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Results of a randomized, double-blind phase II clinical trial of NY-ESO-1 vaccine with ISCOMATRIX adjuvant versus ISCOMATRIX alone in participants with high-risk resected melanoma

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ABSTRACT

Background To compare the clinical efficacy of New York Esophageal squamous cell carcinoma-1 (NY-ESO-1) vaccine with ISCOMATRIX adjuvant versus ISCOMATRIX alone in a randomized, double-blind phase II study in participants with fully resected melanoma at high risk of recurrence.

Methods Participants with resected stage IIc, IIIb, IIIc and IV melanoma expressing NY-ESO-1 were randomized to treatment with three doses of NY-ESO-1/ISCOMATRIX or ISCOMATRIX adjuvant administered intramuscularly at 4-week intervals, followed by a further dose at 6 months. Primary endpoint was the proportion free of relapse at 18 months in the intention-to-treat (ITT) population and two per-protocol populations. Secondary endpoints included relapse-free survival (RFS) and overall survival (OS), safety and NY-ESO-1 immunity.

Results The ITT population comprised 110 participants, with 56 randomized to NY-ESO-1/ISCOMATRIX and 54 to ISCOMATRIX alone. No significant toxicities were observed. There were no differences between the study arms in relapses at 18 months or for median time to relapse; 139 vs 176 days (p=0.296), or relapse rate, 27 (48.2%) vs 26 (48.1%) (HR 0.913; 95% CI 0.402 to 2.231), respectively. RFS and OS were similar between the study arms. Vaccine recipients developed strong positive antibody responses to NY-ESO-1 (p≤0.0001) and NY-ESO-1-specific $CD4^+$ and $CD8^+$ responses. Biopsies following relapse did not demonstrate differences in NY-ESO-1 expression between the study populations although an exploratory study demonstrated reduced $(NY\text{-}ESO\text{-}1)^+$/Human Leukocyte Antigen (HLA) class I^+ double-positive cells in biopsies from vaccine recipients performed on relapse in 19 participants.

Conclusions The vaccine was well tolerated, however, despite inducing antigen-specific immunity, it did not affect survival endpoints. Immune escape through the downregulation of NY-ESO-1 and/or HLA class I molecules on tumor may have contributed to relapse.

INTRODUCTION

NY-ESO-1 is a cancer testis antigen expressed in a variety of tumors, but not in normal tissue, with the exception of testis and placenta.[1] It is expressed in approximately 45% of advanced stage melanomas.[2] Participants with NY-ESO-1-positive tumors who develop anti-NY-ESO-1 antibodies[3 4] often show detectable $CD8^+$[5 6] and $CD4^+$ NY-ESO-1-specific T-cell responses.[7] Although little is known about the biological function of NY-ESO-1, its pattern of expression and demonstrable spontaneous immunogenicity in cancer participants[6] has made it an attractive candidate antigen for cancer immunotherapy and thus, it has been evaluated in numerous clinical trials as a vaccine[6 8–20] and targeted with adoptively transferred T lymphocytes.[21 22]

ISCOMATRIX (CSL Limited, Parkville, Victoria, Australia)[23] is a saponin-based adjuvant that can induce both antibody and T-cell responses and has been previously used as an adjuvant with other vaccines.[24] We previously conducted a phase I placebo-controlled

clinical trial to evaluate the safety and immunogenicity of recombinant NY-ESO-1 protein formulated in ISCOMATRIX adjuvant in participants with melanoma.[10] A total of 46 evaluable participants with fully resected NY-ESO-1-positive tumors received three intramuscular injections of vaccine at 4 weekly intervals. The vaccine was well tolerated and high-titer antibody responses, strong skin reactions and circulating CD8 and CD4 T cells specific for a broad range of NY-ESO-1 epitopes were reported.[10 25] At a later, separate long-term follow-up evaluation, the relapse-free survival (RFS) of the late-stage melanoma participants in this trial appeared to be superior for those vaccinated with NY-ESO-1/ISCOMATRIX compared with those who received placebo or NY-ESO-1 alone.[26] With a median follow-up of 3.9 years, 5/19 (26%) participants relapsed in the cohorts which received NY-ESO-1 protein in combination with the adjuvant, whereas 13/23 (56%) relapsed from cohorts which did not (ie, cohorts receiving either placebo (n=8) or NY-ESO-1 protein alone (n=15)). This apparently substantial difference in outcome could not be explained by differences in recognized prognostic factors.[10 26] In addition, loss of NY-ESO-1 or HLA class I expression in the tumors of those participants who did relapse raised the possibility that immune selective pressure resulted from effective antigen-specific cellular cytotoxicity. As is the case in the current trial, some participants had cancers expressing NY-ESO-1 in a small minority of cells, raising questions as to mechanisms for improved outcomes in such participants. Possibilities include specific expression of NY-ESO-1 in cancer stem-like cells,[27] or 'epitope spreading' to take in more widely expressed antigens.[28]

We undertook a phase II randomized, double-blind clinical trial to determine the clinical efficacy of NY-ESO-1 conjugated with the adjuvant ISCOMATRIX or of ISCOMATRIX alone in participants with resected AJCC stage IIc, IIIb, IIIc or IV melanoma.

METHODS

Eligibility

Participants with resected, histologically confirmed, AJCC stage IIc, IIIb, IIIc or IV melanoma were eligible for enrolment in this study (LUD2003-009) if their tumors showed any expression of NY-ESO-1 antigen by immunohistochemistry. Eligible participants were vaccinated when they had fully recovered, and within 6 months, of surgery for melanoma (allowing a minimum of 2 weeks from the time of the most recent surgery to study entry). Although previous adjuvant therapy for a melanoma was accepted if participants had subsequently relapsed and undergone resection of relapsed disease, they were not eligible if they had prior immunotherapy or systemic therapy for melanoma following their most recent relapse and/or resection. Eligible participants were required to have normal values for laboratory analyses performed within 2 weeks of study entry. Protocol-specified limits allowed hemoglobin >100 g/L, platelets $>100\times10^9$/L, INR ≦2, creatinine ≤0.2 mmol/L, bilirubin ≤30 mmol/L and Alanine aminotransferase (ALT)/Aspartate aminotransferase (AST) <1.5 x Upper Limit of Normal (ULN). Participants were ineligible if they had resected cerebral metastases, primary ocular melanoma or other known malignancy in the three previous years, except for treated non-melanoma skin cancer and cervical carcinoma in situ. Chemotherapy, radiation therapy or participation in any other clinical trial of an investigational agent was not permitted within 4 weeks of the first dose on study.

Treatment plan

The trial was a double-blind phase II trial comparing NY-ESO-1/ISCOMATRIX to ISCOMATRIX alone. Because ISCOMATRIX is reactogenic at injection sites, it was selected as the non-vaccine control in order to maintain study blinding. Briefly, 110 participants enrolled at 18 clinical centers in the UK[11] and Australia/New Zealand (six in Australia, one in New Zealand) were randomized centrally at a 1:1 ratio into two treatment groups and stratified by disease stage (AJCC stage IIc, IIIb, IIIc or IV).

Registration of eligible participants, along with assignment of treatment group, clinical trial training and monitoring were provided by Kendle Australia (Australia/New Zealand) and Kendle International (UK) in collaboration with the Ludwig Institute for Cancer Research (LICR). Versagenics (Morrisville, North Carolina, USA) provided the statistical plan and data analyses.

Each participant received NY-ESO-1 100 µg formulated with ISCOMATRIX adjuvant, 120 µg or 120 µg ISCOMATRIX adjuvant alone. Recombinant NY-ESO-1 and ISCOMATRIX were manufactured in accordance with applicable current Good Manufacturing Practices as previously described[10 29] by LICR and CSL Australia (Parkville, Victoria, Australia). The first three doses were given at 4-week intervals at days 1, 29 and 57, (±3 days of scheduled date) and the fourth injection at month 6 (day 183±3 days). Participants who progressed were withdrawn from treatment but follow-up continued until the end of the study. Dose adjustments or adjustments in the interval of dosing were permitted when, in the opinion of the Investigator at each site, toxicity of sufficient severity attributed to the study agent occurred.

Although the study pharmacists at each site were unblinded, all other personnel associated with the conduct and oversight of the study, including all participants, remained blinded to the treatment allocation and identity of the study drug administered.

Toxicity was assessed every cycle using the National Cancer Institute Common Toxicity Criteria for Adverse Events V.3.0 (NCI CTCAE v3.0). Safety and tolerability of NY-ESO-1/ISCOMATRIX or ISCOMATRIX alone were monitored by adverse event reporting, blood chemistry, urinalysis, physical examination and vital signs. No interim analysis was conducted.

Assessment of efficacy and safety

The primary objective of this study was to compare the clinical efficacy of four intramuscular injections of NY-ESO-1/ISCOMATRIX vaccine and ISCOMATRIX adjuvant alone in eligible participants with resected melanoma at high risk of relapse. The primary endpoint was relapse rate at 18 months among the intention-to-treat (ITT) and two per-protocol (PP) populations, which consisted of all relapses regardless of location. Relapse was defined as the appearance of any new lesion, which had to be confirmed by CT scan. Disease was assessed according to Response Evaluation Criteria in Solid Tumors V.1.0.[30]

The secondary objectives were to evaluate treatment safety, median RFS, overall survival (OS) and NY-ESO-1-specific immunity for participants in each treatment group. After completing all four treatment doses and the final assessments at month 18, each participant was to be followed off-study until disease progression and death. Thus, RFS and OS over the entire period of observation (study defined plus off-study follow-up) among ITT and both PP populations were secondary endpoints for the study. For time to event data, Kaplan-Meier life tables were used to estimate survival rates (for RFS and OS) and determine the survival plots.

Toxicity was defined by NCI CTCAE V.3.0. When the primary endpoint was subsequently found not to have been met, a supplementary protocol was activated to enable collection and analysis of any available tumor tissue at relapse and determine NY-ESO-1 and HLA class I expression.

Statistical analysis

The study was powered on the basis of the observable difference between RFS for the treated and placebo populations in an earlier phase I trial[10 26] and was designed to detect a clinically meaningful reduction in event rates, corresponding to about a 24% difference in recurrence rate at 18 months, and equivalent to detecting an HR of 0.53, assuming a one-sided type I error of 0.05. Assuming that 50% of participants in the control arm (ISCOMATRIX) relapsed by 18 months, 50–55 participants were needed in each arm to achieve 80% power to detect a difference of 24% or more in the rate of RFS between the two arms. The percentage of participants who were relapse free at 18 months was calculated using the HR, the 95% CI and p value. The duration of RFS at 18 months was compared using the Mann-Whitney U test and HR calculated using the Cox regression proportional hazards model.

Exploratory analyses, both univariate and multivariate (Cox regression), were performed to determine the importance of certain covariates (including sex, age, time since diagnosis, primary lesion thickness, disease stage at diagnosis, disease stage at study entry, number of recurrences prior to entry, time since resection prior to study entry), and to identify predictors of time to recurrence. Statistical significance was declared when the p value was found to be less than or equal to 0.05.

Immune monitoring assays

Induction of immune responses against NY-ESO-1, measured by antibody titer and NY-ESO-1-specific T-cell assays, was a secondary endpoint of the study. NY-ESO-1-specific antibodies were measured by ELISA using a validated assay, as previously described.[7 10] Blood samples were collected at baseline, at days 71 and 197, and then at 12 and 18 months (online supplementary figure S1A). Sera from each time point were assayed concurrently to enable direct comparisons of antibody titer. The cut-off for antibody positivity was 1:100. The p values were calculated from a Cochran-Mantel-Haenszel test adjusting for location (UK or Australia/New Zealand).

Peripheral blood mononuclear cells (PBMCs) were isolated as previously described.[31] Cellular immune responses were only evaluated in a subset of participants at selected study sites, based on accessibility to central immune monitoring laboratories in Melbourne, Oxford and Auckland. PBMCs ($5–7\times10^6$) from each of the time points were pulsed with 2 µL of a pool containing all 28 NY-ESO-1 18-mer peptides at 30 µM for 1 hour at 37°C in 200 µL RPMI +10% FCS (RF10). A pool of viral peptide epitopes derived from inFluenza, Epstein-Barr virus and Cytomegalovirus (FEC) that covers the most common HLA class I alleles was kindly provided by the European Vaccine against AIDS (EVA) Center for AIDS Reagents).[32] A FEC-specific T cell internal control, for T cell expansion efficiency, was assessed in parallel. The expanded NY-ESO-1-specific T cells (both $CD8^+$ and $CD4^+$) were then assessed by interferon-γ intracellular cytokine staining as previously described,[10 31] using the same peptide pool as well as the individual peptides within the pool (online supplementary methods and supplementary figure S1B).

Immunohistochemistry for HLA class I and NY-ESO-1

Immunohistochemistry and scoring for NY-ESO-1 and HLA-1 was performed as previously described.[2 26 33] The HC-10 antibody (kindly provided by Brian Tait, Victorian Transplantation and Immunogenetics Service, Melbourne, Australia) preferentially recognizes beta-2-microglobulin-free HLA-A, HLA-B, HLA-C heavy chain and was used at 0.004 µg/L.

Multiplexed immunofluorescence analyses

For the supplemental study LUD2003-009-S, tissue was available for 10 participants who received NY-ESO-1/ISCOMATRIX and nine who received ISCOMATRIX. Eligible participants had histological confirmation of relapse established by surgical biopsy. In order to evaluate HLA class I and NY-ESO-1 coexpression, samples were analyzed according to the previously described method[34] with some modifications (online supplementary methods): Mouse antihuman HLA class I (clone HC-10)[35 36] was used at 1:3000 dilution and mouse antihuman NY-ESO-1 (clone E978)[37] was used at 0.01 µg/mL. Scanning of the entire specimen section was performed with the Vectra V.3.0 automated multispectral quantitative imaging system (PerkinElmer, CLS142338) using

the following filter and exposure settings: DAPI 20 ms, FITC 65 ms, Cy3 35 ms. Analysis was performed on 20 fields for core biopsy samples or 800 fields for tumor sections. Image fields for 20 sections from NY-ESO-1/ISCOMATRIX participants, and 22 sections from ISCOMATRIX alone participants were analyzed and scored for HLA class I or NY-ESO-1 single-positive, HLA class I/NY-ESO-1 double-positive and HLA class I/NY-ESO-1 double-negative cells, using inForm Tissue Finder V.2.2.1 software (PerkinElmer, CLS135783).

RESULTS

Baseline characteristics

Of 623 participants screened at 18 clinical trial sites in Australia, New Zealand and UK for participation in the trial, 111 were randomized: 56 to the NY-ESO-1/ISCOMATRIX arm and 55 to the ISCOMATRIX arm. One participant in the ISCOMATRIX arm did not receive the allocated intervention. Although there were minor differences in baseline characteristics between the treatment groups (table 1), these were not thought to be clinically significant. Participants in the ISCOMATRIX group were more likely to have ulceration of the primary tumor (p=0.024).

Of the 623 participants screened, tumor samples for 242 participants (39%) were positive for NY-ESO-1 expression. Of the 147 eligible participants, 40% had NY-ESO-1 in ≤5% of the tumor cells in the submitted samples, and only 29% had >50% of tumor cells expressing NY-ESO-1 (online supplementary table S1). The study populations were not optimally balanced for antigen distribution. In the NY-ESO-1/ISCOMATRIX group, 41% had tumors expressing NY-ESO-1 in >50% of tumor cells surveyed by IHC, whereas in the ISCOMATRIX alone cohort only 24% had this high level of antigen expression (table 1). After complete eligibility checks, 111 participants were randomly assigned to the two study arms and 110 received one of the study agents (figure 1).

Table 1 Patient characteristics and tumor antigen expression

	Ny-ESO-1/ ISCOMATRIX (n=56)	Iscomatrix (n=54)	P value
Mean age (years)	54.5	53.0	0.261*
Sex, n (%)			0.477†
Female	37 (66.1)	32 (59.3)	
Male	19 (33.9)	22 (40.7)	
Race			1.00‡
White, n (%)	56 (100)	54 (100)	
Eastern Cooperative Oncology Group (ECOG) performance status, n (%)			0.044†
0	51 (91.1)	44 (81.5)	
1	2 (3.6)	8 (14.8)	
2–4	0 (0.0)	0 (0.0)	
American Joint Committee on Cancer (AJCC) stage at study entry, n (%)			0.617†
IIC	2 (3.6)	7 (13.0)	
IIIB	25 (44.6)	18 (33.3)	
IIIC	11 (19.6)	12 (22.2)	
IV	18 (32.1)	17 (31.5)	
Ulceration, n (%)			0.024†
No	27 (48.2)	17 (31.5)	
Unknown	20 (35.7)	19 (35.2)	
Yes	9 (16.1)	18 (33.3)	
Tumor antigen expression			0.114*
≤5%	26 (46.4%)	28 (51.9%)	
6%–25%	5 (8.9%)	10 (18.5%)	
26%–50%	2 (3.6%)	3 (5.6%)	
51%–75%	6 (10.7%)	4 (7.4%)	
>75%	17 (30.4%)	9 (16.7%)	

*From an ANOVA test with factors treatment and location (UK or Australia/New Zealand).
†From a Cochran-Mantel-Haenszel test adjusting for location (UK or Australia/New Zealand).
‡Unable to calculate Cochran-Mantel-Haenszel due to insufficient value variations.
ANOVA, analysis of variance.

Patient disposition and drug exposure

Of the total 111 eligible participants, 110 were randomly assigned and received treatment on protocol (figure 1). All 110 participants comprised the ITT population and of these, 56 were randomized to the NY-ESO-1/ISCOMATRIX arm and 54 to the ISCOMATRIX arm.

Of the 56 participants randomized to the NY-ESO-1/ISCOMATRIX arm, 27 (48.2%) were withdrawn from the study; 24 because of disease progression, and three others because of consent withdrawal, protocol violation and unavailability of drug supply. In the ISCOMATRIX arm, 25 (46.3%) participants were withdrawn from the study; 24 because of disease progression and one due to interruption to drug supply. The treatment doses received by participants in the NY-ESO-1/ISCOMATRIX and ISCOMATRIX cohorts respectively were: dose 1 (day 1): 56 and 54, dose 2 (day 29): 53 (94.6%) and 53 (98.1%), dose 3 (day 57): 50 (89.3%) and 48 (88.9%) and dose 4 (day 183); 37 (66.1%) and 39 (72.2%). Only one participant in the NY-ESO-1 arm required a dose reduction (30% of full dose) at day 183.

Toxicity

The safety analyses were based on all participants who received at least one injection of study drug (ITT population). The vaccine was associated with more frequent injection site reactions (discomfort, erythema, pain, etc) and a variety of other somatic responses (chills, fatigue, influenza-like illness, lethargy, malaise, fever, etc) than ISCOMATRIX alone (table 2). In general, treatment was well tolerated with no participant withdrawing from

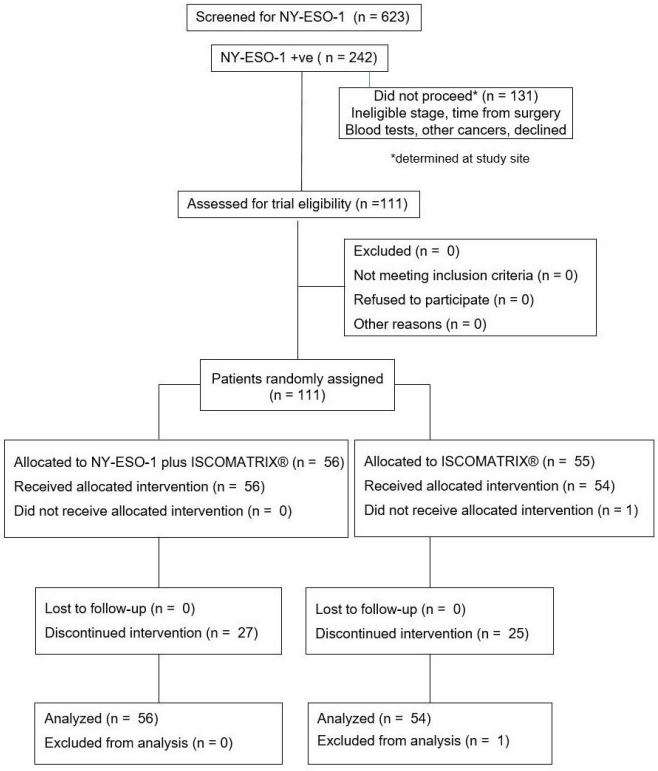

Figure 1 Disposition of participants (CONSORT diagram). CONSORT, Consolidated Standards of Reporting Trials.

either arm due to toxicity. There were no deaths during the 18-month treatment period. The most common toxicities experienced by the majority of participants in each cohort were low-grade local injection site pain and fever. Forty-four (78.6%) and 37 (68.5%) participants experienced ≥4 adverse events during their participation in the study in the NY-ESO-1/ISCOMATRIX and ISCOMATRIX arms, respectively. The maximum CTCAE toxicity grade reported was 3 in 12 (21.4%) in the NY-ESO-1/ISCOMATRIX recipients versus 15 (27.8%) in the ISCOMATRIX arm. Of the reported adverse events, 46 (82.1%) of the participants in the NY-ESO-1/ISCOMATRIX arm, and 28 (51.9%) of those in the ISCOMATRIX arm reported adverse events that were probably or definitely related to the study drug (table 2).

The number of participants who experienced serious adverse events was similar in the two groups: 10 (17.9%) vs 12 (22.2%) in the NY-ESO-1/ISCOMATRIX and ISCOMATRIX treatment arms, respectively. None were related to study drug. The frequency of adverse events leading to discontinuation of study drug was higher in the ISCOMATRIX arm, 7 (13%), than in the NY-ESO-1/ISCOMATRIX arm, 2 (3.6%).

Table 2 Common treatment-related adverse events (incidence ≥5% in either arm)

System organ class/ preferred term	NY-ESO-1/ ISCOMATRIX participants (n=56) n (%)	ISCOMATRIX participants (n=54) n (%)
Blood and lymphatic system disorders		
Lymphadenopathy	4 (7.1)	2 (3.7)
Gastrointestinal disorders		
Nausea	9 (16.1)	7 (13.0)
General disorders and administration site conditions		
Chest pain	3 (5.4)	1 (1.9)
Chills	6 (10.7)	1 (1.9)
Fatigue	13 (23.2)	16 (29.6)
Influenza like illness	26 (46.4)	9 (16.7)
Injection site discomfort	5 (8.9)	1 (1.9)
Injection site erythema	9 (16.1)	1 (1.9)
Injection site pain	28 (50.0)	17 (31.5)
Injection site reaction	16 (28.6)	11 (20.4)
Lethargy	5 (8.9)	3 (5.6)
Malaise	3 (5.4)	0 (0.0)
Fever	6 (10.7)	2 (3.7)
Musculoskeletal and connective tissue disorders		
Arthralgia	8 (14.3)	6 (11.1)
Back pain	6 (10.7)	5 (9.3)
Musculoskeletal pain	3 (5.4)	3 (5.6)
Pain in extremity	9 (16.1)	6 (11.1)
Nervous system disorders		
Headache	18 (32.1)	13 (24.1)
Respiratory, thoracic and mediastinal disorders		
Cough	11 (19.6)	3 5.6)
Nasopharyngitis	4 (7.1)	2 (3.7)
Pharyngolaryngeal pain	3 (5.4)	4 (7.4)
Skin and subcutaneous tissue disorders		
Rash	4 (7.1)	5 (9.3)
Surgical and medical procedures		
Mass excision	6 (10.7)	3 (5.6)

Efficacy

Overall, RFS and OS were not significantly different between the NY-ESO-1/ISCOMATRIX and ISCOMATRIX groups at 18 months, during the entire period of observation or after long-term follow-up (figure 2A,B). The median time to relapse for all participants was 139 days in the NY-ESO-1/ISCOMATRIX arm and 176 days in the ISCOMATRIX arm (p=0.296) at 18 months, and 142 and 176 days (p=0.398), respectively, over the entire period of observation (online supplementary table S2). No significant difference in relapse rate was observed between the NY-ESO-1/ISCOMATRIX arm and the ISCOMATRIX arm, 27 (48.2%) vs 26 (48.1%), respectively (HR 0.913; 95% CI 0.402 to 2.231) at 18 months, or over the entire period of observation: 33 (58.9%) and 29 (53.7%), respectively (HR 0.880; 95% CI 0.532 to 1.455). Similarly, there was no significant difference in RFS between the NY-ESO-1/ISCOMATRIX and ISCOMATRIX arms for

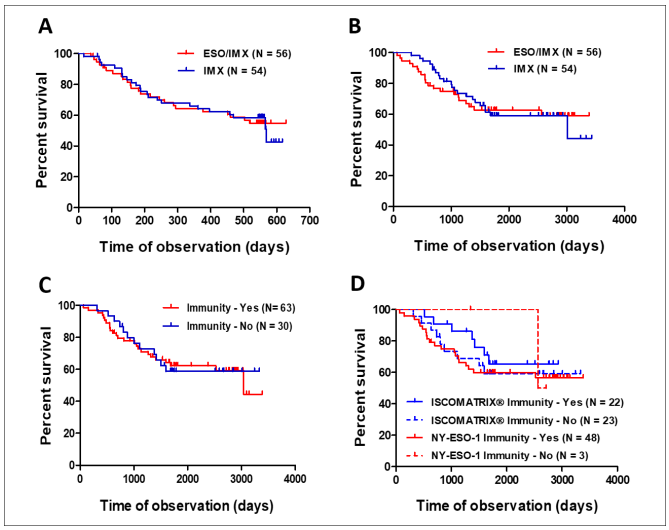

Figure 2 Relapse-free survival (RFS) and overall survival (OS). Kaplan-Meier curves of the ITT population for: (A) RFS over the study defined period, (B) OS over the study period and long-term follow-up, (C) OS by immunity over study period and long-term follow-up and (D) OS by treatment and immunity over study period and long-term follow-up. ESO/IMX: NY-ESO-1/ISCOMATRIX. IMx: ISCOMATRIX. ITT, intention to treat.

the AJCC Stage at study entry, that is, non-stage IV and stage IV groups (online supplementary table S2).

NY-ESO-1 immune responses

As expected, there was no significant (p=0.733) difference in NY-ESO-1 antibody titers between the two treatment groups at baseline prior to receipt of study drug (figure 3 and online supplementary table S3). Vaccination induced a highly significant (p<0.001) difference in antibodies, with 95% of participants in the NY-ESO-1/ISCOMATRIX group demonstrating antibody responses by day 71 vs 7% in the control group (figure 3 and online supplementary table S4). This difference remained for the duration of the 18-month study, although the antibody titer decreased over time. Nonetheless, nearly all participants in the NY-ESO-1/ISCOMATRIX group had elevated antibody titers at the end of the study (figure 3A).

A similar number of participants in each study arm had pre-existing anti-NY-ESO-1 antibodies at baseline, 16 of 51 (31%) in the NY-ESO-1/ISCOMATRIX arm and 13 of 49 (26%) in the ISCOMATRIX arm (online supplementary table S3). NY-ESO-1/ISCOMATRIX induced a strong antibody response in those participants who had negative baseline serology. Of the 31 seronegative participants, 28 (90%) developed vaccine-induced anti-NY-ESO-1 antibodies. Antibody titers were boosted in 9 of the 16 (56%) participants who had pre-existing antibodies. Seroconversion was rare in the ISCOMATRIX arm. Three of the 13 participants (23%) with pre-existing antibody had a boost in antibody levels, and 2 of 32 (6%) seronegative participants developed NY-ESO-1 specific antibodies. Thus, 5 out of 49 participants (10%) developed or increased NY-ESO-1 specific antibodies despite receiving

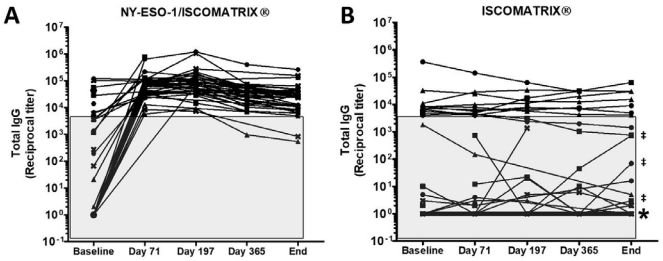

Figure 3 NY-ESO-1-specific antibody responses. Total IgG (reciprocal titer) over the duration of the study in participants vaccinated with (A) NY-ESO-1/ISCOMATRIX (n=51) or (B) ISCOMATRIX alone (n=49). Five participants in each group are excluded due to lack of data. *Thick line encompasses data for 31 participants who did not have antibody responses. Shaded area indicates titers below the limit of quantitation (<5000). ‡Participants in whom antibody titers increased on days 197 or 365, well after the final dose of study drug (day 183).

ISCOMATRIX adjuvant alone. These increases often occurred well after receiving study drug (days 71, 197 and 365). NY-ESO-1 is highly immunogenic and spontaneous antibodies often accompany NY-ESO-1 expression in tumors.[1 3 38] Since clinical relapses occurred in three of these five participants, these were probably spontaneous antibody responses against tumor-derived NY-ESO-1. Participants with NY-ESO-1 immunity did not have better OS than those without (figure 2C,D).

Cellular immunity

Immune monitoring of $CD4^+$ and $CD8^+$ T-lymphocyte responses was performed on a subset of 34 accessible participants: 19 NY-ESO-1/ISCOMATRIX vaccine recipients and 15 control ISCOMATRIX recipients. Using a systematic screening approach with overlapping peptides approach, we have previously detected naturally occurring NY-ESO-1-specific $CD8^+$ and/or $CD4^+$ T cell responses in many melanoma participants.[39] Of the participants studied here, 13 had evidence of pre-existing cellular immunity against NY-ESO-1, 7 prior to NY-ESO-1/ISCOMATRIX vaccine and 6 prior to ISCOMATRIX adjuvant control (figure 4, hatched bars). Administration of the vaccine either boosted pre-existing responses (5/7, 71%) or broadened the T-cell response repertoire to recognize additional peptide epitopes (16/19, 84%) (figure 4, solid bars). In only three vaccine recipients (063–001, 063–004 and 063–005) were no responses detected. Examples of detailed patient immune monitoring data are shown in online supplementary figures S2–S6 and illustrate: (1) a vaccine response with antibody, $CD4^+$ and $CD8^+$ T cells (online supplementary figure S2), (2) a pre-existing antibody and $CD8^+$ and $CD4^+$ T cell response in a vaccinated patient who developed new $CD4^+$ T cell specificities (online supplementary figure S3), (3) Induction of antibody and $CD4^+$ T cells without an apparent $CD8^+$ T response in vaccine recipient (online supplementary figure S4), (4) induction of multiple $CD8^+$ T cell

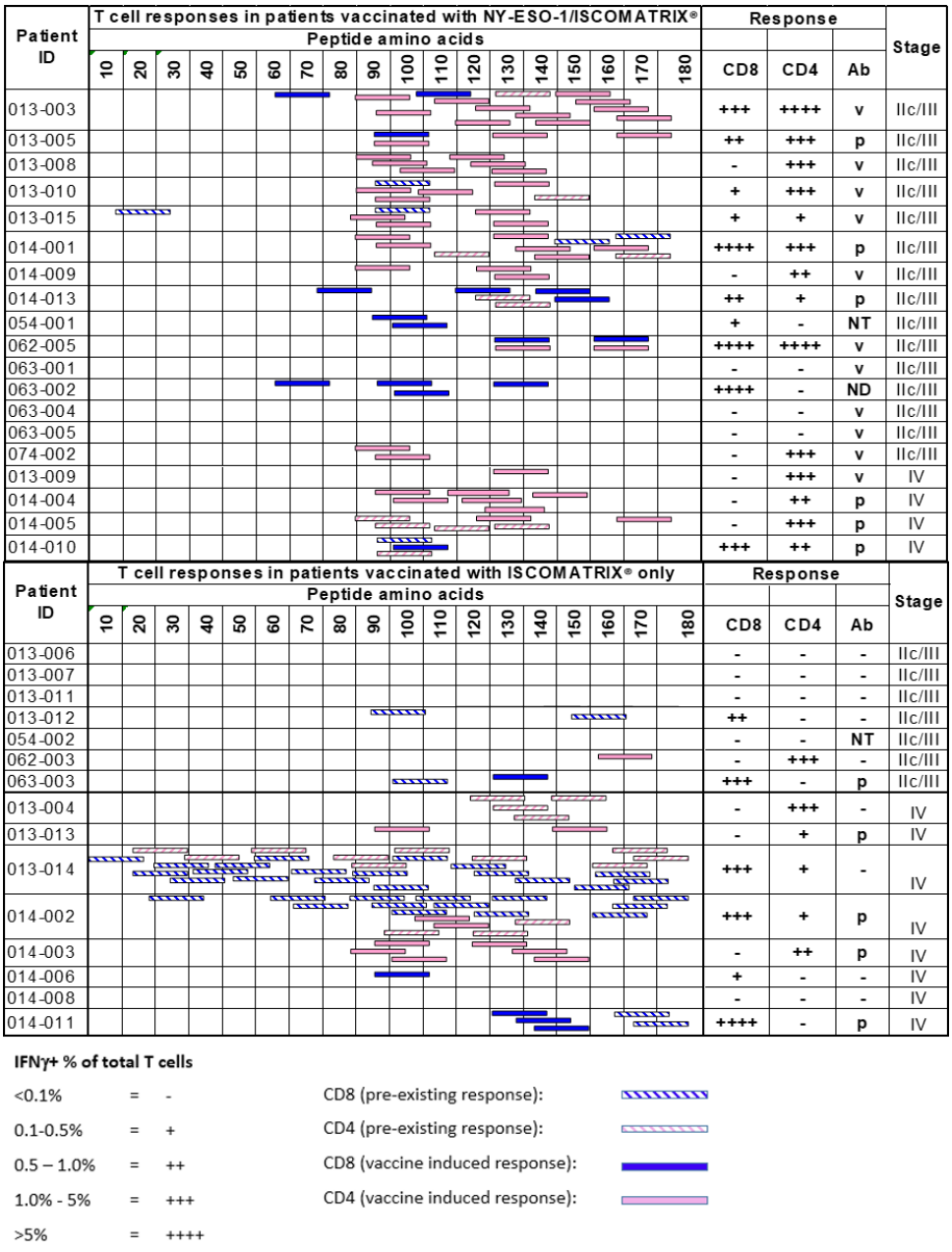

Figure 4 Pre-existing and vaccine-induced T cell responses against NY-ESO-1. T cell responses for 19 participants who received NY-ESO-1/ISCOMATRIX and 15 participants who received ISCOMATRIX only. Responses of interest: 062–003 and 014–006 are difficult to interpret due to weak FACS patterns; 013–013—pre-existing Antibody (Ab), CD4 response at day 365, relapse at day 387, suggestive of a spontaneous response; 014–003—pre-existing Ab and CD8, and a strong but transient CD4 response on days 197 and 365 reflects spontaneous immunity; 063–003—pre-existing Ab and CD8, uninterpretable CD4 response. Antibody titer measured as total IgG: IFNγ, interferon-γ; ND, not determined at baseline; NT, no data for patient; p, pre-existing; v, vaccine-induced; -, no pre-existing Ab or no vaccine-induced Ab response.

specificities in vaccine recipient (online supplementary figure S5).

Additionally, the kinetics and persistence of NY-ESO-1 specific CD4/CD8+T cell responses over time were measured in 31 other patients and these responses are shown in online supplementary figure S7.

In most cases, the vaccine induced $CD4^+$ occurred more frequently than $CD8^+$ T cell responses (figure 4), although there were exceptions such as patient 063–002 who had $CD8^+$ but no evident $CD4^+$ T cell responses (online supplementary figure S5). One possibility reason is that this patient may not have expressed the right MHC class II molecules for presentation of NY-ESO-1 peptides to $CD4^+$ T cells. There are only 10 class II alleles (1 DP and 9 DR) thus far reported to present NY-ESO-1 epitopes to $CD4^+$ T cells (http://archive.cancerimmunity.org/peptidedatabase/tumorspecific.htm). As our study was not intended for detailed immune monitoring, the MHC class II alleles for all participants and some MHC class I alleles for some participants were not typed.

Online supplementary figure S6 shows pre-existing immunity on the basis of a prior antibody response associated with emergence of a $CD4^+$ T cell response that was most apparent after 6 months but not at later time points (days 365 and 547) in a control participant who received ISCOMATRIX adjuvant alone. Apparent responses to NY-ESO-1 in such placebo recipients alone warrants interpretation. Six of the 15 tested had evidence of pre-existing spontaneous immunity (five had detectable antibody at baseline). Seven developed responses against new epitopes (figure 4). In no case does it seem likely that ISCOMATRIX actually induced specific immunity against NY-ESO-1. Two (participants 062–003 and 014–006) showed weak FACS patterns for CD4 and CD8 cells, but CD8 reactivity was already present at baseline so ISCOMATRIX cannot have initiated recognition. In another, two participants (013–013 and 014–003), CD4 responses appeared transiently at a time point that was distantly removed from administration of ISCOMATRIX (ie, days 197 and/or 365). Since both had antibody responses at baseline, these may also be examples of spontaneous T-lymphocyte responses unrelated to the administration of study drug. Similarly, participant 063–003 also had a pre-existing antibody and CD8 response. In the absence of vaccination, these transient T cell responses likely reflect reaction against naturally occurring antigen, and lack of clinical relapse in such patients (eg, participant 014–003) might imply immune control or 'equilibrium'.[40]

Coexpression of HLA class I and NY-ESO-1

Since tumors were heterogeneous for NY-ESO-1 expression, and no clinical benefit was seen despite vaccine-induced immunity, we investigated the possibility that immune responses may have been selectively eradicating NY-ESO-1^+ tumor clones but not those that were antigen negative. A supplementary protocol was written to retrieve tumor blocks on relapse and analyze these for antigen expression. Since $CD8^+$ T cell responses depend on HLA class I, tumors were characterized for the presence of double-positive cells bearing both NY-ESO-1 and HLA class I. This analysis was initially unplanned and it was limited to a relatively small number of available samples. Nonetheless, figure 5 shows that double-positive cells could be quantified and that in the presence of vaccine-induced responses, the abundance of these cells was reduced more than in control subjects (figure 5B). This supports the hypothesis that NY-ESO-1/ISCOMATRIX vaccination induced immune selection against cells expressing HLA class I-restricted NY-ESO-1 epitopes.

CONCLUSIONS

This phase II clinical trial was undertaken to examine the potential clinical impact of a highly immunogenic NY-ESO-1 cancer vaccine in melanoma patients. It was prompted by an unexpectedly encouraging survival trend observed in an earlier phase I trial.[10 26] In that trial, we studied NY-ESO-1 protein complexed with ISCOMATRIX adjuvant. Despite very small numbers, a difference in the rates of relapse between vaccine and placebo recipients was seen to be compelling, such that further evaluation was warranted.

In the phase II trial reported here, NY-ESO-1/ISCOMATRIX vaccine was compared with ISCOMATRIX adjuvant alone. Despite being safe and immunogenic, we were not able to confirm any difference in rate of relapse, RFS or OS. Kaplan-Meier survival estimates for both arms were similar for all time points, and there were no statistically significant predictors of survival among the clinical variables examined for primary or secondary survival endpoints.

The vaccine was immunologically active. Detailed immune monitoring showed that it effectively induced both cellular and antibody responses that were specific for NY-ESO-1. After three immunizations, 73% of participants developed strong specific NY-ESO-1 antibody responses ($p<0.0001$) that often persisted for 12 months or more after the final dose of vaccine (figure 3A). Similarly, in the subset of patients that was monitored for cellular immunity, an increase in antigen-specific $CD8^+$ and $CD4^+$ lymphocytes indicated that humoral immunity was accompanied by cellular immune responses as has been previously described.[4 7 25 39 41 42] Although occasional changes in anti-NY-ESO-1 immunity were seen in ISCOMATRIX adjuvant-alone recipients, these mostly occurred on a background of prior spontaneous NY-ESO-1 immunity (figure 3B, online supplementary figure S6). It is likely that endogenous tumor-associated NY-ESO-1 contributed to this immunity. This is implied from the kinetics of these responses. For instance, in several cases immunity was boosted well after the final dose of study drug on day 365 (figure 3B). Clinical relapses occurred in three of five participants, one 13 months into the study and the other two at the end of the 18 months. These are indicated in figure 3 (‡). For these, the most likely explanation is that endogenous antigen within tumor stimulated the responses. Additionally, when FACS staining patterns are weak and associated with a borderline cell population, such as for patient 062–003, artifacts arising from the methodology can be difficult to distinguish from unequivocal positive responses.

Spontaneous immunity is well documented for NY-ESO-1, and NY-ESO-1-seropositive participants treated with ipilimumab, the anticytotoxic T lymphocyte antigen 4 antibody. In one study, such participants had a greater likelihood of experiencing clinical benefit 24 weeks after ipilimumab treatment than NY-ESO-1-seronegative participants.[43] Our study arms were balanced for this confounding factor; 31% of vaccine and 26% in the placebo recipients had antibodies that preceded vaccine or arose in the absence of vaccination. To assess any impact on clinical outcomes, we related survival to immunity rather than randomization and there was no apparent impact (figure 2C,D).

We have previously proposed that loss of NY-ESO-1 or HLA class I can signify immunoediting following

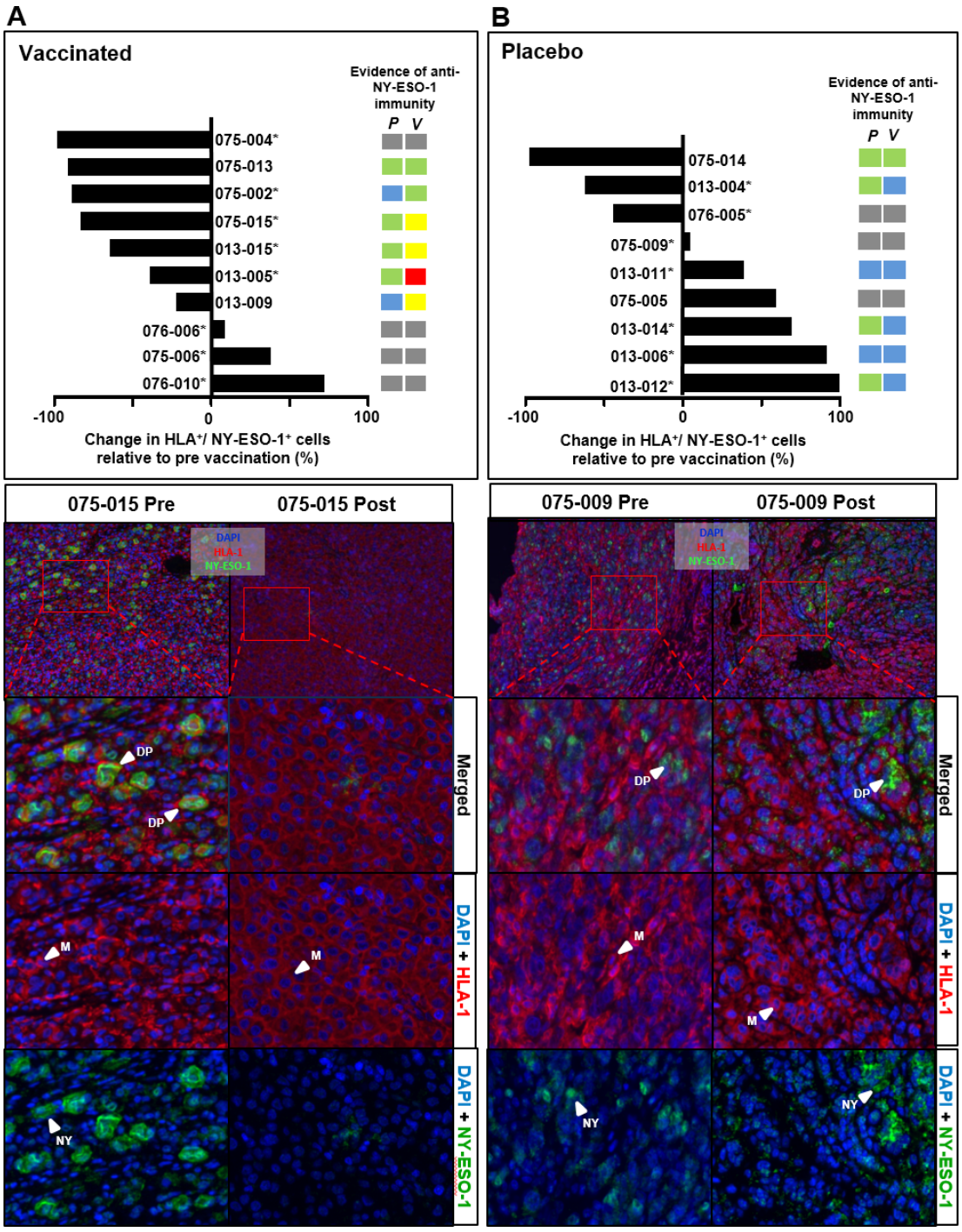

Figure 5 Coexpression of HLA class I and NY-ESO-1. Percentage change (top panels) in the number of double-positive Human Leukocyte Antigen (HLA)$^+$/NY-ESO-1$^+$ cells relative to prevaccination, and evidence of pre-existing (P) or vaccine-induced (V) anti-NY-ESO-1 immunity in a representative number of participants who received (A) NY-ESO-1/ISCOMATRIX or (B) ISCOMATRIX alone. Red: positive response for three of three markers (ie, CD4$^+$, CD8$^+$, Ab$^+$). Yellow: positive response for two of three markers. Green: positive response for one of three markers. Blue: negative response for all three markers. Gray: data not available for this patient. Bottom panels: representative multiplex immunofluorescence for HLA class I (red) and NY-ESO-1 (green) prevaccination and post-vaccination in a patient (075–015) who received NY-ESO-1/ISCOMATRIX, and in a patient (075–009) who received ISCOMATRIX alone. DP, Double positive HLA class I$^+$/ NY-ESO-1$^+$ cells. M, single HLA class I$^+$ cells. NY, single NY-ESO-1$^+$ cells. Cell nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI), magnification: ×20.

NY-ESO-1 immunity.[26] This trial provided the opportunity to test this more formally in vaccinated and placebo groups, and in particular, to assess tumor cells for the simultaneous expression of NY-ESO-1 antigen and HLA class I, thereby characterizing the cells that were potentially targetable. Although the analysis was constrained by the small numbers from whom tissue was available post-relapse, there was a clear trend showing reduced

double-positive cells in vaccine recipients compared with controls.

In summary, this vaccine was safe and immunogenic, however, no apparent clinical benefit was seen in participants with Stage IIc, IIIb, IIIc and IV NY-ESO-1^{+} melanoma. Emergent immunity in the control group indicates that spontaneous NY-ESO-1 immunity can evolve and exploratory studies of NY-ESO-1 and HLA class I expression suggest that antigen display can also evolve in the face of selective pressure in vivo.

As immunotherapy for the treatment of cancer continues to develop with highly effective new agents, the question inevitably turns to defining a role for antigen-specific approaches such as vaccines, alone or in combination with immune checkpoint inhibitors. Despite being immunogenic, further clinical studies of this vaccine as a single agent are not planned. Whether or not combinations with immune checkpoint inhibitors can extend the efficacy of either or both will require evaluation in appropriately designed trials.

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Patient consent for publication Not required.

Ethics approval The study protocol was approved by the institutional review board (IRB) at each participating institution in Australia, New Zealand and UK. A supplementary protocol (LUD2003-009-S) was approved by all relevant IRBs for the collection and analysis of post-relapse tumor tissue, as this had not been included in the original protocol.

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