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Human Tumor Antigen-Specific Helper and Regulatory T Cells Share Common Epitope Specificity but Exhibit Distinct T Cell Repertoire

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Abstract

$CD4^+$ regulatory T cells (Tregs) accumulate at tumor sites and play a critical role in the suppression of immune responses against tumor cells. In this study, we show that two immunodominant epitopes derived from the tumor Ags (TAs) NY-ESO-1 and TRAG-3 stimulate both $CD4^+$ Th cells and Tregs. TA-specific Tregs inhibit the proliferation of allogenic T cells, act in a cell-to-cell contact dependent fashion and require activation to suppress IL-2 secretion by T cells. TRAG-3 and NY-ESO-1–specific Tregs exhibit either a Th1-, a Th2-, or a Th0-type cytokine profile and dot not produce IL-10 or TGF-β. The Foxp3 levels vary from one Treg clone to another and are significantly lower than those of $CD4^+CD25^{high}$ Tregs. In contrast to NY-ESO-1–specific Th cells, the NY-ESO-1–specific and TRAG-3–specific Treg clonotypes share a common TCR CDR3 vβ usage with $Foxp3^+CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells and were not detectable in PBLs of other melanoma patients and of healthy donors, suggesting that their recruitment occurs through the peripheral conversion of $CD4^+CD25^-$ T cells upon chronic Ag exposure. Collectively, our findings demonstrate that the same epitopes spontaneously stimulate both Th cells and Tregs in patients with advanced melanoma. They also suggest that TA-specific Treg expansion may be better impaired by therapies aimed at depleting $CD4^+CD25^{high}$ Tregs and preventing the peripheral conversion of $CD4^+CD25^-$ T cells.

There is ample evidence that melanoma cells express tumor Ags (TAs) that are recognized by T cells isolated from peripheral blood or tumor-infiltrating lymphocytes (TILs) of patients with melanoma (1). However, the presence of TA-specific T cells, even in large numbers, is not consistently correlated with a positive clinical outcome (2). These observations have prompted investigations into the multiple escape mechanisms used by

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tumor cells to evade immune recognition and destruction (3). One major mechanism of peripheral tolerance at the tumor sites appears to be the immunosuppressive functions of CD4$^+$ regulatory T cells (Tregs) (4–6). Tregs are commonly divided into two subsets: the thymic-derived naturally occurring Tregs and the Ag-induced Tregs that acquire regulatory functions under certain conditions of Ag stimulation (7). Human Tregs accumulate at the tumor sites, tumor-draining lymph nodes, and peripheral blood of patients with solid tumors, including melanoma (8–10).

The generation and maintenance of Tregs requires the presence of target Ags (5, 11). However, the identity of these Ags remains largely unknown. One major finding was the identification of TA-derived epitopes encoded by two cancer-germline Ags (CGAs), LAGE-1 and ARTC1, that were recognized by human TA-specific Tregs isolated from TILs of melanoma patients (12, 13). Most recently, the Wilm’s TA overexpressed by leukemias was shown to stimulate TA-specific Treg clones generated in vitro from PBLs of normal donors (14). In addition, immunization with one MAGE-A3 peptide resulted in induction of TA-specific regulatory T cells isolated from PBLs of vaccinated melanoma patients (15).

Whether TA-specific Tregs originate from the peripheral conversion of conventional T cells and/or from the expansion of naturally occurring Tregs, this remains unknown. In this study, we report that the same CGA-derived epitopes from NY-ESO-1 and TRAG-3 stimulate spontaneous circulating TA-specific Th cells and Tregs in PBLs of patients with advanced melanoma and in healthy donors (HDs). We also show that TA-specific Tregs but not the TA-specific Th cell TCR repertoire are shared between naturally occurring Tregs and conventional T cells. Collectively, our findings hold significant implications for the development of combinatorial therapies aimed at impairing the generation of TA-specific Tregs.

Materials and Methods

Subjects and cell lines

Blood samples were obtained from seven melanoma patients (MPs) and from HDs under the University of Pittsburgh Cancer Institute Institutional Review Board approved protocols 96-099 and 00-079. HLA-DR–transfected mouse fibroblast cells, namely, L.DR cells, T2 cells, and EBV-B cells were previously described (16).

Flow cytometric analysis, Abs, and reagents

The following conjugated Abs were used in flow cytometric experiments: anti–GITR-FITC (R&D Systems, Minneapolis, MN), anti–Foxp3-PE (eBioscience, San Diego, CA), anti–CD4-PE, anti–CD3-ECD, anti–CD4-ECD, anti–CD14-ECD, anti–CD19-ECD, anti–CD8-PE-Cy5, anti–CD4-PE-Cy7 (Beckman Coulter, Fullerton, CA), anti–CTLA-4-PE-Cy5, anti–CD25-APC-Cy7 (BD Pharmingen, San Diego, CA) and anti–IL-2-PE (Miltenyi Biotec, Auburn, CA). CFSE Cell Proliferation Kit (Invitrogen, San Diego, CA) was used in proliferation assays. A violet amine reactive dye (Invitrogen) was used to exclude dead cells from analysis. Intracellular Foxp3 was stained using Foxp3 staining kit (eBioscience). FACS events were collected using a FACSAria machine (BD Biosciences, San Diego, CA), and data were analyzed with Flowjo software (Tree Star, Ashland, OR).

In vitro expansion of Ag-reactive CD4$^+$ T cells with peptides and cloning

The expansion of Ag-specific CD4$^+$ T cells in vitro with dendritic cells and peptides was performed as previously reported (16). The expanded Ag-specific CD4$^+$ T cells were cloned by limiting dilution using allogenic PBLs and EBV-B cells as feeders in the presence of IL-2 (PeproTech, Rocky Hill, NJ) and PHA (Sigma-Aldrich, St. Louis, MO), and subsequently tested for specificity in ELISPOT assays as described previously (16). This method was used to generate all TRAG-3 34-48–specific CD4$^+$ T cell clones and NY-ESO-1 119-143–specific CD4$^+$ T cell clones 30/79, 35/67, and 11/4.

Ex vivo NY-ESO-1–specific CD4$^+$ T cell sorting and cloning

CD4$^+$ T cells purified from PBLs of one melanoma patient (MP2) were incubated for 6 h in complete IMDM with autologous CD3-depleted cells as APCs in the presence of 10 μg/ml peptide NY-ESO-1 119-143 or peptide HIVpol 711-725 as negative control. IFN-γ– and IL-5–secreting NY-ESO-1–specific CD4$^+$ T cells were stained using IFN-γ or IL-5 Secretion Assay Detection Kit (Miltenyi Biotec) and sorted by flow cytometry into 96-well plates (one cell per well). IgG isotype control Abs were used to establish the threshold for cytokine-producing T cells. Sorted cells were expanded and tested for specificity in ELISPOT assays. This method was used to generate NY-ESO-1 119-143-specific CD4$^+$ T cell clones 103/3, 103/4, and 107/5.

CFSE proliferation assays

Naive CD4$^+$ and CD8$^+$ T cells were purified from PBLs obtained from HDs by magnetic cell separation using CD45RA microbeads (Miltenyi Biotec) and stained with CFSE (Invitrogen). The 1×10^5 CFSE-labeled naive T cells were cultured for 5 d with 2×10^5 irradiated (5000 rad) autologous CD3-depleted APCs, 0.1 μg/ml anti-CD3 (clone OKT3; eBioscience), IL-2 (50 IU/ml) (PeproTech), and different numbers of TRAG-3 34-48–specific or NY-ESO-1 119-143–specific CD4$^+$ T cell clones. Alternatively, naive T cells and APCs were plated in the lower wells of a 0.4-μm pore size transwell plate (Corning, Corning, NY). CD4$^+$ T cell clones with APCs were added into the inner wells. After 5 d, cells were collected and stained with anti–CD4-PE, anti–CD14-ECD, anti–CD19-ECD, anti–CD8-PE-Cy5, and analyzed by flow cytometry.

Cytokine secretion assays

The 1×10^5 TRAG-3 34-48–specific or NY-ESO-1 119-143–specific CD4$^+$ T cell clones were incubated for 24 h in complete IMDM with HLA-DR matched L.DR cells in the presence of 10 μg/ml cognate peptide or peptide HIVpol 711-725 as negative control. Cytokines present in supernatants were detected using Luminex Lab MAP technology.

IL-2 suppression assays

Tregs were coincubated with Th or CD8$^+$ T cell clones in complete IMDM supplemented with IL-2 (20 IU/ml) for 48 h. L.DR1 cells pulsed with 10 μg/ml relevant peptide TRAG-3

34-48 or irrelevant peptide HIVpol 711-725 were added into the wells containing the HLA-DR1–restricted TRAG-3–specific Treg clone 61/58. Alternatively, L.DR4 cells pulsed with relevant peptide NY-ESO-1 119-143 or irrelevant peptide HIVpol 711-725 were added into wells containing the HLA-DR4–restricted NY-ESO-1–specific Treg clone 103/3. After 24 h, L.DR4 cells pulsed with 10 μg/ml relevant peptide NY-ESO-1 119-143 or irrelevant peptide HIVpol 711-725 were added to wells containing the HLA-DR4–restricted NY-ESO-1–specific Th clone 11/4. Alternatively, T2 cells pulsed with 10 μg/ml relevant peptide MART-1 27-35 or irrelevant peptide HIVpol 476-484 were added to wells containing the HLA-A2–restricted MART-1–specific CD8$^+$ T cell clone 4/43. IL-2 secretion in the culture supernatants was determined by ELISA at the end of incubation. In flow cytometry experiments, CFSE-labeled Th clone 11/4 or CFSE-labeled CD8$^+$ T cell clone 4/43 were coincubated for 24 h with Treg clones 61/58 or 103/3, respectively, in the presence of APCs pulsed with cognate or irrelevant peptides to Treg clones. Then, clones were stained intracellularly for IL-2 after 6-h incubation with APCs pulsed with cognate or irrelevant peptides to CD8$^+$ T cell or CD4$^+$ Th cell clones. Alternatively, CFSE-labeled TA-specific Th clones were assessed for proliferation after 5 d of incubation with TA-specific Treg clones and peptide-pulsed APCs. As control, CFSE-labeled clones 11/4 and 4/43 were also incubated with TRAG-3–specific CD4$^+$ Th clone 62/8 or NY-ESO-1–specific CD4$^+$ T cell clone 107/5, respectively. Clones 11/4 and 4/43 were previously described (16, 17). Clone 11/4 was tested in cytokine production assays and CFSE proliferation assays to confirm its Th functions.

Foxp3 real-time quantitative PCR analysis

Foxp3 quantitative real-time PCR (QRT-PCR) was performed using Applied Biosystems 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) as previously reported (18). All QRT-PCR assays were carried out at two RNA inputs (500 ng and 100 ng), and triplicate reactions were set up for each concentration. Foxp3 expression was measured relative to the expression of endogenous control gene, β-glucuronidase (β-Gus).

TLR-8 expression by Ag-specific CD4$^+$ T cell clones

Total RNA was extracted from CD4$^+$ T cell clones and from PBLs obtained from two HDs, using the RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription was carried out as described previously (19). PCR reactions were performed using the TLR-8–specific primers as described previously (20).

TCR-β gene usage

Total RNA was isolated from 1 × 10^6 CD4$^+$ T cells. TCR-Vβ RT-PCR and sequencing of PCR products were performed as previously described (19).

Clonotypic real-time quantitative RT-PCR

Clonotypic real-time quantitative RT-PCR was performed as previously described (21). The forward (F) and reverse (R) primers and probes (P) used in this study were as follows: CDR3β 103/4F (5′-TGCCCAGTGATCGCTTCTCT-3′), CDR3β 103/4R (5′-CAGTGACTCCTGTCTGCCCG-3′), CDR3β 103/4P (5′-

ACAGCAGGAGGACTCGGCCGTGTATC-3′), CDR3β 107/5F (5′-TTCTCATCAACCATGCAAGCC-3′), CDR3β 107/5R (5′-TGCTCATTGTAGGACCCTCCG-3′), CDR3β 107/5P (5′-TGACAGTGACCAGTGCCCATCCTGAAG −3′), CDR3β 103/3F (5′-CTGAAAGGACTGGAGGGACGTAT-3′), CDR3β 103/3R (5′-GTGCCCCGGGATCCC-3′), CDR3β 103/3P (5′-CCAGCAGCCACCCGGGACA-3′), CDR3β 61/58F (5′-TGCCCAGTGATCGCTTCTCT-3′), CDR3β 61/58R (5′-GCCAAAATACTGCGTATCTGTGTT-3′), CDR3β 61/58P (5′-TGCAGTGCTTGGGGCGGGCT-3′), β-Gus F (5′-CTCATTTGGAATTTTGCCGAT T-3′), (β-Gus)R (5′-CCGAGTGAAGATCCCCTTTTTA-3′), β-Gus P (5′-TGAACAGTCACCGACGAGAGTGCTGG-3′). The sensitivity of real-time RT-PCR in detection of a specific CD4$^+$ T cell clone TCR CDR3β region in PBLs of MPs with NY-ESO-1–expressing tumors and HDs was determined using serial dilutions from each NY-ESO-1–specific or TRAG-3–specific CD4$^+$ T cell clone in PBLs from normal donors (1/10, 1/100, 1/1000, 1/10,000, 1/100,000, and 1/1,000,000). The relative expression of the TCR CDR3β gene region obtained by real-time quantitative RT-PCR was correlated with the different dilution ratios of each NY-ESO-1 119-143–specific or TRAG-3 34-48–specific CD4$^+$ T cell clone in PBLs by a power regression curve. The relative expression values were then used to estimate the number of T cell precursors expressing one specific clonotype in PBLs of MPs and HDs as well as in CD4$^+$CD25^{high} and CD4$^+$CD25$^{—}$ sorted fractions from two melanoma patients (MP1 and MP2).

Foxp3 intron 1 demethylation analysis of CD4$^+$ T cell clones

DNA (500 ng) extracted from T cells was treated with Bisulfite Conversion Kit (Invitrogen) according to the manufacturer’s protocol. The converted DNA was then analyzed by real-time PCR for the methylated and the unmethylated forms of Foxp3 intron 1 as described previously (22). The results are expressed as percentages of unmethylated Foxp3 sequences, calculated as follows: % of demethylation = $2^{\Delta Ct}/(1 + 2^{\Delta Ct})$, wherein ΔCt = (Ct with methylated primers) − (Ct with unmethylated primers).

Results

Functional studies of TRAG-3–specific and NY-ESO-1–specific CD4$^+$ T cell clones generated by in vitro peptide stimulation from PBLs of HDs or patients with advanced melanoma

We have previously reported that two immunodominant epitopes derived from the two CGAs, TRAG-3 34-48 and NY-ESO-1 119143, stimulate spontaneous CD4$^+$ T cells in the majority of patients with NY-ESO-1–expressing and/or TRAG-3–expressing melanoma (21, 23, 24). Using in vitro stimulation with peptides, we have previously isolated TRAG-3–specific CD4$^+$ T cell clones (i.e., clones 61/58, 62/3, and 62/8) and NY-ESO-1–specific CD4$^+$ T cell clones (i.e., clones 30/79, 35/67, and 11/4) from PBLs of two MPs and two HDs. To investigate whether these clones exert suppressive activity, we tested their capability of inhibiting the anti-CD3 Ab-induced proliferation of naive allogenic T cells isolated from PBLs of HDs. TRAG-3–specific clones 61/58 (from MP1) and 62/3, but not clone 62/8 (from HD1), exhibited suppressive effects on the proliferation of naive CD4$^+$ and

$CD8^+$ T cells (Fig. 1A, 1C). NY-ESO-1–specific $CD4^+$ T cell clones 30/79 and 35/67 (from HD2) also suppressed the proliferation of naive T cells (Fig. 1B). In contrast, NY-ESO-1–specific $CD4^+$ T cell clone 11/4 (from MP3), did not suppress the proliferation of T cells (Fig. 1C). The suppressive effect of the TA-specific Tregs appeared to be dose dependent as it decreased at lower E:T ratios. Notably, the suppressive effects of NY-ESO-1–specific and TRAG-3–specific Tregs were not observed in transwell experiments, suggesting that they act in a cell-to-cell contact-dependent fashion (Fig. 1A, 1B).

Collectively, our findings show that the same immunodominant epitopes from NY-ESO-1 and TRAG-3 stimulate TA-specific $CD4^+$ Tregs and Th cells from PBLs of HDs and MPs after in vitro peptide stimulation.

Generation and functional studies of NY-ESO-1–specific $CD4^+$ T cell clones isolated from circulating NY-ESO-1 $CD4^+$ T cells detectable ex vivo

We next wanted to investigate whether TA-specific Tregs could be isolated from spontaneous TA-specific $CD4^+$ T cells that are detectable ex vivo from PBLs of cancer patients with no prior in vitro peptide stimulation. $CD4^+$ T cells were isolated from PBLs of MP2 and stimulated ex vivo with NY-ESO-1 119-143 peptide-pulsed autologous APCs in 6-h IFN-γ and IL-5 cytokine secretion assays prior to cell sorting of cytokine-secreting $CD4^+$ T cells at one cell per well. MP2 exhibited spontaneous IFN-γ-producing NY-ESO-1 119-143–specific $CD4^+$ T cells that were detectable ex vivo; whereas, no IL-5–producing NY-ESO-1–specific $CD4^+$ T cells were detected (Fig. 2A; data not shown). Sorted IFN-γ–producing cells were expanded in vitro without cognate peptide. We obtained thirteen NY-ESO-1–specific $CD4^+$ T cell clones that were tested in CFSE-based proliferation assays. Clone 103/3, but not clones 103/4 and 107/5, inhibited the proliferation of CD3-Ab–stimulated naive CD8+ and $CD4^+$ T cells (Fig. 2B, 2C). Transwell experiments demonstrated that clone 103/3 exerts its suppressive functions in a cell-to-cell contact-dependent fashion.

To exclude the possibility that inhibition of proliferation is the consequence of nonspecific crowding of wells by suppressive T cell clones that otherwise would respond better to anti-CD3 stimulation than nonsuppressive T cell clones, we assessed the proliferation of each individual TA-specific Treg and Th clone. In contrast to TA-specific Th clones, TA-specific Treg clones did not significantly proliferate in response to anti-CD3/IL-2 stimulation, ruling out a possible crowding of wells by Tregs (Fig. 3).

Altogether, our findings show that spontaneous NY-ESO-1–specific $CD4^+$ T cells isolated ex vivo from PBLs of patients with advanced NY-ESO-1–expressing melanoma include both NY-ESO-1–specific Th and Tregs.

Phenotypic characterization of NY-ESO-1–specific and TRAG-3–specific Tregs

To further study the phenotype of the TA-specific Tregs, we performed flow cytometric analysis of the expression of a number of Treg markers by the $CD4^+$ T cell clones. These experiments were performed at least 15 d after the last in vitro stimulation. We found that the large majority of TA-specific Tregs expressed high levels of CD25 and intracellular FoxP3 in sharp contrast with Th clones (Fig. 4A). Four of the five TA-specific Treg clones but none of the three Th clones expressed high levels of GITR. Levels of CTLA-4 expression varied from one Treg clone to another but were consistently higher than that observed for Th clones. Finally, TRAG-3– and NY-ESO-1–specific Treg clones did not express CD127 (data not shown).

We have performed real-time PCR and RT-PCR to further study Foxp3 and TLR-8 expression, respectively, of TA-specific CD4$^+$ Treg clones. All Treg clones expressed higher Foxp3 levels than Th clones or CD4$^+$CD25$^-$ T cells isolated from PBLs of one HD (Fig. 4B). However, these Foxp3 levels remain lower than those observed in naturally occurring CD4$^+$CD25$^+$ Tregs isolated from PBLs of one HD. We have further investigated the methylation status of the Treg-specific demethylated region (TSDR) of Foxp3 gene locus in TA-specific Treg clones because it has been reported that the constitutive expression of Foxp3 in murine and human CD4$^+$CD25$^+$ T cells is correlated with the unmethylated status of CpG dinucleotides located in a conserved region of Foxp3 intron 1 (22, 25, 26). Consistent with their low Foxp3 expression, TRAG-3 and NY-ESO-1–specific Tregs displayed high levels of Foxp3 intron 1 methylation as compared with CD4$^+$CD25$^+$ Tregs isolated from PBLs of one HD (Fig. 4C). Notably, the two NY-ESO-1–specific Treg clones with higher levels of Foxp3 expression in real-time PCR experiments (i.e., clones 35/67 and 103/3) exhibited slightly higher levels of Foxp3 intron 1 demethylation than the other clones tested. None of the NY-ESO-1–specific and TRAG-3-specific Tregs expressed TLR-8. As control, TLR-8 expression was found in PBLs from one HD (Fig. 4D).

Altogether, our findings show that NY-ESO-1–specific and TRAG-3–specific Tregs express the typical Treg markers, including CD25, Foxp3, CTLA-4, and GITR, but not TLR-8.

Cytokine profile of NY-ESO-1–specific and TRAG-3–specific Tregs

To investigate the cytokine profile of TA-specific Tregs upon Ag stimulation, we have performed multiplex cytokine assays for Th1-type and Th2-type cytokines, TGF-β and IL-10 (Fig. 5A). Upon Ag-specific stimulation, TRAG-3–specific CD4$^+$ Treg clone 61/58 produced IFN-γ, GM-CSF, and TNF-α but no Th2-type cytokines, TGF-β, or IL-10. In contrast, NY-ESO-1–specific CD4$^+$ Treg clone 103/3 produced IL-5 and GM-CSF, but little or no Th1-type cytokines, TGF-β, or IL-10. Finally, NY-ESO-1–specific CD4$^+$ Treg clones 30/79 and 35/67 exhibited a Th0-type cytokine profile, producing IFN-γ and/or TNF-α, IL-4 and/or IL-5 cytokines, no TGF-β, and little or no IL-10. In addition, none of the Treg clones expressed membrane-bound TGF-β or latency-associated peptide (data not shown). As controls, Th NY-ESO-1–specific clone 107/5 and TRAG-3–specific CD4$^+$ Th clone 62/8 exhibited a Th1-type and a Th0-type cytokine profile, respectively. Both were found to produce IL-2 and higher amounts of IFN-γ and TNF-α than TA-specific Treg clones. Collectively, our findings show that TA-specific Tregs produce either Th1-, Th2-, or Th0-type cytokines. They did not produce TGF-β and produced no or little IL-10.

NY-ESO-1–specific and TRAG-3–specific Tregs suppress IL-2 secretion of Ag-specific CD8$^+$ and CD4$^+$ T cells upon recognition of specific ligand

We next wanted to define whether TA-specific Tregs suppress immune responses upon recognition of their TA-specific ligand. Therefore, TRAG-3–specific Treg clone 61/58 was coincubated for 48 h with APCs pulsed with relevant peptide (TRAG-3 34-48) or irrelevant peptide (HIVpol 711-725) in the presence of the MART-1 27-35–specific $CD8^+$ T cell clone 4/43 or the NY-ESO-1 119-143–specific $CD4^+$ Th clone 11/4. After 24 h incubation, APCs pulsed with relevant peptide (i.e., MART-1 27-35 for $CD8^+$ clone 4/43 or NY-ESO-1 119-143 for Th clone 11/4) or irrelevant peptide (HIVpol 476-484) were added prior to measuring IL-2 production in the supernatant. As shown in Fig. 5B, TRAG-3–specific Treg clone 61/58 strongly decreased IL-2 production by MART-1–specific CTL clone 4/43 and NY-ESO-1–specific Th clone 11/4 upon recognition of its specific ligand (peptide TRAG-3 34-48) but not in the presence of the irrelevant peptide. Alternatively, the NY-ESO-1–specific Treg clone 103/3 was coincubated with APCs pulsed with relevant peptide (NY-ESO-1 119-143) or irrelevant peptide (HIVpol 711-725) in the presence of $CD8^+$ T cell clone 4/43. After 24 h incubation, APCs that were pulsed with relevant peptide (MART-1 27-35) or irrelevant peptide were added to the wells. We observed that NY-ESO-1–specific Treg clone 103/3 strongly decreased IL-2 production by $CD8^+$ T cell clone 4/43 upon Ag-specific recognition (Fig. 5B). To further demonstrate that Tregs inhibit IL-2 production by Th or CTL clones, we evaluated intracellular IL-2 production by CFSE-labeled NY-ESO-1 119-143–specific $CD4^+$ Th clone 11/4 or CFSE-labeled MART-1 27-35–specific $CD8^+$ T cell clone 4/43 after incubation with TRAG-3–specific Treg clone 61/58 or NY-ESO-1–specific Treg clone 103/3, respectively, in the presence of peptide-pulsed APCs (Fig. 5C). TRAG-3–specific Treg clone 61/58 decreased IL-2 production by NY-ESO-1–specific Th clone 11/4 upon recognition of its cognate Ag but not in the presence of the irrelevant peptide. In addition, NY-ESO-1–specific Treg clone 103/3 decreased IL-2 production by $CD8^+$ T cell clone 4/43 upon Ag-specific recognition. As controls, TRAG-3–specific Th clone 62/8 and NY-ESO-1–specific $CD4^+$ Th clone 107/5 did not inhibit IL-2 production by clones 11/4 and 4/43, respectively, upon recognition of their cognate Ag, and TA-specific Treg clones 61/58 and 103/3 did not produce IL-2 upon stimulation (Fig. 5C). Notably, we observed an impaired proliferation of CFSE-labeled NY-ESO-1–specific Th clone 11/4 after 5 d of incubation with peptide-pulsed APCs and TRAG-3–specific Treg clone 61/58, but not with TRAG-3–specific Th clone 62/8 (Fig. 6). In these assays, TA-specific Tregs also inhibited IL-2 production by TA-specific Ths cells after 5 d of incubation (data not shown). Altogether, our results suggest that NY-ESO-1–specific and TRAG-3–specific Tregs exert their suppressive functions on TA-specific $CD8^+$ and $CD4^+$ T cells after activation with their specific ligand.

NY-ESO-1–specific and TRAG-3–specific Tregs share TCR CDR3 usage with $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells

We next wanted to determine whether NY-ESO-1–specific and/or TRAG-3–specific Tregs share TCR CDR3 usage with $CD4^+CD25^+$ and/or $CD4^+CD25^-$ T cell compartments. To this end, we sequenced the TCR CDR3β region of TRAG-3–specific Treg clone 61/58 isolated from PBLs of MP1, NY-ESO-1–specific Treg clone 103/3, and NY-ESO-1–specific Th clones 103/4 and 107/5 isolated from PBLs of MP2. Each of these clones exhibited a distinct CDR3β region (Table I). We next investigated whether these TCRs could be detected in PBLs obtained from six MPs with spontaneous NY-ESO-1–specific $CD4^+$ T cells (MP1, MP2, MP4, MP5, MP6, and MP7) and in PBLs obtained from four HDs (HD1, HD2, HD3, and HD4). We further investigated whether the clones' TCRs could be detected in the

$CD4^+CD25^{high}$ and/or $CD4^+CD25^-$ T cell FACS-sorted fractions of PBLs from MP1 and MP2 (Fig. 7A). Based on the sequences of the TCR CDR3β regions of the clones, we designed specific primers to perform quantitative clonotypic real-time PCR as described previously (21). To correlate the expression level of CDR3β gene expression with the number of Ag-specific cells present in PBLs, serial dilutions were made of each clone from 10^{-1} to 10^{-6} in PBLs from HDs and clonotypic real-time RT-PCR was performed. Assuming that the signal observed from each pure clone represents 100% of CDR3β gene expression, we expressed the results obtained from the serial dilutions and samples from patients as a fraction of the total CDR3β gene expression obtained from each clone. We detected TRAG-3-specific Treg clone 61/58 CDR3β gene expression and NY-ESO-1–specific Treg clone 103/3 CDR3β gene expression in PBLs of MP1 and MP2, respectively, but not in PBLs of four other MPs with spontaneous NY-ESO-1–specific $CD4^+$ T cells or in HDs. In addition, we observed that MP1 and MP2 had detectable levels of Treg clone 61/58 CDR3β and 103/3 CDR3β gene expression, respectively, both in the $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cell compartments with a precursor frequency of $CD4^+$ T cells of 9×10^{-6} and 8×10^{-6} for clone 61/58, respectively, and 9×10^{-6} and 3×10^{-6} $CD4^+$ T cells for clone 103/3, respectively (Fig. 7B). In contrast, the two NY-ESO-1–specific Th clones, 103/4 and 107/5, were detectable only in the $CD4^+CD25^-$ T cell compartment of MP2 with a precursor frequency of $CD4^+$ T cells of 3.6×10^{-6} and 2.5×10^{-4} $CD4^+$ T cells, respectively (Fig. 7C).

Discussion

We and others have previously shown that CGA-derived epitopes that represent tumor-specific T cell targets (27), give rise to spontaneous Th1-type $CD4^+$ T cells isolated either from PBLs or TILs of cancer patients (24, 28). However, such spontaneous $CD4^+$ T cell responses are found in patients with progressive disease, questioning their role in promoting potent antitumor CTL functions. The paradoxical coexistence of spontaneous TA-specific $CD4^+$ T cell responses with clinical progression has furthered the need to dissect TA-specific $CD4^+$ T cell functions. In the current study, we have shown that two immunodominant CGA-derived epitopes known to stimulate spontaneous Th1-type $CD4^+$ T cells in patients with advanced melanoma also stimulate TA-specific Tregs. In particular, we observed that NY-ESO-1–specific and TRAG-3–specific Tregs can either be obtained after in vitro stimulation with peptide-pulsed APCs or isolated from spontaneous TA-specific $CD4^+$ T cells detectable ex vivo from PBLs of patients with advanced melanoma.

One critical finding is the evidence of TA-specific Th and Tregs directed against the same epitope in the same subject. Our data further add to reports of LAGE-1 and ARTC1-specific Tregs isolated from TILs of patients with melanoma (12, 13). They also add to the recent report of vaccine-induced MAGE-3–specific Tregs detectable only after immunization in PBLs of melanoma patients (15).

TRAG-3– and NY-ESO-1–specific Tregs exhibit either a Th1-, a Th2-, or a Th0-type cytokine profile. We have not found TGF-β– or IL-10–producing TRAG-3– or NY-ESO-1–specific Tregs. Our observation that TA-specific Treg clones display diverse cytokine profiles is in line with previous reports of TA-specific Tregs. François et al. observed that four of five MAGE-A3–specific CD4$^+$ T cell clones produced TNF-α but no IFN-γ, IL-2, IL-4, or IL-5; whereas, one MAGE-A3–specific Treg clone displayed a Th2-type cytokine profile, producing IL-4 and IL-5 (15). Van der Burg et al. isolated HPV-specific Treg clones from lymph node biopsies of cervical cancer patients that produced both IFN-γ and IL-10 (29). Wang et al. isolated LAGE-1-specific Treg clones that produced either IFN-γ, IL-4, and IL-10 or IFN-γ and IL-10 (12). These data represent another illustration of the plasticity of Treg lineage differentiation, which appears to be regulated by epigenetic mechanisms (30, 31). These findings bear significant implications in terms of immunomonitoring of spontaneous and vaccine-induced T cell responses in cancer patients for which measurement of cytokine production remains the main method for defining CD4$^+$ T cell functions. They also stress the need to further refine the monitoring of TA-specific CD4$^+$ T cells with functional studies to differentiate TA-specific Th cells and Tregs.

In humans, the reliability of Foxp3 as a marker of Tregs is still being debated for several reasons. First, Foxp3 expression is tightly linked to TCR-mediated activation (32–38). Second, the Foxp3-specific mAb PCH101, which has been used in most studies, appears to yield nonspecific staining (39). In our study, TA-specific Treg clones express Foxp3 in flow cytometry and real-time PCR. However, their Foxp3 levels assessed by real-time PCR vary from clone to clone and appear modest in comparison with those expressed by freshly isolated CD4$^+$CD25^{high} T cells. We further demonstrated that TRAG-3–specific and NY-ESO-1–specific Treg clones display high levels of TSDR methylation as compared with CD4$^+$CD25^{high} Tregs. This observation appears to contrast with two previous reports of high levels of TSDR demethylation by MAGE-A3–specific CD4$^+$ Treg clones isolated from PBLs of patients with melanoma. However, these studies included a limited number of clones directed against the same MAGE-A3–derived epitope (15, 22). In addition and in line with our findings, one of the five MAGE-A3–specific Treg clones displayed a very low percentage of TSDR demethylation (15). Therefore, additional studies on multiple Tregs directed against multiple TA-derived epitopes are needed to further explore whether high levels of TSDR demethylation are commonly observed in TA-specific Tregs. Notably and in contrast to the freshly isolated CD4$^+$CD25^{high} T cells, the TA-specific Treg clones isolated in our study have been expanded in vitro and two lines of evidence suggest that in vitro culture may impact Foxp3 expression and TSDR methylation status of the Foxp3 gene locus. First, adaptive Foxp3$^+$ Tregs in vitro appear to lose Foxp3 expression on restimulation without TGF-β and their TSDR demethylation levels, although higher than those of Th cells, were much lower than those of CD4$^+$CD25^{high} Tregs (26). Second, progressive loss of Foxp3 expression on repetitive in vitro stimulation has been reported in sorted CD4$^+$CD25^{high} CD127$^-$ Tregs as well as in CD4$^+$CD25^{high} CD45RA$^+$ Treg clones (40). In this study, the loss of Foxp3 expression was associated with the very heterogeneous profiles of Foxp3 expression between Treg clones as is the case for the TA-specific clones in our study. In addition, loss of Foxp3 expression correlated with increased TSDR methylation of the Foxp3 gene locus.

To date, two subsets of human TA-specific Tregs have been reported. The first group includes Tregs that act in a cell-to-cell contact-mediated fashion as is the case for CGA-specific Tregs identified in the current study (12, 13). In contrast, another group of human TA-specific Tregs like WT-1–specific and EBNA1-specific Tregs appear to act in a soluble factor-dependent fashion (14, 41). Our data demonstrate that TA-specific Tregs exert their suppressive functions on polyclonal effector T cells and TA-specific CD8$^+$ and CD4$^+$ T cells after TCR activation by anti-CD3 Abs or upon the recognition of their specific ligand. Of note, TA-specific Treg and Th clones expressed similar levels of CD107a after anti-CD3 stimulation, suggesting that the suppressive capacity of Treg clones is not the consequence of higher cytotoxic capabilities as compared with Th clones (data not shown).

Interestingly, we observed that NY-ESO-1–specific and TRAG-3–specific Tregs were present both in the Foxp3$^+$CD4$^+$CD25^{high} Treg compartment and in the CD4$^+$CD25$^-$ T cell compartment in PBLs. In contrast, NY-ESO-1–specific Th cells were detected only in the CD4$^+$CD25$^-$ T cell compartment. This observation is in line with previous studies in mice and humans showing that CD4$^+$CD25^{high} Tregs use a large unrestricted αβ repertoire that appears distinct from the CD4$^+$CD25$^-$ T cell compartment with an overlap between the two TCR repertoires (42–45). Therefore, our findings most likely support the evidence of peripheral conversion of CD4$^+$CD25$^-$ T cells into Foxp3$^+$CD4$^+$CD25^{high} T cells, occurring possibly upon exposure to TGF-β (46) and/or chronic Ag stimulation (47). In experimental models, tumor cells have been shown to directly convert CD4$^+$CD25$^-$ T cells to Tregs through production of TGF-β, leading to tumor evasion of the immune system (48). Although CGA expression was not initially found in thymus but only in tumor cells, testis, and placenta (27), the isolation of pure thymic stromal cells of human thymus in combination with improved protocol for amplification of small RNA amounts has demonstrated that thymic epithelial cells express a highly diverse selection of genes, including many tissue-specific Ags and several CGAs such as NY-ESO-1 (49). Therefore, we cannot totally exclude that presentation of NY-ESO-1 peptides by thymic epithelial cells does occur, contributing to the generation of NY-ESO-1–specific Foxp3$^+$CD4$^+$CD25^{high} Tregs. However, our findings of shared NY-ESO-1–specific and TRAG-3–specific Treg TCRs between CD4$^+$CD25$^-$ T cells and Foxp3$^+$CD4$^+$CD25^{high} T cells argue against this hypothesis. In addition, we did not detect the NY-ESO-1 Treg TCR and TRAG-3 Treg TCR in PBLs of four other MPs with advanced NY-ESO-1–expressing melanoma or in PBLs of four normal donors, suggesting that the recruitment and expansion of TA-specific Tregs in cancer patients is stochastic and depends on chronic Ag stimulation.

Collectively, our findings demonstrate that spontaneous cytokine-producing CD4$^+$ T cells isolated from PBLs of the same cancer patient and directed against the same TA may be either Th or Tregs. They also demonstrate that TA-specific Tregs contribute to the TCR repertoire overlap between CD4$^+$CD25^{high} Tregs and conventional T cells in cancer patients. Our data support that the impairment of tumor-induced or vaccine-induced TA-specific Treg expansion in cancer patients may be optimally achieved by combinatorial therapies aiming not only at depleting CD4$^+$CD25^{high} Tregs, but also at preventing the peripheral conversion of CD4$^+$CD25$^-$ T cells into TA-specific Tregs.

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Abbreviations used in this paper:

β-Gus	β-glucuronidase
CGA	cancer-germline Ag
HD	healthy donor
MP	melanoma patient
No Stim	no stimulation
QRT-PCR	quantitative real-time PCR
TA	tumor Ag
TIL	tumor-infiltrating lymphocytes
Treg	regulatory T cell
TSDR	Treg-specific demethylated region

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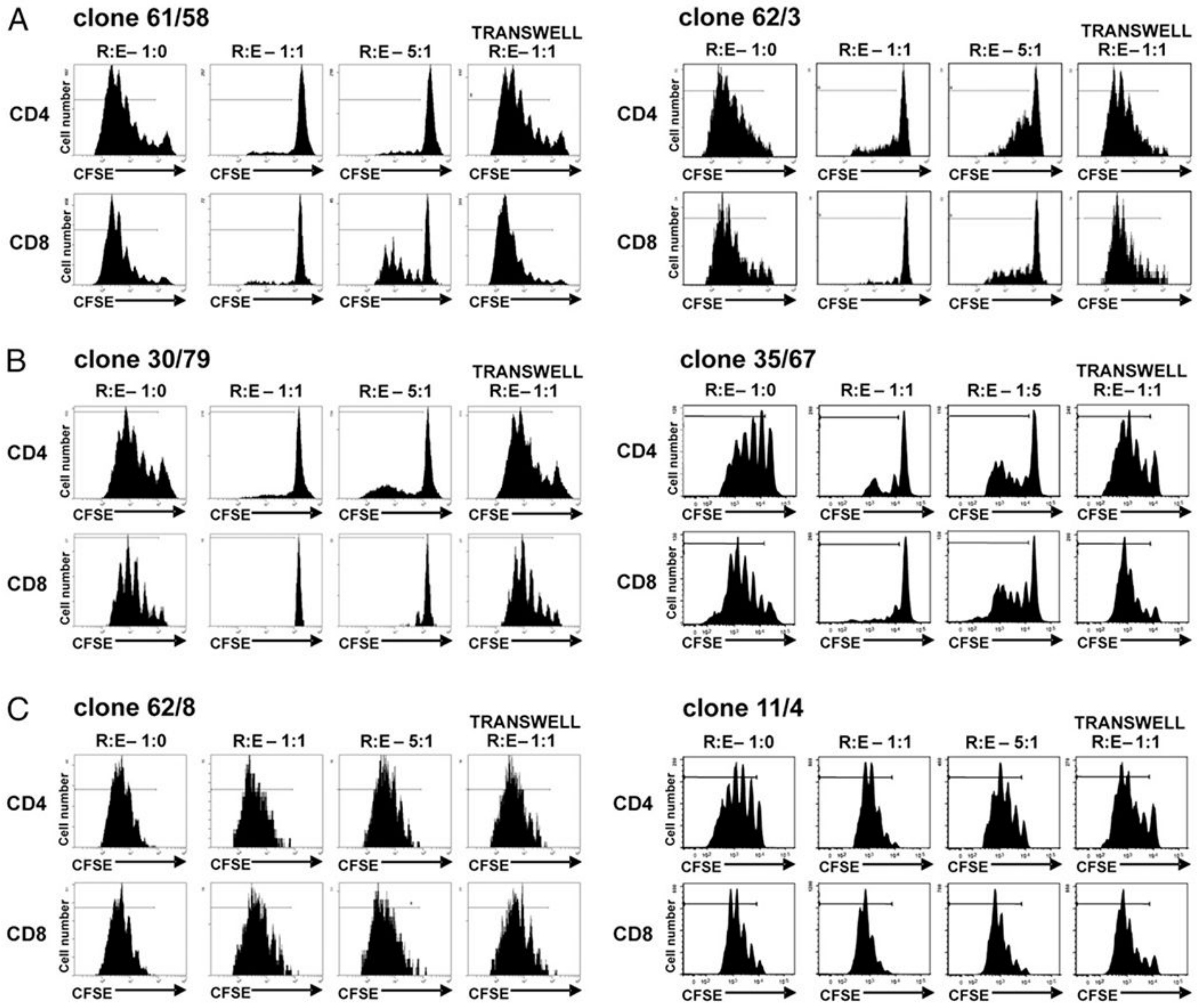

FIGURE 1.

TRAG-3 34-48–specific and NY-ESO-1 119-143–specific CD4$^+$ Treg clones inhibit the proliferation of responder T cells. The proliferation of allogenic CFSE-labeled naive CD45RA$^+$ T cells obtained from blood of HDs was assessed after 5 d of stimulation with soluble anti-CD3 and IL-2 in the presence of autologous CD3-depleted APCs and TRAG-3– or NY-ESO-1–specific CD4$^+$ T cell clones. Flow cytometry histograms are displayed for different ratios of responder CD4$^+$ and CD8$^+$ naive T cells to effector CD4$^+$ T cell clones (R:E). Proliferation of naive T cells was inhibited by the presence of (*A*) TRAG-3 34-48–specific CD4$^+$ T cell clones 61/58 (MP1) and 62/3 (HD1) and (B) NY-ESO-1 119-143–specific CD4$^+$ T cell clones 30/79 and 35/67 (HD2). *C*, Opposite effect was observed with TRAG-3 34-48–specific CD4$^+$ T cell clone 62/8 (HD1) and NY-ESO-1 119-143–specific CD4$^+$ T cell clone 11/4 (MP3). Transwell experiments confirmed that cell–cell contact is required for inhibition of proliferation by Treg clones (*A-C, right panels*). One of three independent experiments is depicted.

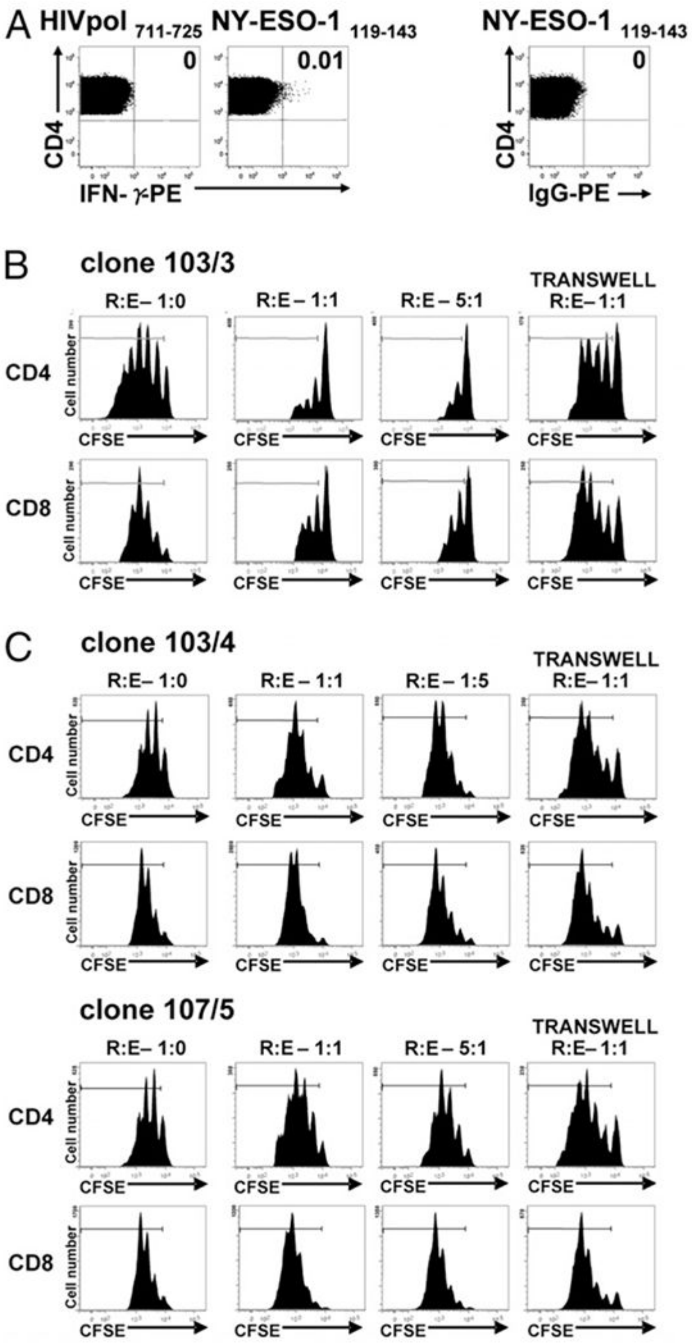

FIGURE 2.

NY-ESO-1–specific CD4$^+$ Tregs are isolated ex vivo from PBLs of melanoma patients. *A*, CD4$^+$ T cells isolated from PBLs of MP2 were incubated ex vivo for 6 h with autologous APCs pulsed with peptide NY-ESO-1 119-143 or peptide HIVpol 711-725 as negative control. CD4$^+$ T cells were stained with an anti–IFN-γ–PE Ab or a PE-labeled IgG isotype control Ab. IFN-γ–positive NY-ESO-1–specific CD4$^+$ T cells were sorted (one cell per well) and expanded in vitro in the absence of peptide. *B*, NY-ESO-1–specific CD4$^+$ T cell clone 103/3 inhibited the proliferation of naive T cells. *C*, NY-ESO-1–specific CD4$^+$ T cell clones

103/4 and 107/5 enhanced the proliferation of responder cells. One of three independent experiments performed is displayed.

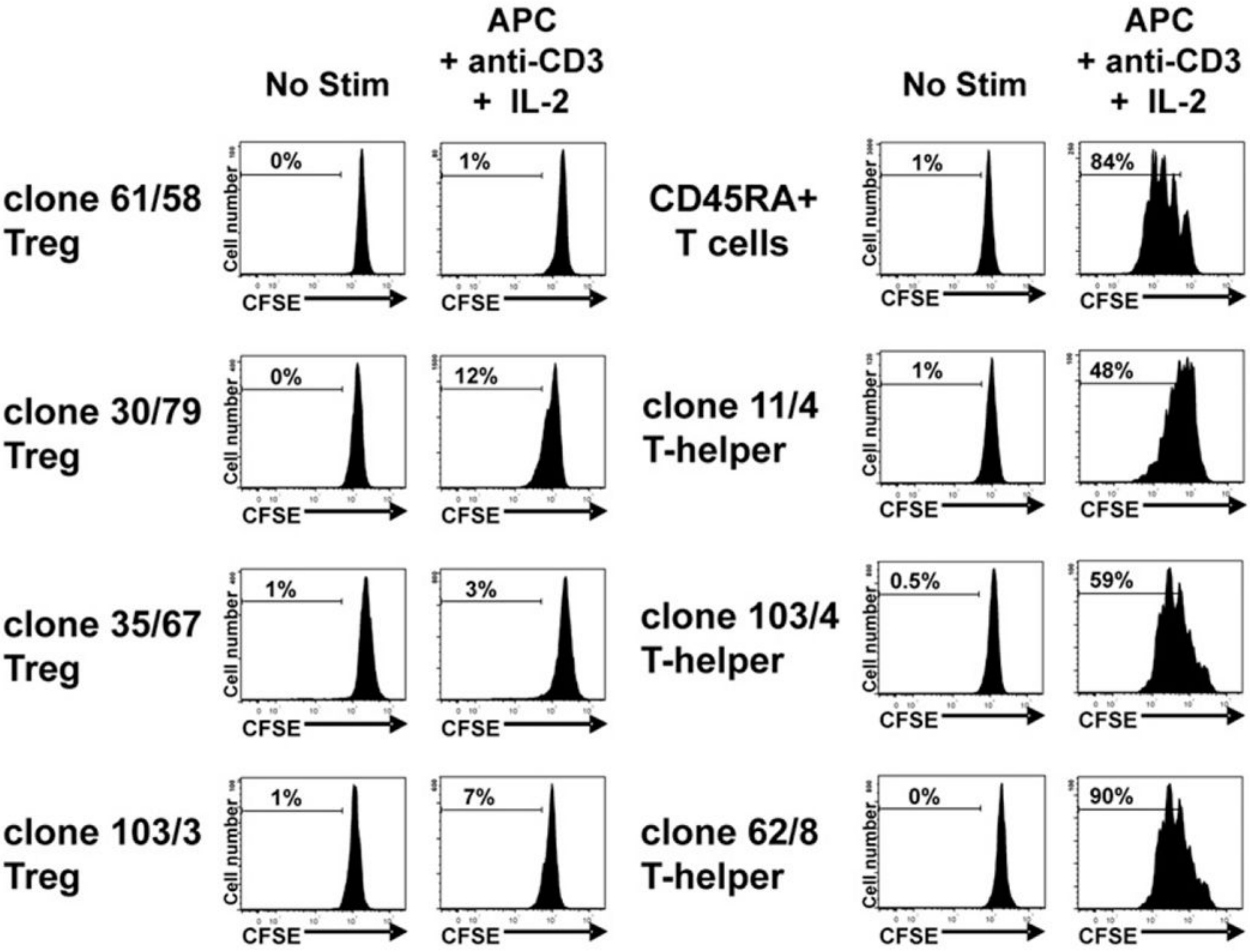

FIGURE 3.

TRAG-3 34-48–specific and NY-ESO-1 119-143–specific CD4$^+$ Treg clones have low proliferative capacity. Proliferation of CFSE-labeled TRAG-3–specific CD4$^+$ Treg clone 61/58 and NY-ESO-1–specific CD4$^+$ Treg clones 30/79, 35/67, and 103/3 was assessed after 5 d of stimulation with soluble anti-CD3 Ab (0.1 μg/ml) and IL-2 (50 IU/ml) in the presence of allogenic CD3-depleted APCs obtained from PBLs of one HD. TRAG-3–specific CD4$^+$ Treg clone 61/58 and NY-ESO-1–specific CD4$^+$ Treg clones 30/79, 35/67, and 103/3 proliferated significantly less than TRAG-3–specific CD4$^+$ Th clone 62/8, NY-ESO-1–specific CD4$^+$ Th clones 11/4 and 103/4 as well as naive CD45RA$^+$ T cells isolated from PBLs of one HD. One of three independent experiments is depicted. No Stim, no stimulation.

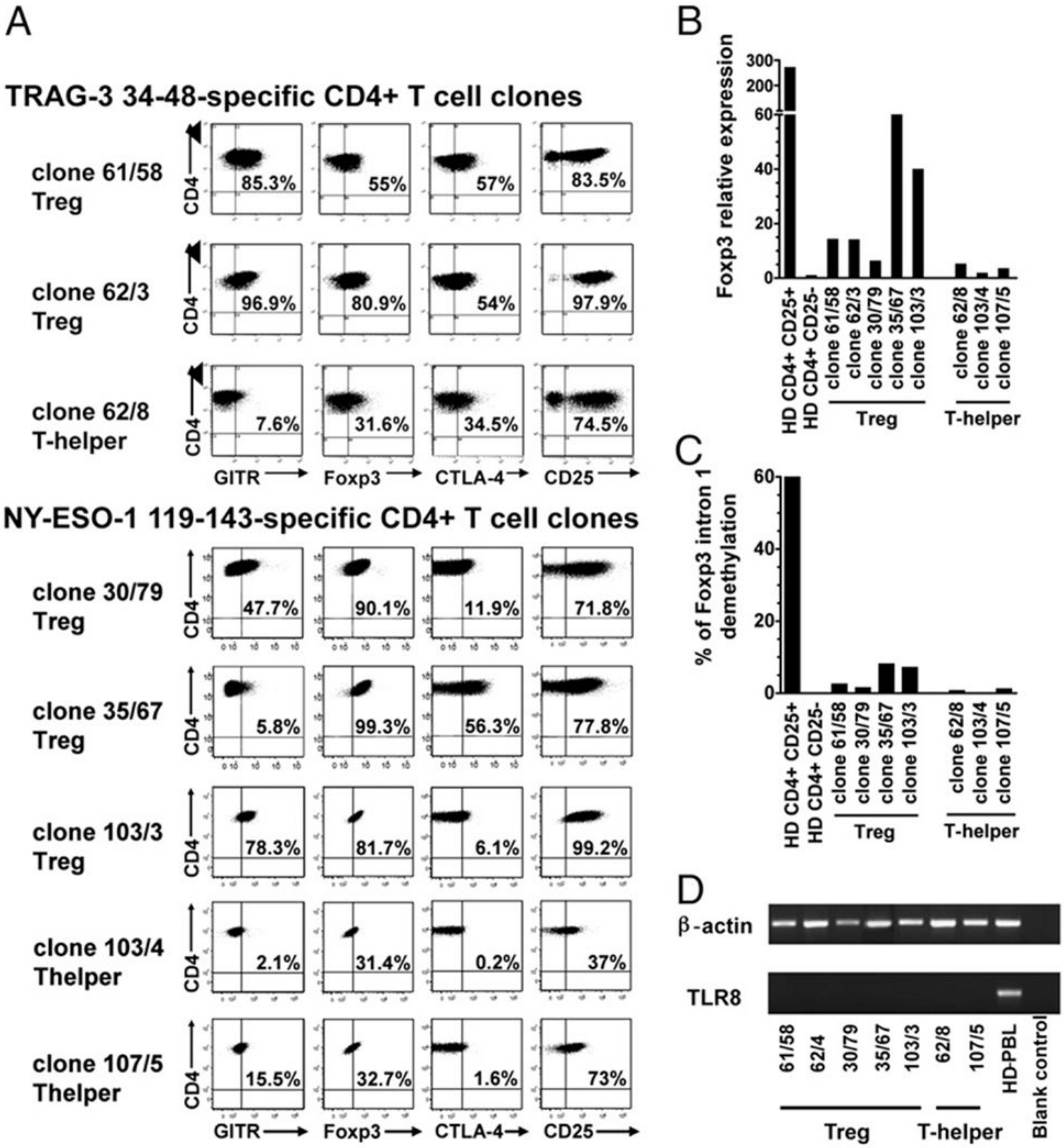

FIGURE 4.

Phenotypic analysis of TRAG-3 34-48–specific and NY-ESO-1 119-143–specific CD4$^+$ Tregs and Th clones. *A*, TRAG-3–specific CD4$^+$ Treg clones 61/58 and 62/3 expressed high levels of GITR and Foxp3 compared with Th clone 62/8 (*upper panel*). Two of three NY-ESO-1–specific CD4$^+$ Treg clones, clones 30/79 and 103/3, expressed high levels of GITR and all Treg clones expressed high levels of Foxp3, compared with Th clones 103/4 and 107/5 (*lower panel*). All Treg clones expressed high levels of CD25 and variable levels of CTLA-4. *B*, The relative expression of Foxp3 by TRAG-3–specific and NY-ESO-1–specific

$CD4^+$ T cell clones was estimated by real-time quantitative PCR and is displayed using the expression of Foxp3 by $CD4^+$ $CD25^-$ T cells of one HD as base line (i.e., value of 1). *C*, The demethylation status of Foxp3 intron 1 from TRAG-3–specific and NY-ESO-1–specific $CD4^+$ T cell clones was assessed by QRT-PCR. The results are expressed as percentages of unmethylated Foxp3 sequences and compared with $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells of one HD. *D*, The expression pattern of TLR-8 in TRAG-3–specific and NY-ESO-1–specific $CD4^+$ T cell clones was determined by RT-PCR. β-actin was used as an internal control. PBLs obtained from one HD served as a positive control (HD-PBL). None of the analyzed TRAG-3 34-48–specific and NY-ESO-1 119-143–specific $CD4^+$ T cell clones showed expression of TLR-8. One of three independent experiments is depicted.

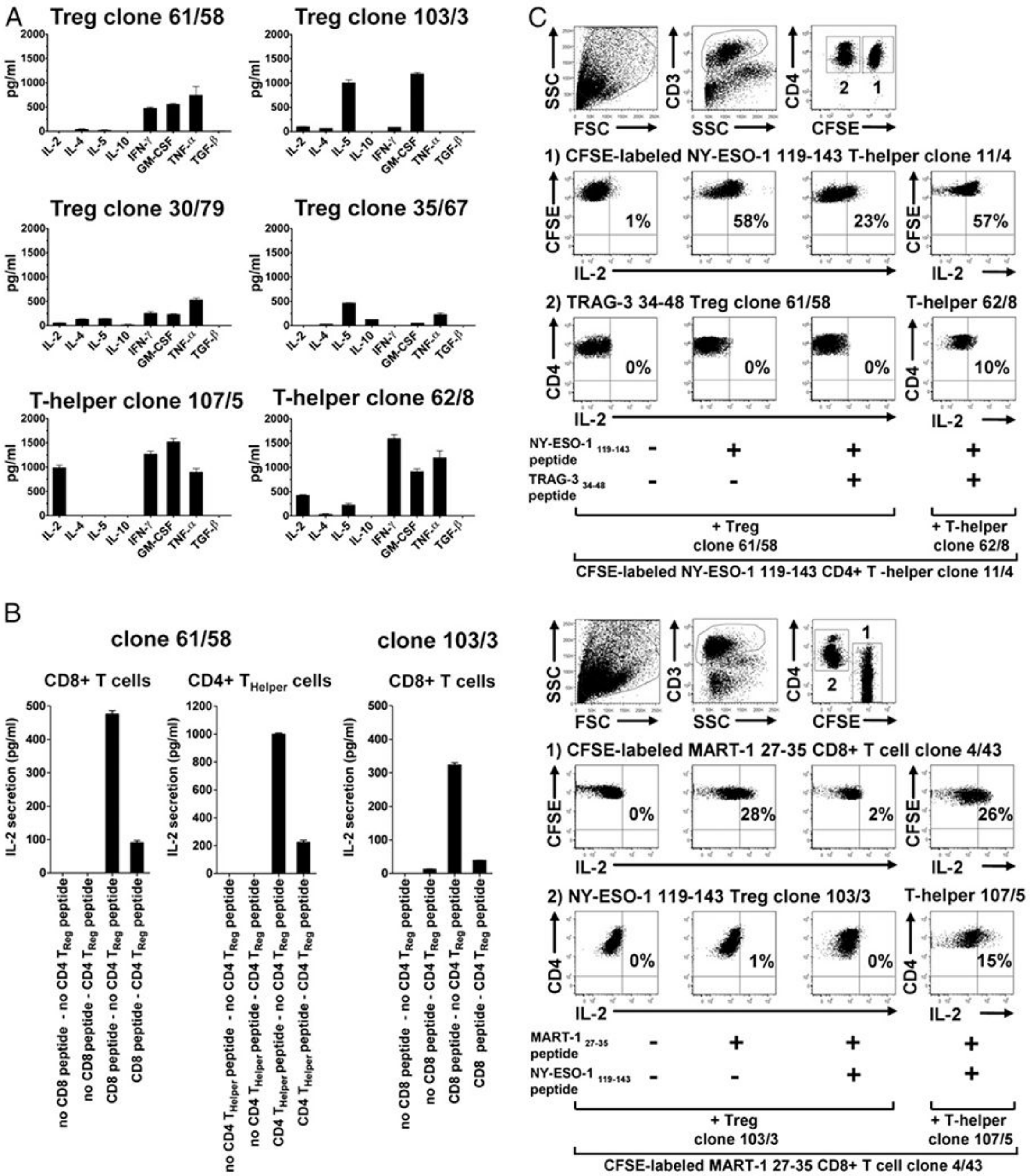

FIGURE 5.

TA-specific CD4$^+$ Tregs produce either Th1-, Th2-, or Th0-type cytokines and suppress IL-2 production by responder T cells. *A*, TA-specific CD4$^+$ Treg and Th clones were stimulated in vitro in the presence of cognate peptide-pulsed APCs prior to measuring cytokine production in supernatants after a 24-h incubation. TRAG-3–specific clone 61/58 secreted Th1-type cytokines. NY-ESO-1–specific CD4$^+$ Treg clones 103/3 displayed a Th2-type cytokine profile; whereas, NY-ESO-1–specific CD4$^+$ Treg clones 30/79 and 35/67 displayed a Th0-type cytokine-profile. NY-ESO-1–specific CD4$^+$ Th clone 107/5 and TRAG-3–

specific CD4$^+$ Th clone 62/8 displayed a Th1-type and a Th0-type cytokine profile, respectively. They produced IL-2 and higher amounts of IFN-γ and TNF-α than TA-specific Treg clones. *B* and *C*, TRAG-3 34-48–specific CD4$^+$ Treg clone 61/58 or NY-ESO-1 119-143–specific CD4$^+$ Treg clone 103/3 were cocultured with CFSE-labeled or unstained MART-1 27-35–specific CD8$^+$ T cell clone 4/43 or NY-ESO-1 119-143–specific CD4$^+$ Th clone 11/4 for 24 h in the presence of APCs pulsed with relevant or irrelevant peptides to Tregs. APCs pulsed with relevant or irrelevant peptides to CD8$^+$ or Th cells were added after 24 h incubation. Supernatants were collected and analyzed for IL-2 by ELISA after an additional 24 h incubation (*B*) or CFSE-labeled CD4$^+$ Th or CD8$^+$ T cell clones were stained intracellularly for IL-2 and analyzed by flow cytometry after an additional 6 h incubation (*C*). Upon cognate Ag activation, TRAG-3-specific CD4$^+$ Treg clone 61/58 inhibited IL-2 production by CD8$^+$ and CD4$^+$ Th cell clones stimulated with relevant peptides, and NY-ESO-1-specific CD4$^+$ Treg clone 103/3 inhibited IL-2 secretion by CD8$^+$ T cell clones. The strategy for gating on CFSE-labeled clones 11/4 and 4/43 as well as on CD4$^+$ Treg clones 61/58 and 103/3 is shown. As controls, Treg clones 61/58 and 103/3 did not produce IL-2 upon Ag specific-stimulation, and TRAG-3-specific Th clone 62/8 and NY-ESO-1–specific CD4$^+$ Th clone 107/5 did not inhibit IL-2 production by clones 11/4 and 4/43, respectively, upon recognition of their cognate Ag (*C*). One of three independent experiments is depicted. The mean ± SD of IL-2 secretion is shown.

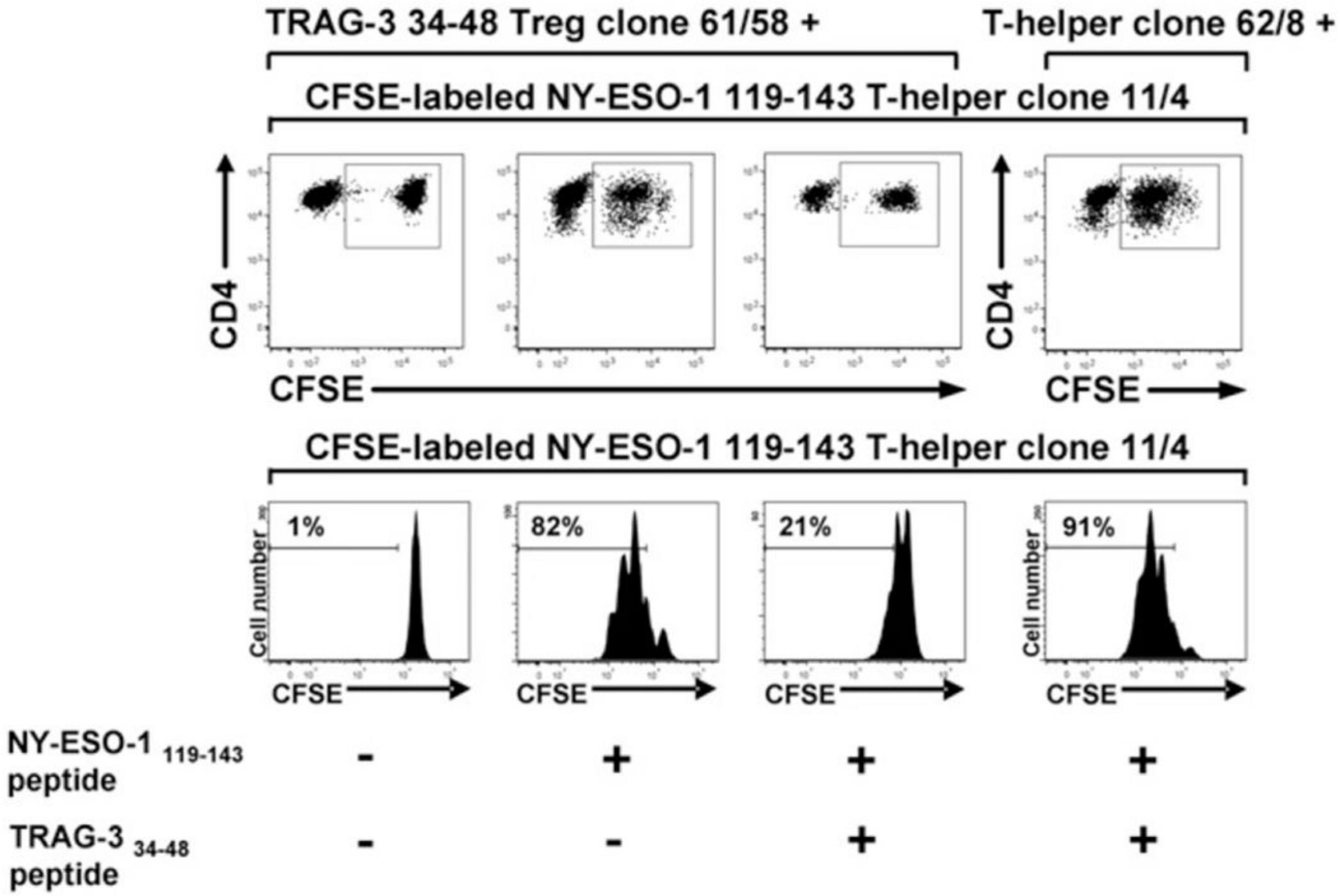

FIGURE 6.

Inhibition of IL-2 production by TRAG-3 34-48–specific CD4$^+$ Treg clone correlates with decreased proliferation of TA-specific Th clones. The proliferation of CFSE-labeled NY-ESO-1–specific CD4$^+$ Th clone 11/4 was assessed after 5 d of incubation with TRAG-3–specific Treg clone 61/58 in the presence of cognate-peptide–pulsed APCs. TRAG-3 34-48–specific Treg clone 61/58 inhibited proliferation of NY-ESO-1 119-143–specific Th clone 11/4 upon recognition of its cognate Ag but not in the presence of the irrelevant peptide. As control, TRAG-3–specific Th clone 62/8 did not inhibit proliferation of clone 11/4 upon recognition of its cognate Ag. One of two independent experiments is depicted.

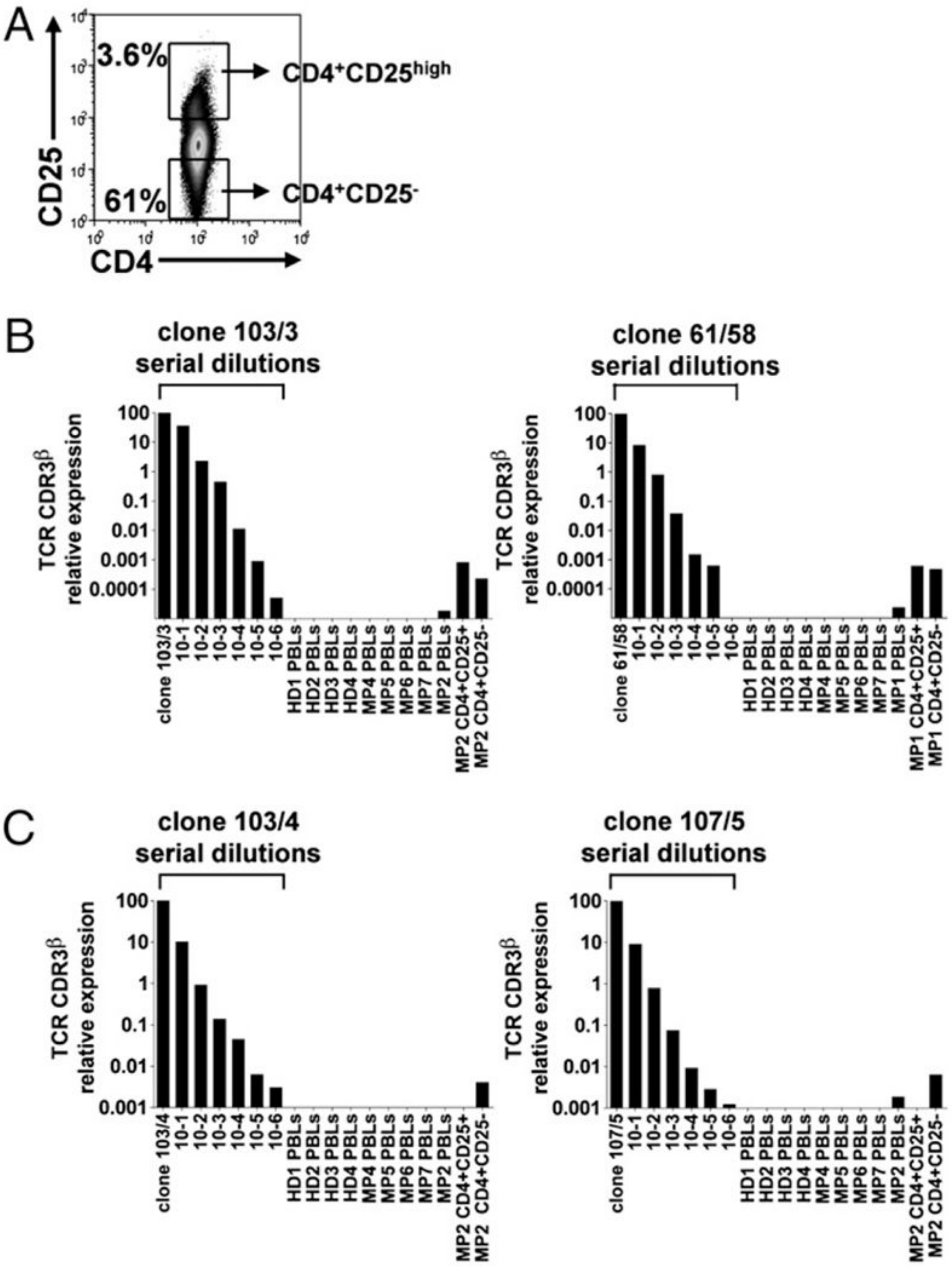

FIGURE 7.

NY-ESO-1–specific and TRAG-3–specific Tregs but not Th cells are detected in both CD4$^+$CD25^{high} and CD4$^+$CD25$^-$ fractions of PBLs isolated from patients with advanced melanoma. *A*, CD4$^+$ T cells were isolated ex vivo from PBLs of MP1 and MP2. CD4$^+$CD25^{high} and CD4$^+$CD25$^-$ fractions were sorted by flow cytometry prior to RNA extraction. *B* and *C*, The relative expression levels of the TCR CDR3β regions of NY-ESO-1-specifc CD4$^+$ Treg clone 103/3, TRAG-3–specific Treg clone 61/58 and NY-ESO-1–specific Th clones 103/4 and 107/5 in total PBLs of four MPs and four HDs and in

$CD4^+CD25^{high}$ and $CD4^+$ $CD25^-$ cells of MP2 or MP1 were evaluated with quantitative real-time RT-PCR and were correlated with different dilution ratios of each $CD4^+$ T cell clone in PBLs from one HD as described in Materials and Methods. Detectable levels of NY-ESO-1–specific Treg clone 103/3 (from MP2) and TRAG-3–specific Treg clone 61/58 (from MP1) CDR3p gene expression were found only in MP2 PBLs and MP1 PBLs, respectively, and in both the $CD4^+CD25^{high}$ and $CD4^+CD25^2$ T cell compartments of MP2 and MP1, respectively (B). NY-ESO-1–specific Th clones, 103/4 and 107/5 (from MP2), were detectable only in the $CD4^+CD25^-$ T cell compartment of MP2 (*C*). One of three independent experiments is depicted.

Table I.

TCR Vβ usage of TRAG-3 34-48–specific and NY-ESO-1 119-143–specific CD4$^+$ T cell clones obtained from PBLs of melanoma

Clones	Function	Vβ[a]	CDR3β		Jβ	
61/58	Treg	2.1	CAS	WGGLN	TDTQYF	2.3
103/3	Treg	22	CASS	HPGQGGSRG	TDTQYF	2.3
103/4	Th	6.7	CAS	GQTGV	TEAFF	1.1
107/5	Th	2.1	CSAR	VPSGG	SYNEQFF	2.1

[a] The TCR Vβ nomenclature is provided by the IMGT (www.imgt.org).