Methods

Study Population

Between 2012 and 2014, patients were recruited in two arms. Arm A required patients to be diagnosed with LS associated CRC and surgically treated within 1 year before vaccination. Arm B recruited patients who were known to be carriers of a germline MMR gene mutation associated with LS and without cancer or more than 5-years cancer-free after primary CRC resection at the time of the study. Being HLA-A*02 positive was an inclusion criteria for both arms. Patients who had any non-CRC malignancy more than 5 years before inclusion, including extracolonic LS-associated malignancies, were also eligible. WHO performance status had to be 0 or 1. Exclusion criteria were: autoimmune diseases, an active viral infection, allergy to shellfish, concomitant use of immunosuppressive drugs, pregnancy or lactation within the study period and laboratory abnormalities (white blood cell count of >3.0x109/L; lymphocytes >0.8x109/L; platelets >100x109/L; serum creatinine <150 μmol/L; and serum bilirubin <25 μmol/L, exception Gilbert's syndrome)

Power of the study

A power analysis was conducted by determining the probability of a positive study outcome, defined as the detection of antigen specific T cells (here CEA) in more than half of the patients (i.e. at least 12 of 23), as a function of the "true" response rate r. Specifically, the number of patients for which an immune response can be detected follows a binomial distribution with parameters 23 and r. An 80% power is achieved when the response rate is ~59%. In a previous study conducted by our group, where 11 patients were vaccinated with the CEA peptide pulsed DCs, CEA specific T cells were observed in 72.7% of the patients[1]; at this response rate, the power of our study is 99%.

Dendritic cell vaccine preparation and characterization

Autologous moDCs were generated from leukapheresis product in compliance with Good Manufacturing Practice in a cleanroom-facility[2]. Plastic adherent monocytes or monocytes isolated by centrifugal elutriation were cultured in X-VIVO-15 medium (Lonza) supplemented with 2% pooled HS (Sanquin, Nijmegen, the Netherlands), IL-4 (500 U/ml), and granulocyte-macrophage-colony stimulating factor (800 U/ml; both CellGenix). Immature DC were pulsed with Keyhole Limpet hemocyanin (KLH) (10 mg/ml, Biosyn) and matured with recombinant tumor necrosis factor-α (TNF-α) (10 ng/ml), recombinant IL-6 (15 ng/ml), recombinant IL-1β (5 ng/ml; all CellGenix) and prostaglandin E2 (10 mg/ml; Pfizer). Mature DC were pulsed with the HLA-A*02:01-restricted peptide of CEACAM5 (protein belonging to the CEA family, henceforth referred to as CEA; peptide sequence: YLSGANLNL) and frameshift derived peptides of TGF-βRII (RLSSCVPVA) and Caspase-5 (FLIIWQNTM) (all Interdivisional GMP Facility LUMC, Leiden, the Netherlands). This protocol gave rise to DCs with a mature phenotype meeting the release criteria: low expression of CD14, high expression of MHC class I, MHC class II, CD83, CD86, CCR7, as analyzed by flow cytometry. Details of antibodies used are summarized in Supplementary Table 1. Patients received at maximum $30x10^6$ DC per vaccination: $20x10^6$ DC intravenously and $10x10^6$ intradermally.

Treatment schedule

The Dutch Central Committee on Research involving Human Subjects (CCMO) has approved the study (NL28985.000.09) and written informed consent was obtained from all patients.

ClinicalTrials.gov identifier of the study is NCT01885702. The study was conducted in accordance with the Good Clinical Practice guidelines and with the provisions of the Declaration of Helsinki (October 9th 2004). Patients were injected intravenously and intradermally with autologous monocyte-derived DC (moDC) loaded with HLA-A*02:01 binding peptides of tumor associated antigen CEA and frameshift derived neoantigens caspase-5 and TGF-βRII according to a schedule of 3 weekly vaccinations. One to two weeks after the last vaccination a delayed-type hypersensitivity (DTH)-skin test was performed. Patients who remained free of disease recurrence were eligible for two maintenance cycles of three weekly vaccinations and a DTH skin test, each at 6-month intervals. After the third round, a colonoscopy was performed.

Proliferative response to KLH

For immunomonitoring purposes and to provide CD4 T cell help, all DCs were loaded with the control antigen KLH to study T cell proliferation. 1 x 10^5 PBMCs, isolated from blood samples before each vaccination, were plated per well of a 96-well tissue culture microplate in the presence of 10 mg/mL KLH or without KLH. After 4 days of culture, 1 µCi/well of tritiated thymidine was added for 8 hours and incorporation of tritiated thymidine was measured in a beta-counter. A proliferative response to KLH was considered positive if the proliferation index (proliferation with KLH / proliferation without KLH) is greater than 2.

Delayed type hypersensitivity skin test

Delayed type hypersensitivity (DTH) challenges were performed within 1-2 weeks after each vaccination cycle[2]. 1x10^6 mature DC loaded with the indicated peptides were injected intradermally at different sites. After 48 hours, 6 mm punch biopsies were taken. Half of the biopsy was manually cut and cultured as described[3]. Lymphocytes cultured from DTH-biopsies were stained with anti-CD8-FITC and tetrameric MHC complexes containing the TGF-βRII, caspase-5 and CEA HLA-A*02:01 epitopes (all Sanquin) and analyzed by flow cytometry. HIV was used as negative control. To test peptide recognition, lymphocytes were challenged with T2 cells pulsed with the indicated peptides or control melanoma peptide gp100:280-288. Production of IFN-γ, IL-2 and IL-5 was measured in the supernatants after 16h by cytometric bead array according to the manufacturer's instructions (eBiosciences).

Generation of (neo)antigen expressing tumor cell lines

Plasmids with vector backbone of pcDNA3.1+/C-(K)DYK (DYK tag replacing the stop codon of the insert) containing cDNA insert of CEA (OHu23459D), wild type *TGFBR2* (OHu26872D), and wild type *CASP5* (OHu04254D), custom generated mutant *TGFBR2* (A-1 at position 458 based on NM_001024847), and custom generated mutant *CASP5* (A-1 at position 202 based on NM_004347) were ordered (all Genscript). Each of the 5 plasmids was first transformed in DH5α bacteria and then single cell colonies were selected post Sanger sequencing using T7 (5' TAATACGACTCACTATAGGG 3') or BGHRev (5' TAGAAGGCACAGTCGAGG 3'): forward and reverse primers respectively (Invitrogen). For each of the 5 plasmids, an HLA-A*02 tumor cell line (BLM) cultured in DMEM (21885108, Gibco) with 10% FCS (Hyclone) was stably transfected using the calcium phosphate transfection kit (K278001, Invitrogen) as per the manufacturer's protocol. Briefly 20µg of transfecting DNA plasmid was precipitated in $CaCl_2$ and HBS and added to 1.5x10^6 BLM cells and given a 10% DMSO (Wak-Chemie Medical GmBH) shock 5 hours after DNA addition to stimulate uptake and incubated

overnight at 37 °C in DMEM with 10% FCS. The cells were then selected with 1mg/mL geneticin or G418 (Gibco) and single cell colonies were cloned through limiting dilution or cloning rings. The cell lines were monitored periodically for expression of the desired antigen. Validation on RT-qPCR was done using SYBR Green reagents on QuantStudio 3 (both Applied Biosystems). Flow cytometry was measured on FACS Verse or FACS Lyric (BD Biosciences) and staining methods for different antigens has been summarized in Supplementary Table 2. For BLM cell lines transfected with mutant *TGFBR2* and wild type and mutant *CASP5*, no antibodies that could be tested on flow cytometry were available, but all cell lines could be shown to highly express the respective antigen indirectly on flow cytometry through the increased expression of the Flag (DYK) tag protein conjugated with the protein of interest. The secondary antibody used for flow cytometry is - Alexa647 Goat anti-mouse IgG (H+L) (1:400, Invitrogen).

In-vitro expansion of (neo)antigen specific T cells

(Neo)antigen specific T cell expansion was based on the protocol by Ali et al.[4]. Briefly, cryopreserved patient PBMCs post vaccination were thawed in RPMI without additives (R8758, Sigma Aldrich) and stained with APC and PE fluorochrome conjugated HLA-A2*02:01 MHC-I dextramers (Immudex) for neoantigen TGF-βRII (WB3302), neoantigen caspase-5 (WB5812) and CEA (WB3277) as per manufacturer's protocol with CD8 antibody (1:1000, BDOptibuild). The dextramer stained cells were bulk sorted with FACSAria (BD Biosciences) in a 96 well round bottom plate on a layer of feeder cells made by irradiating PBMCs at 64Gy on X-RAD 320ix (Precision X-Ray) from three healthy donors (Sanquin, Nijmegen) to facilitate T cell growth. A maximum of 200 dextramer positive cells were sorted in one well containing $2x10^5$ feeder cells. The sorted cells were cultured in X-vivo (Lonza) with 5% HS (Sigma Aldrich) and PHA (1 µg/mL, Remel), IL-2 (850 U/mL, Peprotech) and IL-15 (2 ng/mL, Peprotech). Cells were routinely tested for dextramer specificity during culturing and resorted to a new feeder layer if needed.

Cytotoxicity Assay

Transfected and peptide loaded BLM cell lines were used as target cells in a calcein release assay. For each assay, target cells were treated with IFN-γ (10ng/mL, RayBiotech) and effector CTLs were rested in X-VIVO-15 medium with 2.5% HS, IL-2 (10 U/mL) and IL-15 (0.2 ng/mL) for 48 hours before the assay. In peptide recognition assays, target cells were pre-incubated with 10µM of peptide for 3 hours at 37 °C. Target cells were labeled with calcein AM (2µg/mL, eBioscience) in DMEM (Gibco) without serum in T25 flasks (Greiner) for 30 minutes at 37 °C and then seeded at $1x10^4$ cells per well in a U bottom 96 well plate in 3-6 replicates in phenol red free RPMI with 8% HS. Varying amounts of in-vitro expanded antigen specific patient T cells were then added to the target cells to achieve different effector target ratios in a final volume of 200µL per well. As positive control to measure maximum calcein release, RPMI with 8% HS and 2% Triton (Sigma Aldrich) was added. As negative control to measure spontaneous calcein release, RPMI with 8% HS without T cells was added. W6/32, anti-HLA-ABC class I ascites (ATCC) was used in blocking experiments for 1 hour with ascites containing gp100 antibody (NKI-beteb) as control. After 4 hours of incubation, 100µL of supernatant was used to measure the calcein released on Cytofluor-II (PreSeptive Biosystem) or ClarioStar (BMG Labtech) at Ex:Em of 485:530. Cytotoxicity was calculated as [(Test release-spontaneous release) / (maximum release - spontaneous release)] x 100%. In some conditions, calculated specific lysis values for target (neo)antigen loaded target cells exceeded 100%, likely due to technical variation in

maximum and spontaneous release controls. As cell lysis beyond 100% is biologically implausible, values exceeding 100% were capped at 100% to allow accurate representation and comparison of cytotoxicity across conditions. Conversely, in non-target cell lines, low calcein retention in some replicates was observed, likely due to dye leakage or non-specific effects, and such values were capped at 0%.

Sequencing of tumor and adenoma samples

To assess the microsatellite stability status of *CASP5* and *TGFBR2*, tumor and adenoma samples were sequenced. Formalin fixed paraffin embedded (FFPE) tissues of tumor or adenomas that were resected from the patients before or after the vaccination were available at the Department of Pathology of Radboud university medical center, Nijmegen or requested from Rijnstate hospital in Arnhem, Antoni van Leeuwenhoek hospital in Amsterdam, Jeroen Bosch hospital in 's Hertogenbosch and Elizabeth Tweesteden Ziekenhuis hospital in Tilburg. Four to five sections of 10 µm thickness were cut on a microtome and neoplastic areas were manually isolated with macrodissection. DNA was isolated using the Qiagen AllPrep DNA/RNA FFPE kit as per standard manufacturer's protocol and stored at -20 °C till further use. DNA isolated was quantified using the Qubit fluorometer (Thermo Fisher Scientific) and up to 25ng when available was used in a PCR reaction for amplification of the *CASP5* and *TGFBR2* genes with 0.3 µM primer concentration. For *CASP5* amplification, forward primer 5' CAGAGTTATGTCTTAGGTGAAGG 3' and reverse primer 5' CTTCCTTCAATGTCAGAACATCGTG 3' was used in a PCR reaction using the TaKaRa PrimeStar GXL kit (TaKaRa Bio) with amplification of 42 cycles with 10s at 98 °C, 15s at 57 °C and 30s at 68 °C. For *TGFBR2* amplification, M13 tagged forward primer 5' GTAAAACGACGGCCAGTCCCCAAGCTCCCCTACCATG 3' and reverse primer 5' GGCACAGATCTCAGGTCCCA 3' was used in a PCR reaction using the TaKaRa Ex Premier DNA Polymerase kit (TaKaRa Bio) with amplification of 39 cycles with 10s at 98 °C, 15s at 63 °C and 60s at 68 °C. The PCR products were cleaned using the exoSAP-IT PCR product cleanup reagent or the exo CIP- A and B PCR product cleanup reagent (both Applied Biosystems) as per manufacturer's protocol and sanger sequenced either in house or by Macrogen. For *CASP5* the original PCR primers were used for sequencing. For *TGFBR2*, M13(-20) primer 5' GTAAAACGACGGCCAGT 3' and the original PCR reverse primer were used for sequencing.

Statistical analysis

Descriptive statistics of immunological response in the treatment group was conducted in Rstudio (v4.2.1, RRID: SCR_000432) or GraphPad Prism(v8.0.2, GraphPad Software, Boston, Massachusetts USA). Release of cytokines IFN-γ, IL-2 and IL-5 in response to target (neo)antigen was termed positive if the release of the respective cytokine was at least 5 standard deviations larger than the mean release in response to the irrelevant control peptide. Unpaired t-tests were conducted to assess specific lysis in cytotoxicity assays. Correction for multiple t-tests was applied using the Bonferroni-Dunn method on t-tests conducted per effector-target ratio in cytotoxicity assays. Linear regression model was used to predict the possibility of developing at least one lesion with *TGFBR2* mutation in the presence of mutant TGF-βRII specific T cells (logistic regression is not possible since probability of having a lesion with *TGFBR2* mutation in the presence of mutant TGF-βRII specific T cells is zero in this cohort). Log rank test was done to evaluate the statistical significance in disease free survival Kaplan Meier curves. Statistical significance was defined as $p \leq 0.05$.

References

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2. de Vries, I. J. M. *et al.* Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients. *Clin. cancer Res. an Off. J. Am. Assoc. Cancer Res.* **9**, 5091–5100 (2003).

3. Aarntzen, E. H. J. G. *et al.* Skin-Test Infiltrating Lymphocytes Early Predict Clinical Outcome of Dendritic Cell–Based Vaccination in Metastatic Melanoma. *Cancer Res.* **72**, 6102–6110 (2012).

4. Ali, M. *et al.* Induction of neoantigen-reactive T cells from healthy donors. *Nat. Protoc.* **14**, 1926–1943 (2019).

Supplementary Table 1: Antibodies used in characterization of dendritic cell phenotype

Marker - Conjugate	**Antibody clone**	**Company**	**Dilution used**
CD14 - APC	REA599	Miltenyi Biotech	1:400
HLA ABC - APC	REA230	Miltenyi Biotech	1:80
HLA DR DQ DP - APC	REA332	Miltenyi Biotech	1:80
CD80 - APC	2D10	Miltenyi Biotech	1:80
CD83 - APC	HB15	Miltenyi Biotech	1:160
CD86 - APC	FM95	Miltenyi Biotech	1:160
CCR7 - APC	REA108	Miltenyi Biotech	1:80

Supplementary Table 2: Staining method and antibodies used in monitoring antigen expression in target cell lines used in cytotoxicity assays

	Flow Cytometry		**RT-qPCR**	
	Method	Antibody	Forward primer(5'-3')	Reverse primer(5'-3')
BLM wild type TGF-βRII	Cell surface staining	Primary-Anti TGF-βRII antibody(1:50, ab78419 Abcam)	CAACATCAACCACAACACAGAG	CCGTCTTCCGCTCCTCAG
BLM mutant TGF-βRII	Intracellular staining with Foxp3/transcription Factor staining	Primary-Anti-DYKDDDDK (1:500, Genscript)	CAACATCAACCACAACACAGAG	CTTATCGTCGTCATCCTTGTAATCG

	buffer kit (eBioscience)			
BLM wild type caspase-5	Intracellular staining with Cytofix/cytoperm kit(BD)	Primary-Anti-DYKDDDDK (1:500, Genscript)	ACGGATCAAAAGTCGACCAGT	ACCATGAAGAACATCTTTGCCCAG
BLM mutant caspase-5	Intracellular staining with Cytofix/cytoperm kit(BD)	Primary-Anti-DYKDDDDK (1:500, Genscript)	ACGGATCAAAAGTCGACCAGT	CTTATCGTCGTCATCCTTGTAATCG
BLM CEA	Cell surface staining	Primary-CEACAM5 clone Col-1(1:50 ,abcam)	GACGCAAGAGCCTATGTATG	GGCATAGGTCCCGTTATTA

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