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 Airoldi2, Marina Bagnoli3, Patrizia Pinciroli3, Delia Mezzanzanica3, Maria Grazia Centurion1, Marina Fabbi1*, Silvano Ferrini1*

1Department of Integrated Oncological Therapies, IRCCS AOU San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy;
2Laboratory of Oncology, IRCCS Istituto G. Gaslini, Genoa Italy;
3Department of Experimental Oncology and Molecular Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy;
4Department of Surgery, IRCCS AOU San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy.

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§Grazia Carbotti is enrolled in the Doctorate School of Genetics, University of Genoa, Italy.

*Equal contributors and correspondence: Silvano Ferrini and Marina Fabbi, UOC Terapia Immunologica, IRCCS AOU San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Largo R. Benzi 10, 16132 Genova, ITALY
Phone: 0039-010-5737-372; Fax:-374; Email: silvano.ferrini@istge.it, marina.fabbi@istge.it

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Relevance Statement: IL-18 is an immune-enhancing cytokine, which is being studied in clinical trials of immunotherapy. IL-18BP is a natural antagonist of IL-18 and limits IL-18 biological activity. Here we show that IL-18BP is produced by both tumor cells and tumor-associated leukocytes. Expression of IL-18BP in ovarian cancer cells in vitro is induced by cytokines such as IFN-γ and IL-27, which may play a role in the tumor environment. The high local levels of IL-18BP at the tumor site may limit the induction of an efficient immune response by either endogenous or therapeutic IL-18.
Abstract

PURPOSE: Interleukin (IL)-18 is an immune-enhancing cytokine, which induces Interferon (IFN)-γ production, Th1-responses and anti-tumor effects. In turn, IFN-γ stimulates IL-18 Binding Protein (BP) 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 RT-PCR.

RESULTS: Analysis of gene expression profiles revealed that IL18BP mRNA is increased in EOC tumors compared to normal ovary cells. Release of IL-18BP was detectable in EOC sera and at 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⁺CD14⁻ 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 also IL-27 is involved in IL-18BP up-regulation in EOC cell lines and primary cells through STAT1 activation. Altogether these data indicate that IL-18BP, which is produced in EOC in response to micro-environmental factors, may inhibit endogenous or exogenous IL-18 activity.
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). Several evidences indicate that T lymphocytes capable of recognizing EOC cells are present at the tumor site (2, 3) and that T cell-mediated immunity may impact on clinical course of EOC (3). However, similarly 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 EOC patients 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 serological biomarkers of EOC (11). Among them, IL-18 was proposed as potential biomarker by gene expression profiling (12) and, indeed, IL-18 levels were elevated in serum and ascites of EOC patients (12, 13).

IL-18 is a pro-inflammatory and immune-enhancing cytokine, which induces IFN-γ production by T and NK cells, mediates 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 pre-clinical tumor models mat-IL-18 exhibited anti-tumor properties through the induction of an immune response, whereas pro-IL-18 had no activity (18, 19).

EOC cell lines release pro-IL-18 but not mat-IL-18, due to defective caspase-1 expression or activation, while 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 physiological inducer of IL-18BP, which in turn inhibits IL-18 biological activity in a negative feed-back loop. IL-18BP accumulates in the serum in chronic renal failure, where it may contribute to the defective immune response (26).

To further address the biological role of endogenous IL-18 in human EOC we performed an integrated analysis of IL-18 and IL-18BP in EOC patients. The study of IL-18BP seemed also relevant, as IL-18-based immunotherapy is being evaluated in EOC patients (27, 28) (NCT00659178) and high levels of IL-18BP may interfere with this treatment. We found that indeed IL-18BP levels are elevated in the serum and particularly in the ascites of EOC patients and that IL-18BP is expressed by EOC cells and by reactive leukocytes. Our data also suggest that IL-18BP production may be the outcome of a cross-talk between tumor cells and the microenvironment, involving IFN-γ and IL-27, a member of the IL-12 cytokine family (29, 30). Altogether these data support an immune-regulatory role for IL-18BP in EOC, as it may limit the activity of endogenous or therapeutic IL-18.
Materials and methods

Cells and Cell treatments

The human EOC cell lines SKOV3 (ATCC), A2780 (ICLC), A2774 (IST Genoa) and OVCAR5 (INT Milan) 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 the GeneMapper® software, version 4.0.

Cells (50x10³/well) were seeded in 24-well plates in culture medium. The day after, 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 (mAb228, R&D System). Treatment was carried on 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, 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 performed using the following primers: POLR2A upper primer GACAATGCAGAGAAGCTGG, lower primer GCAGGAAGACATCATCATCC; GAPDH upper primer GAAGGTGAAGGTCGGAGT, lower primer CATGGGTGGAATCATATTGGAA; IL18BP upper primer GTGTCCAGCATTGGAAGTGACC, lower primer GGAGGTGCTCAATGAAGGAACC. Amplification was carried out by the Mastercycler® 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 semi-
quantitative RT-PCR, 2 μl of cDNA were 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 ACCATGAGACACAACCTGGACACCAG, lower primer TTAACCCTGCTGCTGTGGACTGCTG; housekeeping gene *ACTB* upper primer GGCATCGTGATGGACTCCG, lower primer GCTGGAAGGTGGACAGCGA in a Eppendorf Mastercycler ep Gradient S. 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 were with evidence of disease and untreated or off-treatment since at least 2 months (Table 1). Ascitic fluids were collected during surgical procedures. Tumors histopathology, grade and stage were assigned according to the International Federation of Gynecology and Obstetrics (FIGO) criteria.

**EOC Xenotransplant model**

Female homozygous non-obese-diabetic (NOD)/SCID mice (Jackson Laboratory) were bred in-house. Nude mice were from Janvier. The experiments were performed according to the National Regulation on Animal Research Resources and approved by the institutional Review Board. Six-weeks old NOD/SCID animals were injected i.p. with 5x10⁶ 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 2x10⁶ SKOV3 cells. Tumour masses were excised and fixed in 10% buffered formalin.
**ELISA for IL-18, IL-18BP, IFN-γ**

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

**Immunochemistry**

Immunochemistry (IC) detection of IL-18BP was performed on sections of formaldehyde-fixed paraffin-embedded cell pellets or tumors explanted from mice or on commercially available tissue microarrays of EOC patients (Super Bio Chips). Antigen-retrieval was performed with high pH citrate buffer in microwave oven. The sections were immunostained using rabbit anti-IL-18BP (clone EP1088Y, 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’s ematoxylin 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 40x 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 the 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; Iorio dataset included 17 EOC high grade tumors, 2 preparations of normal ovarian surface epithelial cells (OSE) and 6 EOC cell lines. Raw data were downloaded from GEO and normalized through the RMA algorithm (by using the Expression Console software, Affymetrix) except for 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 analysis of variance and appropriate multiple comparison tests were used to compare expression levels between patients and control subjects. The paired Student’s t test was used when appropriate. Parametric methods were used to examine the correlation among gene or protein expression levels (Pearson’s correlation coefficient). An alpha level of 0.05 was used for all statistical tests. GraphPad Prism 5.0d software and R statistical language (URL: 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 10 min at 37°C with or without 20 ng/ml of rIL-27 in 0.5 ml of medium. Cells, were lysed in 100μl of buffer containing 1 mM Na 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-β actin or tubulin mAb (Sigma) and chemiluminescence detection.
Results

Gene expression of IL18BP in EOC tumors

*IL18BP* mRNA was first analysed 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 EOC patients (Table 1), all with evidence of disease and untreated, in 7 patients at relapse, and in 13 age-matched healthy women by ELISA for IL-18BPα, the major isoform of IL-18BP (22). IL-18BP serum levels were higher in both untreated (onset: M±SD=11.06±5.7; Median=9.45 ng/ml, $P=0.03$) and in relapsing EOC patients (M±SD=11.76±3.2; Median=11.21 ng/ml, $P=0.01$) than in healthy women (M±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 to 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 (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 (M±SD=31.9±14.7, Median 32.8 vs M±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 feed-back 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 (Fig. S2A and B).

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 also 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=ns by Pearson’s correlation) and in the ascites (r=0.3, P=ns) of EOC patients (Fig. S2A and B). Moreover, IFN-γ levels were low or undetectable in ascites and sera from EOC patients, in agreement with previous reports, and showed no correlation with IL-18 and IL-18BP (Fig. S2C).

Although microarray data suggest that the IL-18/IFN-γ 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 performed 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 a 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 morphological features and had a non-lymphoid (CD3-CD20-NKP46-) but myeloid (CD13+CD14-low) surface phenotype (Fig. S3). These features may be consistent with “myeloid-derived suppressor cells” (32). Altogether these data indicate that different cell populations express IL-18BP in the EOC microenvironment. By contrast normal ovary and Fallopian tube showed no expression of IL-18BP by immunohistochemistry (Fig. 3C).

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

Different from 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, while IL-18BP was virtually undetectable in the same EOC cell lines in vitro (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 EOC patients, such as IL-6, TNF-α, VEGF-A, EGF, IL-18 and IL-8 could mediate IL-18BP expression in EOC cell lines, but no one 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-B) in four EOC cell lines in a dose-dependent fashion, while IL-35 showed no activity (not shown). IL-27 also increased IL-18BP mRNA expression (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).

As 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; S5D), 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+ EOC and EPCAM- reactive cells (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).

As 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 (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 (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 IC, which revealed IL-27 expression predominantly in a fraction of tumor-associated leukocytes isolated from the ascites and in tissue microarrays, while tumor cell nests appeared negative (Fig. 5C, D). Also EBI3 protein showed a similar distribution both in ascites (Fig. S7A) and within tumor tissues (Fig. S7B). Altogether our data are consistent with a paracrine activation of IL-18BP expression in the tumor microenvironment.
Discussion

In this study we show that IL-18BP levels are elevated in the serum of EOC patients and are even higher in the ascites, reaching 4-fold higher levels than those found in normal serum. This finding suggested a local production of IL-18BP in the microenvironment of EOC. Indeed, immunochemical 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 so far.

Elevated IL-18BP levels were recently described also in serum of patients with prostatic (24) and pancreatic cancer (37). In the latter, the concomitantly elevated levels of free-IL-18 in the serum suggested the existence of a biological 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 SLE patients, where 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 demonstrated that high levels of IL-18 are present in serum and ascites of EOC patients (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, although 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 where alterations of IL-18 processing have been reported (39).
As 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 EOC patients, 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.

Several evidences suggested 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. As 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 up-regulates 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 pro-inflammatory effects through the induction of CXCL10 in macrophages in inflammatory skin disorders (43). However, IL-27 may limit the pro-inflammatory 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, while 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 immunochemistry 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 PFS 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 also STAT1 phosphorylation was increased by IL-27.

Our study may open new perspectives on understanding the role of IL-27 in cancer, since its involvement in the anti-tumor immune response is still poorly understood. In some hematological neoplasia including multiple myeloma (45) and acute leukemias (46, 47) IL-27 displays direct and indirect anti-tumor effects. We thus hypothesize that in EOC IL-27 may be part of an immune-regulatory network, which limits the induction of Th1 responses.
and IFN-γ 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 Treg 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), pre-clinical studies indicated that IL-18 displays anti-tumor activity through its ability to trigger IFN-γ production and to favour the induction of a Th1 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 doxil in advanced stage EOC (NCT00659178) has recently concluded patient recruitment. It might be possible that the high local levels of IL-18BP present in EOC may limit the biological effects of low levels of endogenous IL-18 or of therapeutically administered IL-18, particularly at the tumor site.
Figure Legends

**Figure 1:** Expression of *IL18BP* mRNA and secretion of IL-18BP protein in EOC.  
A: Normalized *IL18BP* gene expression levels in EOC tumors, cell lines and OSE. Expression levels were significantly higher in tumor samples (one-way ANOVA and Bonferroni’s post-test).  
B: *IL18BP* mRNA expression was significantly higher in high-grade tumors (Type II) relative to low malignant potential tumors (LMP), in two independent datasets by two-sided unpaired Student’s t test (Anglesio dataset) and one-way ANOVA and Tukey’s post-test (Tothill dataset). Boxes and whiskers represent median and quartiles with minimum and maximum.  
C: IL-18BP ELISA levels were significantly higher in sera from EOC patients at diagnosis (n. 48) or at relapse (n. 7) than in age matched female controls (n. 13) by Kruskal-Wallis test. IL-18BP levels were similar in stage I/II and stage III/IV patients.  
D: IL-18BP levels were higher in the ascites than in sera simultaneously collected from the same patient (by two-sided paired Student’s t test). Mean and SD are indicated in C and D.

**Figure 2:** Analysis of the correlation between *IL18*, *IL18BP* and *IFNG* mRNA levels in high grade (Type II) tumors.  
A: A significant correlation was found between *IL18* and *IL18BP* in both datasets.  
B: A correlation between *IL18* and *IFNG*, and between *IL18BP* and *IFNG* was also found only in the Tothill dataset. Pearson’s correlation coefficients are shown (r). Lines represent the best fit linear regression analysis with the 95% CI.

**Figure 3:** Immunochemical analysis of IL-18BP expression in EOC and in normal tissue counterparts.  
A: IL-18BP staining of cells isolated from ascites. In the upper panel both tumor cell nests and particularly tumor-associated leukocytes show staining for IL-18BP. Negative control of a contiguous section stained only with secondary antibody is shown. Bar=100μm. Arrows indicate tumor cell nests. Arrowheads indicate tumor-associated...
leukocytes. B: EOC Tissue array staining. Different EOC histotypes show expression of IL-18BP in tumor cells and infiltrating leukocytes. C: Normal ovary and Fallopian tube show virtually no positivity for IL-18BP staining. Negative controls of contiguous sections are shown for each panel.

Figure 4: IL-18BP expression by EOC cell lines. A: IL-18BP secretion is detected by ELISA in culture supernatants following EOC cell treatment with IFN-γ (100 and 1000 U/ml for 24 h. * P<0.05, ** P<0.01 by two-sided unpaired Student’s t-test). B: IFN-γ induced IL18BP mRNA expression in EOC cell lines as detected by RT-PCR analysis. C: Human IL-18BP ascites levels were measured in NOD-SCID mice bearing orthotopic xenotransplants of the SKOV3 (n. 5) and A2774 (n. 2) EOC cell lines.

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 48h P<1E-05 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<1E-05). Black bars show constitutive and IL-27-induced IL-18BP in vitro secretion by cells isolated from patient’s ascites A98, representative of three cases with similar results. B: The conditioned medium of IL-27-stimulated A2780 cells containing 2ng/ml IL-18BP (BP+cm), significantly inhibits IL-18-induced IFN-γ 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+ 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 min with IL-27 (20ng/ml); β-actin was
used as loading control. **D:** In the upper panel tumor-associated leukocytes show staining for IL-27A, while tumor cell nests show no reactivity. Negative control of a contiguous section is shown. Bar=100μm. 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).
References


42. Lucas S, Ghilardi N, Li J, de Sauvage FJ. IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and -independent mechanisms. Proc Natl Acad Sci USA 2003;100:15047-52.


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

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FIGURE 2

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(Log2 expression intensity)
**Figure 4**

(A) Graph showing IL-18BP levels (pg/ml) in response to IFN-γ in different cell lines: A2780, OVCAR5, SKOV3, and A2774. The graph includes error bars and statistical significances indicated by asterisks: ** (p < 0.01) and * (p < 0.05).

(B) Western blot analysis of IL-18BP and actin expression in the same cell lines with and without IFN-γ treatment. The blots show increased IL-18BP expression in the presence of IFN-γ.

(C) Dot plot showing human IL-18BP levels (pg/ml) in SKOV3 and A2774 cells. The plot includes symbols: △ (△) for SKOV3 and ○ for A2774.
Figure 5

A. IL-18BP (pg/ml) levels in different conditions.

B. IFN-γ (pg/ml) levels with varying conditions.

C. Western blot analysis for pSTAT1 and β-actin in different cell lines.

D. Microscopic images of Ascites cells with highlighted areas.

E. Comparison of Endometrioid carcinoma and Serous cystadenocarcinoma with anti-IL-27 Ab treatment.
The IL-18 antagonist IL-18 Binding Protein is produced in the human ovarian cancer microenvironment

Grazia Carbotti, Gaia Barisone, Anna M. Orengo, et al.

Clin Cancer Res  Published OnlineFirst July 19, 2013.