A proportion of cancer patients naturally develop CD4+ T-helper type 1 (Th1) cell responses to NY-ESO-1 that correlate with anti–NY-ESO-1 serum antibodies. To address the role of T-cell regulation in the control of spontaneous tumor immunity, we analyzed NY-ESO-1–specific Th1 cell induction before or after depletion of CD4+CD25+ T cells in vitro. While Th1 cells were generated in the presence of CD25+ T cells in cancer patients seropositive for NY-ESO-1, seronegative cancer patients and healthy donors required CD25+ T-cell depletion for in vitro induction of NY-ESO-1–specific Th1 cells. In vitro, newly generated NY-ESO-1–specific Th1 cells were derived from naive precursors, whereas preexisting memory populations were detectable exclusively in patients with NY-ESO-1 antibody.

Memory populations were less sensitive than naive populations to CD4+CD25+ regulatory T cells. We propose that CD4+CD25+ regulatory T cells are involved in the generation and regulation of NY-ESO-1–specific antitumor immunity.

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Introduction

NY-ESO-1 is a germ-cell protein that is often expressed by cancer cells, but not by normal somatic cells.1 In monitoring a large series of cancer patients, frequent humoral responses to NY-ESO-1 were found and they correlated with NY-ESO-1 expression in tumor cells and with the presence of peripheral CD8+ T cells against NY-ESO-1,

suggesting the involvement of CD4+ helper T cells in coordinating this response. In fact, we have confirmed that NY-ESO-1–specific CD4+ T helper cells are only found in NY-ESO-1 seropositive patients, but not in seronegative patients or healthy individuals.4 Naturally occurring CD4+CD25+ regulatory T cells play an important role in maintaining immunologic balance in hosts by suppressing a wide variety of immune responses.5-8 Although this T-cell population was originally found to suppress the development of autoimmunity,5 it has been shown that depletion of this regulatory T-cell population by antibody enhances the antitumor immune responses9,10 and that stimulation of CD4+CD25+ regulatory T cells by immunization with self-antigens exacerbates tumor growth in mouse studies.11 In human cancer settings, CD4+CD25+ regulatory T cells exist at the local tumor site and contribute to growth of tumors in vivo and are associated with an unfavorable prognosis.12-15

In contrast to our previous data, it has recently been reported that autoreactive T-cell precursors to NY-ESO-1 are present in most healthy individuals, and that autoreactive CD4+CD25+ regulatory T cells play an important role for controlling their activation.16 These observations prompted us to extend this analysis to cancer patients and assess the impact of CD4+CD25+ regulatory T cells on the generation of NY-ESO-1–specific CD4+ T-cell responses in relation to NY-ESO-1 expression in the tumor and presence of specific serum antibody.

Study design

Donor samples

All healthy donors were subjects with no history of autoimmune disease. All cancer patients had NY-ESO-1 expressing melanoma except patients NW2493 with small cell lung cancer and NW1060 with sarcoma. All samples were collected after informed consent as part of a study approved by the Ethics Committee of Landesärztekammer Hessen, Frankfurt.

Antibodies and reagents

Tri-Color–conjugated anti-CD4 and anti-CD45RA and fluorescein isothiocyanate–conjugated anti-CD45RO antibodies were purchased from CALTAG (Burlingame, CA). R-phycoerythrin–conjugated anti-CD25 antibody was purchased from Miltenyi Biotec (Auburn, CA). Synthetic peptides of NY-ESO-1143-154 (RQLQLSISSCLQ), NY-ESO-1157-170 (SLLM-WITQCFPV), and HIV P1737-51 (ASRELERFA VNPGLL)4 were obtained from Bio Synthesis (Lewisville, TX). All peptides were shown to be purchased from Bio Synthesis (Lewisville, TX). All peptides were shown to be restricted by HLA-DRB1*04 or HLA-DRB1*07, except NY-ESO-1157-170 restricted by HLA-DRB1*04.

Generation of NY-ESO-1–specific CD4+ T cells

NY-ESO-1–specific CD4+ T cells were elicited as described previously with some modifications.4 Briefly, CD4+ T cells and CD4+CD25+ T cells were isolated from peripheral blood mononuclear cells (PBMCs) using...
CD4+CD25+ Regulatory T-Cell Isolation Kit (Miltenyi Biotec). In some experiments, CD4+CD25+ T cells (> 96% purity) were further separated into CD45RO-depleted T cells (CD4+CD25+CD45RA- T cells) (> 96% purity) or CD45RA-depleted T cells (CD4+CD25-CD45RO+ T cells) (> 93% purity) using CD45RO or CD45RA Microbeads (Miltenyi Biotec), respectively. Antigen-presenting cells (APCs) were prepared from non-CD4+ cells by allowing them to adhere to tissue culture plates (Corning Inc., Corning, NY) for 2 hours, removing nonadherent cells, and pulsing them with 10 μM of 1 or 2 peptides overnight. After irradiation, 1 x 10^6 APCs were added to round-bottom 96-well plates (Corning) containing 5 x 10^4 unfractionated CD4+, CD4+CD25-, CD4+CD25CD45RO-, or CD4+CD25CD45RA- T cells and were fed with 10 U/mL interleukin (IL)-2 (Roche Molecular Biochemicals, Indianapolis, IN) twice per week.

### Proliferation assay

Presensitized T cells (5 x 10^5) were cultured with 5 x 10^5 irradiated CD3-depleted PBMCs pulsed with 10 μM of peptides overnight in wells of round-bottom 96-well plates. Proliferation was evaluated by pulsing with 0.037 MBq/well (1 Ci/well) [3H]-thymidine for the last 18 hours of a culture, [3H]-thymidine incorporation was measured as a scintillation counter.

### Results and discussion

### NY-ESO-1–specific CD4+ T-cell precursors are present in healthy donors

CD4+ T cells and CD4+CD25- T cells were isolated from PBMCs and were cultured with APCs pulsed with a series of HLA class II-restricted NY-ESO-1 peptides reported previously.15 Fifteen to 20 days later, NY-ESO-1–specific CD4+ helper T-cell induction was analyzed by ELISPOT (Figure 1) and proliferation (Figure S1, available on the Blood website; see the Supplemental Figure link at the top of the online article) assays. IFN-γ–secreting NY-ESO-1–specific CD4+ T cells with proliferative capacity, CD4+ T helper type 1 (Th1) cells, were elicited in 5 of 8 healthy donors, but only in cultures with CD4+CD25+ T-cell depletion, confirming a previous report.16

NY-ESO-1–specific CD4+ T-cell precursors are present in cancer patients with and without NY-ESO-1 antibody

Next, we extended this examination to patients with NY-ESO-1–expressing tumors. Th1 cells were induced in 3 of 3 patients with NY-ESO-1 antibody, without need for CD4+CD25+ T-cell depletion in accordance with our previous results.4 In patients without NY-ESO-1 antibody, although NY-ESO-1–specific Th1 cells were not induced from total CD4+ T-cell populations, they could be induced in 3 out of 4 NY-ESO-1 seronegative patients after depletion of CD4+CD25+ T cells (Figures 1 and S1, summarized in Table 1). Taken together, NY-ESO-1–specific CD4+ T-cell precursors are present in a wider range of patients than formerly thought.4

NY-ESO-1–specific CD4+ T cells are derived from distinct CD4+ T-cell populations between NY-ESO-1 seropositive and seronegative patients

We next asked whether NY-ESO-1–specific CD4+ T-cell precursors were derived from similar or different populations in patients with or without NY-ESO-1 antibody. To address this question, CD4+CD25- T cells were further separated into naive or effector/memory populations according to typical surface-marker molecules CD45RA and CD45RO, respectively (Figure 2A). In patients with NY-ESO-1 antibody, NY-ESO-1–specific Th1 cells were induced from both CD45RA+ and CD45RO+ populations. In contrast, NY-ESO-1–specific Th1 cells were apparently derived only from CD45RA+ population in patients without NY-ESO-1 antibody and healthy donors (Figure 2B). Thus, responses elicited from depletion of CD4+CD25+ T cells are derived from a naive repertoire. Patients with preexisting immunity to NY-ESO-1 still retain the capacity to elicit additional responses from naive precursors upon CD4+CD25+ depletion (as in patient NW2457). Expression of NY-ESO-1 in the tumor is not sufficient to naturally prime NY-ESO-1–specific T-cell responses in patients without NY-ESO-1 antibody.
NY-ESO-1 antibody, since they have no specific precursors with memory phenotype.

CD4+CD25−CD45RO+ T-cell populations are more resistant to CD4+CD25− regulatory T cells for NY-ESO-1–specific Th1 cell induction than CD4+CD25−CD45RA+ T-cell population

To examine the differences between patients with and without NY-ESO-1 antibody, graded amounts of CD4+CD25+ T cells were added to cultures for in vitro stimulation. NY-ESO-1–specific Th1 cells were induced from CD4+CD25−CD45RA+ T-cell population even in the presence of a high percentage of CD4+CD25+ regulatory T cells. However, induction of NY-ESO-1–specific Th1 cells from CD4+CD25−CD45RA+ T-cell population was essentially abrogated in the presence of CD4+CD25+ regulatory T cells (Figure 2C). It appears that NY-ESO-1–specific CD4+ T-cell precursors present in CD4+CD25−CD45RO+ T-cell population of patients with NY-ESO-1 antibody are more resistant to CD4+CD25+ regulatory T cells.

![Diagram](image-url)
We have previously reported that CD4+ T-cell responses are correlated with NY-ESO-1 expression in tumor cells and NY-ESO-1 serum antibody. In this study, we have shown that NY-ESO-1–specific CD4+ T-cell precursors are derived from different populations in patients with or without NY-ESO-1 antibody and that differences in naturally occurringantitumor immunity are defined by distinctive CD4+CD25+ regulatory T-cell sensitivity of each population. It is still unknown if effector/memory T cells become resistant at a single-cell level or whether a relatively high number of precursors within memory population overwhelm suppression. A critical area for future exploration is to explain why some patients can induce memory NY-ESO-1–specific CD4+ T-cell responses even in the presence of naturally occurring CD4+CD25+ regulatory T cells. It is likely that these patients had an effective priming environment (e.g., in which toll-like receptor signaling inhibits the generation/activation of CD4+CD25+ regulatory T cells) during the course of tumor development.

Although it has been shown that presence of CD4+CD25+ regulatory T cells at the local tumor site contributes to tumor growth and is associated with bad prognosis, there is no evidence for a correlation between human antigen-specific antitumor immunity and CD4+CD25+ regulatory T cells. Here, we have clearly shown that CD4+CD25+ regulatory T cells influence the induction of NY-ESO-1–specific antitumor immunity. It has been reported that CD4+CD25+ regulatory T cells require in vitro stimulation to gain their suppressive activity. It is possible that CD4+CD25+ regulatory T cells modulating NY-ESO-1 immunity are activated in an antigen-specific manner, comparable to the specific regulatory T cells generated against NY-ESO-1 gene family member LAGE-1 protein. Alternatively, some CD4+CD25+ regulatory T-cell populations that do not require further in vitro stimulation may exist and suppress induction of immunity.

A unique finding in our study is that CD4+ T-cell responses to new NY-ESO-1 epitopes are induced from naive populations following CD4+CD25+ T-cell depletion, even in patients with preexisting immunity to NY-ESO-1. Our data provide an important hint for effective cancer vaccines during antigen priming through down-modulation of CD4+CD25+ regulatory T-cell function.

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CD4+ CD25+ regulatory T cells control the induction of antigen-specific CD4+ helper T cell responses in cancer patients

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