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mTOR inhibition modulates vaccine-induced immune responses to generate memory T cells in patients with solid tumors

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ABSTRACT

Background Perturbation of the mechanistic target of rapamycin (mTOR) pathway can instruct effector versus memory cell fate of tumor antigen-specific T cells in preclinical models. In this study, we sought to understand the impact of rapamycin (sirolimus), an mTOR inhibitor, on reprogramming vaccine-induced T cells to enhance memory responses in patients with solid tumors following completion of their standard therapy.

Methods We conducted three phase I clinical trials employing New York esophageal squamous cell carcinoma-1 (NY-ESO-1) vaccination approaches, with or without schedule-varied rapamycin. T cell phenotypes, functions, and Vβ usage in peripheral blood were analyzed to ask whether rapamycin influenced the generation of vaccine-induced T cells with memory attributes.

Results The addition of rapamycin to all vaccination approaches was safe and well tolerated. Immediate (days 1–14 postvaccination) or delayed (days 15–28 postvaccination) administration of rapamycin led to a significant increase in the generation of vaccine-induced NY-ESO-1-specific T cells exhibiting central memory phenotypes ($CD45RO^+CD45RA^-$ $CCR7^+$). Moreover, delayed administration resulted in a greater than threefold (p=0.025) and eightfold (p=0.005) increase in the frequency of NY-ESO-1-specific $CD4^+$ T and $CD8^+$ T cells respectively at the time of long-term follow-up, compared with its immediate usage.

Conclusion Our novel finding is that delayed administration of rapamycin to patients during the contraction phase of vaccine-induced antitumor immune responses was particularly effective in increasing the frequency of memory T cells up to 1 year postvaccination in patients with solid tumors. Further studies are warranted to identify the impact of this approach on the durability of clinical remission.

Trial registration number NCT00803569, NCT01536054, NCT01522820.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Preclinical studies have demonstrated that rapamycin, a mechanistic target of rapamycin inhibitor, instructed effector versus memory cell fate of tumor antigen-specific CD8+T cells, and the generation of T cell memory populations and tumor killing efficacy were rapamycin dose and duration dependent.

WHAT THIS STUDY ADDS

⇒ In three phase 1 clinical trials, schedule-varied rapamycin following antigen-specific vaccination consistently altered immune response dynamics and enhanced generation of memory T cells, consistent with preclinical studies.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study lays the foundation for larger clinical trials using rapamycin to enhance vaccine-induced memory T cell responses in patients with solid tumors.

INTRODUCTION

Recent advances in immuno-oncology have led to impressive clinical responses, with particularly notable successes observed for liquid tumors, such as leukemias and lymphomas.[1] Comparable clinical outcomes for immunotherapies in solid tumors have been less forthcoming, with fewer patients responding initially and a short-lived durability of responses. Numerous immunotherapeutic approaches have been examined in solid tumors, including immune checkpoint blockade,[2–4] vaccination against tumor-associated antigens,[5–10] and adoptive T cell therapy,[11 12] but sustained clinical responses remain infrequent. While the lack of prolonged clinical effect could be attributed to several immunosuppressive mechanisms in the tumor microenvironment,[13–17] the inability to generate high frequencies of antigen-specific memory $CD8^+$ T cell

responses for durable tumor control remains a gap in the field.[18]

The nature, intensity, and timing of instructions received by a naïve CD8+ T cell are all critical factors that determine its functional fate. In this regard, the energy-sensitive kinase known as the mechanistic target of rapamycin (mTOR) is a conserved serine/threonine kinase with the unique capacity to control cell fate. It achieves this by sensing and integrating diverse environmental signals, such as nutrients and growth factors, many of which deliver their inputs to the phosphoinositide 3-kinase-AKT pathway.[19] Although the mTOR inhibitor rapamycin (sirolimus) has been considered an immune suppressant,[20] it has also been found to increase the quantity and quality of memory CD8+ T cells during both expansion and contraction phases of the immune response in mouse models of lymphocytic choriomeningitis virus infection, suggesting that achieving optimal T cell fate requires a delicate balance between dose, schedule, and efficacy of rapamycin.[21]

In previous preclinical studies, we demonstrated the ability of mTOR to instruct effector versus memory cell fate of tumor antigen-specific CD8+ T cells, and shifts in T cell memory populations and tumor killing efficacy were rapamycin dose and duration dependent.[22 23] However, the potential role of rapamycin-mediated modulation of cancer vaccine immune responses has never been tested in humans. Therefore, we sought to understand the impact of rapamycin on reprogramming vaccine-induced T cells to boost memory responses and establish durable tumor immunity in patients with solid tumors.

To evaluate the effects of rapamycin dose and scheduling on the generation of tumor-specific immune responses, we focused on the "cancer-testis" antigen New York esophageal squamous cell carcinoma-1 (NY-ESO-1), a prototype tumor antigen for human cancer vaccine studies due to its unique characteristics of tissue-restricted expression and inherent immunogenicity.[24] Several early-phase clinical trials using NY-ESO-1 cancer vaccines reported integrated humoral as well as CD4+ and CD8+ T cell responses, with some reporting encouraging clinical outcomes.[25–32]

Here we report three clinical trials using varied NY-ESO-1 vaccination strategies combined with rapamycin in patients with ovarian and other solid tumors. The primary objectives were to assess the safety of rapamycin in combination with vaccination using either (1) the recombinant canarypox viral vector encoding NY-ESO-1 and the TRIad of CO-stimulatory Molecules (TRICOM) B7.1 (CD80), ICAM-1 (CD54), and LFA-3 (CD58), designated as ALVAC(2)-NY-ESO-1(M)/TRICOM;[7 33 34] or (2) autologous dendritic cells (DCs) pulsed with CDX-1401,[35] a human monoclonal antibody targeting the endocytic DEC-205 receptor fused to the NY-ESO-1 protein (DC+CDX-1401). Secondary objectives were to identify a rapamycin administration schedule that elicits memory T cell responses.

We found that these NY-ESO-1 vaccination approaches in combination with rapamycin were safe and well tolerated, and successfully induced humoral and cellular immune responses. Notably, delayed administration of rapamycin in the postvaccination contraction phase of immune response significantly increased NY-ESO-1-specific T cells with memory attributes compared with immediate or continuous rapamycin administration, suggesting a potential benefit for including rapamycin in cancer vaccination strategies.

METHODS

Clinical trial design

Participants previously received surgery and/or chemotherapy for primary or recurrent disease and would otherwise be entering a standard period of observation with a life expectancy greater than 6 months. All patient tumors expressed NY-ESO-1 or LAGE-1 antigen as determined by reverse transcription-PCR (RT-PCR) or immunohistochemistry.[24]

NCT00803569 (as listed in ClinicalTrials.gov), Trial A (IRB approval identifiers I-125207/NYU 07-790/LUD2007-005): non-randomized, multicenter Phase I study of the cancer vaccine ALVAC(2)-NY-ESO-1(M)/TRICOM(vCP2292), consisting of a replication-defective recombinant canarypox virus (ALVAC(2)) encoding the cancer-testis antigen NY-ESO-1 and TRICOM (B7-1, ICAM-1 and LFA-3), administered subcutaneously (0.5 mL dose containing ≥ 1×10^7 Cell Culture Infectious Dose 50% (CCID50)/mL) on day 1 of a 28-day cycle for up to 6 cycles in patients with epithelial ovarian, fallopian tube, or primary peritoneal carcinoma (online supplemental figure 1). There were 13 participants in this trial. Additionally, granulocyte macrophage-colony stimulating factor (GM-CSF) sargramostim (100 µg) was administered on days 1 through 4, within a 4 cm circumference of the vaccine injection site.

NCT01536054 (as listed in ClinicalTrials.gov), Trial B (IRB approval identifier I-199911): non-randomized phase I study of ALVAC(2)-NY-ESO-1(M)/TRICOM(vCP2292) combined with rapamycin (sirolimus) in patients with epithelial ovarian, fallopian tube, or primary peritoneal carcinoma (online supplemental figure 1) (n=7). The study design mirrored that of Trial A but includes the addition of rapamycin. ALVAC(2)-NY-ESO-1(M)/TRICOM(vCP2292) was administered subcutaneously at a dose of 0.5 mL containing ≥ 1×10^7 CCID50/mL, alongside rapamycin at a dose of 4 mg rapamycin taken orally once daily during the expansion phase of the immune response (days 1–14), repeated every 28 days for 4 cycles. GM-CSF was also administered on days 1–4 of each cycle.

NCT01522820 (as listed in ClinicalTrials.gov), Trial C1/C2 (IRB approval identifier I-191511): non-randomized phase I study of the DC vaccine, autologous DC pulsed with CDX-1401, which is a fusion protein consisting of a fully human monoclonal antibody directed against the endocytic DC receptor, DEC-205, linked to the

tumor-associated antigen NY-ESO-1. The methods to create autologous DC+CDX-1401 vaccines are described in online supplemental material. The vaccine was administered intranodally at a concentration of 1×10^7 cells/mL on days 1, 29, 57, and 113, either without (Trial C1) or with (Trial C2) rapamycin at a fixed dose but varying schedule in patients who have completed standard frontline therapy for ovarian cancer or other solid tumors with high likelihood of recurrence (kidney, bladder, prostate, lungs, esophageal, gastrointestinal, melanoma, brain, hepatocellular, breast, uterine and sarcomas) (online supplemental figure 1). Patients in Trial C1 received only the DC+CDX-1401 vaccine. Trial C2 patients consisted of 3 cohorts: cohort C2a received the DC+CDX-1401 vaccine plus 4 mg of rapamycin taken orally once daily for 14 days immediately following vaccination (corresponding to the expansion phase of immune response: days 1–14, 29–42, and 57–70); cohort C2b received the DC+CDX-1401 vaccine and a delayed dose of 4 mg of rapamycin taken orally once daily for 14 days during the contraction phase of the immune response (days 15–28, 43–56, and 71–84); and cohort C2c received rapamycin at a dose of 4 mg taken orally once daily continuously (days 1–84) postvaccination.

Serum and peripheral blood mononuclear cell collection

Blood samples were collected and processed from patients prior to vaccination and at various time points during and after the course of treatment for immune assessment (online supplemental figure 1), and details are provided in online supplemental materials.

NY-ESO-1 serum antibody titer

Humoral responses to NY-ESO-1 protein and other tumor antigens (LAGE-1/*CTAG2*, p53/*TP53*) were determined by ELISA, and reciprocal titers were calculated from the relative fluorescence units of serially diluted samples as described previously.[36] Specificity was determined as described in online supplemental materials.

NY-ESO-1-specific T cell responses

NY-ESO-1-specific $CD4^+$ and $CD8^+$ T-cell responses were assessed following in vitro presensitization with a pool of NY-ESO-1 overlapping peptides,[27] and IFN-γ ELISpot assay (Mabtech) as described in online supplemental materials. Intracellular cytokine staining and ex vivo phenotyping of isolated $CD4^+$ or $CD8^+$ T cells were performed as described in online supplemental materials.

TCR Vβ sequencing

Total DNA from peripheral blood mononuclear cells (PBMCs) or from $CD4^+$ or $CD8^+$ sorted cells, presensitized with a pool of NY-ESO-1 overlapping peptides, was extracted using the QIAamp DNA kit (QIAGEN). T-cell receptor (TCR) β-chain CDR3 regions were sequenced and analyzed using the ImmunoSEQ platform (Adaptive Biotechnology, Seattle, Washington, USA) as previously described.[37] Additional details are provided in online supplemental materials.

Statistical considerations, analyses, and data availability

For all three phase I studies, primary objectives were to determine the safety of the vaccine regimens with or without rapamycin, and to assess humoral, $CD4^+$ and $CD8^+$ T cell responses. A standard 3+3 design was used for each trial as follows: (1) If 0/3 or 1/3 dose-limiting toxicities (DLTs) are observed, expand the cohort by an additional three patients. If 0/6 or 1/6 DLTs are observed, proceed to the next treatment regimen; (2) If at least 2/6 DLTs are observed, stop and declare the regimen as not tolerable; (3) if at least 2/3 DLTs are observed stop and declare the regimen as not tolerable. Analyses of continuous immunological response endpoints (antibody titers; NY-ESO-1 specific $CD8^+$ and $CD4^+$ frequency and function; frequency of memory T cell populations) were conducted using an analysis of covariance model, with post-treatment levels modeled as a function of pretreatment levels. Statistical analyses for specific experimental approaches are further described in the respective methods sections. Data for all biological assays and patient characteristics are provided as supplemental tables and figures. Under New York State law, TCR Vβ sequencing unprocessed data are not available due to patients not specifically being consented for broad sharing of these data.

RESULTS

Study design and safety profile of vaccine regimens

In total, 38 patients were enrolled in one of three phase I clinical trials (online supplemental table 1 and figure 1). Participants had previously undergone surgery and/or chemotherapy for primary or recurrent disease and were now entering a routine observation period. All tumors were confirmed as positive for NY-ESO-1 or LAGE-1 expression by RT-PCR and/or immunohistochemistry as previously described.[7 8] Trial A and Trial B enrolled patients with stage II–IV ovarian epithelial cancer. In Trial A, patients (n=13) received ALVAC(2)-NY-ESO-1(M)/TRICOM vaccine without rapamycin, while in Trial B, patients (n=7) received the same vaccine with immediate rapamycin administration (days 1–14) during the expansion phase of the immune response. Trial C enrolled patients with any solid tumor at high risk of recurrence, including fallopian tube cancer, melanoma, myxofibrosarcoma, ovarian cancer, peritoneal adenocarcinoma, renal cell carcinoma, small cell lung cancer, squamous cell carcinoma, and uterine sarcoma. In Trial C, patients (n=18) received CDX-1401-pulsed DCs (DC+CDX-1401) vaccination and were divided into Trial C1 (n=3) without rapamycin and Trial C2 (n=15) with rapamycin. There were no major (>grade 2) treatment-related toxicities in any of the trials. Patient characteristics (online supplemental table 1) and adverse event profiles (online

supplemental table 2) across all clinical trials are shown in online supplemental materials.

Rapamycin administered immediately following ALVAC(2)-NY-ESO-1(M)/TRICOM vaccination elicits tumor-specific T cells with memory attributes

To assess the impact of rapamycin on vaccine-induced T cell responses, we compared Trial A (ALVAC(2)-TRICOM-NY-ESO-1) vaccination without rapamycin, with Trial B (ALVAC(2)-TRICOM-NY-ESO-1) vaccination with immediate rapamycin administration (days 1–14). The frequency and specificity of NY-ESO-1-specific $CD8^+$ and $CD4^+$ T cell responses were assessed using peripheral blood from patients before and after vaccination, using an in vitro sensitization protocol. NY-ESO-1-specific T cell activation was measured by IFN-γ ELISpot assays in response to 17 overlapping 20-mer peptides spanning the full-length NY-ESO-1 protein as previously described.[27]

T cell responses to immunodominant regions within NY-ESO-1 were identified consistently across all patients, regardless of the therapy regimen. $CD4^+$ T cell responses were generally broader than $CD8^+$ T cell responses against the peptide epitopes, with prominent $CD4^+$ T cell activation observed against NY-ESO-$1_{81–110}$ and NY-ESO-$1_{119–143}$, and weaker responses against NY-ESO-$1_{41–80}$ and NY-ESO-$1_{151–180}$ (figure 1A,B; online supplemental figure 2). $CD8^+$ T cell activation was more focal, with notable responses observed against NY-ESO-$1_{81–110}$ peptides (figure 1A,B; online supplemental figure 3). These immunodominant peptide regions corroborate findings that we and others have previously reported.[26 27] NY-ESO-1-specific $CD8^+$ T cell frequency was also determined by tetramer staining in patients with HLA-A*02:01 (18 patients), HLA-B*35:01 (7 patients), or HLA-Cw*03:03/03:04 (7 patients) (figure 1C–E).

Pre-existing NY-ESO-1-specific CD8+T cell responses were detected in only one patient across both ALVAC-TRICOM trials. Postvaccination induction of $CD8^+$ T cell responses to NY-ESO-1 were less frequent after treatment with rapamycin (2/7, 29%) compared with ALVAC-TRICOM alone (6/13, 46%); see figure 1B, (online supplemental figure 3 and table 3). In contrast, induction of $CD4^+$ T cell responses to NY-ESO-1 were more robust and widespread across both Trials A and B, although responses were mildly diminished in patients treated with rapamycin (figure 1A, online supplemental figure 2 and table 3). Seven patients across Trials A and B cohorts who were baseline-positive for NY-ESO-1-specific $CD4^+$ T cells remained positive postvaccination. Most patients with undetectable NY-ESO-1-specific $CD4^+$ T cells prior to vaccination demonstrated de novo responses postvaccination. Induction rates were 66.7% for ALVAC-TRICOM alone and 100% for ALVAC-TRICOM with rapamycin (online supplemental table 3).

Percentages of cells exhibiting central memory phenotypes ($CD45RO^+CD45RA^-CCR7^+$) were increased in patients treated with rapamycin (figure 2). Percentages of $CD4^+CD45RO^+CD45RA^-CCR7^+$ T cells also were increased for at least one time point in 1/4 patients from Trial A and 4/7 patients from Trial B (figure 2B). Overall, percentages of $CD4^+$ and $CD8^+$ T cells presenting with a central memory phenotype were significantly higher in postvaccination samples from Trial B compared with those from Trial A (figure 2C; $p=9.73\times10^{-5}$ and 5.39×10^{-4} for $CD4^+$ and $CD8^+$ T cells, respectively). Correspondingly, $CD4^+CD45RO^+CD45RA^-CCR7^-$ effector memory T cell populations were significantly reduced in rapamycin-treated patients in Trial B relative to patients in Trial A (figure 2C; $p=4.21\times10^{-4}$).

Rapamycin diminished the magnitude of humoral responses

Vaccine-induced humoral immune responses were compared between Trial A (vaccination alone) versus Trial B (vaccination plus rapamycin) (figure 3). In Trial A, three patients were baseline seropositive (antibody reciprocal titer>100) for anti-NY-ESO-1 antibody and remained so throughout treatment, with two patients experiencing a boost in antibody levels postvaccination (figure 3A and online supplemental table 3). All other baseline seronegative patients (10/10) became seropositive during treatment, and none reverted to a seronegative state by the end of the monitoring period. Therapy-induced maximum seroreactivity was most observed at middle time points (7/10) rather than early (1/10) or late (2/10) time points ($p<0.05$).

Antibody responses against LAGE-1, which shares 94% homology with NY-ESO-1, were also assessed in Trial A (online supplemental figure 4). Baseline LAGE-1 seroreactivity was predominantly negative, and therapy-induced humoral responses to LAGE-1 were observed in eight patients and mirrored the patterns of NY-ESO-1-induced humoral responses (online supplemental figure 4 and figure 3A). Four patients remained seronegative for LAGE-1 throughout. Next, given that *TP53* mutations are frequent in solid cancers and high frequencies of naturally occurring anti-p53 antibody responses have been previously observed,[38] antibody responses to p53 were also investigated (online supplemental figure 4). Two patients had a baseline humoral response to p53, and only one patient developed anti-p53 antibodies during vaccine therapy.

Patients in Trial B treated with ALVAC(2)-NY-ESO-1(M)/TRICOM and rapamycin (days 1–14) displayed a humoral response profile for NY-ESO-1 reactivity like that of patients in Trial A (figure 3A and online supplemental table 3). Most baseline seronegative patients (4/5) achieved seropositivity at some point postvaccination. Although long-term serological data are not available for Trial A participants, the humoral response in Trial B patients was sustained in one of the four patients who initially exhibited a vaccine-induced response (figure 3A).

Notably, quantitative comparisons between Trial A and Trial B revealed that the addition of rapamycin reduced the magnitude of amplification in the humoral response throughout the monitoring period (median $\log_2$ fold

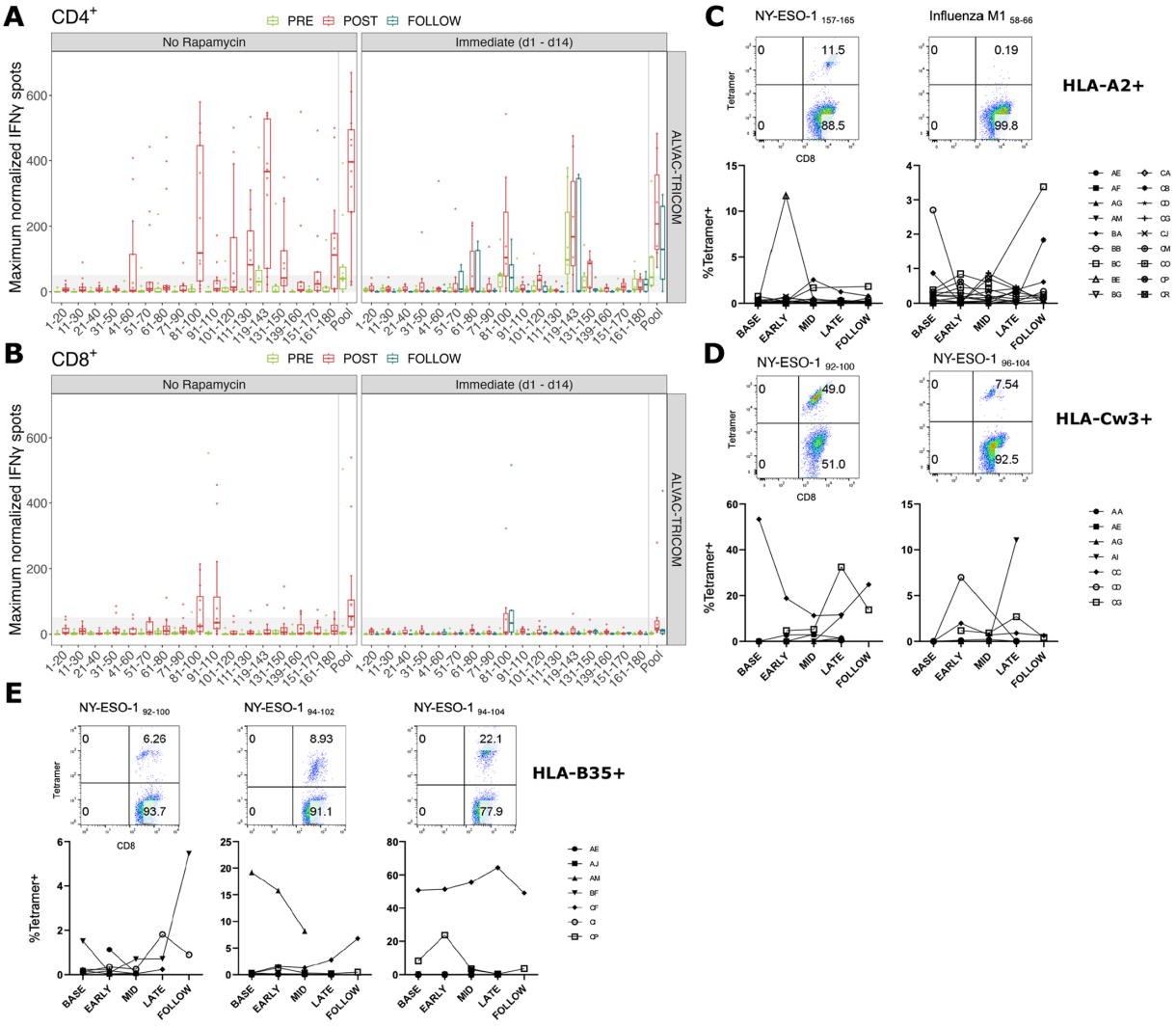

Figure 1 NY-ESO-1 peptide-induced activation of T cells and tetramer frequency in ALVAC-TRICOM-treated patients. PBMC-isolated CD4$^+$ (A) and CD8$^+$ (B) T cell NY-ESO-1-specific activation, reported as IFN-γ spots per 5×10^4 cells obtained in ELISpot assays after incubation with individual or pooled 20mer overlapping NY-ESO-1 peptides in ALVAC-TRICOM trials. The maximum number of normalized IFN-γ spots obtained for each patient at prevaccination (green), postvaccination (red), or follow-up (blue) binned time points are reported. All boxplots display the first quartile, third quartile, and median. (C–E) Isolated CD8$^+$ T cells were presensitized in vitro with a pool of NY-ESO-1 overlapping peptides for 12–14 days and stained with anti-CD8 antibody along with HLA-A*0201/NY-ESO-1$_{157–165}$ and HLA-A*0201/Influenza M1$_{58–66}$ tetramers from HLA-A*0201$^+$ patients (C), HLA-Cw*03/NY-ESO-1$_{92–100}$ and HLA-Cw*03/NY-ESO-1$_{94–106}$ tetramers from HLA-Cw*03$^+$ patients (D), or HLA-B*3501/NY-ESO-1$_{92–100}$, HLA-B*3501/NY-ESO-1$_{94–102}$ and HLA-B*3501/NY-ESO-1$_{94–104}$ tetramers from HLA-B*3501$^+$ patients (E). The representative flow cytometry plot of each tetramer and the change of tetramer frequency for each patient are shown. NY-ESO-1, New York esophageal squamous cell carcinoma-1; PBMC, peripheral blood mononuclear cell; TRICOM, the TRIad of CO-stimulatory Molecules.

change: 7.45 in Trial A vs 3.69 in Trial B), although this difference did not reach statistical significance (p=0.121) (figure 3B).

Delayed administration of rapamycin following vaccination with an autologous DC vaccine (DC+CDX-1401) also elicits tumor-specific T cells with memory attributes

To validate the impact of rapamycin on vaccine-induced immune responses noted in Trials A and B, we tested three additional parameters: (1) impact of rapamycin on a different vaccination modality using autologous DCs pulsed with CDX-1401, a human monoclonal antibody directed against the endocytic DEC-205 receptor fused to NY-ESO-1 protein (DC+CDX-1401) (Trial C); (2) impact of timing of rapamycin administration as immediate (days 1–14), delayed (days 14–28), or continuous (days 1–28) in relation to vaccination (online supplemental figure 1); and (3) impact of rapamycin on vaccine-induced immune responses in patients with non-ovarian solid tumors.

Based on the schema of rapamycin administration in Trial C (DC+CDX-1401, online supplemental figure 1), patient cohorts correspond to Trial C1 (no rapamycin) and Trial C2, which consists of immediate (days 1–14), delayed (days 15–28), or continuous (days 1–28) administration. Pre-existing NY-ESO-1-specific CD4$^+$ T cells were detected

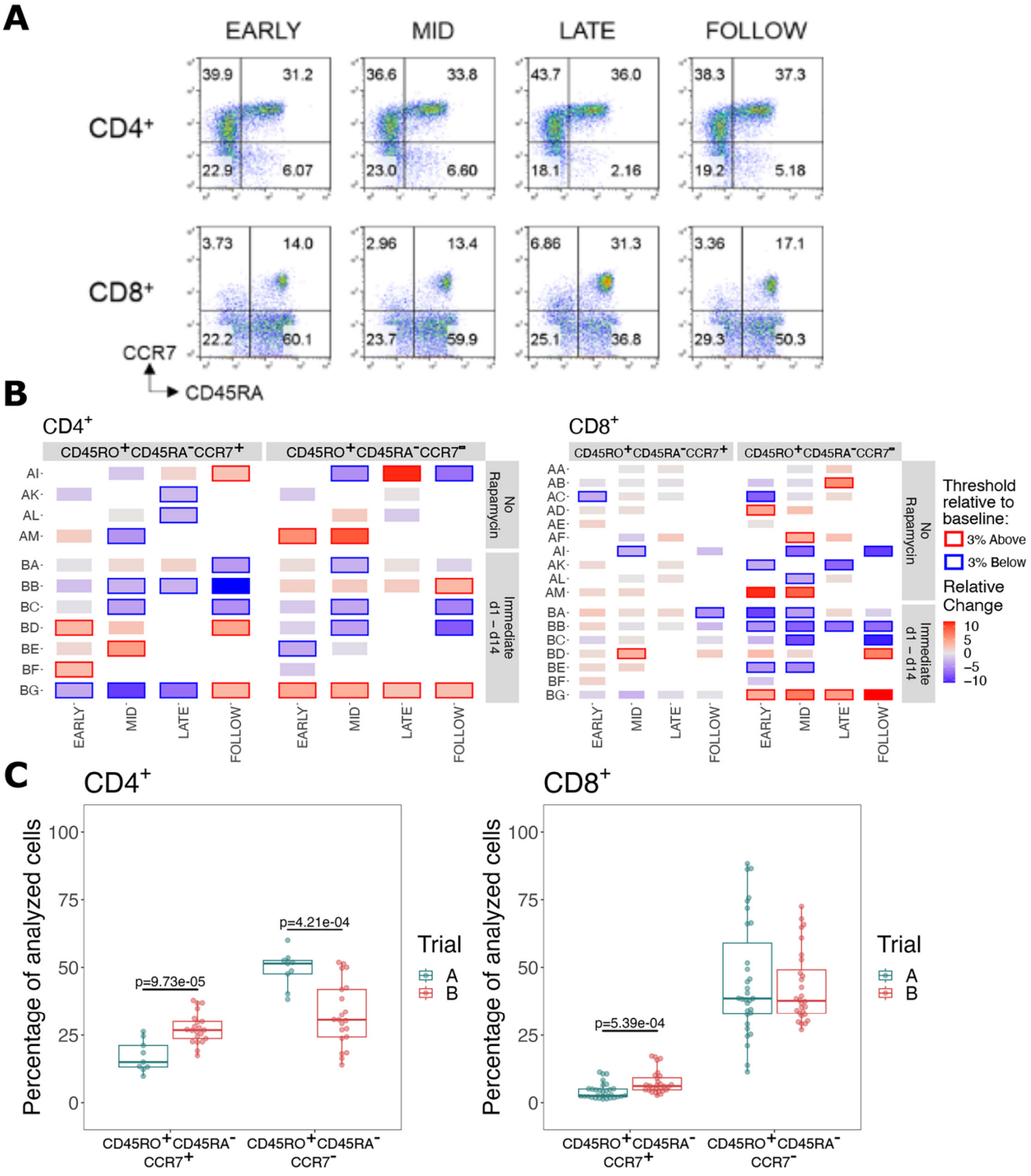

Figure 2 NY-ESO-1 ALVAC-TRICOM vaccination in combination with rapamycin shifts memory T cell populations. (A) Representative flow cytometry scatterplots for CD45RA and CCR7 expression on $CD4^+$ and $CD8^+$ T cells from patient BD at different time points. (B) Patient-specific heatmaps of relative changes in $CD4^+$ and $CD8^+$ central memory ($CD45RO^+CD45RA^-CCR7^+$) and effector memory ($CD45RO^+CD45RA^-CCR7^-$) cell populations relative to baseline, as determined by flow cytometry from available patient samples throughout the ALVAC-TRICOM±rapamycin trials. (C) Comparison of T cell memory populations between vaccination Trial A and Trial B using all postvaccination samples (p value is calculated using ANOVA with post hoc Tukey). Boxplots represent the first quartile, median, and third quartile. ANOVA, analysis of variance; NY-ESO-1, New York esophageal squamous cell carcinoma-1; TRICOM, the TRIad of CO-stimulatory Molecules.

in 10 out of 18 (56%) patients in all Trial C cohorts, and responses were sustained throughout subsequent post-vaccination time points (figure 4A, online supplemental figure 2 and table 3). Additionally, 5 patients achieved de novo induction of an NY-ESO-1-specific $CD4^+$ T cell response after vaccination resulting in a total of 15 out of 18 patients (83%) with NY-ESO-1-specific $CD4^+$ T cell responses. A subset of these patients (3/5) did not exhibit $CD4^+$ NY-ESO-1 activation until long-term follow-up evaluation. NY-ESO-1-specific $CD8^+$ T cell responses were less frequent than $CD4^+$ T cell responses (figure 4A, online supplemental figure 3 and table 3), like patients in Trials

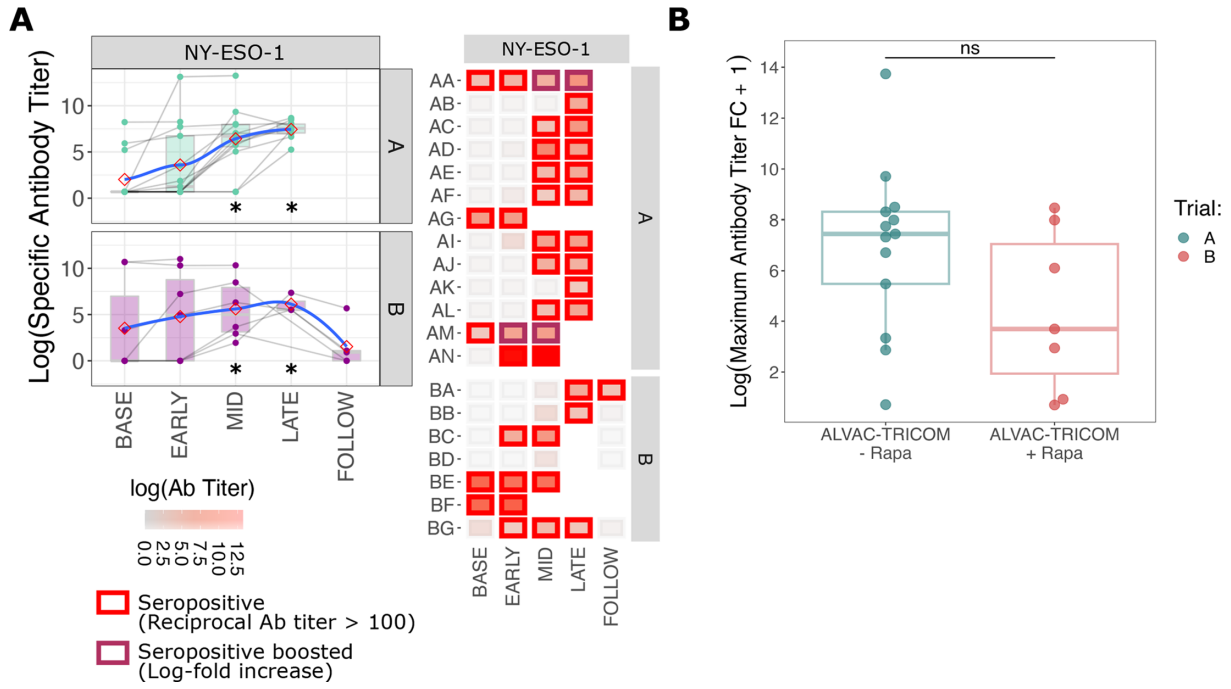

Figure 3 NY-ESO-1 humoral response in ALVAC-TRICOM treated patients. (A) Time-course analysis of serum NY-ESO-1 mean reciprocal antibody titers determined by ELISA relative fluorescence of serially diluted samples at baseline, early, mid, late, and follow-up time groups for ALVAC-TRICOM±rapamycin clinical trials (* indicates p<0.05, linear regression model), including a heatmap of patient-specific mean antibody titers. Thresholds for seropositivity are indicated by a red outline and boosted seropositivity is indicated by purple. An empty cell indicates sample collection was not available at that time point. (B) The maximum fold change of NY-ESO-1 mean reciprocal antibody titer achieved per patient in Trial A (without rapamycin) versus Trial B (with rapamycin), with boxplots representing the first quartile, median, and third quartile (p=0.121, student t-test). Ab, antibody; NY-ESO-1, New York esophageal squamous cell carcinoma-1; TRICOM, the TRIad of CO-stimulatory Molecules.

A and B. Only three patients (17%) exhibited de novo induction of NY-ESO-1-specific $CD8^+$ T cells postvaccination, and five patients (27%) had a pre-existing and sustained $CD8^+$ T cell response. In total, 8 patients (44%) achieved a $CD8^+$ T cell response to NY-ESO-1.

Importantly, the percentages of $CD8^+CD45RO^+CD45RA^-CCR7^+$ central memory T cells in postvaccination patient samples from Trial C2 with rapamycin were significantly increased compared with vaccination with DC+CDX-1401 alone (Trial C1; figure 4B and D; analysis of variance (ANOVA) with post hoc Tukey, p=0.031). Additionally, Trial C2 resulted in a significantly greater number of $CD8^+CD45RO^+CD45RA^-CCR7^-$ effector memory T cells in postvaccination samples compared with Trial C1 (figure 4B, p=0.015). For CD4+T cells, differences in percentages of $CD4^+CD45RO^+CD45RA^-CCR7^+$ central memory cells did not reach statistical significance (figure 4C). However, the addition of rapamycin was associated with an increase in $CD4^+CD45RO^+CD45RA^-CCR7^+$ central memory cells relative to baseline for at least one time point in 6 out of 15 participants (40%) in Trial C2, compared with 0 out of 3 participants (0%) in Trial C1 without rapamycin (figure 4D). These results are generally consistent with findings from Trials A and B, suggesting that rapamycin influenced the generation of memory T cells irrespective of vaccination approach.

We then assessed the impact of the timing of rapamycin administration in Trial C2 relative to vaccination on the induction of T cells with a memory phenotype by comparing cells from patients with immediate (days 1–14), delayed (days 15–28), and continuous (days 1–28) rapamycin administration. We observed that $CD4^+$ T cells from patients receiving rapamycin during the contraction phase (ie, delayed administration) achieved more than a threefold increase in antigen-specific T cell responses compared with samples from patients who received no rapamycin or those who received immediate rapamycin administration (p=0.025, ANOVA post hoc Tukey, figure 4E). Similarly, $CD8^+$ T cells from long-term follow-up samples of delayed rapamycin treatment exhibited an 8-fold and 26-fold increase in average T cell activation rates compared with those with immediate or continuous rapamycin treatment, respectively (p=0.005 and p=0.007, respectively, figure 4F).

Notably, delayed administration of rapamycin during the contraction phase of vaccination resulted in the most substantial persistence of NY-ESO-1-specific T cells at follow-up periods (6 and 12 months post-final vaccination) for both $CD4^+$ and $CD8^+$ T cells (figure 4A, E and F). These findings from our phase I clinical trials are highly consistent with our preclinical studies, which together suggest an ability of rapamycin to polarize T cells toward central memory phenotypes.[22 23]

To provide a more comprehensive presentation of immune responses and patient outcomes, we included Kaplan-Meier analyses of time to progression, stratified

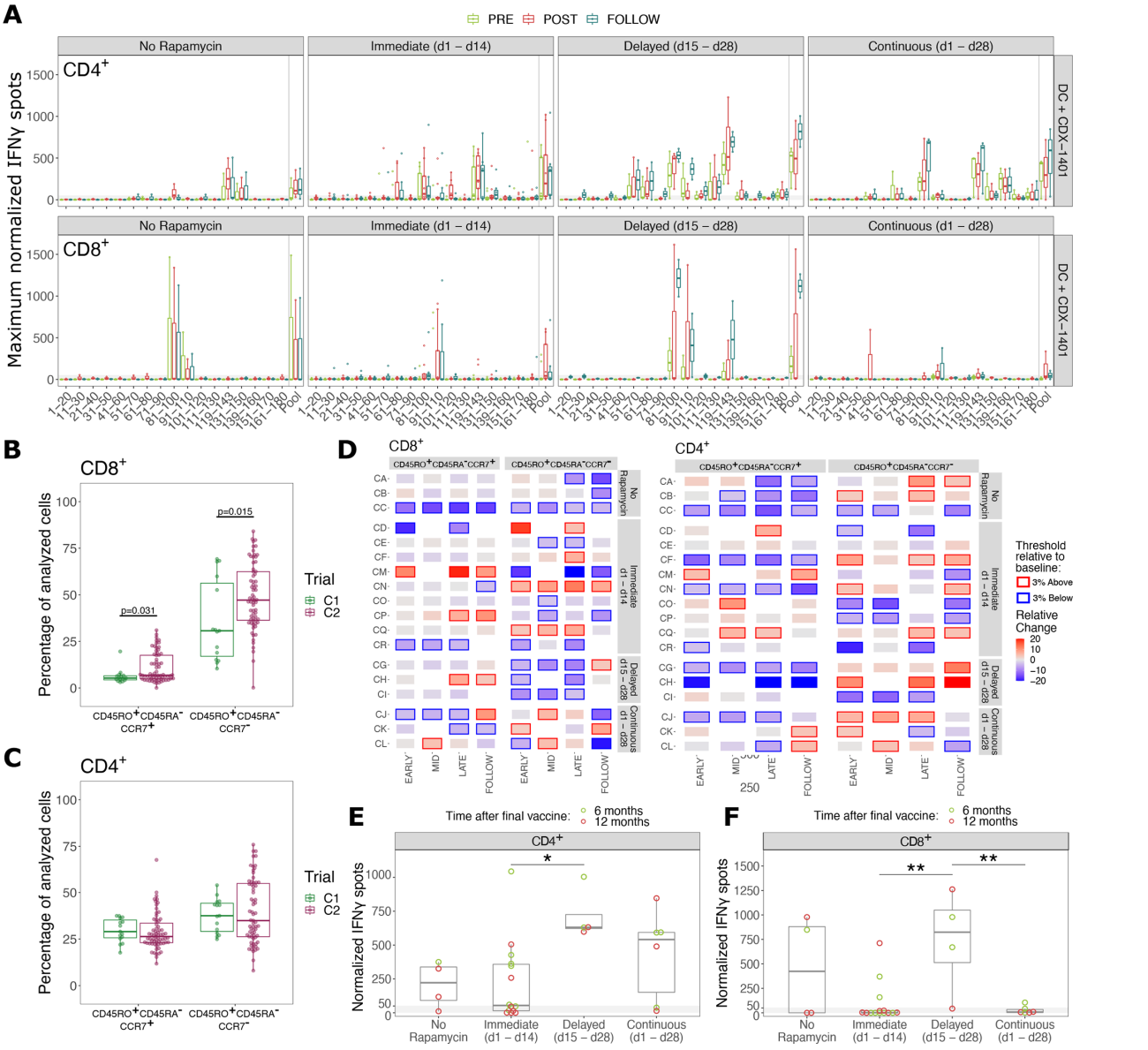

Figure 4 Characterization of NY-ESO-1 specific immune responses in patients treated with DC+CDX-1401, with and without rapamycin. (A) PBMC-isolated CD4$^+$ and CD8$^+$ T cell NY-ESO-1-specific activation, reported as IFNγ spots per 5×10^4 cells in ELISpot assays, after incubation with individual or pooled overlapping NY-ESO-1 peptides in DC+CDX-1401 trials (Trial C2 with rapamycin, and Trial C1 without rapamycin). The highest number of normalized IFNγ spots obtained for each patient at prevaccination (green), postvaccination (red), or follow-up (blue) binned time points are presented. A comparison of CD8$^+$ (B) and CD4$^+$ (C) T cell central memory (CD45RO$^+$CD45RA$^-$CCR7$^+$) and effector memory (CD45RO$^+$CD45RA$^-$CCR7$^-$) populations between Trial C1 and Trial C2 for all postvaccination samples collected (ANOVA with post hoc Tukey). (D) Patient-specific heatmaps show the relative changes in CD4$^+$ and CD8$^+$ central memory (CD45RO$^+$CD45RA$^-$CCR7$^+$) and effector memory (CD45RO$^+$CD45RA$^-$CCR7$^-$) cell populations relative to baseline, as determined by flow cytometry from available patient samples throughout the trials. The NY-ESO-1 pooled peptide ELISpot assay of CD4$^+$ (E) and CD8$^+$ (F) T cells for all follow-up samples collected at 6 and 12 months (green and red, respectively) in DC+CDX-1401 trials is grouped by rapamycin treatment regimens (ANOVA with post hoc Tukey; *p value<0.05, **p value<0.01). All boxplots represent the first quartile, median, and third quartile. ANOVA, analysis of variance; DC, dendritic cell; NY-ESO-1, New York esophageal squamous cell carcinoma-1; PBMC, peripheral blood mononuclear cell.

by the induction of cellular (online supplemental figure 5A,B) or humoral (online supplemental figure 5C) immune responses specific to NY-ESO-1. Although not statistically significant (p>0.05), NY-ESO-1 vaccine-induced activation of CD4$^+$ or CD8$^+$ T cells is associated with a modest progression-free survival benefit. Additionally, the log-fold induction of NY-ESO-1 antibody titers is significantly associated with improved progression-free survival (log rank test, p=0.049).

Specificity and effector functions of vaccine-induced CD8$^+$ and CD4$^+$ T cell responses

Next, we assessed functional capacities of vaccine-induced CD4$^+$ and CD8$^+$ T cells as previously described.[7] Across

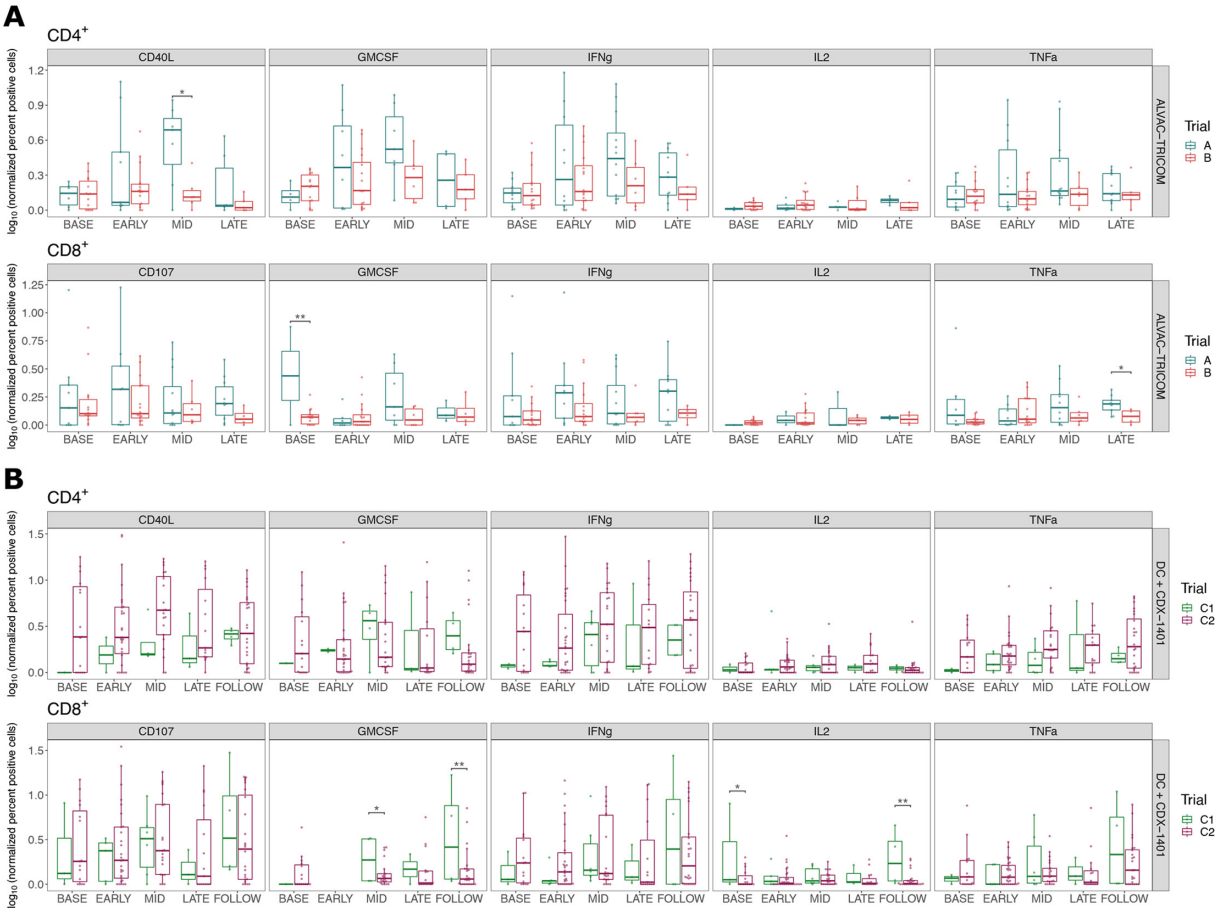

Figure 5 NY-ESO-1-induced cytokine production in CD4+ and CD8+ T cells. Intracellular staining of immune-modulating cytokine in PBMC-isolated CD4+ and CD8+ T cells that were presensitized with autologous NY-ESO-1 peptide-pulsed, CD4+/CD8+ depleted cells. The results were reported as the percentage of staining cells normalized to unpulsed controls in either (A) ALVAC-TRICOM trials (Trials A and B) or (B) DC+CDX-1401 trials (Trials C1 and C2) across all time points of sample collection. Statistical significance was assessed using ANOVA with post hoc Tukey (*p<0.05, **p<0.01). Boxplots represent the first quartile, median, and third quartile. ANOVA, analysis of variance; DC, dendritic cell; GM-CSF, granulocyte macrophage-colony stimulating factor; IL2, interleukin 2; NY-ESO-1, New York esophageal squamous cell carcinoma-1; PBMC, peripheral blood mononuclear cell; TRICOM, the TRIad of CO-stimulatory Molecules.

all clinical trials, when normalized to unpulsed controls, NY-ESO-1-induced T cell cytokine production (figure 5) strongly phenocopies the respective activation profiles observed for CD4+ and CD8+ T cell activation measured by NY-ESO-1 ELISpot assay (figures 1 and 4A). ALVAC(2)-NY-ESO-1(M)/TRICOM vaccination (Trial A) induced greater antigen-specific CD4+ T cell activation than inclusion of rapamycin (Trial B), indicated by a higher percentage of NY-ESO-1 peptide-pulsed cells staining positive for cytokines CD40L, GM-CSF, IFNγ, and TNFα postvaccination; for instance, CD40L expression was significantly reduced (p<0.05, ANOVA post hoc Tukey; figure 5A). CD8+ T cell responses stimulated by ALVAC(2)-NY-ESO-1(M)/TRICOM vaccination were overall less robust than those observed in CD4+ T cells as measured by intracellular cytokine staining (figure 5A). Samples from rapamycin-treated patients (Trial B) displayed fewer cells producing notable cytokine levels compared with non-rapamycin-treated counterparts (Trial A), particularly for CD107, GM-CSF, IFNγ, and TNFα cytokines (ANOVA post hoc Tukey, p<0.05; figure 5A).

In patients vaccinated with DC+CDX-1401 and treated with rapamycin (Trial C2) there was minimal variation in cytokine production across time points (figure 5B). In contrast, CD4+ T cells from patients in Trial C1 (without rapamycin) displayed increased cytokine production indicative of vaccine-induced antigen recognition at early, mid, late, and follow-up time points as compared with baseline for most cytokines assayed (CD40L, GM-CSF, IFNγ, and TNFα; figure 5B). Variations in cytokine production in NY-ESO-1-pulsed CD8+ T cells from patients treated with DC+CDX-1401 were less pronounced compared with that of NY-ESO-1-pulsed CD4+ T cells. Populations of CD8+ T cells producing CD107, IFNγ, and TNFα were comparable between treatments (figure 5B).

Mapping TCR repertoire evolution in response to vaccination with and without rapamycin

TCR β-chain complementary-determining region 3 (TCRβ-CDR3) sequencing revealed characteristics of evolving peripheral T cell repertoires on vaccination and in combination with rapamycin. To identify putative

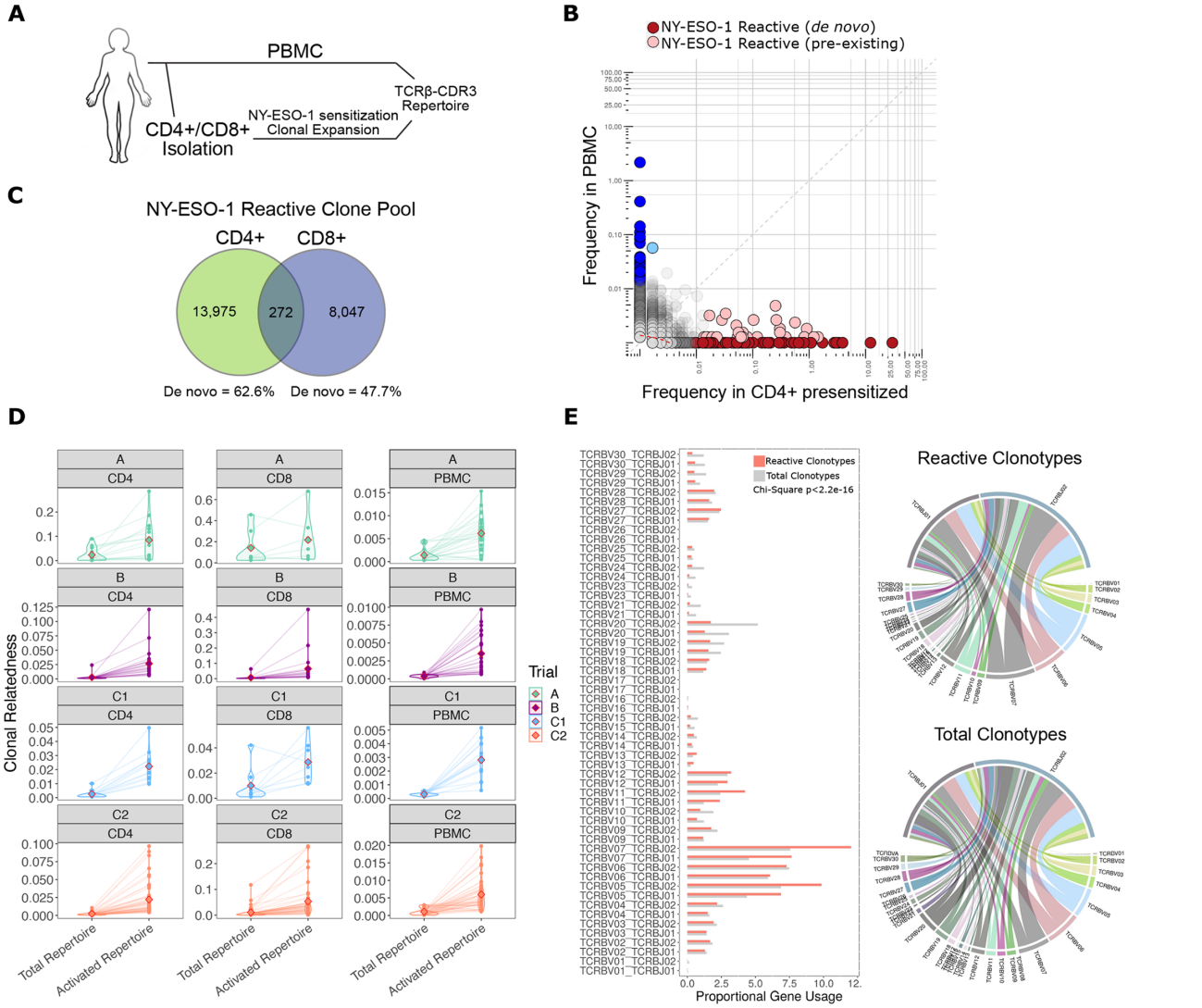

Figure 6 Landscape of TCRβ-CDR3 repertoire on NY-ESO-1 vaccination. (A) Sample preparation for TCRβ-CDR3 sequencing. CD4$^+$ and CD8$^+$ T cells were isolated from PBMCs and presensitized with a pool of NY-ESO-1 overlapping peptides, prior to TCRβ-CDR3 sequencing of both the CD4$^+$/CD8$^+$ populations and PBMCs. (B) Differential abundance of CDR3 clones in PBMC versus the NY-ESO-1 peptide-stimulated and expanded CD4$^+$ T cell pool for a single representative patient and time point. Red points indicate de novo clonotypes identified exclusively in the activated CD4$^+$ T cell repertoire, whereas pink points denote clonotypes present in both the PBMC and CD4$^+$ T cell repertoires. (C) A Venn diagram illustrates the total number of unique NY-ESO-1 reactive CDR3 clones in CD4$^+$ and CD8$^+$ T cells, including the percentage of de novo clones absent in the PBMCs. (D) A CDR3 nucleotide sequence edit distance with a maximum threshold of 10 was used to determine clonal relatedness within samples between the total repertoire and the NY-ESO-1-activated clonal repertoire only. (E) A comparison of VDJ usage between NY-ESO-1 reactive clonotypes (orange) and the total clonotypes (gray) using Circos plots, which identify common VDJ usage patterns in both reactive and total clonotypes. NY-ESO-1, New York esophageal squamous cell carcinoma-1; PBMC, peripheral blood mononuclear cell; TCR, T-cell receptor; VDJ, variable-diversity-joining gene segments.

NY-ESO-1-reactive clonotypes, patient-specific CD4$^+$ and CD8$^+$ T cells were isolated from PBMCs and presensitized with a pool of NY-ESO-1 overlapping peptides prior to TCRβ-CDR3 sequencing (figure 6A,B).

Overall clonality of either peripheral or NY-ESO-1-reactive repertoires did not undergo significant changes throughout the course of treatment in any trial (online supplemental figure 6A,B). A paired assessment of presensitized CD4$^+$ and CD8$^+$ T cell repertoires to their respective PBMC source samples demonstrated significantly increased clonality, suggesting clonal expansion in response to NY-ESO-1 antigen activation (online supplemental figure 6C).

Across all patients, 14,247 CD4$^+$ and 8,319 CD8$^+$ clonotypes were identified at a significantly higher frequency post ex vivo sensitization to the NY-ESO-1 antigen than in their PBMC counterparts, with 272 clonotypes shared between the CD4$^+$ and CD8$^+$ reactive populations (figure 6B,C). The higher number of CD4$^+$ reactive clonotypes was reflective of the higher frequency of activated CD4$^+$ T cells observed in functional studies (figures 1A, 2B and 5A, online supplemental figure 2,3).

Notably, 62.6% of reactive CD4$^+$ and 47.7% of reactive CD8$^+$ clonotypes were not detectable in PBMC, arising de novo in presensitized samples (figure 6B,C). This finding suggests that most NY-ESO-1-reactive clones exist at very low frequencies within the periphery and were expanded by vaccination. Nucleotide sequence edit distance analysis revealed that reactive clonotypes were consistently more similar to each other than expected when compared with sequence similarity within the full clonal repertoire (figure 6D). Furthermore, the reactive clonotype pool showed a significantly distinct variable-diversity-joining rearrangment [V(D)J] gene usage profile compared with the total repertoire observed across patients (figure 6E).

Although clonotypes were more closely related when stratified by NY-ESO-1 activation, peripheral and reactive clonotype repertoires were highly unique to each patient, as determined by the Morisita-Horn (MH) index of similarity. Within-patient heterogeneity of repertoires was less pronounced (figure 7A). For NY-ESO-1-reactive clones in the periphery, temporal progression of cumulative reactive frequencies exhibited marked stochasticity between patients and clinical trials for both CD4$^+$ (online supplemental figure 6D) and CD8$^+$ clonotypes (figure 7B). The MH similarity index captured variability in the evolution of CDR3 repertoires within patients over the course of treatment and underscored interpatient

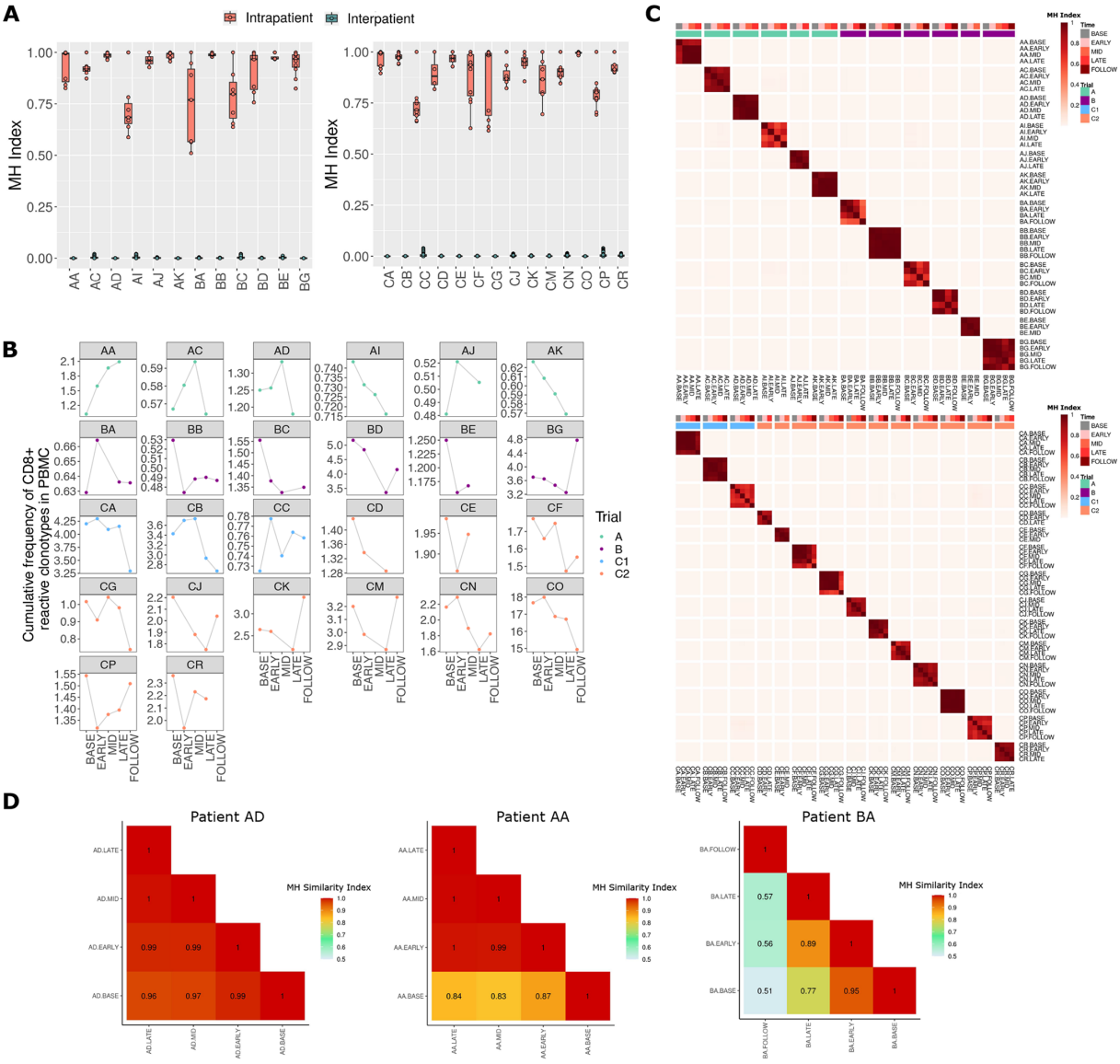

Figure 7 Evolution of the TCRβ-CDR3 repertoire on NY-ESO-1 vaccination. (A) The Morisita-Horn (MH) index as a measure of intrapatient and interpatient similarity of PBMC CDR3 repertoires (orange and green, respectively). (B) The cumulative frequency of reactive clonotypes in PBMC over time, identified in CD8$^+$ NY-ESO-1 reactive T cell pools. (C) The MH similarity matrix comparing peripheral CDR3 repertoires across patients and time points in ALVAC-TRICOM trials (top) and DC+CDX-1401 trials (bottom). (D) Representative patient-specific MH similarity matrices showing distinct patterns of CDR3 repertoire evolution in PBMC over the course of clinical trials (left—no evolution; middle—early evolution; right—progressive evolution). DC, dendritic cell; NY-ESO-1, New York esophageal squamous cell carcinoma-1; PBMC, peripheral blood mononuclear cell; TRICOM, the TRIad of CO-stimulatory Molecules.

heterogeneity of repertoires over time and across clinical trials (figure 7C). Several patients presented with a relatively static repertoire of clonotypes (figure 7D, left; Online supplemental figure 7A), while others showed early or progressive evolution (figure 7D, middle and right, respectively; Online supplemental figure 7A), as repertoires converged in similarity between early and later time points. Patients with early evolving $CD8^+$ CDR3 repertoires produced significantly greater populations of central memory T cells compared with patients with late-evolving or stable $CD8^+$ repertoires (ANOVA with post hoc Tukey, $p<0.05$ for all comparisons; Online supplemental figure 7C). In contrast, patients with stable $CD4^+$ CDR3 repertoires generated significantly larger central memory populations compared with those with late-evolving or early-evolving repertoires (ANOVA with post hoc Tukey, $p<0.05$ for late vs early and stable vs early comparisons; Online supplemental figure 7B). Notably, a substantial subset of patients on rapamycin (patients BG, CJ, CK, CM, and CP) experienced declines in PBMC-detected reactive clone frequencies during treatment, which then rebounded strongly in long-term follow-up (figure 7B), consistent with our hypothesis that vaccination with rapamycin increased induction of antigen-specific memory T cell responses.

DISCUSSION

Multiple NY-ESO-1 vaccination approaches have proven effective in their ability to induce integrated humoral and T cell responses, leading to improved survival for a subset of patients with NY-ESO-1-expressing tumors compared with their non-vaccinated counterparts.[7 8 26–28 30–32] However, a major limitation of these and other vaccines is ineffective generation of memory T cells and eventual relapse of disease. We studied the feasibility of incorporating rapamycin in human clinical trials as an immune modulator to shift T cell differentiation towards central memory populations and enhance durability of tumor-killing responses imparted by NY-ESO-1 vaccines. We conducted three phase I clinical trials to evaluate two distinct NY-ESO-1-based vaccination strategies, each in combination with the mTOR inhibitor rapamycin, administered according to varied dosing schedules.

Similar to previous NY-ESO-1-based vaccination trials, both ALVAC(2)-NY-ESO-1(M)/TRICOM and DC+CDX-1401 vaccination platforms were highly successful in eliciting de novo or amplifying pre-existing NY-ESO-1-specific immune responses in most vaccinated patients. Effects included elevated serum NY-ESO-1 antibody titers, antigen-specific $CD8^+/CD4^+$ T cell activation and cytokine production, and intrapatient shifts in functional TCR repertoires. Overall, patients treated with ALVAC(2)-NY-ESO-1(M)/TRICOM experienced greater vaccine-induced immune responses than those treated with DC+CDX-1401, as measured by seroreactivity, $CD4^+/CD8^+$ T cell activation, and cytokine production. DC+CDX-1401 failed to convert baseline seronegative patients and only sustained or further amplified responses in those who were already NY-ESO-1 seropositive at baseline, except for one patient. Similarly, DC+CDX-1401 treatment resulted in limited de novo induction and further amplification of existing NY-ESO-1-specific $CD4^+/CD8^+$ T cell activation responses in these same patients.

Notably, rapamycin altered immune dynamics of vaccination in a manner consistent with preclinical observations. NY-ESO-1-sensitized $CD4^+$ and $CD8^+$ T cells from rapamycin-treated patients contained higher frequencies of central memory cells compared with their non-rapamycin-treated counterparts. Long-term follow-up samples (6 and 12 months) available for a subset of DC+CDX-1401 patients treated with rapamycin were particularly notable, as they demonstrated functional memory population and, therefore, the potential durability of NY-ESO-1 antigen recall. Compared with other rapamycin dosing regimens, delayed administration postvaccination (days 15–28) resulted in significantly increased numbers of NY-ESO-1 antigen-specific $CD4^+$ and $CD8^+$ T cells up to 1 year later. While we did not observe an elevation in antibody titers attributable to rapamycin, it is possible that rapamycin could lead to qualitative changes in antibody repertoire by promoting B cell class-switching to generate a repertoire of high-affinity antibodies that are more protective, as demonstrated for influenza virus.[39]

Although conventionally employed as an immune suppressant,[20] substantial evidence indicates that rapamycin paradoxically promotes T cell differentiation towards a central memory phenotype through inhibition of the serine/threonine kinase mTOR and its downstream pleiotropic effects.[21–23 40–44] Mechanistically, blocking mTOR unleashes a regulatory switch favoring the maturation of memory T cells by (1) shifting metabolism away from glycolysis towards mitochondrial fatty acid transport and oxidation; (2) disrupting cytokine-induced signal transducer and activator of transcription signaling cascades; and (3) allowing the transcriptional activator Forkhead Box O1 to induce expression of the transcription factor Eomesodermin, which drives central memory T cell formation, while repressing expression of the counteracting T-bet transcription factor, a determinant of effector T cell phenotypes.[18 45]

Our findings are highly consistent with previous reports identifying the timing and duration of rapamycin as key determinants for the quantity and quality of central memory T cell formation.[21 22] Interestingly, in the context of vaccination against viral infection, rapamycin treatment after the initial clonal expansion phase of T cell responses produced the highest quality memory T cell reservoir capable of achieving optimal antigen recall in animal models.[21 22] In our study, consistent with memory attributes, NY-ESO-1-stimulated $CD4^+$ and $CD8^+$ T cells from rapamycin-treated patients exhibited more limited production of key cytokines related to effector T cell function, including CD40L, GM-CSF, IFNγ, TNFα, and CD107 for both vaccination methods.

In all three clinical trials, a highly diverse landscape of TCR repertoires, defined by TCRβ-CDR3 sequencing, contained significant subpopulations that selectively and functionally recognize NY-ESO-1 with no apparent overlap between patients. Overall, the temporal evolution of reactive clonotypes in the peripheral blood was also highly variable. This variability was likely due to the low frequency of reactive clonotypes sampled in PBMC resulting from the timing of sample collection during the T cell contraction phase after a round of vaccination and prior to the start of the next one. Notably, several patients in rapamycin-treated cohorts experienced a rebound in reactive clones detected in the periphery at follow-up time points, suggesting persistence of functional NY-ESO-1-recognizing clones up to 1 year after initial challenge.

While we report important observations regarding clinical use of rapamycin in combination with NY-ESO-1-based vaccination strategies, we recognize that this study has limitations. Our data indicate that while rapamycin had no effect on the frequency of de novo NY-ESO-1 seropositivity in ALVAC-TRICOM trial patients, it delayed induction of these humoral responses. In the DC+CDX-1401 trial patients, we are unable to draw conclusions regarding the effects of rapamycin on seroconversion due to the large number of baseline seropositive patients and the paucity of vaccine-induced immune responses. Additionally, we did not directly assess the effects of rapamycin on DCs in this study. However, considering that DC+CDX-1401 is a DC therapy, other studies have shown that rapamycin-treated DCs are less effective in allostimulation of $CD8^+$ effector T cells and favor generation of regulatory T cells.[46 47] Lastly, these phase I clinical trials were not adequately powered to determine the impact of rapamycin on antitumor effects. Determining which vaccination approach is superior in terms of antitumor effects is beyond the scope of this manuscript due to statistical power limitations and the diverse tumor types included in these phase I trials. Nevertheless, based on the magnitude of de novo induction of NY-ESO-1 antigen-specific CD4 and CD8 T cells, we speculate that ALVAC(2)-NY-ESO-1(M)/TRICOM would probably provide superior antitumor effects. We defer the conclusive answer to this question to future clinical studies with larger sample sizes and conducted in later-phase trials, such as phase II. Other studies have documented that some patients vaccinated against NY-ESO-1 experience disease relapse due to tumor cells downregulating NY-ESO-1 antigen expression[8] or losing the antigen-presenting machinery necessary for T cell recognition.[15]

In summary, adding rapamycin in ALVAC-TRICOM and DC+CDX-1401 vaccination clinical trials altered the dynamics of immune functional responses to NY-ESO-1 vaccination and positively impacted the generation of memory T cells. Furthermore, the inclusion of rapamycin was well-tolerated and safe. The immunomodulating characteristics of rapamycin warrant further investigation in the context of tumor surveillance and immunotherapy, particularly in conjunction with cancer vaccines like the highly immunogenic ALVAC-TRICOM vaccine.

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