

## IL-12 RECEPTOR-MEDIATED UPREGULATION OF FasL IN HUMAN OVARIAN CARCINOMA CELLS

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**The expression and functions of IL-12 receptor (IL-12R) in human ovarian carcinoma cell lines have been investigated. Ovarian carcinoma cells express both the IL-12Rβ1 and the IL-12Rβ2 subunits. IL-12R crosslinking resulted in phosphorylation of Tyk2, p44 (ERK1) and Akt kinases and activation of STATs 2, 3, 4 and 5. IL-12 induced substantial upregulation of Fas ligand (FasL) surface expression in ovarian carcinoma cells paralleled by an increased ability to induce apoptosis in Jurkat cells and PHA-activated lymphocytes. The induction of surface expression of FasL by IL-12 was not due to upregulation of FasL gene expression, but resulted from downregulation of matrix metalloproteinases (MMPs)-3 and -7 and consequently reduced cleavage of FasL from the cell surface. These findings bring new insights into the significance of IL-12-mediated effects in nonlymphoid cancer cells that might be of importance for improving the design of IL-12-based therapies for ovarian cancer.**

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**Key words:** IL-12 receptor; ovarian cancer; Fas ligand; signal transduction; major histocompatibility complex class I; apoptosis; matrix metalloproteinases

Lymphokines play a crucial role in the regulation of natural and adaptive immune responses. Recombinant lymphokines have been widely used for stimulation of the antitumor immune responses in both experimental animals and cancer patients.<sup>1–3</sup> Lymphokines bind to the specific receptors on lymphoid cells and stimulate their proliferation, differentiation and activation. Initially, it was believed that the receptors for lymphokines are expressed only by lymphoid cells (T, B, or NK cells), but recent studies suggest that lymphokine receptors can be found on various malignant cells of nonhematopoietic origin. Human carcinoma lines were shown to express IL-2R.<sup>4–6</sup> IL-4R has been found in normal astrocytes, human breast cancer and head and neck carcinoma cells.<sup>4,7–9</sup> Recently, it was discovered that IL-12R can be expressed by nonlymphoid malignant cells such as human melanoma, osteosarcoma and breast cancer cells and some murine tumor cell lines.<sup>10–13</sup> The biologic significance of IL-12R expression in nonlymphoid cells remains unknown. It is unclear whether this receptor is functionally active and what phenotypic changes in tumors result from IL-12R signaling.

Interleukin-12 (IL-12) is a heterodimeric cytokine composed of p35 and p40 subunits. It exerts biologic effects by binding to specific cell surface receptors (IL-12R). Two human IL-12R subunits, designated IL-12Rβ1 and IL-12Rβ2, have been identified.<sup>14–19</sup> Several studies have shown that signaling by IL-12R occurs through the Jak/STAT signal transduction pathway.<sup>20–23</sup> IL-12 is a potent stimulator of the antitumor immune responses and is able to induce regression of local and metastatic tumors.<sup>24–27</sup> IL-12 stimulates Th1 differentiation, CD8<sup>+</sup> T-cell cytotoxicity and activation of NK, NKT cells and macrophages.<sup>28,29</sup> IL-12-induced activation of T and NK cells is usually associated with upregulation of Fas ligand (FasL) expression by these cells.<sup>30,31</sup> In addition, IL-12 upregulates expression of major histocompatibility complex (MHC) class I molecules.<sup>10</sup> Recent stud-

ies have also demonstrated the ability of IL-12 to inhibit tumor-induced angiogenesis.<sup>32–35</sup>

Numerous clinical trials of recombinant IL-12 or IL-12-producing cells in patients with melanoma,<sup>36–39</sup> renal cell carcinoma,<sup>40</sup> cutaneous T-cell lymphoma<sup>41</sup> and ovarian cancer<sup>42,43</sup> showed, however, only a marginal therapeutic efficacy associated with substantial side effects, despite the frequently observed positive impact on the development of tumor-specific immune responses. Having in mind the discrepancy between the high immunologic effectiveness of IL-12 as immune adjuvant and its low therapeutic efficacy, we addressed the hypothesis that the exposure of tumor cells to IL-12 may have negative consequences by direct modulation of tumor cell functions and increased resistance of tumor cells to the immune attack. Therefore, in the present study, we tested whether IL-12R is functionally active and modulates the functions of ovarian carcinoma cells.

### MATERIAL AND METHODS

#### Cell culture

Two human ovarian adenocarcinoma cell lines, OVCAR3 and SKOV3, were obtained from American Type Culture Collection (ATCC, Manassas, VA). SKOV3 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY). OVCAR3 cell line was grown in RPMI-1640 supplemented with 20% FBS, 10 mM HEPES buffer (Sigma Chemical, St. Louis, MO), 1 mM sodium pyruvate (Gibco-BRL), 4.5 g/L glucose, 1.5 g/L sodium bicarbonate (Mediatech, Herndon, VA) and 0.01 mg/ml human recombinant insulin (Sigma). All experiments were performed on heavily confluent cells.

Normal human T lymphocytes were obtained from peripheral blood mononuclear cells (PBMCs) drawn from healthy volunteers at the Pittsburgh Central Blood Bank. Monocytes were depleted by plastic adherence. Blood lymphocytes were isolated by centrifugation on a Ficoll-Diatrizoate density gradient (ICN, Aurora, OH). Lymphocytes were incubated with 5 μg/ml phaseolus vulgaris agglutinin (PHA) in RPMI media supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and  $5 \times 10^{-5}$  M 2-ME for 3–4 days.

Grant sponsor: the American Cancer Society; Grant number: RSG-01-023-01-CCE.

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Received 20 February 2004; Accepted after revision 28 May 2004

DOI 10.1002/ijc.20482  
Published online 15 July 2004 in Wiley InterScience (www.interscience.wiley.com).

### Reagents and antibodies

Human recombinant IL-12 was a generous gift from Amgen (Seattle, WA). Monoclonal antibodies (Abs), PE-conjugated anti-IL-12R $\beta$ 1, FITC-conjugated anti-MHC I (HLA-A, B, C) (clone G46-2.6) and biotinylated anti-FasL mAb (clone DX2) were purchased from BD Biosciences Pharmingen (San Diego, CA). Specific rabbit polyclonal anti-IL-12R and anti- $\gamma$ -actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against total and phosphorylated p42/44 MAP kinases (Erk1/Erk2) and p38 MAP kinase, and rabbit polyclonal antibodies against Akt kinase and Tyk2 were from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibody against total and phosphorylated Jak2 was from Chemicon (Temecula, CA). Kinase inhibitors, PD 98059, and wortmannin were from Calbiochem (La Jolla, CA). Anti-Fas antibodies, CH11 (activating) and ZB4 (neutralizing), were from Upstate Cell Signaling Solutions (Lake Placid, NY). Purified MMP-3 and broad-spectrum MMP inhibitor N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid (NNGH) were purchased from Sigma Chemical.

### Treatment procedures

Cells were plated at  $5 \times 10^5$  cells/well into 24-well plates. After 3 days in culture, cells were treated with 0–100 ng/ml of IL-12. Based on the results of preliminary dose-response experiments, fixed concentration of 40 ng/ml for IL-12 was employed in most of the experiments. The viability of the cells preceding each treatment was confirmed to be > 90% by Trypan blue exclusion.

### Apoptosis assay

Apoptosis was evaluated in Jurkat cells using annexin V-binding assay according to the manufacturer's instructions (Beckman Coulter, Brea, CA). Cells were double-stained with FITC-conjugated annexin V and CD3 to ascertain their lineage and were immediately analyzed by flow cytometry as described below. Only CD3-positive/annexin V-positive cells were considered in analysis.

### Flow cytometry

Flow cytometry was performed as described.<sup>44</sup> Cells were pelleted and washed with FACS staining buffer containing 1% BSA, 0.1% sodium azide. Cells were incubated with optimal concentrations of FITC- or PE-conjugated primary antibodies for 30 min at 4°C. FasL was stained using biotinylated Ab followed by streptavidin-PE. For all experiments, isotype-matched control IgG antibodies were included. Analysis was performed using the FACScan flow cytometer, and results were analyzed with CellQuest software (both from Becton Dickinson Immunocytometry Systems, San Jose, CA).

### Western blot analysis

Western blot was performed as described.<sup>44</sup> Cells were harvested, and lysates were prepared in a lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 4 mM EDTA, 1% Triton X-100 and protease inhibitors cocktail (Sigma). Total protein concentrations were determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA). Samples containing 100  $\mu$ g of total protein were subjected to SDS-PAGE, transferred onto nitrocellulose membranes (Osmonics, Westborough, MA) and stained with appropriate specific antibodies. The specificity of each staining was confirmed using appropriate blocking peptides. Staining with anti- $\gamma$ -actin antibody was used as a loading control. Proteins were visualized by enhanced chemiluminescence using Western Blotting Luminol Reagent (Santa Cruz Biotechnology).

### Casein zymography of matrix metalloproteinases 3 and 7

Cells were solubilized as described for Western blot but without the addition of DTT. The samples containing 5  $\mu$ g total of protein were electrophoresed in 12% polyacrylamide, 0.1% casein gels. The gels were washed for 30 min on an orbital rocker with 2.5% triton-X-100 to remove SDS, then for 30 min in 50 mM Tris-HCl, pH 7.2, containing 20% glycerol to promote renaturation of the

MMPs. The gels were then incubated at 37°C for 16 hr in 50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 20 mM CaCl<sub>2</sub>. The gels were subsequently stained for 1 hr in 0.2% w/v Coomassie R250 (Bio-Rad) following by destaining in water to reveal cleared areas of casein lysis corresponding to MMP-3 and MMP-7 activity zones.

### RT-PCR

Total RNA was isolated using TRIzol method (Gibco-BRL). For RT-PCR, the ThermoScript RT-PCR System by Qiagen (Valencia, CA) was used. Reverse transcription of 5  $\mu$ g of total RNA to cDNA was done with random hexamers per manufacturer's protocol. The resulting cDNA was diluted 100-fold in sterile water, and aliquots were subjected to RT-PCR. The forward (5'-GAGG-GACTGGTACTGCTTAATCGACTC) and reverse (3'-CCTCACACAGGTTTCATTATGTTAATACGAGTG) primers for IL-12R $\beta$ 2 were designed to span an intron splice site with the Primer Express software package. cDNA was amplified by 35 cycles of denaturation at 94°C for 1 min and annealing/extension at 65°C for 2 min. The predicted size of PCR product was 511 bp. Glyceraldehyde 6-phosphate dehydrogenase (GAPDH) was simultaneously amplified to verify that equal amounts of cDNA were added in each PCR. The primers used for GAPDH were sense primer, 5'-CATCAAGAAGGTGGTGAAGCA-3'; antisense, 5'-TCTACATGGCAACTGTGAGAA-3' (product size, 365 bp). cDNA was amplified by 30 cycles of denaturation at 94°C for 0.5 min, annealing at 60°C for 0.5 min and extension at 72°C for 1.25 min.

### Electrophoretic mobility shift assay (EMSA)

Adherent cells ( $10^6$ ) were grown in 75 cm<sup>2</sup> plastic tissue culture dishes, then incubated for 0–30 min with or without IL-12, washed and harvested by scraping with a rubber policeman. Nuclear extracts were prepared and EMSAs were performed on 4% native polyacrylamide gels as described.<sup>45</sup> Activation of STATs 1–6 was evaluated by using binding reactions with appropriate radiolabeled double-strand STAT consensus binding motifs.<sup>45,46</sup> Quantitation of STAT signal was performed by autoradiography.

### DNA array

OVCAR3 cells were treated with 40 ng/ml IL-12 for 24 hr. Cells were harvested and total RNA was isolated using TRIzol method (Gibco-BRL) according to manufacturer's protocol. We utilized slide-based cDNA microarray comprised of 7,241 nonredundant genes obtained from Research Genetics (Carlsbad, CA). cDNA labeling, array hybridization and data analysis were performed in Fred Hutchinson Cancer Research Center as described.<sup>47</sup> For a given gene, positive ratio corresponded to the normalized value of the intensity of a gene in treated cells divided by the normalized value in untreated cells. Genes that demonstrated ratios  $\geq 2$  were identified for further investigation.

### Statistics

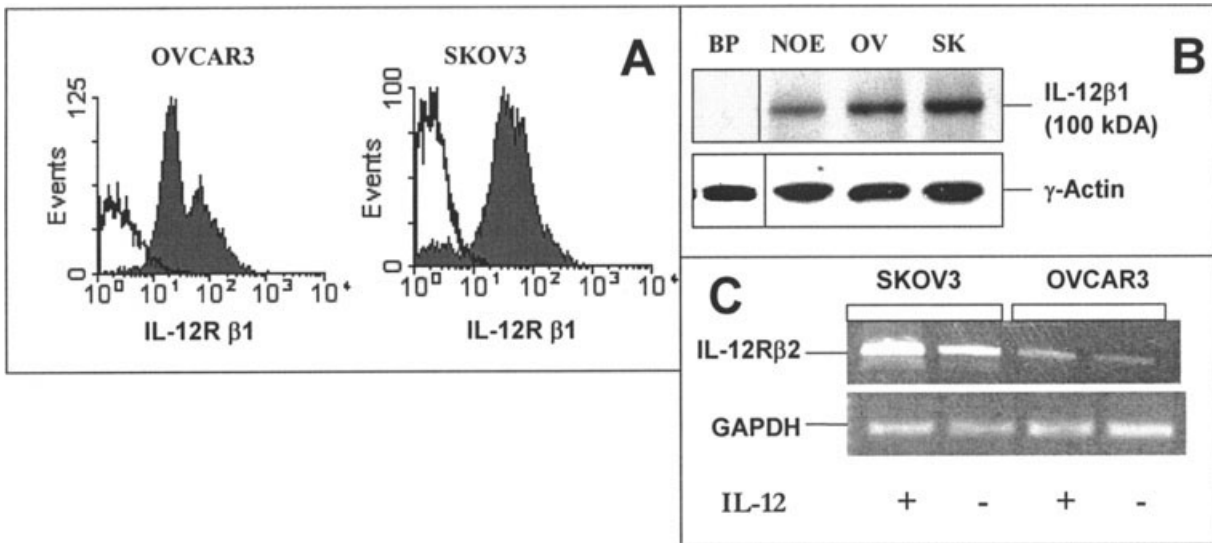
All experiments were performed in duplicate unless otherwise indicated, and mean values were presented as mean  $\pm$  standard error. Comparisons between the values were performed using a 2-tailed Student's *t*-test. A value of *p* < 0.05 was considered statistically significant.

## RESULTS

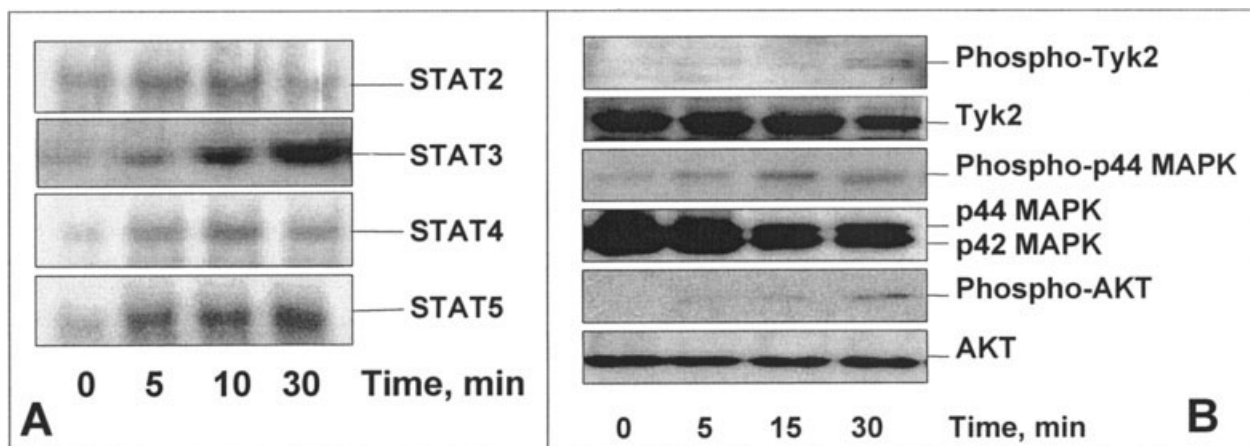
### IL-12R expression by human ovarian carcinoma cells

The surface expression of IL-12R $\beta$ 1 subunit was analyzed using PE-conjugated anti-IL-12R $\beta$ 1 mAb. Both OVCAR3 and SKOV3 lines express high levels of IL-12R $\beta$ 1 (Fig. 1a). Western blot analysis confirmed the presence of IL-12R $\beta$ 1 subunit in total extracts from OVCAR3 and SKOV3 cells (Fig. 1b). IL-12 $\beta$ 1 receptor was also expressed in normal ovarian epithelium (NOE) cells (Fig. 1b).

The expression of IL-12 $\beta$ 2 is important for IL-12-induced signal transduction.<sup>18,19</sup> We therefore examined the expression of IL-12 $\beta$ 2 subunit in ovarian carcinoma cells. Since there is no commercially available antibody against the  $\beta$ 2 subunit of IL-12R,



**FIGURE 1** – Expression of IL-12R in ovarian carcinoma cell lines. (a) Cell-surface expression of IL-12Rβ1. The cells were stained with PE-conjugated anti-IL-12Rβ1 mAb (shaded curve area) or isotype-matched mAbs (IgG; open curve area) and subjected to FACS analysis. (b) Expression of IL-12Rβ1 protein in total cell lysates by Western blot analysis. IL-12Rβ1 expression was visualized using specific monoclonal Ab in OVCAR3 (OV), SKOV3 (SK) and NOE cells. Specificity was confirmed by incubation of the blot with anti-IL-12Rβ1 Ab in the presence of specific blocking peptide (BP). Expression of  $\gamma$ -actin was used as a loading control. (c) Expression of mRNA of IL-12Rβ2 in ovarian carcinoma cells. Cells were treated with or without 40 ng/ml IL-12 for 12 hr; total RNA was isolated and analyzed by RT-PCR. The results of RT-PCR for GAPDH demonstrate the loading of equal amounts of DNA on the gel. The results are representative of at least 3 experiments.



**FIGURE 2** – IL-12 induces signal transduction in ovarian carcinoma cells. SKOV3 cells were treated with 40 ng/ml IL-12 for 0–30 min. (a) EMSA analysis of DNA-binding activity of STATs 2–5. Nuclear extracts were subjected to electromobility shift assay using <sup>32</sup>P-labeled oligonucleotide probes containing appropriate consensus binding motifs for STATs 2–5. (b) Analysis of protein kinase phosphorylation. Whole cell lysates were analyzed by Western blotting with indicated antibodies recognizing phosphorylated forms of Tyk2, p42/p44 and Akt kinases. Blots were stripped and reprobed with Abs recognizing both phosphorylated and nonphosphorylated forms of these kinases. Specificity was confirmed by incubation of the blot with Abs in the presence of specific blocking peptides (not shown). The results are representative of 3 experiments.

the expression of this subunit was evaluated by RT-PCR (Fig. 1c). Figure 1(c) shows that both OVCAR3 and SKOV3 cell lines express IL-12  $\beta$ 2. The expression of IL-12R $\beta$ 2 was much higher in SKOV3 than OVCAR3 cells. Incubation with IL-12 further augmented expression of IL-12R $\beta$ 2 RNA in both ovarian carcinoma cell lines (Fig. 1c).

#### IL-12 activates STAT signaling pathways in ovarian carcinoma cells

Numerous studies demonstrate that engagement of IL-12R on lymphocytes is associated with activation of proteins of the STAT family.<sup>12,13,18,19</sup> We tested whether IL-12 may activate STAT

proteins in ovarian carcinoma cells using EMSA. Incubation of SKOV3 cells with IL-12 led to a substantial activation of STATs 2, 3, 4 and 5 (Fig. 2a). No detectable increase in the activity of STATs 1 and 6 were observed (data not shown). Similar results were obtained with OVCAR3 cells (data not shown). These results further confirm that ovarian cells express functional IL-12R $\beta$ 1 and IL-12R $\beta$ 2 subunits.

#### Protein kinase activity in IL-12-treated ovarian carcinoma cells

IL-12R signaling is mediated via phosphorylation of various protein kinases of JAK, MAP and AKT families.<sup>16,48–51</sup> To clarify the potential involvement of JAK family in IL-12-mediated sig-

naling in ovarian carcinoma cells, the phosphorylation of Jak2 and Tyk2 was analyzed. SKOV3 cells were incubated with IL-12 for 0–30 min. Cell lysates were prepared, Jak2 and Tyk 2 proteins were immunoprecipitated and the level of their phosphorylation was assessed by immunoblotting with anti-pTyr mAb. IL-12 had no effects on tyrosine phosphorylation of Jak2 (data not shown), but induced phosphorylation of Tyk2 in ovarian SKOV3 carcinoma cells (Fig. 2*b*). Similar results were obtained for OVCAR3 cells (data not shown).

To determine whether IL-12 induces activation of the p38 MAPK kinase phosphorylation cascade in ovarian carcinoma cells, we stained for phosphorylated p38 MAPK using mAb that detects p38 MAPK kinase only when activated by dual phosphorylation at Thr180 and Tyr182. Addition of IL-12 did not induce any measurable increase of p38 MAPK activity (data not shown). Likewise, IL-12 did not induce phosphorylation of JNK as detected with monoclonal antibody that recognizes only endogenous p46 and p54 SAPK/JNK dually phosphorylated on threonine 183 and tyrosine 185 (data not shown). We next examined the activation of MAPK kinases, p44 (ERK1) and p42 (ERK2). Treatment of SKOV3 cells with IL-12 (40 ng/ml) produced a marked increase in p44 phosphorylation in SKOV3 cells (Fig. 2*b*). Thus, of the 3 MAPK pathways, only p44 is activated by IL-12 in ovarian carcinoma cells. IL-12 treatment also resulted in phosphorylation of Akt, suggesting that IL-12 directly activates the PI-3K/Akt signaling pathway in human ovarian cells. Together, these results indicate that IL-12R in human ovarian tumor cells is functionally active and is able to transduce intracellular signals. These experiments were performed with OVCAR3 cells and have demonstrated similar results (data not shown).

#### Identification of IL-12-induced gene expression in ovarian carcinoma cells using cDNA microarray

To understand the effect of IL-12-induced signaling on gene expression in ovarian carcinoma cells, we utilized the cDNA array hybridization technique. We set up the cutoff values of > 2-fold normal for upregulation and < 0.5-fold for downregulation. Analysis of the relative expression in intensity between untreated and

IL-12-treated OVCAR3 cells revealed that IL-12 upregulates 646 (8.9%) genes and downregulates 840 (11.6%) genes. Several genes, such as GADD45A, MMP1 (interstitial collagenase), P85SPR (PAK-interacting exchange factor), IRF7 (interferon regulatory factor 7), TNFRSF12 (TNF receptor superfamily, member 12) and CLDN3 (claudin 3), showed 3.8- to 4.7-fold increases in their expression (Table I). The other genes showed more moderate upregulation (2- to 3-fold increase). The magnitude of gene downregulation varied between 0.2- and 0.5-fold. A summary of the gene expression information for 98 of these genes is shown in Table I. Many of these genes have not been previously associated with IL-12 signaling.

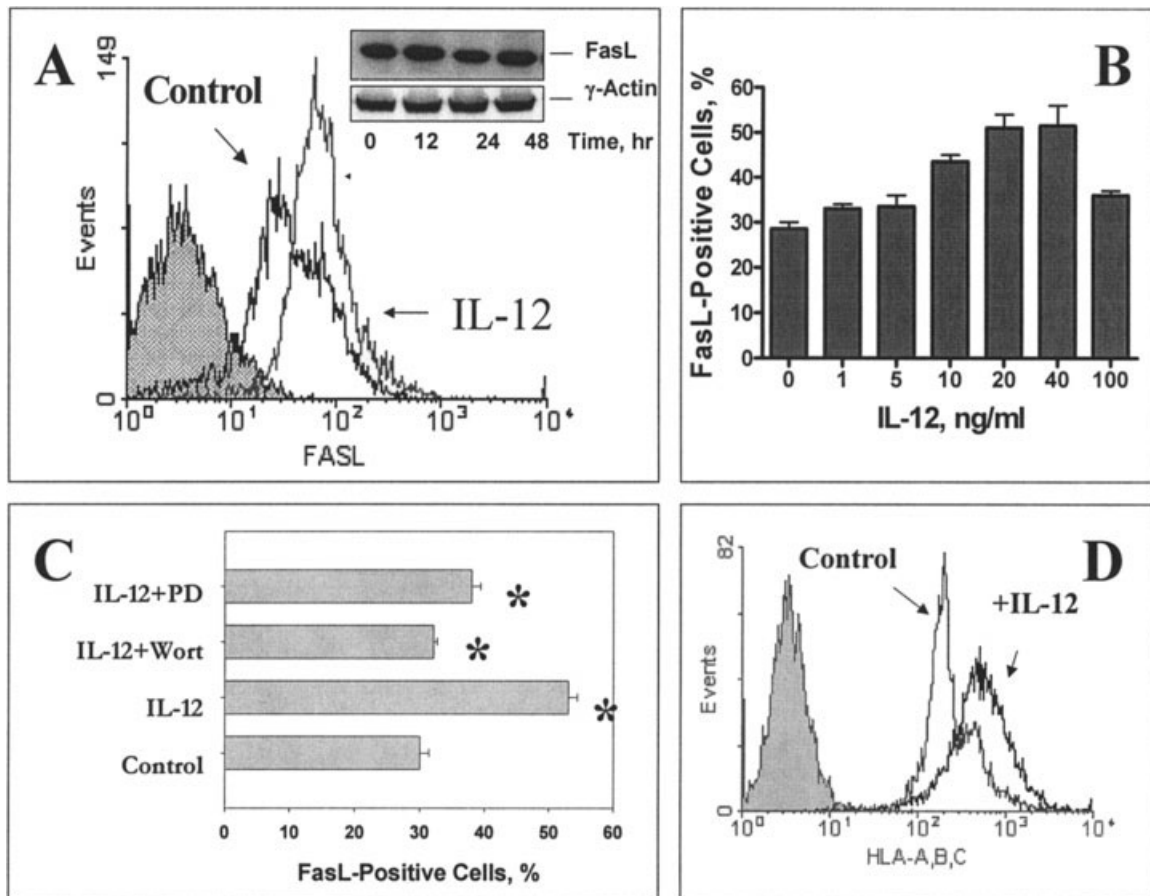
#### IL-12 treatment upregulates expression of FasL and MHC class I on ovarian tumor cells

It has been shown that activation of T and NK cells by various stimuli, including IL-12, results in upregulation of FasL.<sup>30,31</sup> To test whether IL-12R crosslinking leads to surface FasL upregulation on human ovarian carcinoma cells, we have examined FasL expression in OVCAR3 and SKOV3 cells by flow cytometry. Flow cytometric analysis showed that both cell lines express surface FasL molecules (Fig. 3*a* and *c*). Confluent ovarian carcinoma cells expressed much higher levels of surface FasL as compared to proliferating cells (data not shown); therefore, only confluent cells were used for all experiments. Incubation of ovarian carcinoma cell lines with increasing concentrations of IL-12 for 48 hr resulted in a dose-dependent increase in cell surface expression of FasL with maximal induction at 20–40 ng/ml of IL-12. Therefore, for further experiments, IL-12 was used at the optimal concentration of 40 ng/ml. Interestingly, neither expression of FasL protein in total cell lysates by Western blot (Fig. 3*a*, inset) nor FasL mRNA by real-time PCR (data not shown) was induced by IL-12 in ovarian carcinoma cell lines.

To study the mechanisms of upregulation of surface FasL, SKOV3 cells were preincubated with PD 98059 or wortmannin, the inhibitors of p42/p44 MAPK and PI-3/Akt kinases, respectively. Pretreatment of SKOV3 cells with 50  $\mu$ M PD 98059 or with 30  $\mu$ M of wortmannin for 1 hr before incubation with IL-12

TABLE I—SUMMARY OF IL-12 EFFECTS ON GENE EXPRESSION IN OVARIAN CARCINOMA CELLS

Upregulated genes	Fold increase	Downregulated genes	Fold decrease
Signal transduction <i>PTK9, RAB2, PPP1CB, PPP1R2, PPP2CB, PPP2R5C, PTK9</i>	2.0–2.2	<i>IKKE; MAPK4; NOS2A; PI3KCD, G; PLC-E; S100A12; regulator of G-protein signaling 1, 4</i>	2.0–3.0
Transcription factors		<i>STAT3; ZNF35, 135, 202, 219, 220; HOX11; HOXC10; FOXE3</i>	2.0–2.5
Protein synthesis and degradation <i>EEF1E1, 2S1, 2S2, 3S1, 3S6, 3S8, 4G2, 4A2; proteasome 26S ATPase2; proteasome subunits <math>\alpha</math>2, <math>\alpha</math>4, <math>\alpha</math>6, <math>\beta</math>5</i>	2.0–2.5		
Cell cycle <i>CDC 2, 10; CKS 1; cyclins C, E2, H; kinesin 1 (kinesin receptor); RB-binding protein 5, 8; topoisomerase 2<math>\alpha</math>; GADD45A</i>	2.0–3.8	<i>kinesin 2, arrestin 3, GAS 1</i>	2.0–5.0
Apoptosis <i>APR-3; Bcl-2-associated athanogene 2; COX7B, C; PDCD2</i>	2.0–2.5	<i>Bcl-2, caspase-1</i>	2.0–2.5
Cytokines and chemokines <i>IL-13RA1, IL-17B, IL-6, DR3, IRF7, IFRD1, MST1R</i>	2.0–4.3	<i>IL-4; IL-8; IL-13RA2; IL-18R1; CCR1, 2; CX3CR1; TNF-<math>\alpha</math>; BLYS(TNFSF13B); TNFAIP6 IFN-<math>\gamma</math>-inducible proteins 16, 30; MIG</i>	2.0–5.0
Growth factors and oncogenes <i>EGFR, DEK, GRO-1, Abl-1, RAP-1B, c-Myc, RAB2</i>	2.0–2.6	<i>FGF-18, IGFBP1, PDGFR<math>\alpha</math>, Vav-3, c-kit</i>	2.0–3.0
Adhesion/invasion <i>Integrin <math>\alpha</math>V, MMP1, ADADM9, CTNNA1, MCAM</i>	2.1–3.9	<i>Cadherin 11; VCAM1; integrin <math>\alpha</math>8, <math>\alpha</math>10; integrin <math>\beta</math>8; MMP-3, -7, -10, -12, -16, -27; ADAM12; ADAMTS1, 3</i>	2.0–3.0
MHC <i>MHC class I ORF, CIITA</i>	2.4–2.7		



**FIGURE 3**—IL-12 effects on the surface expression of FasL and MHC class I molecules in ovarian carcinoma cells. (a) Flow cytometric analysis of FasL expression by OVCAR3. Cells were untreated (control) or treated with 40 ng/ml IL-12 for 48 hr (IL-12), stained with biotinylated monoclonal anti-FasL mAb (open curve area) or isotype-matched mAbs (IgG<sub>1</sub>; shaded curve area), followed by streptavidin-FITC and subjected to FACS analysis. Inset: Expression of FasL protein in total cell lysates by Western blot analysis. OVCAR3 cells were incubated with 40 ng/ml IL-12 for 0–48 hr. FasL expression was visualized using CH11 mAb. Specificity was confirmed by incubation of the blot with CH11 anti-Fas mAb in the presence of specific blocking peptide (data not shown). (b) Dose-curve of surface FasL expression in IL-12-treated OVCAR3 cells. Cells were treated with 0–100 ng/ml IL-12 for 48 hr, stained with biotinylated anti-Fas Ab and subjected to FACS analysis. (c) Evidence of the involvement of PI-3/Akt and p42/p44 MAP kinase pathways in the regulation of FasL by IL-12. Cultured SKOV3 cells were pretreated for 1 hr with 30  $\mu$ M PD98059 (PD), 30  $\mu$ M wortmannin (Wort) or DMSO, then stimulated with 40 ng/ml IL-12 for 48 hr. Untreated DMSO-pretreated SKOV3 cells served as control. Combined results represent the mean  $\pm$  SEM from 3 independent experiments. Asterisk,  $p < 0.05$  for IL-12 compared to untreated cells or for IL-12 + inhibitors compared to the value of IL-12 alone. (d) Effect of IL-12 on MHC I expression in OVCAR3 cells. Cells were untreated (control) or treated with 40 ng/ml IL-12 for 48 hr (IL-12), stained with FITC-conjugated anti-MHC I (HLA-A, B, C) mAb (open curve areas) or FITC-conjugated isotype-matched mouse IgG<sub>1</sub> (shaded curve area) and subjected to FACS analysis. Isotype-matched IgG binding did not differ between untreated and IL-12-treated cells (data not shown).

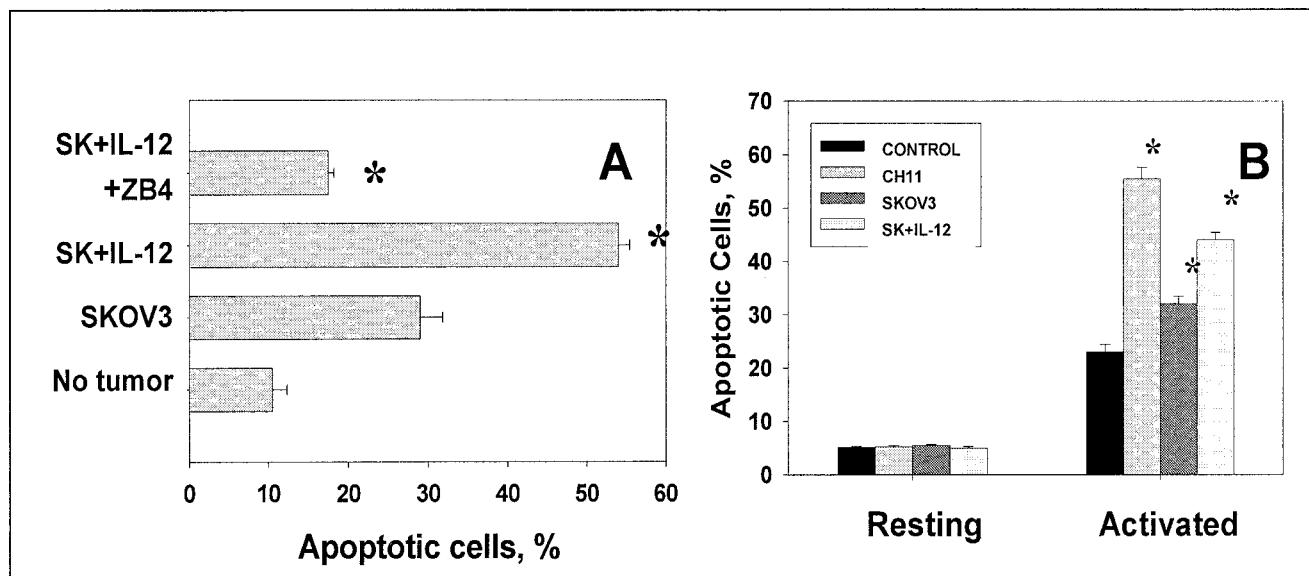
abrogated the increase in FasL expression in ovarian carcinoma cells (Fig. 3c). Treatment of SKOV3 cells with these inhibitors in the absence of IL-12 did not affect the baseline expression of FasL (data not shown). The results were similar for OVCAR3 cells (data not shown). These data indicate that the IL-12-induced upregulation of surface FasL is mediated via p42/p44 MAPK and PI-3/Akt kinase pathway.

Since it has been previously demonstrated that IL-12R crosslinking upregulates MHC class I molecules,<sup>50</sup> we tested whether IL-12 is able to upregulate MHC class I molecules in ovarian carcinoma cells. Incubation of OVCAR3 and SKOV3 cells with IL-12 (40  $\mu$ g/ml) for 24–48 hr resulted in obvious increase in the expression of MHC class I molecules (Fig. 3d for OVCAR3 cells; results for SKOV3 cells not shown). IL-12 is the most potent inducer of IFN- $\gamma$  production in T cells,<sup>50</sup> which could be responsible for the upregulation of MHC I expression in lymphoid and nonlymphoid cells. However, we did not observe any expression or induction of IFN- $\gamma$  in the IL-12-treated ovarian carcinoma cells by flow cytometry, Western blot, or RT-PCR analyses (data not

shown), suggesting that IL-12-induced upregulation of MHC class I molecules is mediated via the IFN- $\gamma$ -independent mechanisms.

#### *IL-12-treated ovarian carcinoma cells become more efficient in inducing T-cell apoptosis*

To ascertain the physiologic implications of IL-12-mediated upregulation of FasL, we evaluated the ability of ovarian carcinoma cells treated and untreated with IL-12 to induce apoptosis of Jurkat T-cell lymphoma cells. After 48 hr of treatment with IL-12 (40 ng/ml), OVCAR3 and SKOV3 cells were incubated with Jurkat cells for 24 hr. Nonadherent cells were harvested and stained with annexin V and the percentage of apoptotic Jurkat cells was determined by flow cytometry. Population of Jurkat cells normally contains about 10% of apoptotic cells. Incubation of Jurkat cells with ovarian carcinoma cell lines resulted in increase of apoptosis to the level of 29%. Pretreatment of ovarian carcinoma cells with IL-12 led to further increase in the killing of Jurkat cells up to 53% (Fig. 4a for SKOV3 cells; data for OVCAR3 cells not shown). Preincubation of Jurkat cells for 1 hr with 100 ng/ml



**FIGURE 4** – Increased apoptotic activity of IL-12-treated ovarian carcinoma. (a) SKOV3 cells were pretreated with or without 40 ng/ml IL-12 for 48 hr, washed and incubated with Jurkat cells for 24 hr in the presence or absence of 100 ng/ml of neutralizing anti-Fas Ab, ZB4. Floating cells were harvested, stained with anti-CD3-PE Ab and annexin V-FITC and analyzed by flow cytometry. (b) Normal human T lymphocytes were isolated from peripheral blood of healthy volunteers. Resting or PHA-stimulated T lymphocytes were incubated with or without 10 ng/ml of activating anti-Fas Ab (CH11). Alternatively, T lymphocytes were cocultured for 24 hr with SKOV3 cells or with SKOV3 cells pretreated with 40 ng/ml IL-12 (SK<sup>+</sup> IL-12). Lymphocytes were collected, stained with annexin V-FITC and analyzed by flow cytometry. The data are presented as mean percentages  $\pm$  SD from 3 experiments. Asterisk,  $p < 0.001$ .

of neutralizing anti-Fas Ab (ZB4) completely abrogated apoptosis ( $p < 0.001$ ) induced by IL-12-treated SKOV3 cells (Fig. 4a), indicating that IL-12-induced killing of Jurkat cells by ovarian carcinoma cells utilizes Fas/FasL pathway. These apoptotic effects require close cell-to-cell contacts, since no killing was observed when Jurkat cells were separated by a cell culture insert (data not shown).

We next tested the ability of ovarian tumor cells to induce apoptotic death in normal lymphocytes. Peripheral blood mononuclear cells were activated by culturing with PHA (5  $\mu$ g/ml) for 3 days. Naive and PHA-activated lymphocytes were incubated with IL-12-treated SKOV3 cells and their susceptibility to apoptosis via Fas/FasL pathway was examined as follows. Lymphocytes were treated with 100 ng/ml of agonistic anti-Fas Ab, CH11, and apoptosis was evaluated by annexin V method as described above. In agreement with published evidence,<sup>52</sup> crosslinking of Fas receptor by anti-Fas antibody induced apoptosis in activated but not resting lymphocytes (Fig. 4b). We therefore tested the ability of SKOV3 cells to induce apoptosis in PHA-activated lymphocytes. When the activated lymphocytes were incubated with SKOV3 cells for 24 hr, about 32% lymphocytes became apoptotic, whereas only 22% of control lymphocytes were found to be apoptotic. Following IL-12 treatment, the apoptotic effects of SKOV3 cells against activated lymphocytes were further augmented and the percentage of the apoptotic lymphocytes increased to 43% (Fig. 4b). These results indicate that only activated T lymphocytes are susceptible to tumor-induced apoptosis and that IL-12 is able to upregulate apoptotic effects of ovarian tumor cells against activated lymphocytes.

#### Role of MMP-3 and -7 in IL-12-induced upregulation of FasL

IL-12 treatment of ovarian carcinoma cells showed no changes in expression of total FasL protein (Fig. 3a, inset) and mRNA (data not shown). The observed IL-12-induced increase in cell surface expression of FasL could be due to changes in the membrane trafficking or the cleavage and shedding of FasL from the cell surface. Experimental evidence indicates that MMP-3 (stromelysin) and MMP-7 (matrilysin) can cleave FasL from the cell membrane.<sup>53,54</sup> Interestingly, our cDNA array showed that IL-12 treat-

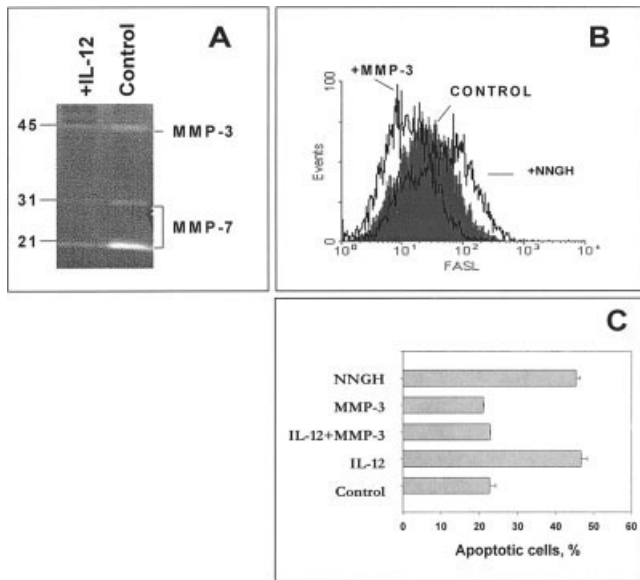
ment substantially downregulated MMP-3 and -7 (Table I). We measured the effect of IL-12 treatment on activities of these MMPs in ovarian carcinoma cell lines. Incubation of SKOV3 cells with 40 ng/ml of IL-12 for 48 hr resulted in substantial inhibition of activities of MMP-3 and -7 as measured by gel zymography (Fig. 5a). Therefore, IL-12-mediated downregulation of MMP-3 and -7 may reduce FasL cleavage, resulting in a higher cell membrane expression of FasL.

To confirm the effect of MMPs on FasL cell membrane expression, SKOV3 cells were incubated with purified MMP-3 (2  $\mu$ g/ml) for 4 hr or with 0.25  $\mu$ M MMP inhibitor, NNGH, for 12 hr. Figure 5 demonstrates that treatment of SKOV3 ovarian carcinoma cells with MMP-3 resulted in reduction of FasL expression, probably due to increased FasL cleavage. Consistent with these results, treatment of IL-12-induced ovarian carcinoma cells with MMP-3 completely abrogated IL-12-stimulated killing of Jurkat cells (Fig. 5c). Furthermore, inhibition of MMP-3 activity with NNGH had an opposing effect, yielding increased FasL expression and Jurkat killing in tumor cells (Fig. 5b and c).

#### DISCUSSION

Our data demonstrate that human ovarian cancer cells OVCAR3 and SKOV3 express IL-12R. Normal nonactivated T cells express only IL-12R $\beta$ 1 chain, whereas IL-12R $\beta$ 2 subunit usually appears in CD4<sup>+</sup> T cells following their differentiation into Th1 cells, but not Th2 cells.<sup>18,19</sup> We have found that human ovarian cancer cells express both IL-12R $\beta$ 1 and IL-12R $\beta$ 2 chains. Thus, in this regard, the IL-12R in human ovarian cancer cells mimic Th1 rather than Th2 cells.

It was previously shown that IL-12 activates STAT1, STAT3 and STAT4 and induces production of IFN- $\gamma$  only in Th1 but not in Th2 cells that lack IL-12R $\beta$ 2 chain.<sup>18,19,55</sup> Our data demonstrate that, in ovarian cancer cells, IL-12 was able to activate STATs 2–5 without obvious changes in STAT1 and STAT6. Activation of STAT4 in lymphocytes was found to be important for IL-12-induced secretion of IFN- $\gamma$  because IL-12 fails to induce IFN- $\gamma$



**FIGURE 5** – (a) The effects of IL-12 on MMP-3 and -7 activities in ovarian carcinoma cells. SKOV3 cells were incubated with 40 ng/ml IL-12 for 48 hr. Cells were harvested and total cell lysates were analyzed by casein zymography. Top bar, MMP-3, active form (43/45 kDa). The proform is not visible. Bottom bar, MMP-7, proform (31 kDa) and active form (21 kDa). (b) The effects of MMP-3 and MMP inhibitor on FasL expression in ovarian carcinoma cells. SKOV3 cells were incubated with 2  $\mu$ g/ml purified MMP-3 for 4 hr or with 0.25  $\mu$ M NNGH for 12 hr. Control, untreated cells. Cells were stained with biotinylated FasL mAb followed by streptavidin-FITC and analyzed by flow cytometry. (c) The effects of IL-12, MMP-3 and MMP inhibitor on apoptotic activity of ovarian carcinoma cells. SKOV3 cells were treated as above and coincubated with Jurkat cells for 24 hr. Apoptosis of Jurkat cells was analyzed by annexin V staining as above.

production in STAT-4<sup>-/-</sup> mice.<sup>56,57</sup> Although IL-12 activated STAT-4 in human ovarian cells, it did not result in upregulation of IFN- $\gamma$  gene expression and IFN- $\gamma$  production. This paralleled with a failure of IL-12 to phosphorylate the p38 MAPK in ovarian cancer cells. It was previously shown that in Th1 lymphocytes, IFN- $\gamma$  production is mediated via the p38 MAP kinase signaling pathway.<sup>49,58</sup> Some reports indicate that IL-12 is unable to stimulate ERK1/2 signaling pathway in activated Th1 lymphocytes.<sup>49,58</sup> However, in human ovarian cells, IL-12 phosphorylated

Tyk2, ERK1 (p44) and Akt kinases. Thus, our results showed that human ovarian cancer cells express functional IL-12 receptors, which is efficient in signal transduction via pathways different from those in lymphocytes.

We did not find that induction of surface FasL expression by IL-12 was due to the increased transcription or protein synthesis. It could therefore be a result of increased extracellular trafficking or decreased shedding of FasL from the cell surface. Some reports point out that MMP-3 and -7, but not other MMPs, can cleave FasL from the cell membrane.<sup>53,54</sup> The results presented in this report demonstrate that IL-12 signaling leads to transcriptional downregulation of MMP-3 and -7 activity and reduction of FasL cleavage with concomitant accumulation of FasL on cell surface.

Using the kinase inhibitors wortmannin and PD 98059, we were able to abrogate IL-12-induced upregulation of FasL. This may indicate the involvement of PI3/Akt and ERK1/2 pathways in IL-12-mediated upregulation of FasL. Based on the mechanism outlined above, it is unlikely that wortmannin and PD 98059 directly control FasL expression, but rather inhibit IL-12R signaling, thus preventing downregulation of MMP-3 and -7 and FasL cleavage.

In mouse models of ovarian cancer, IL-12 therapy led to substantial improvement.<sup>59,60</sup> This was attributed to the ability of IL-12 to stimulate the antitumor immune and antiinflammatory responses. As we demonstrate here, crosslinking of IL-12R on ovarian carcinoma cells results in upregulation of MHC class I molecules, which could further increase recognition and destruction of cancer cells by immune effector cells. However, clinical efficacy of IL-12 administered intraperitoneally (IP) was tested in ovarian cancer patients and have demonstrated low response rate.<sup>42</sup> It was shown previously that human ovarian cancer cells express FasL and are able to induce apoptosis in Jurkat cells and activated lymphocytes.<sup>61</sup> Our data demonstrate that IL-12 treatment of ovarian cancer cells could further increase FasL expression and their ability to induce apoptosis in lymphocytes. In agreement with our data, lymphocyte apoptosis was demonstrated in patients receiving IP IL-12 in phase 2 clinical trial.<sup>43</sup> Therefore, IL-12 therapy in ovarian cancer could result in upregulation of FasL by tumor cells, thus triggering an apoptotic death of immune lymphocytes during their interaction with ovarian cancer cells. This could lead to decreased efficacy of IL-12 therapy in ovarian cancer patients.

#### ACKNOWLEDGEMENTS

The authors thank Drs. Walter Storkus, Pawel Kalinski and Ruth Modzelewski for critically reading the manuscript and for stimulating discussions. Supported by the American Cancer Society grant RSG-01-023-01-CCE (to A.E.L.)

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