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Influence of CD4⁺CD25⁺ Regulatory T Cells on Low/High-Avidity CD4⁺ T Cells following Peptide Vaccination

Hiroyoshi Nishikawa,* Feng Qian,† Takemasa Tsuji,* Gerd Ritter,* Lloyd J. Old,* Sacha Gnjatic,2,3* and Kunle Odunsi2,3†

We have recently reported that NY-ESO-1-specific naive CD4⁺ T cell precursors exist in most individuals but are suppressed by CD4⁺CD25⁺ regulatory T cells (Tregs), while memory CD4⁺ T cell effectors against NY-ESO-1 are found only in cancer patients with spontaneous Ab responses to NY-ESO-1. In this study, we have analyzed mechanisms of CD4⁺ T cell induction following peptide vaccination in relation to susceptibility to Tregs. Specific HLA-DP4-restricted CD4⁺ T cell responses were elicited after vaccination with NY-ESO-1157–170 peptide (emulsified in IFA) in patients with NY-ESO-1-expressing epithelial ovarian cancer. These vaccine-induced CD4⁺ T cells were detectable from effector/memory populations without requirement for in vitro CD4⁺CD25⁺ T cell depletion. However, they were only able to recognize NY-ESO-1157–170 peptide but not naturally processed NY-ESO-1 protein and had much lower avidity compared with NY-ESO-1-specific pre-existing naive CD4⁺CD25⁻ T cell precursors or spontaneously induced CD4⁺ T cell effectors of cancer patients with NY-ESO-1 Ab. We propose that vaccination with NY-ESO-1157–170 peptide recruits low-avidity T cells with low sensitivity to Tregs and fails to modulate the suppressive effect of Tregs on high-avidity NY-ESO-1-specific T cell precursors. The Journal of Immunology, 2006, 176: 6340–6346.

 Naturally occurring CD4⁺CD25⁺ regulatory T cells (Tregs) play an important role in maintaining immunological balance in hosts by suppressing a wide variety of immune responses (1–4). Although this T cell population was originally found to suppress the development of autoimmune (1), it has been shown that depletion of Treg populations by Ab enhances antitumor immune responses (5, 6) and that stimulation of CD4⁺CD25⁺ Tregs by immunization with self-Ags enhances chemically induced primary tumor development (7) and increased number of pulmonary metastasis following injection of transplantable tumor cells (8). Ab depletion of CD4⁺CD25⁺ Treg populations also enhances vaccine-induced specific T cell induction and therapeutic effects in a murine cancer vaccine model (9, 10). In human cancers, the presence of high numbers of Tregs at the local tumor site is correlated with poor prognosis (11, 12). In addition, the existence of Tregs has also been reported in patients receiving melanoma Ag vaccine (13). However, the detailed mechanism of action of Tregs in modulating the effects of cancer vaccines remains unclear.

NY-ESO-1 is a germ cell protein that is often expressed by tumor cells, but not normal somatic cells (14); it was discovered by serological identification of Ags by recombinant expression cloning using the serum of an esophageal cancer patient (14, 15). The frequent finding of humoral and cellular immune responses against this Ag in cancer patients with NY-ESO-1-expressing tumors makes it one of the most immunogenic human tumor Ags (16, 17). In monitoring a large series of cancer patients, humoral responses to NY-ESO-1 were found to be correlated with the presence of peripheral CD8⁺ T cells against NY-ESO-1, suggesting the involvement of CD4⁺Th1 cells in coordinating these responses (16–18). It was indeed confirmed that effector CD4⁺Th1 cell responses to NY-ESO-1 were only detectable in cancer patients who had Abs against NY-ESO-1. However, it has recently been reported that NY-ESO-1-specific CD4⁺Th1 cell precursors actually exist at relatively high frequency in healthy individuals and that Tregs play a critical role in keeping NY-ESO-1-specific precursors under control (19, 20). In healthy donors and in patients with NY-ESO-1-expressing tumors but without specific Ab, NY-ESO-1-specific CD4⁺Th1 cells are detected only after in vitro stimulation of CD4⁺CD25⁻ T cells isolated from PBMC by depletion of Tregs. Although the repertoire of these regulated T cells specific for NY-ESO-1 is exclusively naive (CD4⁺CD25⁻CD45RA⁺), including in seronegative cancer patients with tumors expressing NY-ESO-1, the majority of CD4⁺ T cells in seropositive patients are memory (CD4⁺CD25⁻CD45RO⁺) and readily detectable even in the presence of Tregs (20).

So far, a number of different cancer vaccine strategies targeting NY-ESO-1 have resulted in the development of measurable immune responses (21–23). These results prompted us to analyze the origin and nature of the peptide vaccine-induced NY-ESO-1-specific T cell repertoire in relation to Treg control. In the present study, we examined CD4⁺ T cell responses in epithelial ovarian cancer patients following vaccination with HLA-DPB1*0401/0402-restricted NY-ESO-1157–170 peptide (24) in the presence of IFA. We also measured the avidity of CD4⁺ T cells against NY-ESO-1 in relation to surface markers for naive/memory phenotype.
in patients seronegative and seropositive for NY-ESO-1. Furthermore, to elucidate the impact of Treg action during peptide immunization, vaccine-induced NY-ESO-1-specific T cells were analyzed before and after depletion of Tregs and we found that Tregs suppressed high-avidity pre-existing naive NY-ESO-1-specific T cell precursors throughout vaccination with NY-ESO-1\textsubscript{157-170} peptide.

Materials and Methods

Donor samples

Epithelial ovarian cancer patients undergoing debulking surgery at the Roswell Park Cancer Institute (Buffalo, NY) consented to participate in a pilot clinical study, LUD2002-011, sponsored by the Ludwig Institute for Cancer Research. Ovarian tumor specimens were analyzed for the expression of NY-ESO-1 by RT-PCR and immunohistochemistry. Four HLA-DP\textsuperscript{b}0401/0402 patients (two baseline seronegative and two baseline seropositive) with NY-ESO-1-expressing tumors were vaccinated for five cycles every 3 wk with clinical-grade NY-ESO-1\textsubscript{157-170} peptide (see below) emulsified in Montanide (IFA). PBMC and serum samples were collected before, during, and after vaccination under a protocol approved by the Institutional Review Board of Roswell Park Cancer Institute.

Reagents

Synthetic peptides of NY-ESO-1\textsubscript{87-98} (LLEFYLAMPFAT), NY-ESO-1\textsubscript{121-132} (VLLKEFTVSGNI), NY-ESO-1\textsubscript{143-154} (RQLQLSISSCLQ), and HIV P17\textsubscript{37-51} (ASRELRAFAVNPGLL) (18) were obtained from Bio Synthesis. Recombinant NY-ESO-1 or SSX-2 protein were prepared using similar procedures described previously (25).

Generation of NY-ESO-1-specific CD4\textsuperscript{+} T cells

NY-ESO-1-specific CD4\textsuperscript{+} T cells were elicited as described previously (20). Briefly, CD4\textsuperscript{+} T cells and CD4\textsuperscript{+}CD25\textsuperscript{-} T cells were isolated from PBMC using CD4\textsuperscript{+}CD25\textsuperscript{-} Regulatory T Cell Isolation Kit (Miltenyi Biotec). In some experiments, CD4\textsuperscript{+}CD25\textsuperscript{-} T cells were further separated into CD45RO-depleted T cells (CD4\textsuperscript{+}CD25\textsuperscript{-} CD45RO\textsuperscript{-} T cells) or CD45RA-depleted T cells (CD4\textsuperscript{+}CD25\textsuperscript{-} CD45RA\textsuperscript{-} T cells) using CD45RO Microbeads or CD45RA Microbeads (Miltenyi Biotec), respectively (20). The purity of selected populations was confirmed to be >96%. Non-CD4\textsuperscript{+} cells retained in the MACS separation column were flushed out and were used to prepare APC. These non-CD4\textsuperscript{+} cells were allowed to adhere to tissue culture plates (Corning Glass) for 2 h, and nonadherent cells were removed by washing. The adherent cells pulsed with 10 \( \mu \)M of one or two peptides overnight were used as APC. After irradiation, 1 \( \times 10^{6} \) APC were added to round-bottom 96-well plates (Corning) containing 5 \( \times 10^{4} \) unfractionated CD4\textsuperscript{+}, CD4\textsuperscript{+}CD25\textsuperscript{-}, CD4\textsuperscript{+}CD25\textsuperscript{-}CD45RO\textsuperscript{-}, or CD4\textsuperscript{+}CD25\textsuperscript{-}CD45RA\textsuperscript{-} T cells and were fed with 10 U/ml IL-2 (Roche Molecular Biochemicals). Subsequently, one-half of medium was replaced by fresh medium containing IL-2 (20 U/ml) twice per week.

ELISPOT assay

The number of IFN-\( \gamma \)-secreting Ag-specific CD4\textsuperscript{+} T cells was assessed by ELISPOT assays as described previously (18, 20, 26). Briefly, flat-bottom, 96-well nitrocellulose-coated microtiter plates (Millipore) were coated with anti-IFN-\( \gamma \) Ab (1-D1K; Mabtech). The presensitized T cells and phytohemagglutinin (HA15; Murex Diagnostics) activated CD4\textsuperscript{+} T cells or dendritic cells (DC) pulsed with 10 \( \mu \)M of peptides or 25 \( \mu \)g/ml protein overnight were added to each well and incubated for 24 h. Spots were developed using biotinylated anti-IFN-\( \gamma \) Ab (7-B6-1-biotin; Mabtech), alkaline phosphatase-conjugated streptavidin (Roche Diagnostics), and 5-bromo-4-chloro-3-indolyl phosphate (NBT; Sigma-Aldrich) and counted with CTL Immunospot analyzer and software (Cellular Technologies).

Proliferation assay

A total of 5 \( \times 10^{4} \) presensitized T cells was cultured with 5 \( \times 10^{4} \) irradiated CD3-depleted PBMC pulsed with 10 \( \mu \)M peptides overnight in round-bottom 96-well plates. Proliferation was evaluated by pulsing cells with 1 \( \mu \)Ci/well \( ^{3}H \)thymidine for the last 18 h of 72 h culture. \( ^{3}H \)Thymidine incorporation was measured by a scintillation counter.

Preparation of monocyte-derived DC

Monocyte-derived DC were generated from PBMC of vaccinated patients as previously described with some modifications (27). Briefly, CD14\textsuperscript{+} monocytes were enriched by positive selection using CD14 Microbeads (Miltenyi Biotec). Monocytes were cultured in the presence of 1000 U/ml GM-CSF (ImmuneX) and 20 ng/ml IL-4 (R&D Systems) in X-VIVO-15 serum-free medium (Cambrex). Medium was replaced by fresh medium containing cytokines 3 days later. On day 6, DC were harvested and pulsed overnight with 10 \( \mu \)M peptide or 25 \( \mu \)g/ml protein.

FIGURE 1. NY-ESO-1-specific CD4\textsuperscript{+} T cell responses are suppressed in seronegative patients but present in seropositive patients before peptide vaccination. The presence or absence of NY-ESO-1\textsubscript{157-170} specific CD4\textsuperscript{+} T cells and CD4\textsuperscript{+}CD25\textsuperscript{-} T cells was analyzed by ELISPOT (left) and proliferation (right) assays following a 15–20 day culture with APC pulsed with indicated NY-ESO-1 peptides (see Materials and Methods). Responses were analyzed in patients SN1 (A) and SN2 (B) seronegative for NY-ESO-1 and in patients SP1 (C) and SP2 (D) seropositive for NY-ESO-1. These experiments were performed independently at least twice with similar results. Data are expressed as mean \( \pm \) SD.
**Results**

**NY-ESO-1-specific CD4⁺ T cell precursors are present before vaccination**

Whole CD4⁺ T cells and CD4⁺CD25⁻ T cells were isolated from PBMC of epithelial ovarian cancer patients before peptide vaccination. Cells were derived from patients seronegative (SN1, SN2) or seropositive (SP1, SP2) based on the status of NY-ESO-1-specific Ab and were cultured with autologous APC pulsed with a series of previously reported NY-ESO-1 peptides in pools of two (18, 20). Fifteen to 20 days later, peptide-specific CD4⁺ Th cell induction was analyzed by ELISPOT and proliferation assays (Fig. 1). In accordance with our previous report (20), IFN-γ-secreting specific CD4⁺ T cells with proliferative capacity, CD4⁺ Th1 cells, were only elicited after CD4⁺CD25⁻ T cell depletion in seropositive patients, and can thus be described as pre-existing precursors (Fig. 1, A and B). In contrast, specific CD4⁺ Th1 cells were found without the need for CD4⁺CD25⁻ T cell depletion in seropositive patients (18, 20), and can thus be described as spontaneously induced effectors (Fig. 1, C and D).

**NY-ESO-1 peptide-specific CD4⁺ T cell responses are induced in seronegative patients after NY-ESO-1157–170 peptide vaccination**

We next asked whether vaccination with NY-ESO-1157–170 peptide could induce NY-ESO-1157–170-specific CD4⁺ Th1 cells in patients without Ab to NY-ESO-1. Seronegative patient SN1 had pre-existing CD4⁺ T cell precursors specific for NY-ESO-1157–170 throughout the study, but developed NY-ESO-1157–170-specific CD4⁺ Th1 effectors detectable without need for CD4⁺CD25⁻ T cell depletion on day 85 after vaccination with NY-ESO-1157–170 peptide (Fig. 2A). Similarly, seronegative patient SN2 also had specific pre-existing CD4⁺ T cell precursors against NY-ESO-1157–170 but developed NY-ESO-1121–132-specific CD4⁺ Th1 cells detectable without CD4⁺CD25⁻ T cell depletion on days 43 and 85 after vaccination with NY-ESO-1157–170 peptide (Fig. 2B). These data indicated that NY-ESO-1157–170 peptide vaccination was able to induce NY-ESO-1157–170 peptide-specific CD4⁺ Th1 cells in epithelial ovarian cancer patients. Though vaccine-induced CD4⁺ T cells may express the CD25⁺ molecule as a result of activation, we could not detect specific T cell response from purified CD4⁺CD25⁻ T cell populations (data not shown).

**NY-ESO-1 peptide-specific CD4⁺ T cell responses are also induced by NY-ESO-1157–170 peptide vaccination in seropositive patients**

We examined the time course of NY-ESO-1 peptide-specific CD4⁺ Th1 cell responses during vaccination in patients with Ab to NY-ESO-1. Before vaccination, seropositive patient SP1 had spontaneously induced CD4⁺ T cell effectors against NY-ESO-1187–98 as well as pre-existing CD4⁺ T cell precursors against NY-ESO-1157–170 (Fig. 1C). As shown in Fig. 2C, vaccination with NY-ESO-1157–170 peptide did not affect the spontaneous immune response against NY-ESO-1187–98. In contrast, detection of the NY-ESO-1157–170-specific CD4⁺ T cell response from PBMCs collected before vaccination required the depletion of CD4⁺CD25⁻ T cells, whereas NY-ESO-1157–170-specific CD4⁺ Th1 cell effectors were readily detectable without the need for CD4⁺CD25⁻ T cell depletion on day 85 after vaccination with NY-ESO-1157–170 peptide. Another seropositive patient, SP2, had a spontaneous immune response against NY-ESO-1157–170 along with pre-existing CD4⁺ T cell precursors against NY-ESO-1121–132 (Fig. 1D). Surprisingly, the spontaneous immune response against NY-ESO-1157–170 in this patient did not appear to be significantly boosted by vaccination with NY-ESO-1157–170 peptide, while pre-existing NY-ESO-1157–170-specific CD4⁺ T cell precursors were expectedly unchanged by vaccination with NY-ESO-1157–170 peptide (Fig. 2D).

**NY-ESO-1157–170 peptide vaccine-induced CD4⁺ Th1 cells are not able to recognize naturally processed NY-ESO-1 protein**

It has been reported that peptide vaccine-induced CD8⁺ T cells have lower avidity than spontaneously induced CD8⁺ T cells (28). These data prompted us to examine whether peptide vaccine-induced NY-ESO-1121–132-specific CD4⁺ Th1 cells could recognize naturally processed NY-ESO-1 protein. Whole CD4⁺ T cells and CD4⁺CD25⁻ T cells derived from ovarian cancer patients after vaccination with NY-ESO-1157–170 were presensitized with NY-ESO-1 peptides and analyzed for their capacity to recognize naturally processed Ag by ELISPOT assays using protein-pulsed DC as APC. NY-ESO-1187–98-specific CD4⁺ Th1 cells derived from patient SP1 and NY-ESO-1157–170-specific CD4⁺ Th1 cells derived from patient SP2, both of which were spontaneously induced and detected before vaccination from either whole CD4⁺ T cell or CD4⁺CD25⁻ T cell populations, were able to recognize both NY-ESO-1 peptide-pulsed and protein-pulsed DC in accordance with our previous reports (18, 29) (Fig. 3, C and D). However, peptide vaccine-induced NY-ESO-1157–170-specific CD4⁺ Th1 cells elicited from whole CD4⁺ T cell populations derived from patients SP1, SN1, or SN2 were...
not able to recognize NY-ESO-1 protein-pulsed DC (Fig. 3, A–C). Surprisingly, pre-existing NY-ESO-1 157–170-specific CD4+ T cell precursors detected from CD4+CD25+ T cell populations (in which CD4+CD25+ T cells were depleted) of patients SP1, SN1, or SN2 were able to recognize NY-ESO-1 protein-pulsed DC (Fig. 3). The responses against control peptide and protein were marginal. There is thus a repertoire of detectable NY-ESO-1 protein-reactive T cells in all patients that is suppressed by Tregs in the absence of spontaneously induced immunity to NY-ESO-1. Taken together, these data suggest either that Tregs are responsible for abrogating the recognition of naturally processed NY-ESO-1 protein by vaccine-induced CD4+ effector cells or that peptide vaccine-induced CD4+ Th1 cells are recruited from a low-avidity repertoire not susceptible to CD4+CD25+ T cell regulation.

Tregs suppress high-avidity pre-existing NY-ESO-1-specific CD4+ Th1 cell precursors in vitro

To examine the difference in avidity of NY-ESO-1-specific CD4+ Th1 cell responses derived from whole CD4+ T cell populations or from CD4+CD25+ T cell populations within a same patient, graded doses of peptides were pulsed on APC and specific IFN-γ secretion was analyzed by ELISPOT assays. Spontaneously induced NY-ESO-1 187–98-specific CD4+ Th1 cells from patient SP1 and NY-ESO-1 157–170-specific CD4+ Th1 cells from patient SP2 were both high-avidity, and could recognize as little as 0.1 μM peptide in the presence or absence of CD4+CD25+ T cells (Fig. 4, C and D). In contrast, peptide vaccine-induced NY-ESO-1 157–170-specific CD4+ Th1 cell precursors elicited from whole CD4+ T cell populations derived from patients SP1, SN1, or SN2 had much lower avidity compared with pre-existing NY-ESO-1 157–170-specific CD4+ Th1 cell precursors elicited from the CD4+CD25+ T cell population (Fig. 4, A–C). These results indicate that Tregs suppress high-avidity pre-existing NY-ESO-1-specific CD4+ T cell precursors, despite vaccination, probably explaining the differential ability to recognize protein in unseparated CD4+ T cell populations.

Peptide vaccination alone generates low-avidity memory NY-ESO-1-specific CD4+ T cells

Next, we asked whether peptide vaccination was able to tap into the repertoire of pre-existing NY-ESO-1-specific CD4+ T cell precursors. To address this question, CD4+CD25+ T cells from NY-ESO-1 seronegative donors were further separated into naive or effector/memory populations according to typical surface marker molecules CD45RA or CD45RO, respectively (20). We found that...
high-avidity NY-ESO-1,157–170-specific CD4+ T cells were detected from pre-existing CD4+CD25−CD45RA+ naive precursors, sensitive to CD4+CD25+ T cell regulation (20), and not affected by vaccination with NY-ESO-1,157–170 peptide (Fig. 5). In contrast, NY-ESO-1,157–170-specific CD4+ T cell effectors elicited after vaccination with NY-ESO-1,157–170 peptide were detected from CD4+CD25−CD45RO+ memory populations (Fig. 5). These T cells had low-avidity against NY-ESO-1,157–170 similar to the avidity of NY-ESO-1,157–170-specific CD4+ T cell precursors but rather expanded a repertoire of low-avidity effector/memory CD4+ T cells less sensitive to Tregs. These results are summarized in Table I.

Discussion
We have shown that vaccination with NY-ESO-1,157–170 peptide elicited HLA-DPB1*0401/0402-restricted CD4+ T cell responses to NY-ESO-1,157–170 peptide in patients with NY-ESO-1-expressing epithelial ovarian cancers. These responses were detectable without the need for CD4+CD25+ T cell depletion and were found only after vaccination in patients without spontaneously induced immunity to NY-ESO-1. Although these vaccine-induced CD4+ T cells were able to recognize NY-ESO-1,157–170 peptide and displayed an effector/memory phenotype, they failed to recognize naturally processed NY-ESO-1 protein due to low avidity for their target epitope. In contrast, we also showed the presence of pre-existing but suppressed NY-ESO-1,157–170-specific naive CD4+ T cell precursors in the peripheral blood, which were detected after depletion of CD4+CD25+ T cells in vitro. These cells efficiently recognized the NY-ESO-1 protein and peptide with high avidity, but were not affected or recruited by peptide vaccination. It is likely that vaccination with NY-ESO-1,157–170 peptide predominantly expanded a low-avidity CD4+ T cell repertoire, either from naive precursors with weak reactivity to peptide or possibly from cross-reactive memory T cells specific for other Ags, with lesser sensitivity to CD4+CD25+ Tregs (20). This is in stark contrast with spontaneously induced CD4+ T cell effectors of patients with naturally occurring immunity to NY-ESO-1, which are likely elicited by Ag from the tumor with a more adequate stimulation context. These effector/memory populations were previously shown to be more resistant to CD4+CD25+ T cell regulation than naive CD4+ T cells (20). These data suggest that vaccination with NY-ESO-1,157–170 peptide fails to activate pre-existing NY-ESO-1,157–170-specific naive CD4+ T cell precursors that have the capacity to recognize naturally processed Ag. This failure reflects, in turn, the inability of peptide vaccination with NY-ESO-1,157–170 to overcome Treg action, along with an inability to induce NY-ESO-1 Ab and CD8+ T cell responses even though this peptide is recognized by both CD8+ and CD4+ T cells (22).

Table I. Categories of NY-ESO-1-specific CD4+ T cell responses

<table>
<thead>
<tr>
<th>Memory Population (CD45RO+)</th>
<th>Naive Population (CD45RA+)</th>
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<tbody>
<tr>
<td>High avidity (recognition of naturally processed NY-ESO-1 protein or 0.1 μM peptide)</td>
<td>Spontaneously induced CD4+ T cell effectors (in seropositive patients)</td>
</tr>
<tr>
<td>Low sensitivity to Tregs</td>
<td>Pre-existing CD4+ T cell precursors (in donors or patients)</td>
</tr>
<tr>
<td>Low avidity (recognition of &gt;1 μM peptide but not naturally processed NY-ESO-1 protein)</td>
<td>Peptide vaccine-induced CD4+ T cell effectors (in vaccinated patients)</td>
</tr>
<tr>
<td>Low sensitivity to Tregs</td>
<td>Not detected</td>
</tr>
</tbody>
</table>
After depletion of Tregs in cell cultures from vaccinated patients, high-avidity NY-ESO-1-157-170-specific T cells from naive CD4+ T cell precursors are still detected even though low-avidity effector/memory CD4+ T cells with the same specificity are also present. It is possible that high-avidity naive CD4+ T cells dominantly expand in the absence of Tregs while effector/memory CD4+ T cells grow faster than naive CD4+ T cells in culture conditions containing Tregs. It is also possible that high-avidity CD4+ T cells compete for epitope recognition with low-avidity CD4+ T cells and inhibit their growth, allowing expansion/activation of self-Ag-specific CD4+ T cells after depletion of Tregs.

Whether Tregs suppress T cells directly or through APC remains to be determined (30, 31). In our culture condition, Treg may directly suppress Th1 cell induction or may inhibit APC, such as DC maturation and differentiation to DC to elicit Th1 cells. These possibilities are not necessarily mutually exclusive. Additional experiments are required to elucidate the mode of action of Tregs.

Despite thymic-negative selection, normal individuals still harbor self-reactive T cell clones and Tregs have an important role to keep self-reactive T cells that are sufficiently pathogenic in check (4). Cancer-testis Ags like NY-ESO-1 are considered to be only expressed in cancer cells and testis, but not in normal somatic cells (14). It has, however, been reported that some cancer-testis Ags are expressed in medullary thymic epithelial cells (32). It is plausible that cancer-testis Ags like NY-ESO-1 could be considered self-Ags during ontological education, resulting in a repertoire of NY-ESO-1-specific T cells that are either subject to central tolerance or kept in check in the periphery. The latter possibility is consistent with the finding that activation of NY-ESO-1-specific CD4+ T cell precursors in PBMC of healthy individuals are controlled by Tregs (19, 20).

Somewhat, a proportion of cancer patients seem to be able to naturally bypass this control and generate high-avidity immune responses to NY-ESO-1. Activation of high-avidity T cells may require an adequate tumor environment in cancer patients, where presence of signals with the capacity to suppress Treg activity, like TLR ligands, may be critical (33–35). Taken together, vaccine strategies targeting depletion of Tregs or abrogation of suppression by Tregs may be required for activation of NY-ESO-1-157-170-specific pre-existing naive high-avidity CD4+ T cell precursors.

Recently, it has been shown that certain types of immunization methods or DC stimulations could elicit regulatory T cells in vivo (36–40). It has also been reported that a NY-ESO-1-related molecule, LAGE-1, is a target of regulatory T cells (41). Our vaccination method using NY-ESO-1-157-170 peptide emulsified in IFA might induce the expansion of NY-ESO-1-specific Tregs and these Tregs might suppress the induction of Ag-specific CD4+ T cells. However, this is less likely because a peptide-induced Treg population should display suppressive activity in an Ag nonspecific manner (42), and this has not been observed: spontaneously induced CD4+ T cell effectors against NY-ESO-1-157-170 and Ab responses in two seropositive patients were not suppressed after peptide vaccination (Fig. 2C and data not shown).

To achieve immunologic control of cancer, cancer vaccine trials are currently receiving much attention (43–45). A number of promising tumor Ags have been identified and are being incorporated into cancer vaccines (44, 45). However, only a minority of treated patients experienced favorable clinical responses to date (46, 47). Though the sample size of patients analyzed in this study was relatively small and warrants cautious interpretation, we showed that control of Tregs represents a critical element to induce high-avidity Ag-specific CD4+ T cells and avoid low-avidity peptide-specific CD4+ T cells. Our data provide an important hint for effective cancer vaccines during Ag priming, through modulation of Treg function.

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Disclosures

The authors have no financial conflict of interest.

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