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Discovery of chemotherapy-associated ovarian cancer antigens by interrogating memory T cells

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According to the immunogenic cell death hypothesis, clinical chemotherapy treatments may result in $CD8^+$ and $CD4^+$ T-cell responses against tumor cells. To discover chemotherapy-associated antigens (CAAs), T cells derived from ovarian cancer (OC) patients (who had been treated with appropriate chemotherapy protocols) were interrogated with proteins isolated from primary OC cells. We screened for immunogenicity using two-dimensional electrophoresis gel-eluted OC proteins. Only the selected immunogenic antigens were molecularly characterized by mass-spectrometry-based analysis. Memory T cells that recognized antigens associated with apoptotic (but not live) OC cells were correlated with prolonged survival in response to chemotherapy, supporting the model of chemotherapy-induced apoptosis as an adjuvant of anti-tumor immunity. The strength of both memory $CD4^+$ and $CD8^+$ T cells producing either IFN-γ or IL-17 in response to apoptotic OC antigens was also significantly greater in Responders to chemotherapy than in nonresponders. Immunogenicity of some of these antigens was confirmed using recombinant proteins in an independent set of patients. The T-cell interrogation system represents a strategy of reverse tumor immunology that proposes to identify CAAs, which may then be validated as possible prognostic tumor biomarkers or cancer vaccines.

Key words: ovarian cancer, chemotherapy associated antigens, Th1 cells, Th17 cells, reverse immunology, tumor biomarkers

Abbreviations: Background: BG; CAAs: chemotherapy associated antigens; CM: Central Memory; CFSE: carboxyfluorescein diacetate succinimidyl ester; DCs: dendritic cells; 2DE: two-dimensional electrophoresis; EIF5A: eukaryotic translation initiation factor 5A; ELISPOT: enzyme-linked immunosorbent spot assay; EM: Effector Memory; EMRA: Effector Memory RA; FABPE: fatty acid binding protein; FITC: fluorescein isothiocyanate; HD: Healthy Donor; ICS: intracellular cytokine staining; MS: mass-spectometry; OC: ovarian cancer; PBMCs: peripheral blood monuclear cells; PMA: phorbol 12-myristate 13-acetate; r: recombinant; RAB7: Ras-related protein; SEM: standard error of mean; SPSS: Statistical Product and Service Solution

Additional Supporting Information may be found in the online version of this article.

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What's new?

According to the immunogenic cell-death hypothesis, chemotherapy can induce $CD8^+$ and $CD4^+$ T-cell responses against tumor cells. The trigger is thought to be the release of additional tumor antigens from dying tumor cells. This paper describes a "reverse immunology" technique called a T-cell interrogation system, for identifying chemotherapy-associated antigens (CAAs) in ovarian cancer patients. Memory T cells that recognized antigens associated with apoptotic (but not live) OC cells were also correlated with prolonged survival in response to chemotherapy. This suggests that immune responses induced by immunogenic cell death may enhance current therapy.

Ovarian cancer (OC) is typically diagnosed at an advanced stage when the tumor is widely metastatic.[1] Although improvements in systemic therapy have been achieved, OC is the fifth most common cancer in women and the fifth most common cause of cancer-related death, thus necessitating the development of new therapies such as immunotherapy.

Several studies have demonstrated that T cells infiltrating OC exert clinically relevant pressure against tumor progression.[2,3] However, in the majority of the cases, immune pressure against OC is not sufficient. The incapacity of the immune system to eradicate tumors is attributed to various (not mutually exclusive) causes, including local immunosuppressive effects, dysfunction/exhaustion of tumor-specific T cells, accumulation of a variety of suppressor cells.[4–9] The "cancer immunoediting" hypothesis implies that symptomatic tumors represent a failure of the immune system, which occurs after a long period of equilibrium between tumor progression and immune surveillance.[10] Recently, the application of unbiased approaches by using advanced genome- or proteome-wide analyses has been extremely informative with respect to tumor biomarkers or tumor associated antigens.[11,12] However, to date, they remain strictly descriptive and do not allow the physical recovery of proteins at adequate amounts for functional assays aimed at establishing mechanisms of correlation (requiring concentration of the order of milligram per single protein).

An additional category of tumor antigens has been suggested by the immunogenic cell death hypothesis that is tumor cells can became immunogenic as a consequence of chemotherapy.[13] Previous evidences showed that specific chemotherapy affects antigen presentation leading to an improved T-cell response, however, which antigen is recognized was not identified.[14] Several experimental models suggest that these therapies elicit a multistep process in dying (apoptotic, necrotic, autophagic, etc.) cancer cells, including the release of "find-me" signals (such as fractalkine, nucleotides ATP) that attract phagocytes or dendritic cells (DCs), the expression of "eat-me" signals (such as phosphatidylserine and calreticulin) that facilitate recognition by phagocytes or DCs, and finally the release of "danger-associated molecular patterns" (such as high mobility group box 1 protein) that enable apoptotic cells to lose the propensity to induce tolerance and to stimulate powerful anticancer immune responses.[15,16] An additional factor that is involved in the success of the immunogenic chemotherapy may emerge from the capacity of caspases to cut and release apoptotic cell-associated antigenic fragments, thus facilitating their processing and cross-presentation by DCs ("digest-me" signals).[17] The final goal of these multiple checkpoints is to elicit a wide repertoire of memory tumor-specific T cells in patients undergoing tumor regression in response to appropriate chemotherapeutic regimes.

Building on these concepts, we hypothesized that memory T cells against OC antigens could be induced by chemotherapy treatments and that this T-cell response could recognize new antigens specific for apoptotic cells. Therefore, we interrogated memory T cells derived from OC patients showing prolonged survival in response to chemotherapy—responders compared with nonresponders—so as to recognize and discriminate the immunogenic OC proteins among the hundreds that had been isolated and recovered in apoptotic cells by two-dimensional electrophoresis (2DE) gels. Although 2DE analysis has a limited resolution and it has been essentially replaced by mass-spectometry (MS)-based proteomics,[12] it allows to obtain adequate amounts of proteins for various functional immunological assays. Immunogenic 2DE gel-proteins defined by T-cell responses were molecularly identified by mass spectrometry, thus focusing the analysis only on functionally-defined (immunogenic) proteins derived from primary tumors. Among the CAAs identified by this method, the immunogenicity of the recombinant form of some of these proteins was confirmed in an independent set of OC patients. Finally, using a panel of 2DE-derived proteins we could establish an association with response to therapy (responders *vs.* nonresponders). This approach, referred here as the *T cell interrogation system*, represents an extension of "reverse immunology,"[18] in which T cells have been used as a read-out system to recognize CAAs.

Material and Methods

Study population

The study cohort included 16 OC patients (median age 51 years, range 38–65 years), in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and a priori approval of the ethics committee at our institutions (Supporting Information Table 1). Informed consent was obtained from all patients. Patients were classified according to the demographic characteristics, cancer histology, FIGO (International Federation of Gynecology and Obstetrics) stage and patients' outcome (Supporting Information Table 1). Enrolled patients underwent cytoreductive surgery followed by six cycles (one cycle every 21 days) of platinum and taxane combination chemotherapy. There has been no clinical evidence of cardiotoxicity in all

patients. Patients were defined as Responders to therapy if they showed absence of disease progression (serum level of cancer antigen CA-125 constantly <35 U/ml, absence of metastasis as evaluated by positron emission tomography and computed tomography imaging) for 6 months after the last chemotherapy treatment. In addition, the disease-free interval was calculated in all patients: this value corresponded to the total number of months of survival without disease progression from the end of the chemotherapy treatment. Age-matched healthy donors were studied as controls.

Preparation of T cells and DCs

Peripheral blood mononuclear cells (PBMCs) were isolated and T-cell lines were generated as previously described.[19] $CD4^+$, $CD8^+$, or $CD14^+$ cells were purified from PBMCs by immunomagnetic positive selection according to manufacturer's instructions (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany) as previously described.[20] Flow cytometry analysis demonstrated greater than 99% $CD4^+$, $CD8^+$, or $CD14^+$ cells in the positively purified population and less than 5% in the respective depleted populations. To generate T-cell lines, purified $CD4^+$ or $CD8^+$ T cells were incubated with $CD14^+$ monocytes in the presence of OC antigens for 15 d in IL-2-conditioned medium. iDCs were derived from $CD14^+$ peripheral monocytes purified as described above. In particular, $CD14^+$ cells were incubated for 5 days in RPMI1640 medium containing 5% FCS, 2 mM glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 50 μg/ml kanamycin (Life Technologies Inc Gibco/Brl Division, Grand Island, NY), 50 ng/ml recombinant (r)GM-CSF (Novartis Pharma AG, Basel, Switzerland), and 1,000 U/ml rIL-4 (gently provided by A. Lanzavecchia, Bellinzona, CH). The definition of monocyte-derived DCs was based on their surface phenotype profile by staining with anti-CD14 monoclonal antibody (mAb), anti-CD86 mAb (Caltag Laboratories, Burlingame, CA), anti-CD1a mAb, anti-CD1c mAb, anti-CD11c mAb, anti-CD32 mAb, anti-CD80 mAb (BD Biosciences Pharmingen, San Diego, CA), Annexin-V (ApoAlert Apoptosis Kit, Clontech Laboratories Inc), Propidium Iodide (PI) (Sigma-Aldrich Biotechnology, St. Louis, MO), and the appropriate labeled secondary antibodies (BD PharMingen).

Isolation and characterization of OC cells

Neoplastic ascites from six patients (two at the stage IIIA, two stage IIIB, and two stage IIIC) were centrifuged and the resulting OC cell pellets underwent immunomagnetic selection with anti-ErbB2 mAb coupled to magnetic beads (Miltenyi Biotec). To purify cancer cells we relied on ErbB2 expression since it was expressed on 100% of cancer cells by flow cytometry analysis (data not shown) in agreement with published data.[21] OC cells were then characterized by standard immunochemistry techniques as previously described.[21]

Preparation of apoptotic OC cells

OC cells were incubated with 2 μM of mitoxanthrone (1,4-dihydroxy-5,8-bis[2-(2-hydroxyethylamino)ethylamino]-anthracene-9,10-dione) (Sigma-Aldrich Biotechnology) for 5 days at 37°C. Mitoxanthrone is a type II topoisomerase inhibitor, which inhibits DNA synthesis and DNA repair in both healthy and cancer cells. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin-V and PI (BD Biosciences Pharmingen) and apoptosis was measured by flow cytometry analysis. To measure caspase activation, cells were washed, fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences Pharmingen) at 4°C for 20 min, re-washed with Perm Wash Buffer (BD Biosciences Pharmingen) and stained at the intra-cellular level by a polyclonal antibody to cleaved caspase-3 (Cell Signaling Inc., Danvers, MA) for 30 min at 4°C. Cells were then washed and stained with the appropriate secondary FITC-labeled antibody (BD Biosciences Pharmingen), acquired by flow cytometry analysis with FACSCanto and analyzed with FACSDiva software. Finally, apoptotic OC cells were isolated by positive selection with annexin-V coupled to magnetic beads (Miltenyi Biotec) as previously described.[17] Control cells were represented by OC cells that were lysed by repeated freezing and thawing.

Two-dimensional electrophoresis

2DE procedure is described in the Supporting Information Data.

Preparation of 2DE gel-eluted proteins for immunological assays

Preparation of 2DE gel-eluted proteins is described in the Supporting Information Data.

MALDI-TOF/TOF-MS and database searching

These procedures are described in the Supporting Information Data.

Enzyme-linked immunosorbent spot assay

To determine the effector function of specific T cells, electroeluted proteins (20 μg/ml) were transferred in duplicate into 96-well Enzyme-linked immunosorbent spot nitrocellulose-backed plates (ELISPOT plate, MAHA S4510, Millipore) that had been previously coated with capture anti-IFN-γ mAb (BD Biosciences Pharmingen). Then, PBMCs (1×10^5) were directly added to each single well of the ELISPOT plate in duplicate, in the presence of purified mAb to CD28 (2 μg/ml), and incubated for 48 h at 37°C. Polyclonal activation of T cells was evaluated with Dynabeads Human T-activator CD3/CD28 (Life Technologies Inc Invitrogen Molecular Probes Division, Grand Island, NY). Ultimately, the IFN-γ spot formation was promptly revealed and visualized with the AID ELISPOT Reader (AID GmbH).

Intracellular cytokine staining and T-cell proliferation

Fresh PBMCs, or T cell lines were stimulated directly with immunogenic 2DE gel-electroeluted proteins, whereas $CD4^+$ or $CD8^+$ T cell lines with autologous monocytes had been previously pulsed with the same antigens. For validation

experiments, we choose among the more immunogenic proteins the following human recombinant (r)proteins : Fatty acid binding protein (FABPE, ab63112) and Ras-related protein (RAB7, ab103507) (Abcam, Cambridge, UK), eukaryotic translation initiation factor 5A (EIF5A, pro-674) (ProSpec, NJ). A cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin was used to induce polyclonal cytokine production *in vitro*. At the second hour of incubation at 37°C, 10 μg/ml brefeldin-A (Sigma-Aldrich Biotechnology) was added. FACS analysis was carried out as previously described using the following antibodies: PE-Cy7-labeled anti-CD4, APC-labeled anti-CD8 (BD Biosciences Pharmingen), PE-labeled anti-CD197 (CCR7) (BD Biosciences Pharmingen), FITC-labeled anti-CD45RO (Caltag Laboratories), eFluor605NC-labeled anti-CD45RA (eBioscience, CA), and a cocktail of labeled-mAbs and -reagents (APC-Cy7-labeled mAbs to CD14, CD16, CD19, and CD56 [Biolegend, San Diego, CA]) and Fixable Viability Dye eFluor 780 [eBioscience, San Diego, CA]) (dump channel) for 20 min at 4°C. Cells were washed, fixed, and permeabilized with Cytofix/Cytoperm solution (BD Biosciences Pharmingen) at 4°C for 20 min. Then, PBMCs were washed with Perm/Wash buffer (BD Biosciences Pharmingen), and the intracellular cytokine staining (ICS) was performed using in different combination PE-labeled anti-IFN-γ and FITC-labeled anti-IL-17 mAbs or PE-labeled anti-IL-2 and FITC-labeled anti-IL-4 mAbs (BD Biosciences Pharmingen), for 30 min at 4°C. Negative controls were obtained by staining cells with irrelevant isotype-matched mAbs. They were then acquired with a FACSCanto or LSRFortessa cytometer (Becton Dickinson) and analyzed with FlowJo software version 7.5.5 (Tree Star, Inc. San Carlos, CA). Cytokine-producing cells were analyzed in $CD8^+$, $CD4^+$ cells (or relative $CCR7^+CD45RA^-$, $CCR7^-CD45RA^+$, $CCR7^-CD45RA^-$) after exclusion of B cells, monocytes, NKT cells, NK cells (dump channel). To detect T cell proliferation, highly purified $CD4^+$ or $CD8^+$ T cells were labeled with 1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Life Technologies), for 10 min at 37°C, stopped with serum for 10 min at 37°C, washed with complete medium, and cultured (5×10^4) in a 96-well plate, in the presence of autologous immature (i) DCs that had been previously pulsed or not with 2DE gel-electroeluted proteins, in the presence or absence of a blocking anti-programmed death ligand 1 (PD-L1) antibody (R&D Systems, MN). After 7 days stimulation, cells were stained with different combinations of labeled mAbs to CD4, CD8, CD45RO, and CCR7, and analyzed with a FACSCanto (Becton Dickinson) and analyzed with FlowJo software version 7.5.5 (Tree star, Inc. San Carlos, CA).

Statistical analysis

Statistical analyses were performed with both Statistical Product and Service Solution (SPSS) software, version 11.0 (SPSS Inc.) or Prism 5 software (GraphPad). To verify the linear relationship between analyzed variables Pearson's correlation test was applied, while nonparametric two-tailed Mann-Whitney *U* test was used to compare value differences between two independent groups. The differences were considered significant at $p < 0.05$ and standard error of mean (SEM) applied.

Results

2DE of apoptotic or live OC cells

Six independent OC cell populations were isolated from single patients and characterized by immunochemistry techniques: greater than 90% of them were positive for keratin 7, MOv-18 and calretinin. They then underwent apoptosis induction with mitoxanthrone that belongs to the anthracycline family and is a well established "immunogenic cell death" inducer.[15,16] Apoptosis was detected both by staining with annexin-V and PI and by visualizing caspase activation looking at cleaved caspase-3 (Supporting Information Fig. 1). Live or apoptotic OC cells were promptly lysed and compared with subtractive analysis by 2DE to identify modified proteins.[17] Multiple 2DE gel analyses of the respective OC cells were comparable and approximately 300 spots were resolved by Coomassie blue staining (Supporting Information Fig. 2). Of these spots, 61 were exclusively detected in protein patterns of apoptotic OC cells, 49 were exclusively detected in protein patterns of live OC cells, and 153 shared between live and apoptotic OC cells (28 of them were at least twice as abundant in apoptotic OC cells as in live OC cells, and 125 were at least twice as abundant in live OC cells as in apoptotic OC cells).

T-cell responses to purified OC proteins

One hundred eighty-two OC cell-derived proteins (55 exclusive of apoptotic OC cells, 43 exclusive of live OC cells, and 84 shared by both apoptotic and live OC cells) were directly recovered from 2DE gels and each electro-eluted protein was selectively collected in duplicate wells of 96-well plates (2DE gel-eluted protein library). To verify their immunogenicity, PBMCs from OC patients (responders or nonresponders to chemotherapy) and healthy donors were tested for the capacity to form IFN-γ spots within 48 h of contact with about 20 μg/ml of the single 2DE gel-eluted proteins in the ELISPOT assay. In a preliminary screening, 59 2DE gel-eluted proteins (21 exclusive of apoptotic OC cells, 12 exclusively of live OC cells, and 26 shared by both apoptotic and live OC cells) out of 182 2DE-eluted OC-associated proteins tested, induced IFN-γ spot formation by T cells at values at least twofold higher than background (BG): notably, the mean number of IFN-γ spots formed by T cells in response to the total 2DE gel-eluted proteins derived from apoptotic OC cells was significantly more elevated in Responders than in non-Responders to chemotherapy, whereas the mean of the responses to the total 2DE gel-eluted proteins derived from live OC cells or to those shared by both live and apoptotic OC cells were significantly more elevated in nonresponders than in responders (Supporting Information Fig. 3). However, when we selected 24 super-immunogenic 2DE

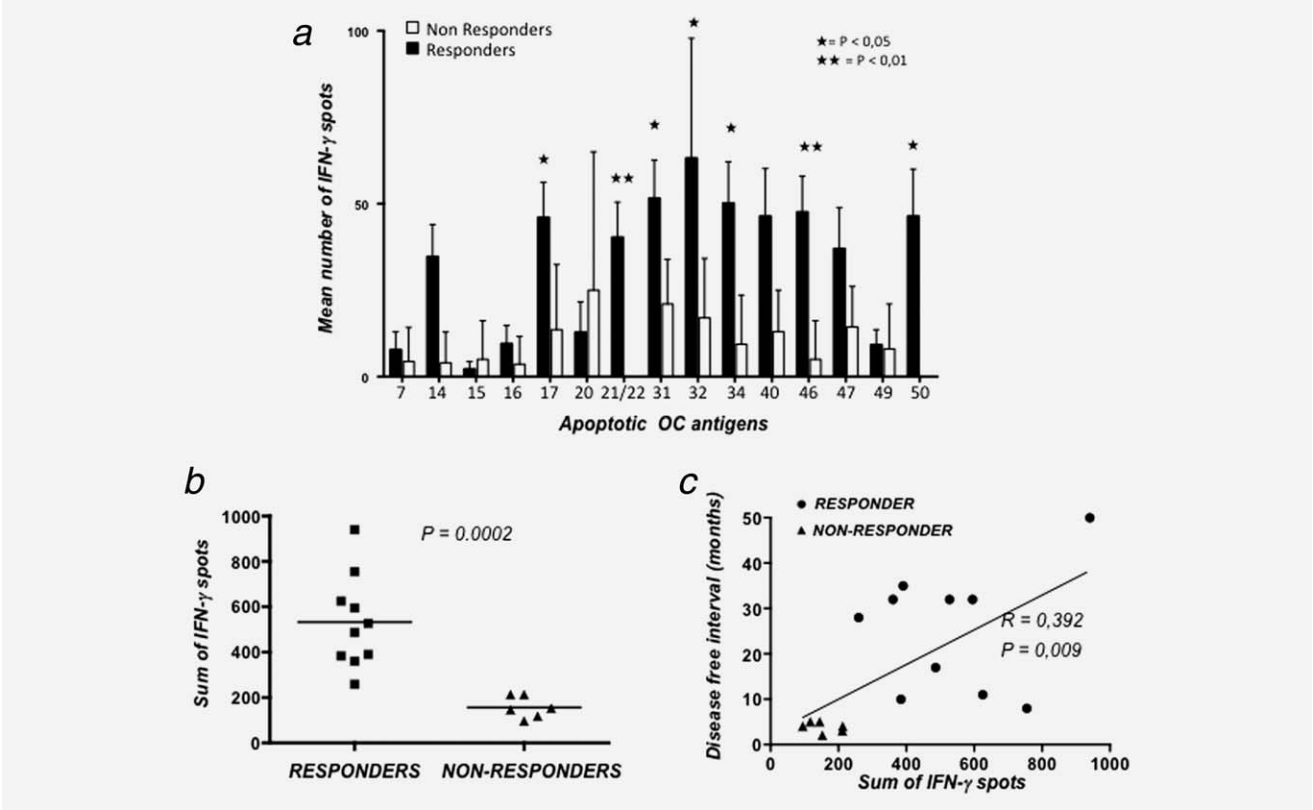

Figure 1. T cells identifying immunogenic antigens derived from apoptotic OC cells. (*a*) Mean number of IFN-γ spots formed (at values of at least fivefold higher than background, as detected by ELISPOT assay) by fresh T cells from 10 responders (filled bars) or 6 nonresponders (empty bars) to chemotherapy within 48 h of contact with about 20 μg/ml of each single apoptotic OC antigen (Ag) that was eluted and purified from 2DE gels. The numbers indicating the apoptotic OC proteins corresponded to those shown in the Table 1. Statistical analysis was performed with nonparametric Mann-Whitney *U*-test for unpaired data and SEM is shown. (*b*) Sum of IFN-γ spots formed by T cells from each single responder or nonresponder in response to total apoptotic OC antigens. Each symbol represents a single patient. Statistical analysis was performed with non-parametric Mann-Whitney *U*-test for unpaired data. (*c*) Correlation between disease-free interval (months) and the sum of IFN-γ spots formed by T cells from each single responder or nonresponder in response to the total apoptotic OC antigens. Each symbol represents a single patient. Values are shown with the background subtracted. Statistical analysis was performed with Pearson's correlation test. N.S. = not significant.

gel-eluted proteins (among the 182 tested in the ELISPOT assay) that were capable to induce IFN-γ spot formation by T cells at values at least fivefold higher than BG, 15 of them were exclusive of apoptotic OC cells (apoptotic OC antigens), 9 were shared by both apoptotic and live OC cells, and none were exclusive of live OC cells. In addition, both the mean number of IFN-γ spots formed by T cells from all patients in response to 7 out of the 15 super-immunogenic apoptotic OC antigens (Fig. 1*a*), and the sum of IFN-γ spots formed by T cells from each single patient in response to all these apoptotic OC antigens (Fig. 1*b*), were significantly more elevated in responders to chemotherapy than in nonresponders. Interestingly, the sum of IFN-γ spots formed by T cells from each single patient in response to these seven apoptotic OC antigens was directly correlated with the disease-free interval time (survival) (Fig. 1*c*). By contrast, no difference was observed between Responders and non-Responders in response to the nine super-immunogenic antigens shared by both apoptotic and live OC cells (Supporting Information Figs. 4*a*–4*c*). All patients analyzed (responders or nonresponders) were similarly responsive to unspecific stimulation with anti-CD3/CD28 mAbs (data not shown). Altogether these data indicate the strength of T-cell responses to apoptotic OC antigens is associated with a better prognosis of OC upon chemotherapy treatment. None of the 15 healthy donors exhibited significant responses against any of the 2DE-eluted OC proteins evaluated (Supporting Information Figs. 4*d*–4*e*).

Effector and central memory T cells specific to apoptotic OC cells

To define T cell subpopulation responding to 2DE-derived antigens and their functional properties, FACS analysis was performed with immunogenic proteins, as previously determined by ELISPOT assay. PBMCs underwent ICS assay to evaluate the production of various cytokines (IFN-γ, IL-17, IL-2, IL-4) within 18 h of stimulation. IFN-γ, IL-4 and IL-17 were used for characterizing type-1, type-2 and type-17

Table 1. Immunogenic OC proteins isolated only from apoptotic OC cells as identified by MALDI-TOF/TOF-MS and database searching

N. spots[1]	aa	AN	ID	Description	pI	Mr theoretic	Mr found	Ubiquitous	Tissue-specific	Intensity of spots in live OC cells	Intensity of spots in apoptotic OC cells
SPOT 7	142–569	Q9BRR9	ARHGAP9	Rho-GTPase-activating protein 9 (750aa)	8.41	72.5	83.8	Yes	No	−	+
SPOT 14	46–55	P29508	SERPINB3	Squamous cell carcinoma antigen 1 fragm.(390 aa)	8.48	35	27	−	TAA (Squamous cells, hepatocellular Ca cells)	−	+
SPOT 15	18–224	P47974	ZFP36L2	Zinc finger protein 36 fragm.(494 aa)	8.6	35	27.3	Yes	No	−	+
SPOT 16	12–216	P04179	SODM	Superoxide dismutase (mn) mitochondrial precursor (222aa)	8.35	24.8	22.8	Yes	No	−	±
SPOT 17	12–174	P02511	CRYAB	Alpha crystallin B chain (alpha (B)-crystallin) (175aa)	6.76	20.1	18.7	Yes	No	−	+
SPOT 20	12–72	P62987	UBA52	Ubiquitin and ribosomal protein L40 (128aa)	6.97	16.8	15	Yes	No	−	++
SPOT 21/22	21–118	P02261	H2AC	Histone H2A-IV (129aa)	10.9	13.8	11.1	Yes	No	−	±
SPOT 31	18–81	Q01469	FABPE	Fatty acid-binding protein, epidermal (135aa)	6.6	15.4	6.9	−	Keratinocites	−	±
SPOT 32	23–128	P25398	RS12	40S ribosomal protein S12 (132aa)	6.3	14.5	11.9	Yes	No	−	±
SPOT 34	38–203			RAB7 (207aa)	6.47	22.1	23.4	Yes	No	−	+
SPOT 46	55–120	P63241	IF5A	Eukariotic translation initiation factor 5A (153aa)	5.0	16.9	7.1	Yes	No	−	+
SPOT 40	19–127	P09382	LEG1	Galectin-1 (135aa)	5.34	14.9	11.9	−	TAA (muscle, neurons, thymus, kidney…)	−	++
SPOT 47	1–140	P09211	GSTP1	Glutathione S-transferase P (210aa)	5.44	23.4	15.1	Yes	No	−	+
SPOT 49	20–221	P09936	UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1 (223aa)	5.33	25.1	23.1	Yes	No	−	±
SPOT 50	4–188	P04792	HSPB1	heat shock protein beta-1 (heat shock 27kDa protein) (205aa)	5.98	22.8	20.2	Yes	No	−	++

[1]Spot number corresponding to those shown in 2DE analysis.
Abbreviations: aa, amino acids; AN, access number in Swiss-Prot database; ID, identity; fragm., fragment; pI, isoelectric point; Mr, relative molecular mass; spot intensity was measured as described in materials and methods and graded according to the following scale: −, absent; ±, low intensity; +, medium intensity; ++,high intensity. The SPOTS in bold correspond to the super-immunogenic proteins (see Fig. 1).

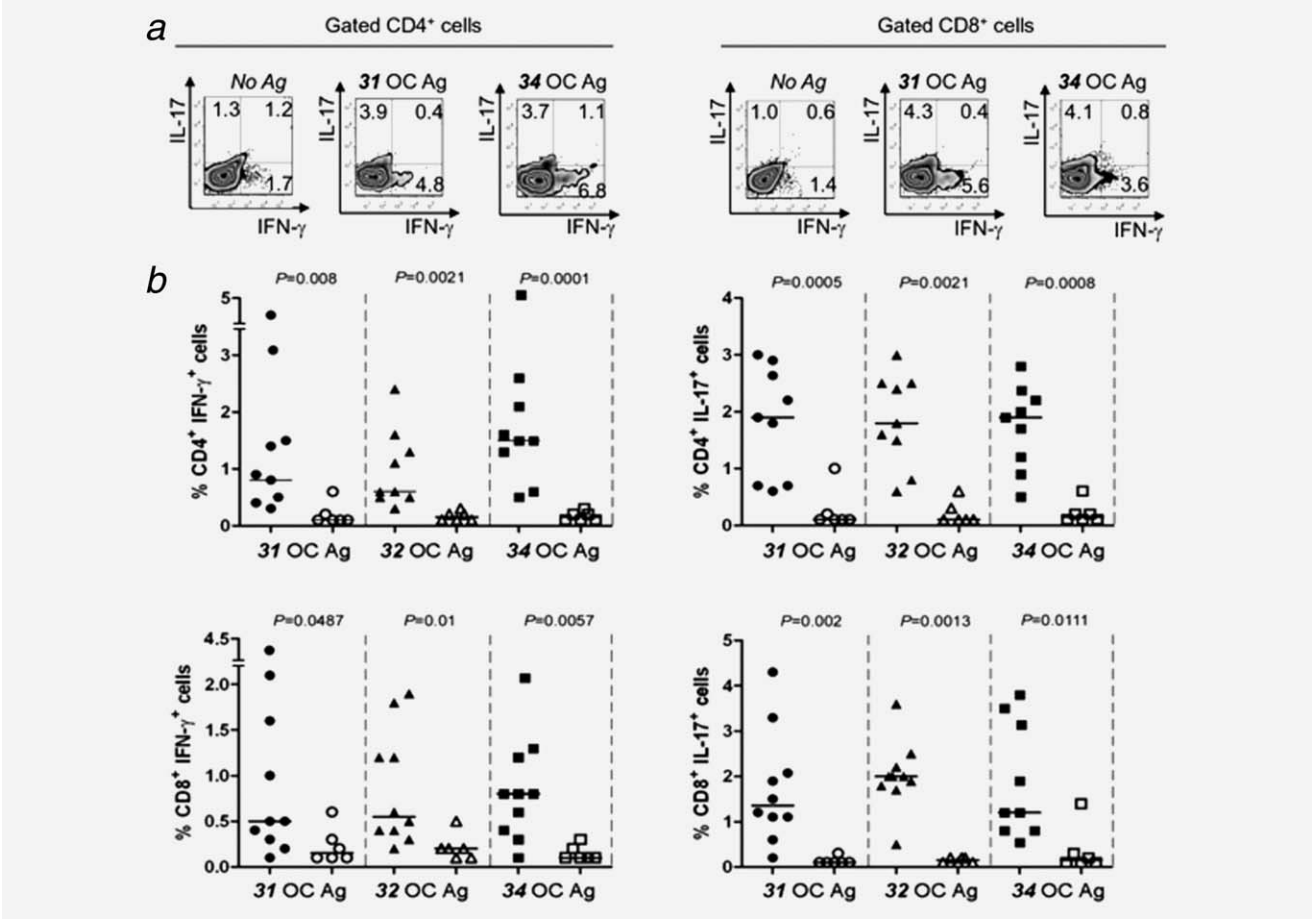

Figure 2. Production of IL-17 and IFN-γ by $CD4^+$ or $CD8^+$ T cells in response to apoptotic OC antigens. (*a*) Representative fresh PBMCs from a responder were stimulated with the indicated apoptotic OC antigens (Ag) (numbers indicating the apoptotic OC proteins correspond to those shown in Table 1) for 18 h, and then processed for the detection of IL-17 and IFN-γ by ICS assay with the relevant mAbs. Counterplot analyses are gated on $CD4^+$ or $CD8^+$ cells and show percentages of cytokine-producing cells. The percentage of cells is reported in each quadrant. (*b*) Percentage of fresh $CD4^+$ or $CD8^+$ T cells producing IL-17 or IFN-γ in response to the indicated apoptotic OC antigens in all responders (filled symbols) or nonresponders (empty symbols) tested. Values are shown with the background subtracted. Statistical analysis was performed with nonparametric Mann-Whitney *U*-test for unpaired data. In both (*a*) and (*b*), the numbers indicating the apoptotic OC proteins correspond to those shown in Table 1: in particular,***31*** OC Ag (circle symbols) represents the fatty acid-binding protein; *32* OC Ag (triangle symbols) represents the 40S ribosomal protein S12; *34* OC Ag (square symbols) represents RAB7.

$CD4^+$ or $CD8^+$ T cells, respectively. In response to the selected apoptotic OC antigens, both $CD4^+$ and $CD8^+$ T cells promptly produced IFN-γ and IL-17 in amounts that were significantly higher in Responders than in nonresponders (Figs. 2*a* and 2*b*), suggesting that a wide repertoire of T cells arise in response to OC antigens in Responders. By contrast, no significant difference was observed in the IL-2 or IL-4 production (Supporting Information Fig. 5). The lower IL-2 or IL-4 levels, as compared with IFN-γ and IL-17 produced by OC antigen-specific T cells, suggest that the latter are strongly polarized type-1 or type-17 cells particularly producing their own corresponding cytokine ((i.e., IFN-γ and IL-17…). Due to the limited amount of PBMCs available we could not analyze other relevant cytokines such as IL-10 or IL-23 for immunosuppressive or Th-17 response, respectively. Taken together, these data suggest that T-cell responses to apoptotic OC antigens were significantly more diverse in responders than in nonresponders. Cytokine production was almost undetectable when PBMCs of 15 healthy individuals were stimulated with this procedure (data not shown). Notably, these mixed polyfunctional effector responses occurred because of the parallel presence in the same PBMC population of different antigen-specific $CD4^+$ and $CD8^+$ T cell subsets, each of which produced a single cytokine (Fig. 2*a*). Indeed, a tiny number of the $CD4^+$ and $CD8^+$ T cell subsets simultaneously produced IFN-γ and IL-17 (Fig. 2*a*), or both IL-2 and IL-4 (data not shown). Cells promptly producing cytokines were confined within a $CD45RO^+CCR7^-$ cell population, supporting that they expressed an effector memory T (T_{EM}) cell phenotype (Figs. 3*a*–3*c*). The HLA-restriction was demonstrated by blocking these $CD4^+$ and $CD8^+$ T-cell responses with appropriate anti-class I and anti-class II MHC mAbs, respectively (data not shown). To determine the T cell memory capacities (in terms of T-cell expansion and effector

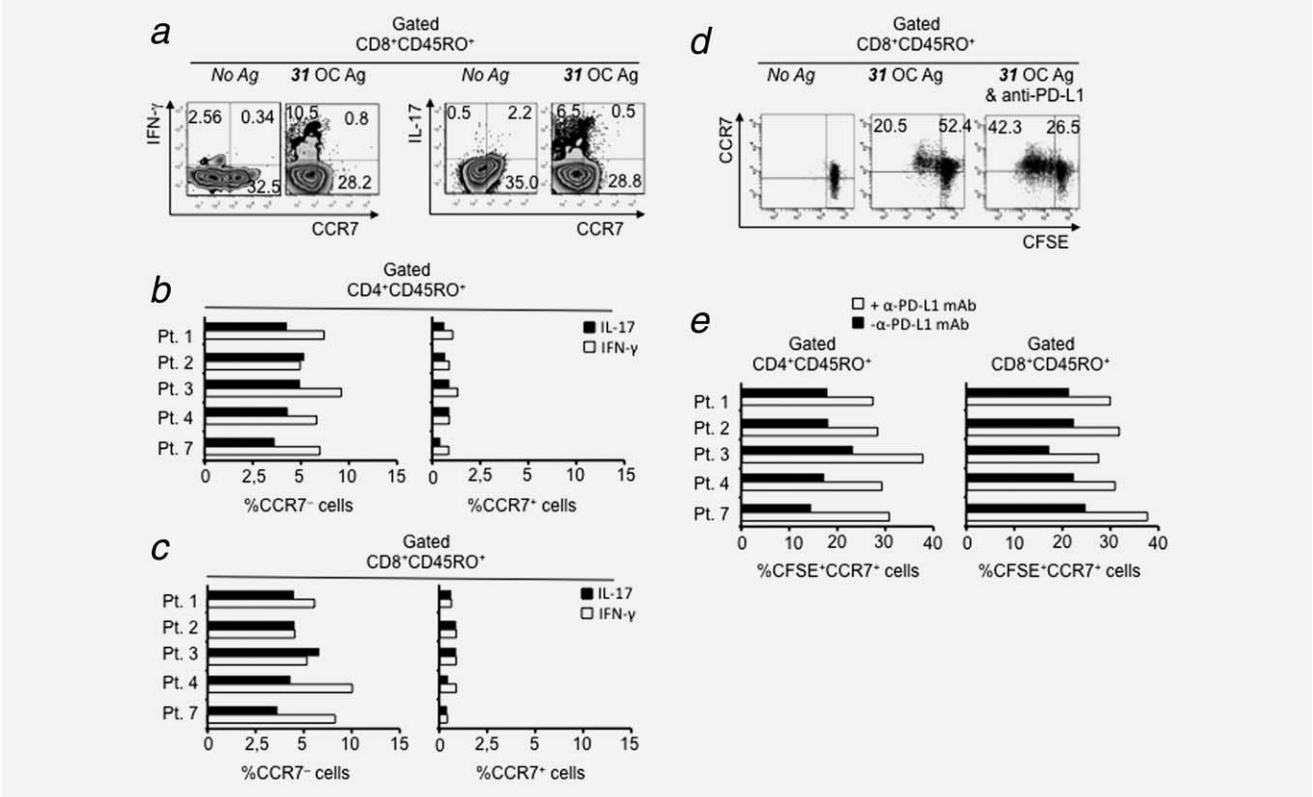

Figure 3. Functional memory T cells specific to apoptotic OC antigens. (*A*) Representative analysis of $CD8^+$ T_{EM} cells ($CD45RO^+CCR7^-$). Fresh PBMCs from a responder (Pt. 3 and Pt. 4) were stimulated with apoptotic ***31*** OC antigen for 18 h and stained with mAbs to CD4, CD8, CD45RO, and CCR7. Cells were processed for the detection of IL-17 and IFN-γ by ICS assay with the relevant mAbs. In this representative experiment, counterplot analyses are gated on $CD8^+CD45RO^+$ cells and show percentages of cytokine-producing $CCR7^+$ or $CCR7^-$ cells. The percentage of cells is reported in each quadrant. (*b* and *c*) Cumulative experiments in five responders showing the percentage of $CCR7^+$ or $CCR7^-$ cells in $CD4^+CD45RO^+$ (B) or $CD8^+CD45RO^+$ (C) cells producing IL-17 (filled bars) or IFN-γ (empty bars) by ICS assay in response to apoptotic *31* OC antigens. (*d*) Functional $CD4^+$ or $CD8^+$ T_{CM} cells ($CD45RO^+CCR7^+$) specific to apoptotic OC antigens. Representative freshly isolated $CD8^+$ T cells from a responder were stained with CFSE and stimulated with autologous DCs that had been pulsed with apoptotic *31* OC antigen, in the presence or in the absence of blocking anti-PD-L1 antibody. After 7 days, cells were stained with mAbs to CD4, CD8, CD45RO, and CCR7, and analyzed with a flow cytometer. In this representative experiment, dot plot analyses are gated on $CD8^+CD45RO^+$ cells and show percentages of $CCR7^+$ $CFSE^+$ or $CFSE_{dim/low}$ cells. The percentage of cells is reported in each quadrant. (*e*) Cumulative flow cytometry analyses in the five responders, performed as described in (*d*), showing the percentage of T_{CM} CD4 or CD8 proliferating cells (CFSElow/-) in response to apoptotic *31* OC antigen, in the presence (empty bars) or absence (filled bars) of an anti-PD-L1 mAb. Values are shown with the background subtracted. Pt. = Patient.

cell renewal *in vitro*), highly purified $CD4^+$ and $CD8^+$ T cell populations from six responders and four nonresponders were stained with CFSE and stimulated for 7 days with autologous DCs that had been pulsed with the relevant apoptotic OC antigens. Under these conditions, we observed only in responders (but not in nonresponders [data not shown]) a notable expansion of CFSE-labeled $CD45RO^+CCR7^+$ T central memory T_{CM} cells (Figs. 3*d* and 3*e*). Interestingly, the capacity to proliferate by OC antigen-specific T_{CM} cells markedly improved in the presence of a blocking antibody specific to the ligand of programed death (PD)-1 (PD-L1) (Figs. 3*d* and 3*e*), supporting the possibility that tumor-specific T cells are limited by PD-1 and their effector function can be further improved by PD-1-blockade immunotherapy.[6] Importantly, the resulting T cell lines derived from the relative T_{CM} cells produced high levels of cytokines in response to a short-term contact with autologous monocytes or DCs that had been previously pulsed with either the appropriate apoptotic OC antigens (Figs. 4*a* and 4*b*), or the entire apoptotic OC cells (Supporting Information Fig. 6). By contrast, no response was shown when DCs cross-presented lysed OC cells (Supporting Information Fig. 6).

MALDI-TOF/TOF-MS analysis

The 2DE gel-eluted proteins that had been previously identified as immunogenic through the T-cell interrogation system (immunogenic OC antigens) were afterwards molecularly characterized by MALDI-TOF/TOF-MS. Of the 25 immunogenic OC antigens that were recognized as known proteins by combining peptide mass fingerprinting and MS/MS approaches, 15 belonged to the apoptotic 2DE-eluted OC proteins (Table 1), whereas 10 belonged to the shared

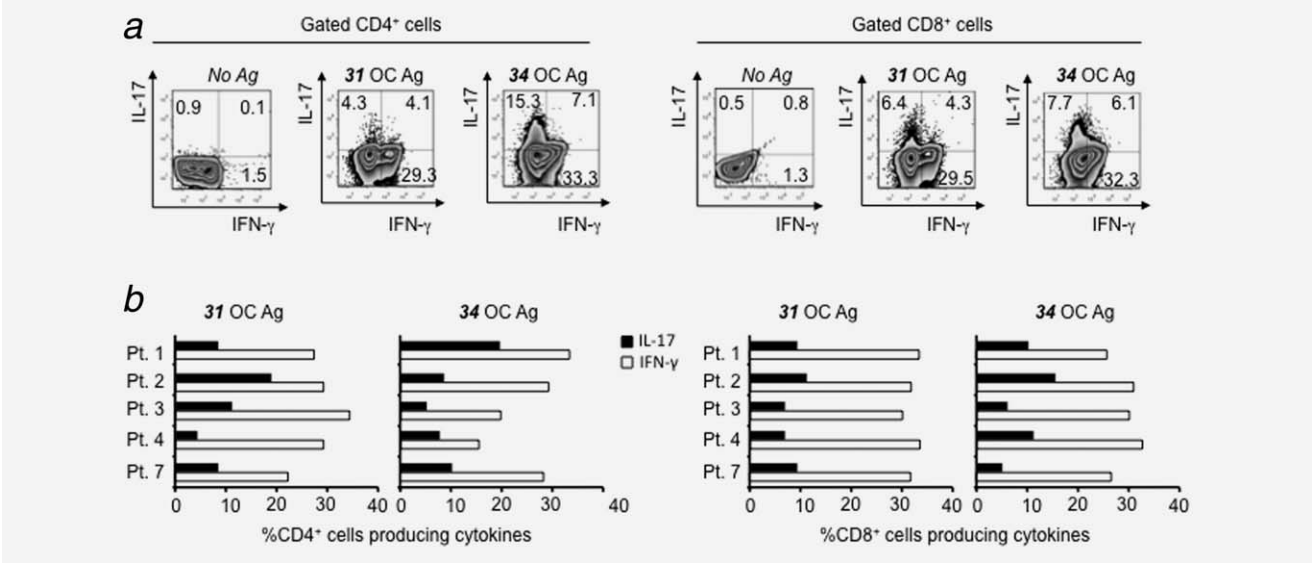

Figure 4. Renewal of $CD4^+$ or $CD8^+$ T_{EM} cells from relative T_{CM} cells specific to apoptotic OC antigens *in vitro*. (*A*) Representative $CD4^+$ or $CD8^+$ T cell lines that were derived from a Responder (Pt. 1 and Pt. 3), upon antigen stimulation for 7 days, were then restimulated for 18 h with autologous monocytes, which had been previously pulsed with the indicated apoptotic OC antigens. Then, they were processed for the detection of IL-17 and IFN-γ by ICS assay with the relevant mAbs. Counterplot analyses are gated on $CD4^+$ or $CD8^+$ cells and show percentages of cytokine-producing cells. The percentage of cells is reported in each quadrant. (*b*) Cumulative experiments in five responder cell lines, performed as described in A, showing the percentage of $CD4^+$ or $CD8^+$ T cells producing IL-17 (filled bars) or IFN-γ (empty bars) by ICS assay in response to the indicated apoptotic OC antigens. Values are shown with the background subtracted. Pt. = Patient.

apoptotic and live 2DE-eluted OC proteins (Supporting Information Table 2).

Immune responses to recombinant OC proteins

To confirm the observed immunogenicity of the proteins identified by the proteomic analysis, some experiments were repeated with three related recombinant proteins (rRAB7, rEIF5A, and rFABPE) in some OC patients (six new responders described in Supporting Information Table 3 and four nonresponders). These recombinant proteins were chosen on the basis of the finding that the corresponding 2DE-gel eluted proteins (31, 32, and 34) induced IFN-γ and IL-17 production by both $CD4^+$ and $CD8^+$ T cells in an extremely higher fashion in Responders than in non-Responders (see Fig. 2). Further analyses including recombinant proteins mimicking the other 2DE-gel eluted proteins showing higher differences between Responders and non-Responders in the ELISPOT assay (see Fig. 1) will be performed to support the immunogenicity of these proteins. We asked whether the recombinant proteins analyzed were recognized by $CD8^+$ or $CD4^+$ T cells differently in Responders, non-Responders or HDs looking at IFN-γ and IL-17 expression by ICS (Fig. 5). It is striking that the Responders analyzed showed the highest responses against rRAB7 and rEIF5A antigens by $CD8^+$ T cells (Figs. 5*a* and 5*c*), as well as against rRAB7, rEIF5A, and rFABPE by $CD4^+$ T cells (Figs. 5*b* and 5*d*). More detailed analyses showed that these responses were performed by both $CD8^+$ T_{EM} cells—in agreement with the data obtained with 2DE-derived proteins—and terminally-differentiated effector T cells, namely the effector memory RA^+ T (T_{EMRA}) cells (Supporting Information Fig. 7). In line with data obtained with the 2DE-derived proteins, specificity of $CD4^+$ and $CD8^+$ T cell lines was confirmed against Rab7 and FABPE (data not shown).

Discussion

In this study, for the first time, chemotherapeutics-induced memory T-cell responses against CAAs were identified in OC patients. To this end, we took advantage of a modified reverse immunology approach, by which immunogenic OC antigens were first selected by interrogating memory T cells derived from OC patients and then molecularly characterized through a MS-based approach (Supporting Information Fig. 8).

We found that memory T cells that recognized apoptotic OC antigens exhibited both a wider repertoire and significant stronger effector responses (that were correlated with a significant more prolonged survival) in OC responder patients than in nonresponders. Interestingly, the apoptotic OC antigens tested were efficiently recognized by both $CD4^+$ and $CD8^+$ T cells. Because of the exogenous form of our OC antigens, we assumed that $CD8^+$ T cells were induced through cross-presentation of OC antigens by professional antigen-presenting cells (APCs) (i.e., DCs) *in vivo*.[22–24] In the case of chemotherapy-induced immunostimulatory apoptosis, the presence of various danger signals can influence the switch from tolerogenic DCs to DCs with high migratory and stimulatory capacity that can prime or cross-prime

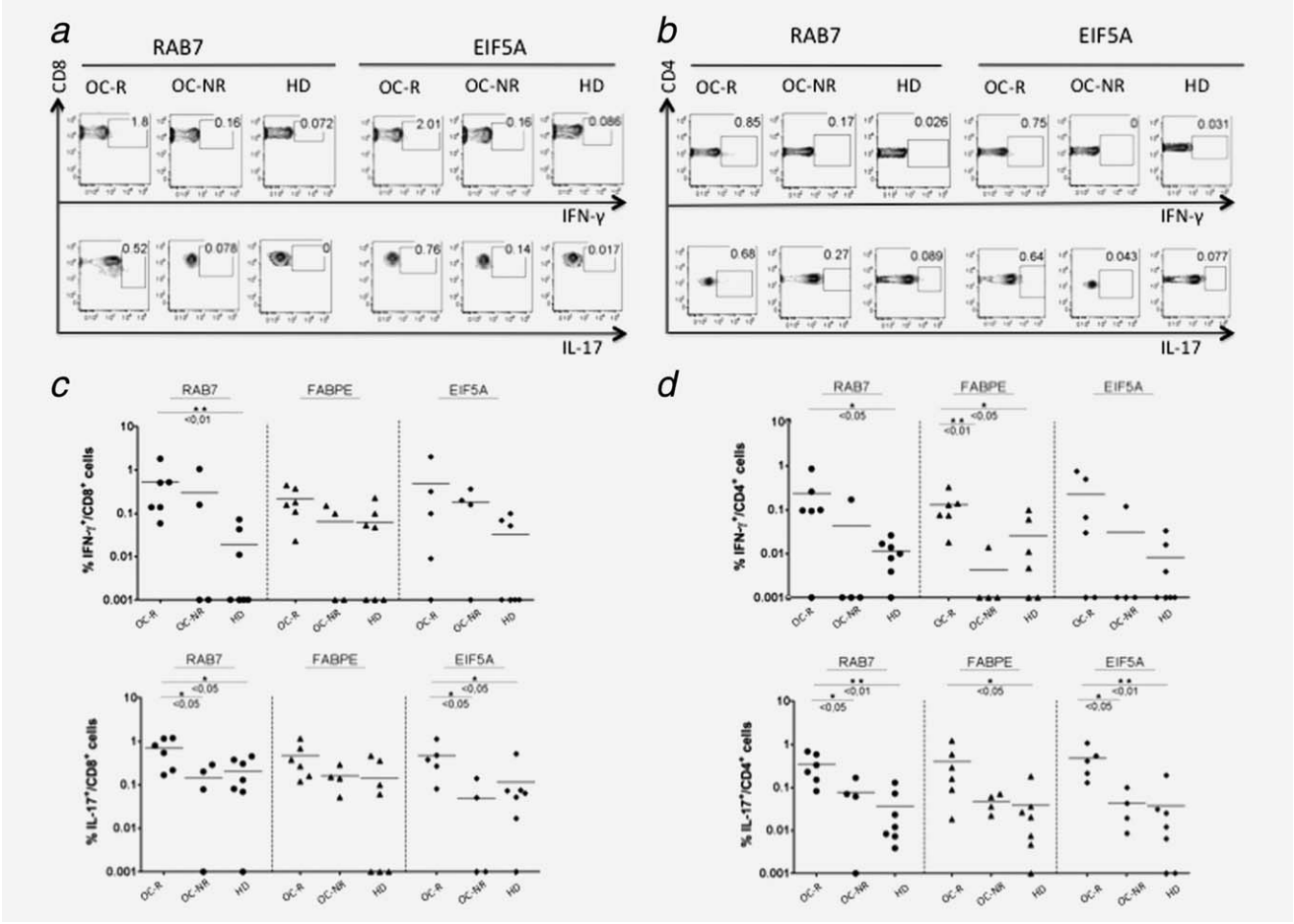

Figure 5. Production of IL-17 and IFN-γ by $CD4^+$ and $CD8^+$ cells in response to recombinant proteins. PBMCs from OC Responder patients (OC-R), OC non-Responder patients (OC-NR) and healthy donors (HD) were stimulated with recombinant proteins (RAB7, FABPE, EIF5A) and anti-CD28 mAb for 18 h at 37°C. Then cells were stained with mAb to CD8 or CD4 and the dump channel reagents so as to exclude monocytes, B cells, NK cells and dead cells from the analysis. Then cells were processed for the detection of IL-17 and IFN-γ by ICS assay with the relevant mAbs. (*a* and *b*) Representative flow cytometry analysis of $CD8^+$ (*a*) or $CD4^+$ (*b*) T cells (from an OC-R, an OC-NR and an HD) producing IFN-γ or IL-17 in response to rRAB7 or rEIF5A. Counterplot analyses are gated on $CD8^+$ or $CD4^+$ T cells and show percentages of cytokine-producing cells. (*c* and *d*) Cumulative results of CD8 (*c*) or CD4 (*d*) T cells (from six OC-R, four OC-NR and seven HD) producing IFN-γ or IL-17 in response to rRAB7, rFABPE or rEIF5A. Values are shown with the background subtracted. Statistical analysis was performed with nonparametric Mann-Whitney *U*-test for unpaired data.

efficient T cell responses.[16,17,25] Consistent with this hypothesis, both $CD4^+$ and $CD8^+$ T_{EM} cells ($CD45RO^+CCR7^-$) from Responders promptly produced significant amounts of proinflammatory cytokines (IFN-γ and IL-17) in response to the apoptotic OC antigens tested (compared with T cells from nonresponders), suggesting that a wide repertoire of functional $CD4^+$ and $CD8^+$ T_{EM} cells can emerge in response to apoptotic OC antigens. Notably, this immunocompetence in responders (but not in nonresponders) was supported by the simultaneous presence of $CD4^+$ and $CD8^+$ T_{CM} cells ($CD45RO^+CCR7^+$) that were capable to expand and provide renewed effector cell responses *in vitro*. This data suggests that responders with efficient T cell effector responses to OC antigens harbor expandable T_{CM} cells that are capable to deliver new effector cells on demand. The chemotherapy efficacy in Responders might overcome the tumor-dependent immune suppression and favor tumor regression through the induction of vigorous IFN-γ- and/or IL-17-mediated T_{EM} cell responses.[7,26–29] In addition, the combination with "biologicals" blocking immunosuppressive molecules, such as PD-1 might increase the beneficial effects in the treatment of tumors.[30] This possibility is supported by the evidence that the expansion of T_{CM} cells specific to apoptotic OC antigens improved, when they were disengaged from the PD-1-dependent control in our *in vitro* system. Further investigations are required to establish if the mechanistic basis of the superior immune responses to OC antigens, including the compelling theory, suggesting that the dominance and functional properties of memory T cells that are specific to apoptotic OC antigens might be influenced by the initial frequency of antigen-specific precursors,[31–34] as well as the possibility of differences in the generation of regulatory T

cells (Tregs) by OC antigens between responders and nonresponders. Analysis of Tregs is an important issue to address in future experiments where timing of blood retrieval is closer to chemotherapy allowing a short-term effect on Tregs.

The evidence that the superimmunogenic antigens, whose T-cell response was significantly more elevated in responders than in nonresponders, were identified among those specifically expressed in apoptotic cells (7/55) and none among those co-expressed or exclusively expressed in live cells (0/125), further supports the immunogenic strength of antigens unveiled by the chemotherapy-induced apoptosis. The selective correlation of the super-immunogenic apoptotic antigens with protection may be due to the fact that the latter are less tolerogenic, as compared with the shared that are equally represented by live cells, known to be more biased to induce tolerance. In this context, our findings merit a particular attention. Indeed, we induced apoptosis of primary OC cells with mitoxanthrone (an anthracycline family member), because of its well known capacity to establish "immunogenic cell death".[15–17,25] However, our patients showed significant T cell responses against apoptotic OC proteins, despite they were treated with a combination of platinum and taxane (without anthracycline). This observation strongly supports that the immunogenic chemotherapy effect is not limited to the classical "immunogenic cell death" inducers (including anthracyclines, doxorubicin, alkylating agents, gemcitabine, or tyrosine kinase ihibitors),[15–17,25] but that other drugs (*e.g.*, platinum-based chemotherapeutics) can induce T-cell responses to CAAs that correlate with survival.[35,36] Although our findings clearly demonstrate the generation of T-cell responses against mitoxanthrone-induced apoptotic OC proteins in patients treated with platinum-based chemotherapeutics *in vivo*, experiments are in progress to assure that similar apoptotic proteins are unveiled by platinum-induced apoptosis *in vitro*.

Another finding from our MS-based analysis is that most of apoptotic CAAs identified corresponded to ubiquitous proteins. This finding is consistent with a previous observation showing that in murine spontaneous prostate cancer, $CD8^+$ T cell recognize an ubiquitous histone H4 epitope.[37] In this context, epigenetic changes may lead to the overexpression of a series of histones, including H2A found in our apoptotic OC cells. These results indicate that CAAs correspond not necessarily to tumor cell-specific antigens, but also to ubiquitous proteins that, under normal conditions, are sequestered in cell structures that limits their processing and presentation to T cells.[37] However, as a result of the chemotherapy effects within a tumor microenvironment, apoptosis of tumor cells can induce upregulation of a wide range of ubiquitous proteins in a sufficient amount for subsequent processing and presentation by DCs that could thus prime the corresponding specific T cells. Consistent with this hypothesis, T cell lines specific for individual apoptotic OC proteins were stimulated by cross-presentation of the entire apoptotic OC cells (unveiling the appropriate antigens), but not of lysed OC cells (representing the proteome of lived OC cells). In the prospect of a possible vaccine building, apoptotic antigens that are common in all individuals may elicit "public" anti-tumor responses (universal vaccines) by both the by-stander killing phenomenon—by which $CD8^+$ T_{EM} cells migrating into tumor tissue recognize cognate antigens on APCs cross-presenting apoptotic OC cells, and then they kill neighboring live OC cells in a nonantigen-specific manner—[38,39] and the subsequent production of proinflammatory cytokines that favor the recruitment of inflammatory cells. By contrast, the direct recognition of the huge number of mutated tumor-associated epitopes on live OC cells likely might only elicit "private" anti-tumor responses (personal vaccines) for each single patient.[40]

Although our experimental design did not aim to define the possible role of the OC-associated CAAs in the tumorigenesis, the biological function of these antigens deserve further attention. Three of the seven immunogenic apoptotic OC associated proteins—HSPB1,[41] FABPE,[42] and H2AC—[43,44] had been previously related to OC development or progression, but their immunogenicity was unknown. In addition, two of them—CRYAB[45] and RS12—[46] have been previously associated with development of other tumors, but not with OC.

Importantly, the observation that effector T-cell responses to some of the recombinant proteins tested are significantly increased in OC patients (and in particular in responders) supports the concept that chemotherapy can result in T-cell response against specific CAAs. The limited number of OC patients studied in these experiments may explain the significant IL-17 and/or IFN-γ expression in response to some, but not to all the recombinant CAAs utilized (as compared with the corresponding 2DE-gel eluted OC proteins). Since endotoxin contamination in our 2DE-gel eluted proteins can be reasonably excluded because of the purification process (TCA/acetone) and LAL test, the presence of residual danger signals supplied by apoptotic tumor cells[15–17,25] might explain the stronger immunogenic power of 2DE gel-eluted proteins, as compared to the related recombinant ones. Studies on a larger OC patient population are required to validate the prognostic value of immune responses to recombinant OC proteins.

In conclusion, the T-cell interrogation system represents a valid reverse immunology model that may be directly exploitable in patients with tumors. Because this system selectively identifies immunogenic OC proteins by interrogating T cells from patients with OC (the response of which are correlated with patients' survival), we are tempted to propose these antigens for cancer biomarkers or even vaccines.

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