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Mammaglobin B (SCGB2A1) is a novel tumour antigen highly differentially expressed in all major histological types of ovarian cancer: implications for ovarian cancer immunotherapy

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Background: We studied the genetic fingerprints of ovarian cancer and validated the potential of Mammaglobin b (*SCGB2A1*), one of the top differentially expressed genes found in our analysis, as a novel ovarian tumour rejection antigen.

Methods: We profiled 70 ovarian carcinomas including 24 serous (OSPC), 15 clear-cell (CC), 24 endometrioid (EAC) and 7 poorly differentiated tumours, and 14 normal human ovarian surface epithelial (HOSE) control cell lines using the Human HG-U133 Plus 2.0 chip (Affymetrix). Quantitative real-time PCR and immunohistochemistry staining techniques were used to validate microarray data at RNA and protein levels for SCGB2A1. Full-length human-recombinant SCGB2A1 was used to pulse monocyte-derived dendritic cells (DCs) to stimulate autologous SCGB2A1-specific cytotoxic T-lymphocyte (CTL) responses against chemo-naive and chemo-resistant autologous ovarian tumours.

Results: Gene expression profiling identified *SCGB2A1* as a top differentially expressed gene in all histological ovarian cancer types tested. The CD8+ CTL populations generated against SCGB2A1 were able to consistently induce lysis of autologous primary (chemo-naive) and metastatic/recurrent (chemo-resistant) target tumour cells expressing SCGB2A1, whereas autologous HLA-identical noncancerous cells were not lysed. Cytotoxicity against autologous tumour cells was significantly inhibited by anti-HLA-class I (W6/32) monoclonal antibody. Intracellular cytokine expression measured by flow cytometry showed a striking type 1 cytokine profile (i.e., high IFN-γ secretion) in SCGB2A1-specific CTLs.

Conclusion: *SCGB2A1* is a top differentially expressed gene in all major histological types of ovarian cancers and may represent a novel and attractive target for the immunotherapy of patients harbouring recurrent disease resistant to chemotherapy.

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Ovarian carcinoma remains the cancer with the highest mortality rate among gynaecological tumours. In 2012, 22 280 new cases of ovarian carcinoma were predicted in the United States, with 15 500 deaths secondary to this disease (Siegel *et al*, 2012). At the time of diagnosis, two-thirds of patients had advanced disease, and although the majority of these women initially experience a response to platinum and taxane chemotherapy, ovarian cancer will recur in a majority of them and their prognosis remains dismal. Overall, the 5-year survival rate for FIGO (International Federation of Gynaecologists and Obstetricians) stage III disease is 20–25% and for stage IV disease is only 5% (DiSaia and Creasman, 2002). These figures illustrate the dire need for the development of novel, effective approaches for the eradication of chemotherapy-resistant ovarian cancer.

The prospects for immunological treatment of cancer have risen sharply in the past few years, based on concerted efforts to identify tumour-specific antigens that may serve as immune targets, and on a better understanding and application of dendritic cells (DCs) as powerful inducers of tumour antigen-specific T-cell responses (Mellman *et al*, 2011). Consistent with this view, recent work has shown that (1) the presence of ovarian tumour-infiltrating T cells (TILs) correlate with improved progression-free survival and overall survival in patients with advanced ovarian cancer; (2) specialised immune cells, known as cytotoxic T lymphocytes (CTLs), when activated by DCs, can recognise tumour-specific antigens produced by ovarian tumour cells; and (3) ovarian antigen-specific CTLs are capable of killing ovarian cancer cells (Santin *et al*, 2000; Zhang *et al*, 2003; Bellone *et al*, 2009, 2009a; Mellman *et al*, 2011). Importantly, because T-cell responses are able to eliminate tumour cells independently of their proliferative state as well as their resistance to chemotherapy, ovarian cancer immunotherapy may represent an attractive treatment option for patient harbouring recurrent chemotherapy-resistant disease.

Large-scale gene expression analysis, using such techniques as high-density oligonucleotide and cDNA microarrays, represents a powerful tool to identify genes involved in ovarian carcinogenesis. In this study, with the ultimate goal being to identify potential novel targets for ovarian cancer immunotherapy, we used oligonucleotide microarrays that interrogate the expression of some 47 000 human transcripts (Human HG-U133 Plus 2.0 chip; Affymetrix Santa Clara, CA, USA) to profile 70 primary epithelial ovarian carcinomas (including 24 serous (OSPC), 15 clear-cell (CC), 24 endometrioid (EAC) and 7 poorly differentiated ovarian tumours) and 14 normal human ovarian surface epithelial (HOSE) control cell lines. Because mammaglobin-B (*secretoglobin, family 2A, member 1* (*SCGB2A1*), *MGB-2*), a uteroglobin gene family member originally isolated in human endometrium (Becker *et al*, 1998), was found as a top differentially expressed gene in all histological types of epithelial ovarian cancer, with over 905-fold of mean upregulation relative to human ovarian normal tissues (HOSE), we next evaluated the potential of SCGB2A1 as a novel tumour-rejection antigen to target advanced/chemotherapy-resistant disease. Using autologous SCGB2A1-pulsed DCs, we provide the first evidence that *in vitro* stimulated SCGB2A1-specific CTLs are able to recognise and kill primary (i.e., chemo-naive) as well as recurrent chemotherapy-resistant autologous ovarian cancer cells *in vitro*. Moreover, we demonstrate a striking type 1 cytokine profile (i.e., high IFN-γ secretion) in SCGB2A1-specific CTLs. These results highlight for the first time SCGB2A1 as a novel and attractive target for the immunotherapy of the common spectrum of histological types of epithelial ovarian cancer.

MATERIALS AND METHODS

Ovarian tissue samples for gene expression profiling. A total of 70 snap-frozen epithelial ovarian carcinomas of histologically proven ovarian origin were obtained from the Division of Gynecologic Oncology at the University of Brescia, Italy, from consented patients according to the institutional guidelines. The study has been performed following the Declaration of Helsinki Principles and it has been approved by the Research Review Board – the Ethic Committee – of the Spedali Civili, Brescia, Italy, and Yale University. Patient clinical and pathological characteristics are shown in Table 1. Briefly, ovarian tumour tissues were identified, sharp dissected and snap frozen in liquid nitrogen within 30 min from resection. The samples were embedded in OCT medium, microdissected and the frozen sections were stained with H&E to check epithelial purity. Each sample was histologically analysed by a staff pathologist and only tumour samples containing at least 70% tumour epithelial cells were retained for further total RNA extraction.

Establishment of HOSE primary cell lines for gene expression profiling. A total of 14 primary ovarian cell lines (HOSE) were established after sterile processing of samples from surgical biopsies as previously described (Bignotti *et al*, 2006). The HOSE cell lines were derived from normal ovarian epithelial tissues of patients undergoing surgery for benign pathologies including uterine fibromas or prolapses. Pathological examination confirmed the absence of any neoplastic disease. The total length of *in vitro* culture was <14 days for all samples. Normal cell cultures were collected for RNA extraction at 70–80% confluence without being subcultured (passage 0). Only cell cultures composed of at least 99% epithelial cells were retained for RNA extraction.

Total RNA extraction and GeneChip hybridisation. Total RNA was obtained from a total of 84 samples including 24 flash-frozen serous OSPC, 15 CC, 24 EAC and 7 poorly differentiated tumours

Table 1. Clinical and pathological characteristics of patients

			Age			Grading			Stage			
Pathology	**Histotype**	**Number of Patients**	**Range (Y)**	**Mean (Y)**	**s.d.**	**1**	**2**	**3**	**I**	**II**	**III**	**IV**
None	Normal ovary	14	37–61	53	6	—	NA	—		—	NA	—
Primary EOC	Serous	24	41–84	60	12	—	2	22	—	3	12	9
	Endometrioid	24	39–71	56	10	4	8	12	7	7	8	2
	Clear cell	15	34–74	60	12	—	—	15	6	5	4	—
	Undifferentiated	7	38–84	55	16	—	—	7	—	1	4	2

Abbreviations: EOC = epithelial ovarian carcinoma; NA = not available; Y = years.

as well as 14 HOSE cell lines by using TRIZOL reagent (Life Technologies, Inc., Carlsbad, CA, USA) and then further purified using RNeasy Min-elute Clean-up Columns (Qiagen Inc., Valencia, CA, USA) as previously described by our group (Bignotti *et al*, 2006). Labelling of samples and hybridisation to the Affymetrix Human HG-U133 Plus 2.0 oligonucleotide microarrays chip containing over 54 000 probe sets covering 47 000 transcripts were performed following the manufacturer's protocols, as described (Bignotti *et al*, 2006).

Data processing. All data used in our analyses were derived from Affymetrix 5.0 software. GeneChip 5.0 output files are given as a signal that represents the difference between the intensities of the sequence-specific perfect-match probe set and the mismatch probe set, or as a detection of present, marginal, or absent signals as determined by the GeneChip 5.0 algorithm. Gene arrays were scaled to a median signal of 1500 and then analysed independently. Signal calls were transformed to their base-2 logarithms, and each sample was normalised to have its mean and variance equal to the grand mean and average variance across all samples.

Gene expression data analysis. Statistical analyses of gene expression data were performed with SAS version 9.2 (SAS Institute, Cary, NC, USA) and SAM version 3.05 (Tusher *et al*, 2001). Genes were selected for analysis based on detection and expression. In each comparison of a cancer to HOSE, a gene was selected for analysis via SAM if the higher-expressing group had $\geqslant$75% 'present' detection calls and an average normalised signal call of at least 64. SAM was used to establish FDR *q*-values and fold changes, and genes were retained if they showed a fold change of >4.0.

Validation of gene expression by quantitative RT–PCR. Real-time PCR was performed on a sub-group of 38 ovarian cancer samples including 11 OSPC, 14 EAC, 10 CC and 3 undifferentiated and 14 HOSE control samples in triplicate by using primer sets and probes specific for *SCGB2A*1, *claudin-3* (*CLDN3*), *claudin-4* (*CLDN4*), human trophoblast cell-surface marker (*TROP-2*) and lipophilin B (*SCGB1D2*). All the reactions were carried out on the ABI PRISM 7000 Sequence detection System (Applied Biosystems, Applera UK, Warrington, UK) using the TaqMan Universal PCR master Mix and the following Assays on Demand (Applied Biosystems): Hs00267180_m1 (SCGB2A1), CLDN3 (Hs00265816_s1), CLDN4 (Hs00533616_s1), TROP-2 (Hs00242741_s1) and SCGB1D2 (Hs00255208_m1) as previously described (Bignotti *et al*, 2006). The data were normalised using *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as reference gene. Spearman's rank correlation was used to estimate the degree of association between microarray and qRT–PCR data for SCGB2A1, CLDN3, CLDN4, TROP-2 and SCGB1D2.

Immunohistochemistry on formalin-fixed tissues. To confirm protein expression levels in ovarian cancer, immunohistochemical staining for MGB-2 was performed on 10 representative ovarian carcinoma samples. As controls, surface epithelia obtained from normal ovaries was used. Briefly, immunohistochemistry (IHC) was performed on 4 μm FFPE tissue sections that were deparaffined and rehydrated in graded solutions of ethanol and distilled water. Antigen retrieval was performed in microwave oven (3 $\times$ 5-min cycles at 750 W) in EDTA buffer, pH 8.0. Mammaglobin primary antibody (rabbit, clone 31A5, Zeta Corporation, Sierra Madre, CA, USA) was applied diluted 1 : 50. The reaction was revealed using Novolink Polymer (Leica Mycrosystems, Newcastle upon Tyne, UK) followed by diaminobenzidine (DAB, Dako, Carpinteria, CA, USA) as chromogen. Sections were then counterstained with Mayer's haematoxylin.

Full-length mammaglobin B protein production. Briefly, purified SCGB2A1 protein was generated by cloning the full-length MGB-2 DNA into a PET16 vector with 10 histidine tags at the N terminal end in BL21 *E. coli* bacteria. The protein expression was induced in cultures at an optical density at 600 nm of 0.6 by the addition of isopropyl-β-D-thiogalactoside (IPTG; final concentration, 0.4 mM). Cultures were grown for 4 h, cells were pelleted and resuspended in binding buffer (Guanidine 6 M, Tris 20 mM, NaCl 0.5 M, Imidazole 20 mM, β-mercaptoethanol 5 mM and Triton X-100 1%) with proteases inhibitors (EDTA free) and lysozyme. After 30 min of incubation, the bacteria were disrupted by sonication on ice in short bursts. The bacterial lysate was centrifuged at 15 000 r.p.m. for 15 min at 4 °C, and the supernatant was purified with Ni-NTA Agarose in accordance with the procedures suggested by the manufacturer (Qiagen Inc.). Cleavage of the protein was achieved using PBS with imidazole 200 mM and PBS with imidazole 400 mM. The protein was dialyzed overnight in PBS and the purity was analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Coomassie blue staining, whereas quantification was obtained spectrophotometrically by the protein assay of Bio-Rad Laboratories (Hercules, CA, USA). Preparations were typically $>$98% pure MGB-2.

Cell line cultures. Primary autologous serous ovarian cancer cell lines were established after sterile processing of the samples from surgical biopsies as previously described for ovarian carcinoma specimens (Bellone *et al*, 2009, 2009a). Tumour specimens used in cytotoxicity studies were obtained from a 67-year-old patient harbouring a stage IV high-grade serous carcinoma of the ovary before chemotherapy (i.e., primary chemo-naive cell line established from an ovarian tumour biopsy collected at the time of the primary tumour debulking) and at the time of disease progression after multiple regimens of chemotherapy (i.e., metastatic chemotherapy-resistant cell line established from pleural effusion), under approval of the Institutional Review Board. The *in vivo* chemotherapy resistance of the metastatic/recurrent tumour was confirmed *in vitro* by measuring chemotherapy resistance as percentage cell inhibition (PCI) by ChemoFx (Precision Therapeutics, Pittsburgh, PA, USA) (Cross *et al*, 2010). Both tumour lines were cultured in RPMI-1640 supplemented with HEPES buffer, L-glutamine, penicillin and 10% heat-inactivated FBS. The epithelial nature and the purity of primary tumour cultures was verified by IHC staining and flow cytometric analysis with antibodies against cytokeratin and vimentin as previously described (Bellone *et al*, 2009, 2009a). Only primary cultures that had at least 90% viability and contained $>$99% epithelial cells were used for cytotoxicity assays.

Small interfering RNA (siRNA) knockdown experiments. Both SCGB2A1-specific siRNA oligonucleotides (ID s8730) and non-specific siRNA duplexes used as negative control were purchased from Ambion (Austin, TX, USA). Briefly, SCGB2A1-positive metastatic ovarian carcinoma cells were cultured in six-well plates and transfected with anti-SCGB2A1 siRNA duplexes at 10 nM in conjunction with 5 μl Lipofectamine RNAiMAX (Invitrogen, Grand Island, NY, USA) following the manufacturer's instructions. Mock transfections and nonspecific siRNA duplexes were used as negative controls. Tumour cells were treated for 72 h (i.e., the time we found required for maximal downregulation of SCGB2A1, based on qRT–PCR), after which they were used as targets in cytotoxicity assays as described below.

Isolation of peripheral blood mononuclear cells (PBMCs) and generation of DCs. Autologous PBMCs were separated from heparinised venous blood by Ficoll-Hypaque (Sigma, St Louis, MO, USA) density gradient centrifugation and either cryopreserved in RPMI-1640 (Gibco-BRL, Grand Island, NY, USA) plus 20% DMSO, 30% autologous plasma or immediately used for DC generation. Briefly, PBMCs obtained from 42 ml of peripheral blood were placed into six-well culture plates (Costar, Cambridge, MA, USA) in AIM-V (Gibco-BRL) at 0.5–1 $\times$ 10^6 cells in 3 ml per

well. After 2 h at 37 °C, nonadherent cells were removed, and the adherent cells were cultured at 37 °C in a humidified 5% CO_2/95% air incubator, in medium supplemented with recombinant human GM-CSF (800 U ml^{-1}; Immunex, Seattle, WA, USA) and IL-4 (500 U ml^{-1}; Genzyme Cambridge, MA, USA) (Santin *et al*, 2001). Every 2 days, 1 ml of spent medium was replaced by 1.5 ml of fresh medium containing 1600 U ml^{-1} GM-CSF and 1000 U ml^{-1} IL-4 to yield final concentrations of 800 U ml^{-1} and 500 U ml^{-1}, respectively (Santin *et al*, 2001). After 5 days of culture, DC maturation was induced by addition of TNFα, IL-1β (both from R&D Systems, Minneapolis, MN, USA) and PGE_2 (Sigma) for 48 h as described (Santin *et al*, 2001). After 7 days of culture, DCs were harvested for pulsing with SCGB2A1 as described below.

DC pulsing. Following culture, DCs were washed twice in AIM-V and added to 50 ml polypropylene tubes (Falcon, Oxnard, CA, USA). The cationic lipid DOTAP (Boehringer Mannheim, Indianapolis, IN, USA) was used to deliver SCGB2A1 protein into cells. SCGB2A1 (50 μg ml^{-1}) and DOTAP (20 μg ml^{-1}) were mixed in 12 × 75 mm polystyrene tubes at room temperature for 20 min. The complex was added to the DCs in a total volume of 2–5 ml of AIM-V and incubated at 37 °C in an incubator with occasional agitation for 3 h. The cells were washed twice with PBS and resuspended in AIM-V as described below.

***In vitro* generation of SCGB2A1-specific CTLs.** Fresh or cryopreserved responder PBMCs were washed and resuspended in AIM-V at 10–20 × 10^6 cells/well in six-well culture plates (Costar) with SCGB2A1 protein-pulsed autologous DCs (ratios from 20 : 1 to 30 : 1 responders PBMC/DC). After 14 days, responder T cells were collected and restimulated with MGB-2-pulsed DCs. For the second and third DC stimulations, the medium was supplemented with 50 U ml^{-1} IL-2 (Chiron Therapeutics, Emeryville, CA, USA), and the culture period extended to 21 days. After three restimulations, CD8+ T cells were recovered by positive selection with anti-CD8 magnetic beads (Life Technologies, Grand Island, NY, USA). The cytotoxicity of generated CTL lines was assessed using a standard 5-h chromium release assay (^{51}Cr release). The MHC restriction of lytic activity and SCGB2A1 as target antigen conferring lysis specificity were tested by a blockade of the killing of the tumour using the anti-pan-HLA mAb W6/32 compared with the isotype-matched control and by downregulation of SCGB2A1 expression using siRNA. As negative control targets, autologous lymphoblasts were prepared by 3-day stimulation with PHA (GIBCO-BRL; 1 μg ml^{-1}) in RPMI-1640 plus IL-2 (100 U ml^{-1}) and 5% autologous plasma, whereas EBV-transformed autologous lymphoblastoid B-cell lines (LCL) were established by coculture of PBMCs with EBV-containing supernatant from the B95.8 cell line in the presence of 1 μg ml^{-1} cyclosporin A (Sandoz, Camberley, UK) and were maintained in AIM-V supplemented with 10% human AB serum (Gemini Bioproducts, Calabasas, CA, USA). Specific lysis of target cells is presented as percentage of specific ^{51}Cr release, calculated from the formula: $(E - S/T - S) \times 100$, where E is experimental ^{51}Cr release, S is the spontaneous ^{51}Cr release and T is the total ^{51}Cr release by 1% SDS.

Phenotypic analysis of T cells. Enriched cultures of CD8+ T cells were phenotyped at the time of first cytotoxicity and thereafter in order to correlate cytolytic specificity with a particular lymphoid subset. Flow cytometry was performed using mAbs directly conjugated against the following human leukocyte antigens: Leu-4 (CD3, pan T cells); Leu-3 (CD4, T helper/inducer); Leu-2a (CD8, T cytotoxic/suppressor); Leu-19 (CD56, NK/K cells); Tac (CD25, the IL-2R); anti-HLA-DR (L-243); anti TcR-$\alpha\beta$ or TcR-$\gamma\delta$ (Becton Dickinson, San Jose, CA, USA) and analysed on a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

Cytokine assays. Intracellular cytokine expression was measured by flow cytometry after overnight co-culture of T cells with anti-CD3 monoclonal antibody (solid-phase OKT-3, 10 μg ml^{-1}; Ortho Pharmaceutical Corporation, Raritan, NJ, USA) and/or 6 h stimulation with PMA and ionomycin, as previously described (Bellone *et al*, 2009, 2009a). Negative controls included T cells cultured alone or stained with isotype control antibodies. Fluorescence was measured with a FACSCalibur and data were analysed with CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis. Statistical analyses of gene expression data were performed as described above. Statistical analysis of other data was performed with the software packages SPSS10.0 (SPSS, Chicago, IL, USA). Cytotoxicity results were analysed using Student's *t*-test. All data were expressed as mean percentages of positive cells + s.d. In all tests, the difference was considered significant when *P*-values were <0.05.

RESULTS

Gene expression analyses of serous ovarian carcinomas, clear-cell ovarian carcinomas, endometrioid ovarian carcinomas and poorly differentiated ovarian cancers *vs* HOSE. Comprehensive gene expression profiles of 70 snap-frozen ovarian carcinomas and 14 HOSE cell lines were generated using high-density oligonucleotide. In our first comparison, we evaluated differences in gene expression profiling between 24 OSPC *vs* HOSE. After filtering out most 'absent' genes, analysis via SAM revealed a total of 2187 probe sets showing >4-fold change, 2186 of which had FDR $q<0.05$. Out of 2187 genes, 1075 were found upregulated in OSPC when compared with HOSE (Table 2 depicts the 15 most frequently upregulated genes whereas Supplementary Table 1a and b lists all upregulated and downregulated genes, respectively). As shown in Table 2, *SCGB2A1* was the second most overexpressed gene (789-fold) in OSPC *vs* HOSE. *Claudin 3* and *claudin 4*, the two genes encoding for the receptors of the *Clostridium perfringens* enterotoxin (CPE), a potent cytolytic toxin showing promise as novel local/regional therapy in ovarian cancer (Santin *et al*, 2005), were also found highly differentially overexpressed in OSPC when compared with HOSE (Supplementary Table 1a, 213-fold and 34-fold, respectively). In our second comparison, we evaluated 24 EAC *vs* HOSE. Analysis via SAM revealed a total of 2188 probe sets showing >4-fold change, 2183 of which had FDR $q<0.05$. Out of 2188 genes, 1054 were found upregulated in EAC when compared with HOSE (Table 3 depicts the top 15 upregulated genes whereas Supplementary Table 2a and b lists all upregulated and downregulated genes, respectively). As shown in Table 3, *SCGB2A1* was the top overexpressed gene (1844-fold) in EAC *vs* HOSE. *Claudin 3* and *claudin 4* were also highly differentially expressed in EAC when compared with HOSE (Supplementary Table 2a, 186-fold and 36-fold, respectively). In our third comparison, we evaluated 15 ovarian CC carcinoma *vs* HOSE. Analysis via SAM revealed a total of 2043 probe sets showing >4-fold change, all but 5 of which had FDR $q<0.05$. Out of 2043 genes, 1077 were found upregulated in CC when compared with HOSE (Table 4 depicts the top 15 upregulated genes whereas Supplementary Table 3a and b lists all upregulated and downregulated genes, respectively). As shown in Table 4, *SCGB2A1* was the top overexpressed gene (773-fold) in CC *vs* HOSE. *Claudin 3* and *claudin 4* were also highly differentially expressed in CC tumours when compared with HOSE (Supplementary Table 3a, 136-fold and 42-fold, respectively). Finally, in our last comparison, we evaluated 7 undifferentiated ovarian carcinoma (UOC) *vs* HOSE. The SAM analysis revealed a total of 2225 probe sets showing >4-fold change, 2169 of which (97.5%) had FDR $q<0.05$.

Table 2. Fifteen most frequently upregulated genes in OSPC *vs* HOSE

U133 Plus 2.0 probe set	Gene symbol	Fold change	Gene name
216834_at	*RGS1*	976.89	*Regulator of G-protein signalling 1*
205979_at	*SCGB2A1*	788.77	*Secretoglobin, family 2A, member 1*
1558034_s_at	*CP*	282.25	*Ceruloplasmin (ferroxidase)*
225645_at	*EHF*	244.63	*Ets homologous factor*
225846_at	*RBM35A*	226.47	*RNA binding motif protein 35A*
203953_s_at	*CLDN3*	213.31	*Claudin 3*
221884_at	*EVI1*	213.07	*Ecotropic viral integration site 1*
219768_at	*VTCN1*	194.11	*V-set domain containing T cell activation inhibitor 1*
231192_at	*LPAR3*	193.62	*Lysophosphatidic acid receptor 3*
204846_at	*CP*	192.74	*Ceruloplasmin (ferroxidase)*
214677_x_at	*IGLV1–44*	191.07	*Immunoglobulin λ locus 1–44*
213993_at	*SPON1*	163.23	*Spondin 1, extracellular matrix protein*
228377_at	*KLHL14*	131.84	*Kelch-like 14 (Drosophila)*
211430_s_at	*IGHG1 || IGHG2 || IGHM || IGHV4–31*	131.10	*Immunoglobulin heavy locus*
227253_at	*CP*	128.69	Ceruloplasmin (ferroxidase)

Abbreviations: HOSE = human ovarian surface epithelial control cell line; OSPC = ovarian serous papillary carcinoma.

Table 3. Fifteen most frequently upregulated genes in endometrioid EOC *vs* HOSE

U133 Plus 2.0 probe set	Gene symbol	Fold change	Gene name
205979_at	*SCGB2A1*	1843.78	*Secretoglobin, family 2A, member 1*
216834_at	*RGS1*	418.89	*Regulator of G-protein signalling 1*
225645_at	*EHF*	336.68	*Ets homologous factor*
203953_s_at	*CLDN3*	186.30	*Claudin 3*
225846_at	*RBM35A*	183.31	*RNA binding motif protein 35A*
221884_at	*EVI1*	165.92	*Ecotropic viral integration site 1*
206799_at	*SCGB1D2*	156.66	*Secretoglobin, family 1D, member 2*
227235_at	*GUCY1A3*	139.50	*Guanylate cyclase 1, soluble, α3*
219768_at	*VTCN1*	135.86	*V-set domain containing T cell activation inhibitor 1*
1558034_s_at	*CP*	126.31	*Ceruloplasmin (ferroxidase)*
209173_at	*AGR2*	123.32	*Anterior gradient homolog 2 (Xenopus laevis)*
232361_s_at	*EHF*	123.23	*Ets homologous factor*
231007_at	—	122.48	*Transcribed locus*
226147_s_at	*PIGR*	118.02	*Polymeric immunoglobulin receptor*
240304_s_at	*TMC5*	114.63	*Transmembrane channel-like 5*

Abbreviations: EOC = epithelial ovarian carcinoma; HOSE = human ovarian surface epithelial control cell line.

Out of 2225 genes, 985 were found upregulated in UOC when compared with HOSE (Table 5 depicts the top 15 upregulated genes, whereas Supplementary Table 4a and b lists all upregulated and downregulated genes, respectively). As shown in Table 5, *SCGB2A1* was the third top overexpressed gene (219-fold) in UOC *vs* HOSE. *Claudin 3* and *claudin 4* were also found highly differentially expressed in OSPC when compared with HOSE (Supplementary Table 4a, 43-fold and 25-fold, respectively).

Validation of gene expression by qRT–PCR. The *SCGB2A1*, *CLDN3*, *CLDN4*, *TROP-2* and *SCGB1D2* gene expression results in OSPC, CC, EAC, UOC and HOSE were validated using qRT–PCR analysis in a total of 38 ovarian carcinomas and 14 HOSE controls. The quantitative real-time PCR data and microarray data for all the validated genes were highly correlated (*SCGB2A1*, $r = 0.85$; *CLDN3*, $r = 0.83$; *CLDN4*, $r = 0.80$; *TROP-2*, $r = 0.81$; and *SCGB1D2*, $r = 0.85$; $P < 0.01$) as estimated from paired samples. The qRT–PCR results for *SCGB2A1*, *CLDN3*, *CLDN4*, *TROP-2* and *SCGB1D2* expression in OSPC, CC, EAC, UOC *vs* HOSE are shown in Figure 1. All epithelial ovarian cancers, regardless of their histology, overexpressed *SCGB2A1*, *CLDN3*, *CLDN4*, *TROP-2* and *SCGB1D2* mRNA at significantly higher levels when compared with HOSE ($P < 0.001$). The expression levels of *SCGB2A1*, *CLDN3*, *CLDN4*, *TROP-2* and *SCGB1D2* showed variability among

Table 4. Fifteen most frequently upregulated genes in clear-cell EOC *vs* HOSE

U133 Plus 2.0 probe set	Gene symbol	Fold change	Gene name
205979_at	*SCGB2A1*	773.01	*Secretoglobin, family 2A, member 1*
216834_at	*RGS1*	294.06	*Regulator of G-protein signalling 1*
221884_at	*EVI1*	291.28	*Ecotropic viral integration site 1*
231007_at	—	256.42	*Transcribed locus*
218960_at	*TMPRSS4*	227.29	*Transmembrane protease, serine 4*
219768_at	*VTCN1*	225.97	*V-set domain containing T cell activation inhibitor 1*
1558034_s_at	*CP*	189.84	*Ceruloplasmin (ferroxidase)*
228692_at	*PREX2*	162.60	*phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2*
204846_at	*CP*	162.27	*Ceruloplasmin (ferroxidase)*
209173_at	*AGR2*	159.77	*Anterior gradient homolog 2 (Xenopus laevis)*
210397_at	*DEFB1*	149.24	*Defensin, β1*
225846_at	*RBM35A*	140.15	*RNA binding motif protein 35A*
202992_at	*C7*	137.24	*Complement component 7*
203953_s_at	*CLDN3*	136.34	*Claudin 3*
219607_s_at	*MS4A4A*	133.21	*Membrane-spanning 4-domains, subfamily A, member 4*

Abbreviations: EOC = epithelial ovarian carcinoma; HOSE = human ovarian surface epithelial control cell line.

Table 5. Fifteen most frequently upregulated genes in undifferentiated EOC *vs* HOSE

U133 Plus 2.0 probe set	Gene symbol	Fold change	Gene name
216834_at	*RGS1*	462.81	*Regulator of G-protein signalling 1*
214677_x_at	*IGLV1–44*	225.64	*Immunoglobulin λ locus 1–44*
205979_at	*SCGB2A1*	218.68	*Secretoglobin, family 2A, member 1*
231192_at	*LPAR3*	215.95	*Lysophosphatidic acid receptor 3*
225645_at	*EHF*	208.35	*Ets homologous factor*
225846_at	*RBM35A*	182.76	*RNA binding motif protein 35A*
213993_at	*SPON1*	182.33	*Spondin 1, extracellular matrix protein*
213975_s_at	*LYZ*	159.39	*Lysozyme (renal amyloidosis)*
1558034_s_at	*CP*	139.02	*Ceruloplasmin (ferroxidase)*
211430_s_at	*IGHV 4–31*	135.28	*Immunoglobulin heavy locus*
221884_at	*EVI1*	124.92	*Ecotropic viral integration site 1*
219768_at	*VTCN1*	103.35	*V-set domain containing T cell activation inhibitor 1*
213994_s_at	*SPON1*	102.09	*Spondin 1, extracellular matrix protein*
204846_at	*CP*	98.24	*Ceruloplasmin (ferroxidase)*
201839_s_at	*TACSTD1*	97.61	*Tumour-associated calcium signal transducer 1*

Abbreviations: EOC = epithelial ovarian carcinoma; HOSE = human ovarian surface epithelial control cell line.

EOCs belonging to different histological types; however, these differences were not statistically significant (Figure 1). Thus, qRT–PCR data suggest that most array probe sets are likely to accurately measure the levels of the intended transcript within a complex mixture of transcripts.

Validation of protein expression by IHC staining. To confirm gene expression results at the protein level, IHC for SCGB2A1 was carried out on 10 formalin-fixed tumours and 5 normal samples. As representatively shown in Figure 2, a strong cytoplasmic staining for SCGB2A1 was detected in all different histological types of ovarian cancer showing SCGB2A1 over-expression at the mRNA level. In contrast, in agreement with previous results reported by our group (Tassi *et al*, 2007), all normal ovaries tested by IHC were negative for SCGB2A1 expression (data not shown).

Tumour-specific CD8 + cytotoxic T-cell responses. Cytotoxicity assays were conducted after multiple *in vitro* stimulations of autologous T cells with SCGB2A1-pulsed DC as described in the Materials and Methods. Strong HLA class I-restricted lysis of autologous chemotherapy-naive and highly chemotherapy-resistant tumour cells at different effector/target cell ratios were seen (Figure 3), whereas lymphocytes stimulated with DCs in the absence of SCGB2A1 failed to generate specific responses against autologous tumour cells (data not shown). The results presented in

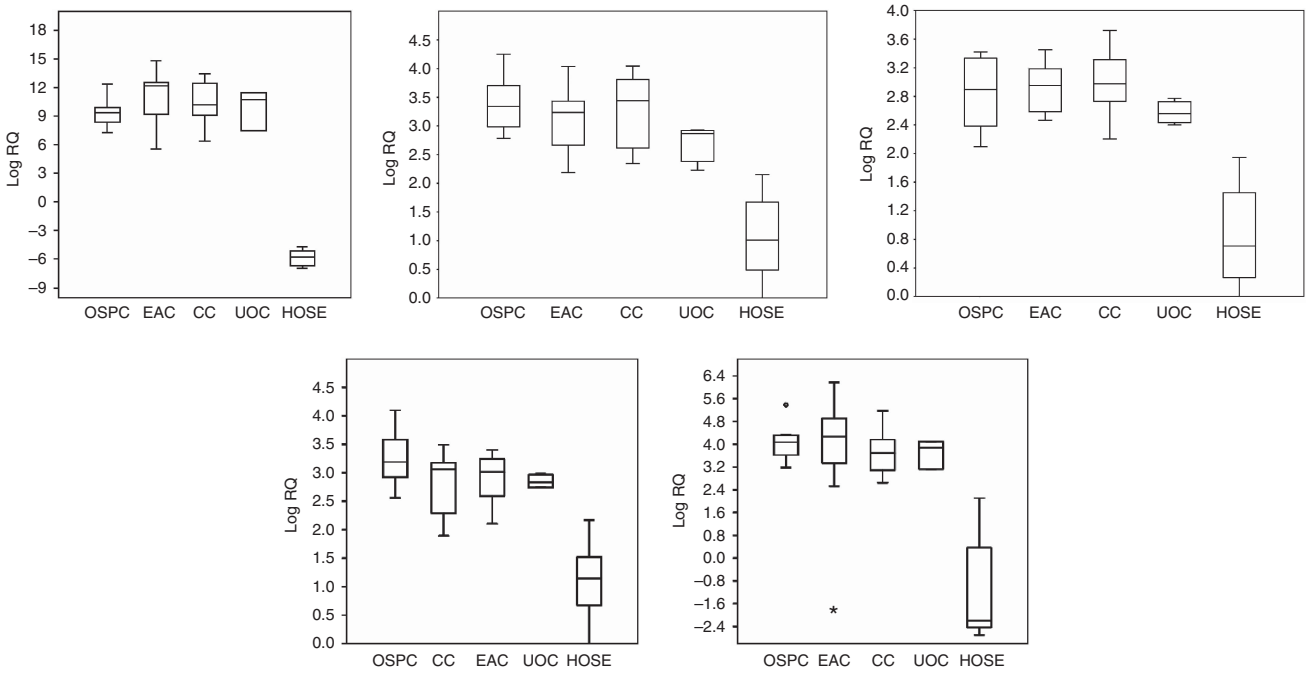

Figure 1. Quantitative RT–PCR analysis of *SCGB2A1*, *CLDN3*, *CLDN4*, *TROP-2* and *SCGB1D2* expression in ovarian carcinoma. The box plots of the relative quantification (RQ) expression values for each histological type of ovarian cancer when compared with HOSE in log scale. CC, clear-cell ovarian carcinoma; EAC, endometrioid ovarian carcinoma; HOSE, normal human ovarian surface epithelial control cell lines; OSPC, ovarian serous papillary carcinoma; UOC, poorly differentiated ovarian carcinoma. The qRT–PCR data were highly correlated to the microarray data ($P<0.01$).

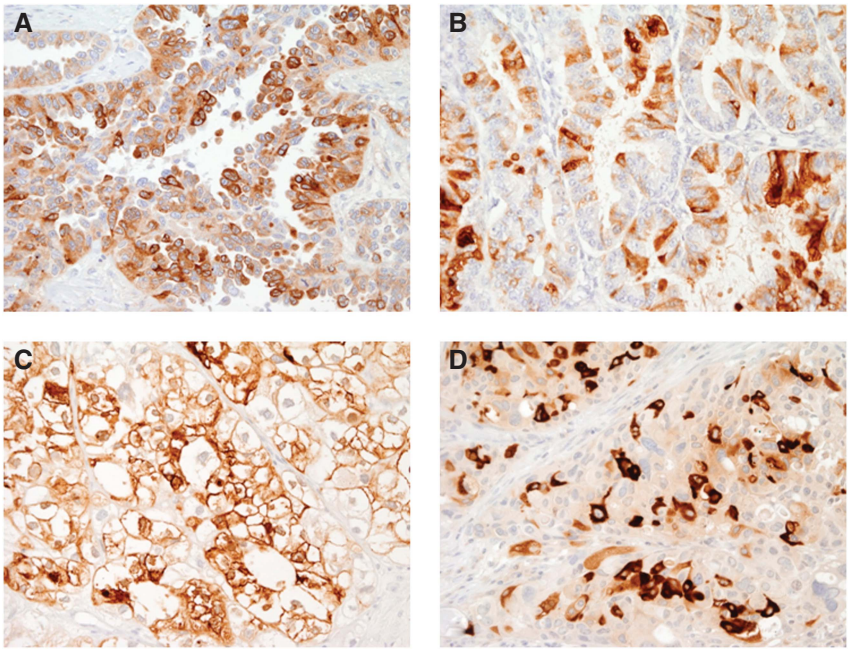

Figure 2. Representative patterns of SCGB2A1 immunoreactivity in the different histological types of ovarian cancer tested. (**A**) High-grade serous carcinoma, (**B**) endometrioid carcinoma, (**C**) clear-cell carcinoma and (**D**) undifferentiated carcinoma. Original magnification $\times 400$.

Figure 3 represent the mean of more than 8 assays, with ranges of 11% to 21% lysis against the primary chemotherapy-naive tumour cell line and 20% to 56% lysis against the metastatic chemotherapy-resistant tumour cell line in a 10 : 1 ratio (Figure 3). In all cases, minimal cytotoxic activity was observed against autologous PHA-stimulated lymphoblasts (control (CTR)) (Figure 3) or EBV-transformed LCL (not shown). Blocking studies indicated that in all cases tumour-specific lysis by CD8+ T cells was significantly inhibited by mAb specific for HLA class I, with the range of inhibition being from 55% to 69% against the chemotherapy-naive tumour cell line and 51% to 64% against the chemotherapy-resistant tumour cell line (Figure 3, upper and middle panel, $P<0.01$). The siRNA downregulation of SCGB2A1 expression in tumour cells (i.e., 95% to undetectable level by RT–PCR, data not shown) significantly decreased tumour cell killing by SCGB2A1-specific CTLs (Figure 3, lower panel, $P<0.03$).

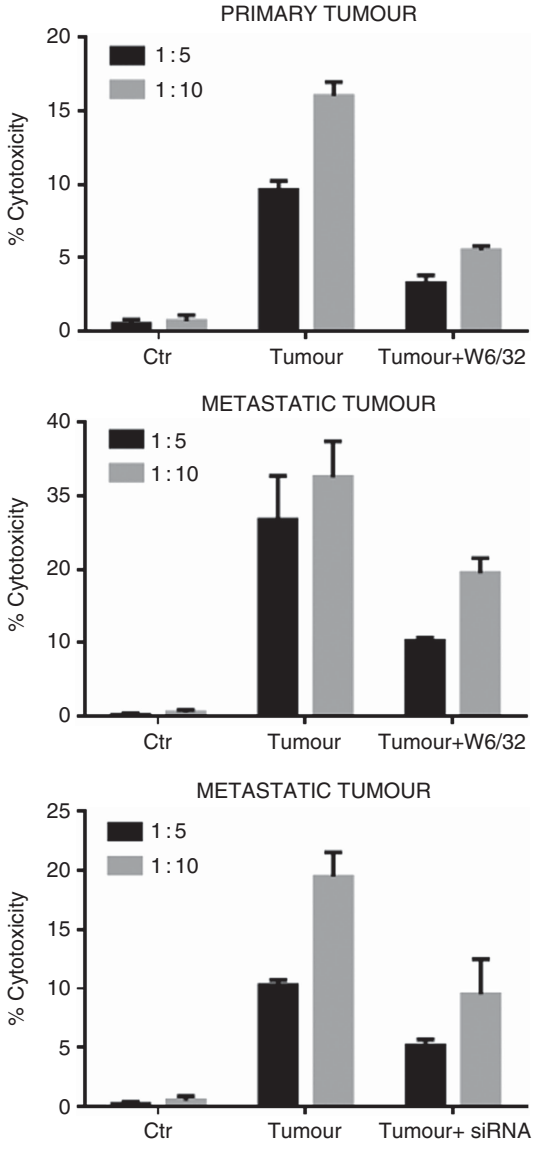

Figure 3. Tumour-specific CD8+ CTL responses induced by SCGB2A1-pulsed DCs against autologous chemotherapy-naive (upper panel) and metastatic/chemotherapy-resistant (middle and lower panels) primary cell lines, measured in a 5 h ^{51}Cr release assay. Percentage lysis (+s.d.) at a 5 : 1 and 10 : 1 effector/target cell ratios are shown. Anti-HLA class I blocking antibody (W6/32) was used at 50 μg ml^{-1}. Ctr, control.

Phenotypic analysis. Flow cytometric analysis was used to determine the phenotype of the populations of DC− SCGB2A1-stimulated CD8+ T cells. All the cells were CD3/CD8+ and CD4−. Further analysis revealed the populations to be TCR$\alpha\beta$+ (95–98%), TCR-$\gamma\delta$+ (2–5%), CD25+, HLA-DR+ and CD16− (data not shown).

Intracellular cytokine expression by SCGB2A1-specific T cells. To evaluate whether cytokine expression from SCGB2A1-stimulated CD8+ T cells segregated in discrete IFN-γ+/IL-4− and IFN-γ−/IL-4+ subsets, we took advantage of flow cytometric techniques for the detection of intracellular cytokine expression at the single cell level. Two-colour flow cytometric analysis of intracellular IFN-γ and IL-4 expression by CTLs was performed after 6 weeks of culture and thereafter as described in the Materials and Methods section. As shown in Figure 4, the striking majority

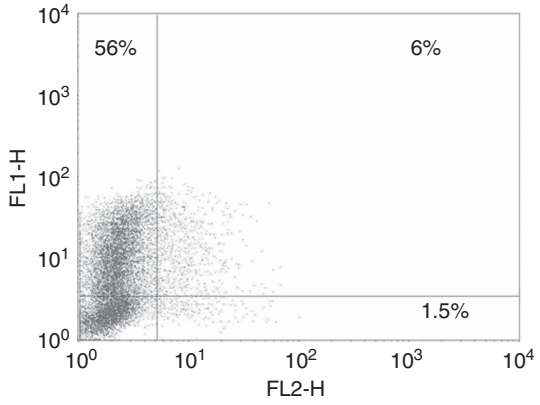

Figure 4. Representative dot plot analysis of intracellular IFN-γ and IL-4 expression by SCGB2A1-specific CTL. T cells were tested at ~6 weeks after priming, after resting for 14 days after the last antigen stimulation before activation by PMA and ionomycin as described in the Materials and Methods. Numbers in the quadrants represent the percentage of CD8+ cytokine-positive T lymphocytes. FL1-H channel: IFN-γ; FL2-H channel: IL-4.

of CD8+ T cells contained intracellular IFN-γ but not IL-4, whereas a small subset contained both intracellular IFN-γ and IL-4 and a second minor subset contained only IL-4. Similar results were consistently obtained in several repetitive *in vitro* priming analyses, suggesting no bias in favour of a type 2 cytokine profile. Unactivated (i.e., resting) CD8+ T cells failed to stain for IFN-γ or IL-4 (data not shown).

DISCUSSION

Ovarian tumour-specific immunotherapy may offer the prospect of an effective treatment for patients with refractory or residual disease after completion of primary standard treatment (i.e., cytoreductive surgery plus adjuvant chemotherapy). With the goals of identifying genes differentially expressed in multiple histological types of ovarian carcinoma and using this knowledge for the development of novel immunotherapeutic strategies to prevent disease progression following surgical and chemotherapy treatment, we used oligonucleotide microarrays to profile 70 highly purified primary epithelial ovarian carcinomas including 24 OSPC, 15 CC, 24 EAC and 7 poorly differentiated tumours and 14 normal HOSE. Our study identified a large number of genes highly differentially expressed in the diverse histological types of epithelial ovarian cancer when compared with HOSE. Of great interest was *SCGB2A1*, which was consistently found as the most frequently differentially expressed gene in EAC and CC tumours and the second and third most differentially expressed gene in the OSPC and undifferentiated subtype, respectively. These data confirm and extend our previously reported gene expression profiling results using a less comprehensive Affymetrix chip (i.e., U95Av2) and limited to high-grade serous ovarian cancer, which also showed *SCGB2A1* as a top differentially expressed gene (Bignotti *et al*, 2006). Other validated genes in our qRT–PCR experiments included *Claudin-3* and *Claudin-4*, the epithelial receptors for CPE (Santin *et al*, 2005), *TROP-2*, a surface glycoprotein targeted by the humanised antibody hRS7 (Varughese *et al*, 2011), and *SCGB1D2*, an additional member of the mammaglobin family of proteins (Jackson *et al*, 2011).

The *SCGB2A1* gene sequence is highly homologous to mammaglobin A (MGB1), a gene preferentially expressed in

breast tissue and upregulated in breast cancer (Becker *et al*, 1998). Although normal expression of *SCGB2A1* has been described in secretory mucosal epithelia of breast, uterus and lacrimal glands, *SCGB2A1* has been reported to be overexpressed in primary breast cancer tissues and in occult breast metastases (Aihara *et al*, 1999; O'Brien *et al*, 2002) and it is considered one of the most informative breast cancer markers to detect micrometastatic disease in the circulation (Mercatali *et al*, 2006) and lymph nodes (Ooka *et al*, 2000; Nissan *et al*, 2006). Of interest, previous reports have shown that MGB1-loaded DCs can stimulate mammaglobin A-specific T cells *in vitro* (Jaramillo *et al*, 2004; Viehl *et al*, 2005), whereas vaccination with MGB1 cDNA may induce response of established human breast tumours in *in vivo* animal models (Narayanan *et al*, 2004). These data combined with our results demonstrating a high differential expression of *SCGB2A1* in multiple histological types of epithelial ovarian cancer suggest that *SCGB2A1* may represent an attractive and potentially universal tumour antigen for ovarian cancer immunotherapy.

Consistent with this view, in this study we have cloned the full-length *SCGB2A1* human gene in an expression vector and demonstrated for the first time that SCGB2A1-pulsed autologous DCs can stimulate specific CD8+ cytotoxic T-cell responses that are capable of killing autologous chemotherapy-naive as well as highly chemotherapy-resistant ovarian tumour cell lines. Importantly, a large proportion of the autologous tumour-specific cytotoxicity detected in our experiments was inhibited by anti-HLA class I antibodies. These data, therefore, indicate that most of the cytotoxicity against autologous tumour cells was mediated by antigen-specific HLA class I-restricted CTLs. Autologous LCL or PHA-activated blasts were not killed by tumour-specific CTLs, confirming that, although these CTLs were highly cytolytic for autologous tumour cells expressing SCGB2A1, they failed to kill autologous normal cells. Finally, downregulation of SCGB2A1 by siRNA in chemotherapy-resistant ovarian cancer cells significantly reduced the cytoxicity by SCGB2A1-activated CTLs, suggesting that SCGB2A1 expression in tumour cells was indeed responsible for most of the recognition and killing. These results obtained using SCGB2A1 as a tumour target antigen are consistent with recent publications from our group demonstrating that peptides derived from CA125 and TADG-12, the two proteins encoded by other genes known to be differentially expressed in ovarian cancer, may also represent novel potential tumour rejection antigens in patients harbouring advanced disease in progression after chemotherapy (Bellone *et al*, 2009, 2009a).

T-cell-mediated protection from tumours is thought to be promoted by type 1 cytokine responses and impaired by type 2 cytokine responses (Romagnani, 1992). In general, type 1 T cells express IL-2, IFN-γ and TNF $\alpha\beta$, and are cytotoxic, whereas type 2 T cells express IL-4, IL-5, IL-6, IL-10 and IL-13, provide efficient help for B cell activation and are noncytotoxic. Consistent with this view, IL-2- and IFN-γ-producing type 1 T cells are believed to promote the development of cell-mediated immunity against neoplasms. In this study we took advantage of a flow cytometric technique for detecting intracellular cytokine expression at the single cell level in SCGB2A1-stimulated CD8+ cells. Two-colour flow cytometric analysis of intracellular IFN-γ and IL-4 expression by CD8+ SCGB2A1-specific T cells demonstrated that these T cells showed a major type 1 bias in cytokine expression. Indeed, the majority of cytokine-expressing T cells showed IFN-γ expression, whereas a minority expressed only IL-4. These findings support the view that even in patients with ovarian cancer in progression after multiple regimens of chemotherapy, presentation of SCGB2A1 by DCs is still able, at least *in vitro*, to activate a strong type 1 T-cell response, and that type 1 cytokine expression is associated with high cytotoxic activity against the autologous tumour cells by CD8+ T cells.

Despite the great potential of immunotherapeutic approaches, tumour immunity in cancer patients is thought to be hindered *in vivo* by an overall suppressive tumour microenvironment that includes expression of a variety of cell surface molecules known as immune checkpoint modulators on the tumour and infiltrating immune cells (i.e., B7-1/CD80, B7-2/CD86, B7-H1/PDL1, B7-H2/L-ICOS, B7-DC, B7-H3 and B7-H4) (Mellman *et al*, 2011). Consistent with this hypothesis, blockade/modulation of the PD-L1/PD-1 and CTLA4 co-inhibitory pathways has already shown great promise for the treatment of multiple human solid tumours refractory/resistant to chemotherapy (Mellman *et al*, 2011). On the basis of this recent evidence, we may speculate that the most successful therapeutic vaccination strategy in ovarian cancer patients will likely require the integration of SCGB2A1-targeted T-cell immunotherapy with the blockade/modulation of the PD-L1/PD-1 and B7-H4 co-inhibitory pathways. The future design and implementation of clinical trials will ultimately determine the validity of this combined approach.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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