BP mRNA expression in two different EOC datasets. Interestingly, high EBI3 expression correlated with a shorter progression-free survival in type II tumors. The finding that IL18BP gene expression had no significant correlation with relapse-free survival of patients with type II tumors may reflect the multiplicity of components driving clinical outcome. A correlation with Il27A mRNA expression could not be found (data not shown), but this may relate to technical limitations, as only one probeset was present in the arrays. The possible role of IL-27 in vivo was reinforced by the detection of constitutive STAT1 activation in both neoplastic and reactive cells isolated from the ascites, in the absence of measurable IFN-γ levels. Moreover, these ascites cells showed spontaneous secretion of IL-18BP in culture, which could be further enhanced by the addition of exogenous IL-27. Consistently, STAT1 phosphorylation also was increased by IL-27.

Our study may open new perspectives on understanding the role of IL-27 in cancer, as its involvement in the antitumor immune response is still poorly understood. In some hematologic neoplasia, including multiple myeloma...
(45) and acute leukemias (46, 47). IL-27 displays directly and indirect antitumor effects. We thus hypothesize that in EOC IL-27 may be part of an immune-regulatory network, which limits the induction of T\textsubscript{H}1 responses and IFN-\gamma production in the microenvironment by inhibiting IL-18 activity. In support of this concept, studies in murine models highlighted a predominant role of IL-27 as an immune-regulatory and anti-inflammatory agent that generates and maintains T-regulatory cell functions (48) and induces IL-10 production by T lymphocytes (49).

Although the role of IL-18 in tumor cell biology has been debated (50), preclinical studies indicated that IL-18 displays antitumor activity through its ability to trigger IFN-\gamma production and to favor the induction of a T\textsubscript{H}1 response (18, 19). Therefore, recombinant IL-18 is undergoing testing in clinical trials of cancer immunotherapy (27, 28) and in particular a clinical phase I study of IL-18 and doxorubicin in advanced-stage EOC (NCT00659178) has recently concluded patient recruitment. It is possible that the high local levels of IL-18 present in EOC may limit the biologic effects of low levels of endogenous IL-18 or of therapeutically administered IL-18, particularly at the tumor site.

References


The IL-18 Antagonist IL-18–Binding Protein Is Produced in the Human Ovarian Cancer Microenvironment

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