Circulating tumor antigen-specific regulatory T cells in patients with metastatic melanoma

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Although it is accepted that regulatory T cells (T regs) contribute to cancer progression, most studies in the field consider nonantigen-specific suppression. Here, we show the presence of tumor antigen-specific CD4\(^+\) T regs in the blood of patients with metastatic melanoma. These CD4\(^+\) T regs recognize a broad range of tumor antigens, including gp100 and TRP1 (melanoma tissue differentiation antigens), NY-ESO-1 (cancer/testis antigen) and survivin (inhibitor of apoptosis protein (IAP) family antigen). These tumor antigen-specific T regs proliferate in peripheral blood mononuclear cells (PBMC) cultures in response to specific 15-mer peptides, produce preferentially IL-10 and express high levels of FoxP3. They suppress autologous CD4\(^+\)CD25\(^+\) T cells responses in a cell contact-dependent manner and thus share properties of both naturally occurring regulatory T cells and type 1 regulatory T cells. Such tumor antigen-specific T regs were not detected in healthy individuals. These tumor antigen-specific T regs might thus represent another target for immunotherapy of metastatic melanoma.

FoxP3 | IL-10 | NY-ESO-1

Molecular identification of human cancer antigens in the last decade, for example MART-1/MelanA or NY-ESO-1, has ushered in a new era of antigen-specific cancer immunotherapy targeting these antigens. Despite promising data in animal models, the use of therapeutic cancer vaccines in humans has not yet lived up to its promises. The improved understandings of antigen presentation, and most specifically in dendritic cell (DC) biology, represent a new avenue for research in the field. Ex vivo-generated and antigen-loaded DCs have been used as vaccines to improve immunity in patients with cancer (1). We have vaccinated patients with metastatic melanoma with ex vivo-generated dendritic cells loaded with either HLA-A\(^*\)0201-restricted peptides derived from melanoma-associated antigens (2) or killed allogeneic melanoma cells (3). Despite some remarkable clinical responses, the overall objective tumor regression rate remains low (1). Because insufficient antitumor immunity might be due to immunosuppression driven by regulatory T cells (T regs), we engaged in identifying such cells in our patient population.

T regs are specialized in the control of responsiveness to self. They are composed of subsets with distinct ontogeny and functions. Naturally occurring CD4\(^+\)CD25\(^{hi}\) T regs are produced in the thymus (4), and express FoxP3, a transcriptional factor critical for their development and function (5, 6). Their depletion results in the development of autoimmune diseases in murine models (7, 8). Conversely, their adoptive transfer inhibits the occurrence of autoimmune diseases in disease-prone mice (9). CD4\(^+\)CD25\(^{hi}\) T regs suppress the immune responses in a cell contact-dependent manner through mechanisms that remain to be identified (10). T regs are also generated in the periphery from nonregulatory T cells (11–16). These include regulatory type 1 (Tr1) (12) and Th3 (11) cells, both of which preferentially secrete regulatory cytokines, IL-10, and/or TGF\(\beta\) and do not express FoxP3 (12, 13). These T regs suppress immune responses in a soluble factor-dependent manner, without requiring cell-to-cell contact. Another CD4\(^+\) Treg subset that expresses CD25 and FoxP3 is induced in the periphery de novo from FoxP3\(^-\) nonregulatory T cells in the mouse (14–16). Their existence in humans is still debated. T regs inhibit both the development and effector functions of tumor-specific T cells. CD4\(^+\)CD25\(^{hi}\) T regs accumulate at the tumor site (17–19), where they appear to directly suppress cytotoxic T cell responses against the tumors (20, 21). Indeed, their depletion permits immune-mediated tumor rejection in murine models of cancer (22). CD4\(^+\)CD25\(^{hi}\) T regs are also increased in metastatic lymph nodes (18) and peripheral blood (23) of patients with various types of cancers, including metastatic melanoma. However, thus far, only two studies reported the isolation, from tumor-infiltrating lymphocytes, of T reg clones specific for tumor antigens: a nonmutated antigen LAGE1 (24) and mutated peptide generated from ARTC1 (25). Because mouse studies have indicated the superior suppressive capacity of antigen-specific T regs over non-antigen-specific T regs (26–28), it is important to determine whether patients display a broad repertoire of tumor antigen-specific T regs. Here, we show that metastatic melanoma patients have circulating IL-10-producing CD4\(^+\) T regs specific for a broad range of tumor antigens.

Results

IL-10-Inducing Peptides of NY-ESO-1. To examine the antigen-specific T cell repertoires, 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled peripheral blood mononuclear cells (PBMCs) from metastatic melanoma patients were exposed to overlapping peptides from libraries covering the full length of four antigens associated with melanoma (gp100 and TRP1) and other cancers as well (NY-ESO-1 and survivin). Cytokine levels were measured at day 2, and T cell proliferation was analyzed at day 6 (3).

PBMCs from patient #094-004 were cultured with overlapping peptides encoding NY-ESO-1 (43 peptides). Two NY-ESO-1 peptides, p#21 (NY-ESO-1\(\_181\-185\)) and p#23 (NY-ESO-1\(\_241\-245\)), were found to induce IL-10 secretion (Fig. 1A). These two NY-ESO-1 peptides also induced the proliferation of a fraction of CD4\(^+\) T cells (p#23 in Fig. 1B and p#21, data not shown). The CD4\(^+\) T cell proliferation was inhibited by blocking mAb against HLA-class II molecules (Fig. 1C), demonstrating that the peptide-induced CD4\(^+\) T cell proliferation depended on antigen-presenting cells. The culture with NY-ESO-1 p#23 yielded two distinct populations in CFSE-diluted CD4\(^+\) T cells: one with a low level of FoxP3 and another with a high level of FoxP3 (Fig. 1D). The CFSE-diluted FoxP3low CD4\(^+\) T cells were likely activated non-T regs (29–31), because their level of FoxP3 was comparable to that of CD4\(^+\) T cells responding to tetanus toxoid (TT) (Fig. 1D and E). In contrast, the CFSE-diluted FoxP3\(^{hi}\) CD4\(^+\) T cells expressed even higher levels...
of FoxP3 than CFSE nondiluted FoxP3\(^+\) CD4\(^+\) T cell population (Fig. 1D).

To examine whether the difference in FoxP3 levels in CFSE-diluted CD4\(^+\) T cell population was due to the differential activation of T cells, the expression of FoxP3 was followed until day 10 of culture. CFSE-diluted CD4\(^+\) T cells in response to TT lost FoxP3 expression at day 10 of culture (Fig. 2A and B), which is consistent with previous reports (30, 31). In contrast, a fraction of CFSE-diluted cells in response to NY-ESO-1 p\#23 maintained FoxP3 expression (Fig. 2A and B). Thus, CD4\(^+\) T cells responding to NY-ESO-1 p\#23 contain CD4\(^+\) T cells constitutively express high levels of FoxP3, which are distinct from activated non-T regs that transiently express FoxP3.

We next tested whether the presence of NY-ESO-1 peptides promoting IL-10 secretion in PBMC cultures was restricted to patient #094-004, or represented a frequent event in metastatic melanoma patients. As shown in Table 1, the PBMCs of 8 of 16 patients (before DC vaccination) produced IL-10 in response to one or more NY-ESO-1 peptides. Notably, the PBMCs of patients #025-002 and #025-003 reacted with five and four distinct peptides, respectively. These represent a minimum of three distinct epitopes for CD4\(^+\) T cells in each patient. In contrast, PBMCs from nine healthy individuals did not produce IL-10 (<20 pg/ml) in response to any of the NY-ESO-1 peptides (data not shown).

NY-ESO-1 p\#23-Specific CD4\(^+\) T Cell Lines. To further characterize the NY-ESO-1 p\#23-responding CD4\(^+\) T cells in Pt# 094-004, oligoclonal CD4\(^+\) T cell lines were established by sorting CFSE-diluted CD4\(^+\) T cells into 96-well plates at 3–10 cells per well. Cells were expanded for 3 weeks with irradiated mouse fibroblasts coexpressing CD3\(\delta\) and ICOS-ligand, in the presence of anti-CD3 mAb and IL-2 (Fig. 3A). At the end of the cell culture, higher amounts of IL-10 were detected in the cultures of NY-ESO-1 p\#23-responding CD4\(^+\) T cells than in those of TT-responding CD4\(^+\) T cells (Fig. 3B).

To test their peptide-specificity, CD4\(^+\) T cells were pooled from wells containing >400 pg/ml IL-10 (IL-10\(^{high}\) CD4\(^+\) T cells) and stimulated overnight with autologous DCs loaded with NY-ESO-1 p\#23 or an irrelevant 15-mer peptide. Pooled CD4\(^+\) T cells produced higher amounts of IL-10 when stimulated with DCs loaded with NY-ESO-1 p\#23 than with a control peptide (Fig. 3C), indicating their specificity for NY-ESO-1 p\#23. The pooled CD4\(^+\) T cells produced only minute amounts of IL-2, IL-5, or IFN\(\gamma\) (Fig. 3C) and are distinct from Th1 or Th2 cells capable of secreting IL-10.

NY-ESO-1 p\#23-Specific IL-10\(^{high}\) CD4\(^+\) T Cells Have Suppressive Functions. The pooled IL-10\(^{high}\) CD4\(^+\) T cells proliferated poorly in response to CD3/CD28 stimulation when compared with autologous CD4\(^+\)CD25\(^-\) T cells (Fig. 4A). The pooled IL-10\(^{high}\) CD4\(^+\) T cells actually suppressed the proliferation of CD4\(^+\)CD25\(^-\) T cells (Fig. 4A) in a dose-dependent manner (Fig. 4B). In contrast, pools of CD4\(^+\) T cells producing low levels of IL-10 (IL-10\(^{low}\) CD4\(^+\) T cells) did not suppress cell proliferation (Fig. 4A). The pooled IL-10\(^{high}\) CD4\(^+\) T cells also suppressed IL-2 and IFN\(\gamma\) production of CD3/CD28-activated CD4\(^+\)CD25\(^-\) T cells (Fig. 4C). No suppression was observed when cells were separated from CD4\(^+\)CD25\(^-\) T cells in transwell cultures (Fig. 5A). Strikingly, the IL-10\(^{high}\) CD4\(^+\) T cells suppression of T cell proliferation was only marginally blocked by an anti-IL-10 mAb (Fig. 5B).

A fraction of pooled IL-10\(^{high}\) CD4\(^+\) T cells coexpressed high levels of FoxP3 and intracytoplasmic CTLA-4 (Fig. 5C). In contrast, pooled IL-10\(^{low}\) CD4\(^+\) T cells did not include such cells. Therefore, despite their capacity to produce IL-10, NY-ESO-1-specific T regs require cell-to-cell contact for their suppressive function and, thus, differ from Tr1 cells.
### Patients

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<th>Pt. ID</th>
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<th>Stage at diagnosis</th>
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<td>53</td>
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**NY-ESO-1** or gp100-specific CD4⁺ T regs were found in 9 of 16 patients with metastatic melanoma. Eight of the nine patients showed NY-ESO-1-specific CD4⁺ T regs. Survivin and TRP-1-specific CD4⁺ T regs were searched only in Pt#094-004. HLA restriction elements of each patient for each peptide were predicted by using average relative binding (ARB) matrix binding prediction method, available at the web site of The Immune Epitope Database and Analysis Resource (IEDB), http://tools.immuneepitope.org/tools/matrix/iedb. Addditional antigens need to be tested to determine whether these patients do not display any antigen-specific CD4⁺ T regs.

## Discussion

Here, we demonstrate the presence of circulating tumor-antigen specific T regs in patients with metastatic melanoma. Importantly, these T regs were identified, without the need for T cell cloning, by their capacity to secrete IL-10 upon exposure to a specific peptide. These tumor antigen-specific CD4⁺ T regs contained FoxP3⁺ cells and were able to suppress T cell proliferation in a cell contact-dependent manner. This study identified CD4⁺ T regs specific for four tumor-associated antigens, including gp100 and TRP1 (melanoma tissue differentiation antigens), NY-ESO-1 (cancer/testis antigen), and survivin (inhibitor of apoptosis protein (IAP) family antigen).

NY-ESO-1-specific T regs were present in 8 of 16 examined patients with metastatic melanoma. It is intriguing that far more T reg epitopes were found in NY-ESO-1 than gp100 (Table 1). NY-ESO-1 (one of the cancer/testis antigens) has been discovered by using the SEREX approach (32). NY-ESO-1 is highly immunogenic and elicits spontaneous antibody and T cell responses in a fraction of cancer patients (33). Whether this is associated with the presence of NY-ESO-1-specific antibodies remains to be established. Some patients appear to display T regs reacting with at least three epitopes from the same protein (e.g., Pt#094-013 and 094-017), whereas others display T regs reacting with at least three epitopes from different proteins (e.g., Pt#094-004). In 7 of 16 patients, we could not find IL-10-secreting CD4⁺ T regs against NY-ESO-1 or gp100. Additional antigens need to be tested to determine whether these patients do not display any antigen-specific CD4⁺ T regs.

NY-ESO-1-specific CD4⁺ T reg epitopes identified in this study can be divided into four groups according to their location: namely, epitopes approximately (i) the 60–85 region, (ii) the 80–105 region, (iii) the 120–145 region, and (iv) the 160–180 region (Fig. 7). Interestingly, many known CD4⁺ helper T cell epitope are localized in the three regions including 80–105, 120–145 and 160–180.
pooled from wells containing irrelevant peptide, and secreted cytokines were measured (a representative result of 8).

Fig. 3. Expanded CFSE-diluted CD4+ T cells produce IL-10 in response to NY-ESO-1 p#23 stimulation. (A and B) NY-ESO-1 p#23-responding CD4+ T cells secrete higher amounts of IL-10 than TT-responding CD4+ T cells. CFSE-diluted CD4+ T cells in response to IL-10-inducing peptides or TT were sorted into 96-well plates at 3–10 cells per well, and cultured for 21 d with irradiated ICOS-ligand transfectant and anti-CD3 mAb in the presence of rhIL-2 (1,000 units/ml). IL-10 levels secreted during the cell expansion in each well were measured by ELISA. (A) is a representative result of 12. (B) Peptide-specific IL-10 secretion. CD4+ T cells pooled from wells containing >400 pg/ml IL-10 at 3 weeks of culture were stimulated overnight with autologous DCs incubated with NY-ESO-1 p#23 or an irrelevant peptide, and secreted cytokines were measured (a representative result of 8).

CD4+ T cell population in response to the IL-10-promoting peptides. The expanded Tregs possessed potent suppressive functions and inhibited the proliferation and cytokine secretion of autologous CD4+CD25+ T cells. They preferentially secrete IL-10, but not IL-2, IL-5, or IFN-γ. Despite their capacity to secrete IL-10, the suppressive function of tumor antigen-specific Tregs was largely independent of IL-10, because it required cell-to-cell contacts and was virtually unaffected by blocking with anti-IL-10 receptor mAb. Thus, tumor antigen-specific Tregs are distinct from Tr1 cells and IL-10-secreting Th1 or Th2 cells. Rather, tumor antigen-specific Tregs appear to share the properties with CD4+CD25high Tregs.

Fig. 4. Expanded NY-ESO-1 p#23-specific IL-10high CD4+ T cells have suppressive functions. (A) Inhibition of CD4+CD25+ T cell proliferation. The pooled IL-10high CD4+ T cells were cultured alone or together with autologous CD4+CD25+ T cells (1:1 ratio) in the presence of CD3/CD28 stimulation. [3H]Thymidine incorporation was analyzed on day 3 (a representative of >10). (B) Titration of pooled IL-10high CD4+ T cells for suppressive function. Indicated number of the pooled cells was added to the autologous CD4+CD25+ T cell culture stimulated with CD3/CD28 mAb. (C) The pooled CD4+ T cells abrogate cytokine production from CD4+CD25+ T cells. Culture supernatants were harvested at day 2 of the suppression assay shown in A, and secreted cytokines were measured (a representative of 8).

Fig. 5. NY-ESO-1-specific IL-10high Tregs require cell-to-cell contact for their suppression. (A) Soluble factor is not responsible for the suppression. CD4+CD25+ T cells were separated from the pooled IL-10high CD4+ T cells by transwell in suppression assays (a representative of 6). (B) IL-10 plays only a minor role in the suppression. An IL-10Rα blocking mAb was added in the suppression assays. Each dot represents an independent experiment. (C) The pooled IL-10high CD4+ T cells contain FoxP3highCTLA4high T cells. Pooled IL-10high CD4+ T cells and IL-10low cells were examined for their expression of FoxP3 and CTLA4.
IL-10-secreting oligoclonal CD4+ T cells are generated in CFSE-labeled PBMCs cultured for 6 days with the indicated T reg epitopes. The FoxP3 expression of CD4+ T cells in CFSE-labeled PBMCs cultured for 6 days with the indicated T reg epitopes is shown (Middle) (representatives of 6 on each). Suppression assays with pooled IL-10-secreting oligoclonal CD4+ T cell lines (IL-10high CD4+ T cells) are shown on the right (representatives of >10 on each). There, the pooled IL-10high CD4+ T cells were cultured alone or together with autologous CD4+CD25+ T cells (1:1 ratio) in the presence of CD3/CD28 stimulation. [3H]Thymidine incorporation was analyzed on day 3.

Fig. 6. Identification of other tumor antigen-specific T regs. Survivin54-67 (Survivin p#15) and TRP-1 449–463 (TRP-1 p#113) were found as CD4+ T reg epitope in Pt#094–005. IL-10 production was measured with ELISA (BD Biosciences). CD4+ T cell immunity by elimination of epitopes yielding CD4+ T reg cells could be beneficial to patients and incorporated in the generation of novel vaccines.

Materials and Methods

Patient Samples. Blood samples were obtained from 16 stage IV melanoma patients who signed informed consent forms from institutional review board-reviewed protocols. Clinical parameters are shown in Table 1.

Analysis of Peptide-Specific CD4+ T Cell Repertoires with Overlapping Peptides. NY-ESO-1, TRP-1, gp100, and survivin overlapping peptides (15-mers, with a 4-aa gap) were generated at Mimotopes (Clayton) or were gifts from R. Blanck, and Laure Bourdery [Baylor Institute for Immunology Research (BIIR) Flow Core]; Jennifer Shay and Yuan Xu (Luminex Core); Cindy Coquery (BIIR Flow Core); and the Baylor Health Care Systems Foundation and the Falk Foundation and by National Institutes of Health Grants R01 CA78846, R01 CA85540, P01 CA84512, and U1 AI-57234 (to J.B.). J.B. holds the W. W. Caruth, Jr., Chair for Transplantation Immunology Research. A.K.P. holds the Ramsay Chair for Cancer Immunology.

Fig. 7. CD4+ T reg epitopes of NY-ESO-1. CD4+ T reg epitopes identified in this study are shown in green. Previously identified CD8+ T cell epitopes are shown in red, and previously identified CD4+ helper T cell epitopes are shown in blue. Refs: a, Cancer Immunity peptide database, www.cancerimmunity.org/peptidedatabase/Tcellpeptide.htm, and refs. 35 and 36; b, ref. 37.

Suppression Assay. The pooled IL-10-secreting CD4+ T cells (5 × 104 cells) were cultured for 72 h with autologous CD4+CD25+ T cells at a ratio of 1:1 in the presence of anti-CD3/CD28 mAb-coated beads (0.1 μg/ml) in 200 μl of CM. One microcurie of [3H]thymidine (PerkinElmer) was added during the last 18 h of the culture. In blocking experiments, the CD4+CD25+ T cells were preincubated for half an hour with 20 μg/ml IL-10 blocking antibodies (R & D Systems) and put together with the pooled IL-10-secreting CD4+ T cells before adding the anti-CD3/CD28 mAb-coated beads.

Specificity Assay. Autologous DCs were generated by culturing monocytes with GM-CSF and IL-4 for 6 days. At day 8, DCs were incubated for 24 h with a 20 μM concentration of the corresponding peptide or a control peptide. Pooled IL-10-secreting CD4+ T cells (5 × 104 cells) were cultured with peptide-loaded DCs (5 × 103 cells) in 96-well plates for 15 hours, and the produced cytokines were measured.

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