Priority Brief

Cancer Immunology Research

CD4$^+$ T Effectors Specific for the Tumor Antigen NY-ESO-1 Are Highly Enriched at Ovarian Cancer Sites and Coexist with, but Are Distinct from, Tumor-Associated Treg

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Abstract

Whereas tumor infiltration by T effectors is generally associated with a more favorable prognosis, the accumulation of CD4$^+$ regulatory T cells (Treg) within tumors is instead often associated with poor disease outcome. Because approaches to improve antitumor immunity aim, on one hand, at expanding tumor antigen-specific T cells and, on the other, at eliminating or inactivating Treg, an outstanding question is whether, and to what extent, tumor antigen-specific CD4$^+$ T effectors present at tumor sites overlap with tumor-associated Treg. Here, we used MHC class II/peptide tetramers incorporating an immunodominant peptide from the human tumor-specific antigen NY-ESO-1 to assess antigen-specific CD4$^+$ T cells among conventional CD4$^+$ T effectors and Treg at sites of ovarian cancer. We found that, in patients who spontaneously respond to the antigen, the frequency of NY-ESO-1 tetramer$^+$ cells detected *ex vivo* was highly enriched in tumors as compared with the periphery. At tumor sites, NY-ESO-1 tetramer$^+$ cells were detected concomitantly with high proportions of Treg but were distinct from the latter and displayed characteristics of T_H1 effectors. Thus, even in the presence of high proportions of Treg, tumor antigen-specific CD4$^+$ T cells can accumulate in ovarian tumors and maintain an effector phenotype. *Cancer Immunol Res; 1(5); 303–8. ©2013 AACR.*

Introduction

The balance between T-cell subsets that play opposite immune functions is a determinant factor in antitumor immunity. The presence of high proportions of T effectors at tumor sites is generally associated with efficient antitumor responses (1), whereas that of regulatory/suppressor CD4$^+$ T cells (Treg) has been often reported to be associated with less favorable clinical outcomes (2). Some studies, however, have instead reported an association between high levels of FOXP3$^+$ Treg and improved clinical outcomes (3, 4), a contradictory finding that could possibly result from the fact that certain subsets of Treg comigrate with T effectors and are therefore enriched in tumors that are also highly infiltrated by T effectors (5). In support of their favorable role in antitumor immunity, T effectors in tumors have been found to contain cells that recognize antigens expressed by cancer cells, leading to their elimination (6).

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doi: 10.1158/2326-6066.CIR-13-0062-T

On the basis of this evidence, strategies to improve antitumor immunity currently aim at increasing tumor antigen-specific T effectors in patients with cancer through various approaches, including vaccination, adoptive cell transfer therapy, and/or checkpoint blockade (7–10). Other potentially complementary approaches aim instead at impacting Treg by eliminating/inactivating them or converting them into effectors (11). However, because the antigen specificity of Treg infiltrating human tumors is largely unknown, an outstanding question in the development of all these approaches is whether, and to what extent, tumor antigen-specific CD4$^+$ T effectors present at tumor sites overlap with tumor-infiltrating Treg. This, however, has been difficult to assess, partly because Treg are anergic, i.e., unable to proliferate or produce cytokines following T-cell receptor (TCR)-mediated stimulation, which complicates the assessment of their antigen specificity.

NY-ESO-1, a human tumor-specific antigen of the cancer/testis group expressed in germ cells and in cancer, but not in normal somatic tissues (12), is a major candidate for the development of immunotherapy in cancers of different histologic types. NY-ESO-1 is frequently expressed in high-grade serous ovarian cancer, a tumor type in which Treg are present in high proportions and may significantly contribute to dampen antitumor immunity (2, 13, 14). We have previously identified an immunodominant peptide, NY-ESO-$1_{119\text{-}143}$, recognized by NY-ESO-1–specific CD4$^+$ T cells, and have generated MHC class II/NY-ESO-1 peptide tetramers that allow the direct assessment of antigen-specific CD4$^+$ T cells by flow cytometry, independent of their function (15). Here, we have used the tetramers to directly assess NY-ESO-1–specific cells e*x vivo*

among conventional $CD4^+$ T cells and Treg present at sites of ovarian cancers in patients with spontaneous serologic responses to the antigen.

Materials and Methods

Patient samples and phenotypic assessment of $CD4^+$ T cells

Surgical tumor specimens and peripheral blood samples were obtained at the Institut de Cancérologie de l'Ouest (Nantes-Saint Herblain, France) and Roswell Park Cancer Institute (Buffalo, NY) from patients with ovarian cancer upon approval by the institutional review boards and signed informed consent. Tumor specimens were processed by mechanical dissection to single-cell suspensions. Cells from ovarian cancer ascites were isolated by centrifugation. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient sedimentation using LSM 1077 lymphocyte separation medium (PAA Laboratories GmbH). $CD4^+$ T cells were enriched by positive selection using magnetic cell sorting (Miltenyi Biotec) from PBMC and single-cell suspensions from ovarian solid tumors or ascites. Isolated $CD4^+$ T cells were assessed phenotypically by staining with fluorochrome-labeled monoclonal antibodies (mAb; from BD Biosciences unless indicated otherwise) specific for CD4, CD25 (Beckman Coulter), CD127 and FOXP3 (eBioscience), as indicated, and analyzed by flow cytometry (FACSAria, BD Biosciences). For assessment of NY-ESO-1–specific cells, isolated $CD4^+$ T cells were stained with phycoerythrin-labeled HLA-DR52b/His-tag-NY-ESO-$1_{119–143}$ tetramers (TCMetrix) and anti-CD4, -CD25, and -CD127 mAb as previously described (15). For *ex vivo* flow cytometry cell sorting, enriched $CD4^+$ T cells were stained with mAb specific for CD4, CD25, and CD127 and separated into Tconv ($CD4^+CD25^{low}$) and Treg ($CD4^+CD25^{high}CD127^{low}$) to high purity (>97%) by flow cytometry cell sorting (FACSAria, BD Biosciences).

Functional assessment of $CD4^+$ T cells

The ability of tumor-associated $CD4^+$ T cells to produce IFN-γ was assessed in a 6-hour intracellular cytokine staining assay using mAb specific for IFN-γ and FOXP3 following stimulation with PMA (100 ng/mL; Sigma-Aldrich) and ionomycin (1 μg/mL; Sigma-Aldrich), as previously described (5). The suppressive capacity of *ex vivo*-sorted tumor-associated Treg and Tconv was assessed by coculture with CFSE-labeled responder $CD4^+$ T cells in the presence of irradiated monocytes and phytohemagglutinin (PHA; Sigma-Aldrich). Growth of responder cells was assessed by flow cytometry analysis of CFSE dilution in day-5 cultures. The growth (percentage of divided cells) of the wells with the test population (experimental group) was compared with that of the wells without the test population (control). The percentage of suppression was determined as follows: 100 − [(growth of experimental group/growth of control) × 100; ref. 5]. NY-ESO-1-$tetramer^+$ T-cell lines were obtained by stimulation of $tetramer^+$ cells, isolated by flow cytometry cell sorting from tumor-associated $CD4^+$ T cells, with PHA and irradiated allogeneic feeder cells as previously described (15). To assess antigen recognition, the lines were stimulated with serial dilutions of NY-ESO-$1_{119–143}$ in the presence of HLA-DR52b$^+$ antigen-presenting cells (APC), and secreted IFN-γ was measured in 24-hour supernatants by ELISA. The ability of the lines to produce cytokines was assessed in a 4-hour intracellular cytokine staining assay following stimulation with PMA/ionomycin using mAb specific for IFN-γ, TNF-α, interleukin (IL)-2,, IL-4, IL-10, and IL-17 (eBioscience).

Results and Discussion

A phenotypic and functional analysis of ovarian cancer-associated conventional $CD4^+$ T effectors (Tconv) and Treg was initially carried out using cell suspensions obtained *ex vivo* from ovarian cancer ascites (TAL) or from mechanically dissociated ovarian tumor tissue tumor-infiltrating leukocytes (TIL). Treg were identified among $CD4^+$ T cells as $CD25^{high}CD127^{low}$, and Tconv were identified as $CD25^{low}$ (Fig. 1A). Treg identified by this phenotype in tumor samples expressed high levels of FOXP3 and were highly suppressive *ex vivo* (Fig. 1B and C). In contrast, Tconv had little expression of FOXP3 and no suppressive activity. Tumor-associated Tconv stimulated *ex vivo* produced, for the most part, high levels of IFN-γ, whereas most tumor-associated $FOXP3^+$ Treg failed to secrete IFN-γ (Fig. 1D). Thus, whereas tumor-associated Treg were highly suppressive *ex vivo*, most tumor-associated $CD4^+$ Tconv instead displayed characteristics of effector cells.

Integrated immune responses to NY-ESO-1, including specific antibodies, $CD4^+$ and $CD8^+$ T cells, spontaneously develop in patients with ovarian cancer bearing antigen-expressing tumors (13). Using MHC class II (DR52b/DRB3*0202)/NY-ESO-1 peptide tetramers, we have previously shown that NY-ESO-1-$tetramer^+$ cells are detected among circulating $CD4^+$ T cells from patients with spontaneous antibody responses to the antigen, but not in healthy donors or in NY-ESO-1–seronegative patients (15, 16). By assessing a group of 215 patients with ovarian cancer, 20 patients with detectable levels of circulating NY-ESO-1 antibodies were identified. For 2 patients who were $DRB3^*0202^+$, cryopreserved PBMC and cell suspensions from TAL and TIL were available. From these samples, $CD4^+$ T cells were enriched *ex vivo* by magnetic cell sorting and stained with tetramers in combination with anti-CD25 and -CD127 mAb. NY-ESO-1-$tetramer^+$ cells were detected *ex vivo* in all samples (Fig. 2A). For patient OC1, $tetramer^+$ cells were detected among $CD4^+$ T cells in TAL and TIL at a frequency of 0.3% and 1%, respectively, i.e., about 20- and 60-fold higher than in circulating $CD4^+$ T cells (Fig. 2B). Similarly, for patient OC2 the frequency of $tetramer^+$ cells among $CD4^+$ T cells in TAL and TIL was 0.1% and 0.2%, respectively, i.e., 5- and 10-fold higher than in the periphery.

We have previously reported that NY-ESO-1-$tetramer^+$ cells in circulating $CD4^+$ T cells from patients with both spontaneous and vaccine-induced immune responses to NY-ESO-1 are found among Tconv and not Treg (15, 16). Consistent with this, we found that NY-ESO-1-$tetramer^+$ T cells in circulating $CD4^+$ T cells from patients OC1 and OC2 were $CD25^{low}CD127^{high}$ (Fig. 3A and B). Similarly, NY-ESO-1-$tetramer^+$ cells in both TAL and TIL were distinct from

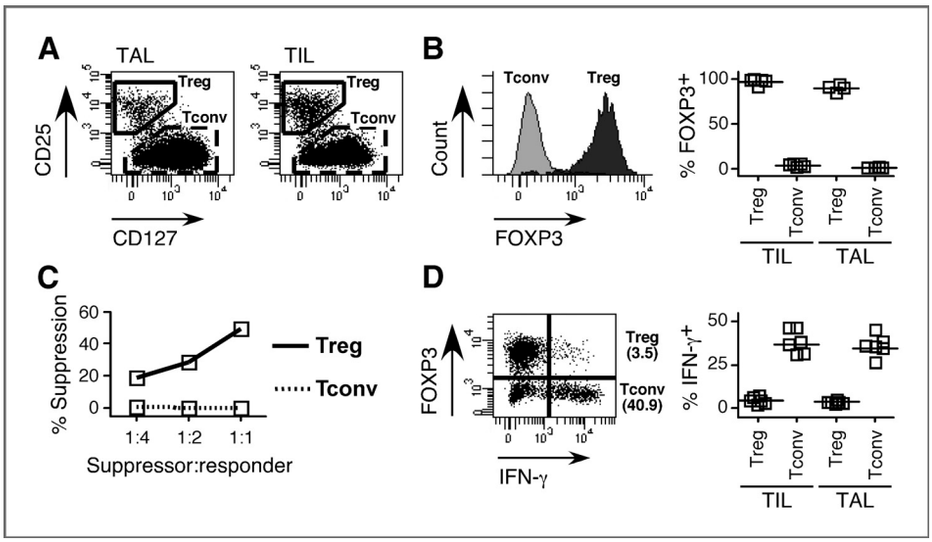

Figure 1. Phenotype and function of Tconv and Treg in ovarian cancer. A and B, CD4$^+$ T cells isolated from TAL and TIL were stained with mAb specific for CD4, CD25, CD127, and FOXP3 and analyzed by flow cytometry. Within the CD4$^+$ T-cell gate, Treg are identified as CD25^{high}CD127^{low} and Tconv as CD25^{low} (A). Gated Treg and Tconv were examined for intracellular FOXP3 expression (B). Examples of dot plots are shown for 1 TIL and 1 TAL in A. Examples of histograms are shown for 1 TIL in B, and data obtained for all samples are summarized in B. C, Treg and Tconv isolated *ex vivo* from TIL were cultured with CFSE-labeled responder CD4$^+$ T cells in the presence of PHA and APC for 5 days. The percentage of suppression was calculated as detailed in Materials and Methods. D, CD4$^+$ T cells isolated from tumors were stimulated *ex vivo* with PMA/ionomycin for 6 hours followed by labeling with FOXP3- and IFN-γ–specific mAb. A dot plot corresponding to CD4$^+$ T cells from 1 TIL is shown, and data obtained with all samples are summarized. Numbers correspond to the percentage of IFN-γ$^+$ cells in FOXP3$^+$ (Treg) and FOXP3$^-$ (Tconv) cells.

tumor-associated CD25^{high}CD127^{low} Treg, as they were largely CD25^{low} and expressed various levels of CD127 (Fig. 3A and B), a phenotype that has been reported to identify differentiated effector cells producing IFN-γ (17).

To assess tumor-associated NY-ESO-1–specific CD4$^+$ T effectors functionally, we isolated NY-ESO-1-tetramer$^+$ T cells from TIL and TAL and expanded them *in vitro* by mitogen stimulation to generate NY-ESO-1-tetramer$^+$ T-cell lines (Fig. 4A and B). The lines obtained following this strategy exhibited a clear T_H1 effector profile as they produced high levels of IFN-γ, TNF-α, and IL-2 but did not produce cytokines associated with other CD4$^+$ T-cell subsets, such as IL-17, IL-4, or IL-10 (Fig. 4C).

In sum, our data provide direct evidence that high frequencies of CD4$^+$ T cells specific for NY-ESO-1 are found at sites of ovarian cancers of patients with spontaneous immune responses to the antigen, where they coexist with high proportions of Treg. However, NY-ESO-1–specific CD4$^+$ T cells in tumors are clearly distinct from Treg and exhibit instead characteristics of T_H1 effectors. Because tumor-associated Treg are highly suppressive *ex vivo*, and despite the capacity of NY-ESO-1–specific T_H1 cells to expand at tumor sites, it is likely that Treg still affect them and limit their full growth potential and/or effector functions, particularly in solid tumor tissues, where Treg are present in high numbers.

It is noteworthy that, because only a few MHC class II tetramers incorporating peptides from human tumor-specific antigens have been developed thus far, the data reported here represent the first direct *ex vivo* assessment of tumor antigen-specific CD4$^+$ T cells at tumor sites of patients with spontaneous immune responses to a tumor-specific antigen. In our

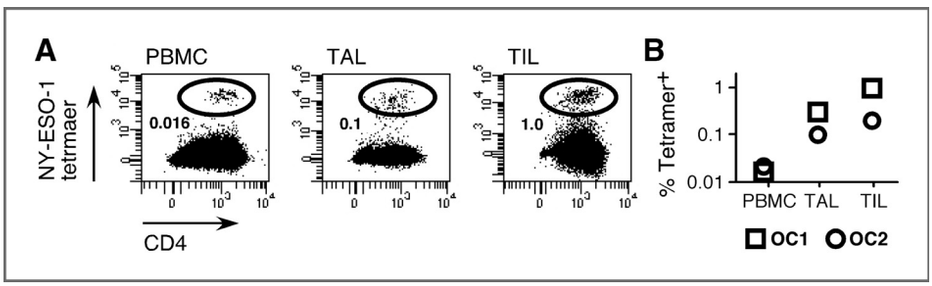

Figure 2. CD4$^+$ NY-ESO-1-tetramer$^+$ T cells are highly enriched in tumors from DRB3*0202$^+$ patients with spontaneous immune responses to the antigen. CD4$^+$ T cells isolated from PBMC, TAL, and TIL were stained with NY-ESO-1 tetramers and with anti-CD4 mAb and analyzed by flow cytometry. Examples of dot plots are shown in A; numbers correspond to the percentage of tetramer$^+$ cells) and data are summarized in B.

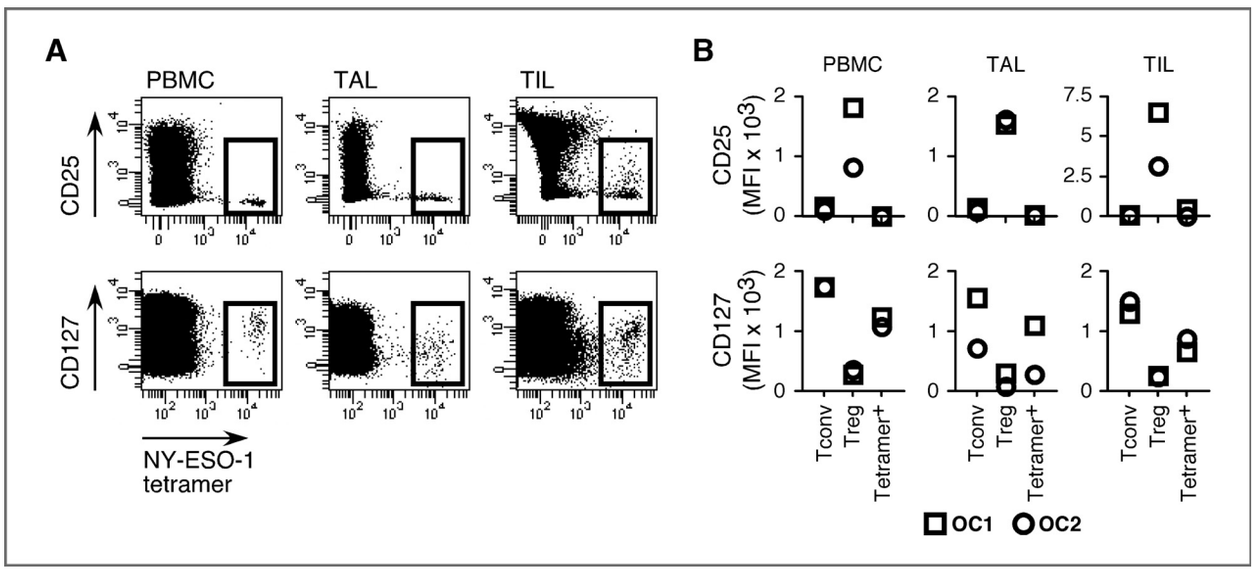

Figure 3. CD4$^+$ NY-ESO-1-tetramer$^+$ T cells at tumor sites are distinct from tumor-associated Treg. CD4$^+$ T cells isolated from PBMC, TAL, and TIL were stained with NY-ESO-1 tetramers and with anti-CD4, -CD25, and -CD127 mAb and analyzed by flow cytometry. Examples of dot plots, gated on CD4$^+$ cells, are shown in A. The mean fluorescence intensities (MFI) of CD25 and CD127 staining in Tconv (CD25^{low}), Treg (CD25^{high}CD127^{low}), and NY-ESO-1-tetramer$^+$ cells (gated as shown in A) are summarized for all samples in B.

previous assessments of NY-ESO-1-tetramer$^+$ cells in circulating CD4$^+$ T lymphocytes from patients with cancer with spontaneous or vaccine-induced immune responses (15, 16), and, in this study, in tumor-associated CD4$^+$ T cells, we have consistently failed to detect NY-ESO-1–specific Treg. Our results are at variance with those of other studies reporting that a fraction of NY-ESO-1–specific CD4$^+$ T cells that spontaneously develop in patients with cancer or are induced through vaccination are Treg (18, 19). These studies, however, did not use *ex vivo* assessments by tetramers, but indirect strategies that may have confounded results and conclusions.

The origin and specificity of tumor-associated Treg is currently under debate. One study has proposed that accumulation of Treg in tumors is the consequence of an early

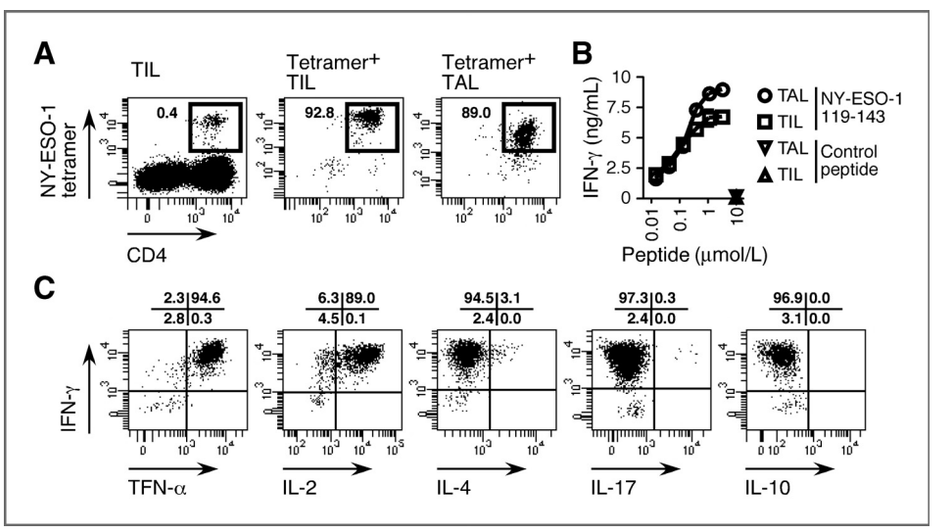

Figure 4. Tumor-associated NY-ESO-1-tetramer$^+$ CD4$^+$ T cells exhibit a T_H1 cytokine secretion profile. A, TIL and TAL, grown *in vitro* for 2 weeks in the presence of IL-2, were stained with NY-ESO-1 tetramers and anti-CD4 mAb (left dot plot), and tetramer$^+$ CD4$^+$ cells were sorted by flow cytometry and expanded by mitogen stimulation to generate NY-ESO-1–specific CD4$^+$ T-cell lines. Samples of the T-cell lines were stained with tetramers and anti-CD4 mAb and analyzed by flow cytometry (middle and right dot plots). B, T-cell lines were assessed for antigen recognition by incubation with serial dilutions of NY-ESO-$1_{119–143}$ or control peptide in the presence of DRB3*0202$^+$ APC, and IFN-γ was measured in 24-hour supernatants by ELISA. C, the T-cell lines were assessed for cytokine production in a 4-hour intracellular cytokine staining assay. Data obtained with the TIL-derived T-cell line are shown. Similar results were obtained with the TAL-derived T-cell line.

recruitment/expansion at tumor sites of Treg specific for self-antigens (20) presumably of thymic origin (called natural or nTreg). Other studies, however, have suggested that the accumulation of Treg in tumors results from the local conversion of Tconv, including those directed against tumor-specific antigens, into induced Treg (iTreg; ref. 21). In line with the concept that Treg infiltrating ovarian cancers are mostly nTreg, we have recently reported that a large fraction of them express Helios, a transcription factor of the Ikaros family that has been proposed to distinguish nTreg from iTreg (5, 22). Interestingly, based on their comparison of the TCR sequences of tumor-infiltrating Treg in two different mouse models, Sainz-Perez and colleagues recently reported that Treg–Teff conversion is not an active process at tumor sites (23), a conclusion that is in line with the results of the present study.

Although our results do not rule out that iTreg specific for certain types of tumor-associated antigens, for example tissue differentiation antigens, could be represented among tumor-associated Treg, they nevertheless show that tumor-associated CD4$^+$ T cells directed against a highly tumor-specific and immunogenic antigen such as NY-ESO-1 are largely distinct from tumor-associated Treg. The results of this study are highly relevant to the development of cancer immunotherapy in several respects. First, they show that CD4$^+$ T cells that recognize highly specific tumor antigens can accumulate in tumors and, even in the presence of very high proportions of Treg, are not converted into Treg, but maintain instead an effector phenotype. These results therefore imply that vaccination strategies based on NY-ESO-1 or other tumor-specific and immunogenic antigens have a high potential for expanding antitumor effectors and not Treg *in vivo*, even when these cells migrate at tumor sites. On the other hand, because tumor antigen-specific CD4$^+$ T effectors at tumor sites are distinct from Treg, our results suggest that a selective elimination of Treg, if achievable, would not erode the tumor antigen-specific component of antitumor immune responses, but could rather potentiate it, and contribute to remove the brakes that prevent antitumor effectors from completely eradicating tumor cells, *in vivo*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Ayyoub, D. Valmori
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Pignon, J.-M. Classe, K. Odunsi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Ayyoub, D. Valmori
Writing, review, and/or revision of the manuscript: M. Ayyoub, D. Valmori
Study supervision: M. Ayyoub, D. Valmori

Grant Support

The study was supported by the Cancer Research Institute, the Ludwig Institute for Cancer Research, and La Ligue contre le Cancer.

Received May 28, 2013; revised August 9, 2013; accepted August 12, 2013; published OnlineFirst August 19, 2013.

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