

Assessment of vaccine-induced CD4 T cell responses to the 119-143 immunodominant region of the tumor-specific antigen NY-ESO-1 using DRB1*0101 tetramers

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Statement of translational relevance

The development of reagents allowing rapid and efficient immunological monitoring of vaccine-induced CD4 T cell responses to human tumor antigens is instrumental for the development of anti-cancer vaccines. Whereas MHC class I fluorescent tetramers incorporating tumor antigen peptides, allowing direct analysis of specific CD8 T cells, are widely used, only few MHC class II tetramers have been generated so far. Here, we generated DRB1*0101 tetramers incorporating an immunodominant peptide from the tumor-specific antigen ESO, presently viewed as an important model antigen for the development of generic anti-cancer vaccines, and used them to characterize vaccine-induced CD4 T cell responses in vaccinated patients expressing DR1. DR1/ESO₁₁₉₋₁₄₃ tetramers specifically identified vaccine-induced CD4 T cells both in peptide-stimulated cultures and *ex vivo*, allowing their direct quantification and phenotyping. The development of DR1/ESO₁₁₉₋₁₄₃ tetramers will be instrumental for the assessment of spontaneous and vaccine-induced immune responses to ESO in DR1⁺ patients.

Abstract

Purpose: NY-ESO-1 (ESO), a tumor specific antigen of the Cancer/Testis group, is presently viewed as an important model antigen for the development of generic anti-cancer vaccines. The ESO₁₁₉₋₁₄₃ region is immunodominant following immunization with a recombinant ESO vaccine. In this study, we have generate DRB1*0101/ESO₁₁₉₋₁₄₃ tetramers and used them to assess CD4 T cell responses in vaccinated patients expressing DR1.

Experimental design: We generated tetramers of DRB1*0101 incorporating peptide ESO₁₁₉₋₁₄₃ using a previously described strategy. We assessed ESO₁₁₉₋₁₄₃ specific CD4 T cells in peptide-stimulated post-vaccine cultures using the tetramers. We isolated DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ cells by cell sorting and characterized them functionally. We assessed vaccine-induced CD4⁺ DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ T cells *ex vivo* and characterized them phenotypically.

Results: Staining of cultures from vaccinated patients with DR1/ESO₁₁₉₋₁₄₃ tetramers identified vaccine-induced CD4 T cells. Tetramer⁺ cells isolated by cell sorting were of T_H1 type and efficiently recognized full-length ESO. We identified ESO₁₂₃₋₁₃₇ as the minimal optimal epitope recognized by DR1-restricted ESO-specific CD4 T cells. By assessing DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ cells using TCR V β specific antibodies, we identified several frequently used V β . Finally, direct *ex vivo* staining of patients CD4 T cells with tetramers allowed the direct quantification and phenotyping of vaccine-induced ESO-specific CD4 T cells.

Conclusions: The development of DR1/ESO₁₁₉₋₁₄₃ tetramers allowing the direct visualization, isolation and characterization of ESO-specific CD4 T cells will be instrumental for the evaluation of spontaneous and vaccine-induced immune responses to this important tumor antigen in DR1 expressing patients.

Introduction

Active elicitation of immune responses to tumor-specific antigens through vaccination is currently explored as a strategy that could complement standard cancer therapy to stabilize disease and prevent recurrence (1-3). One promising approach is to use molecularly defined synthetic vaccines incorporating well-characterized recombinant tumor antigens administered with strong adjuvants (4, 5). These vaccines can elicit integrated antibody and cellular immune responses, but their ability to eradicate cancer cells, particularly in the case of intracellular tumor antigens, relies on the elicitation of antigen specific T cells. Although cytotoxic CD8 T cells (CTL) are considered the main anti-tumor effector cells, CD4 T cell responses are key to the development of efficient anti-tumor immunity, both by providing help for the development of CTL and by directly exerting different effector functions (6-10). A rapid and hopefully successful development of anti-cancer vaccines is therefore dependent on the availability of methods that allow the efficient and reliable monitoring of vaccine-induced tumor antigen-specific T cells. In this context, the development of soluble fluorescent MHC-peptide oligomers (commonly referred to as tetramers), allowing the direct visualization, enumeration and characterization of antigen specific T cells, has represented a major advance (11, 12). Hundreds of tetramers corresponding to different MHC class I alleles incorporating peptides from pathogen and self-antigens, including tumor antigens, have been generated and widely used in recent years (12-14). The development of MHC class II-peptide tetramers, instead, has been much more limited, and has been successful only in a minority of cases (12, 15-17).

NY-ESO-1 (ESO), a tumor-specific antigen of the cancer/testis group frequently expressed in tumors of different histological types but not in normal somatic tissues is an important candidate for the development of generic anti-cancer vaccines (18, 19). Several candidate

anti-cancer vaccines using ESO-based immunogens are currently under trial (4, 20). Following vaccination with a recombinant ESO protein (rESO) administered with Montanide ISA-51 and CpG ODN 7909, we have obtained induction of CD4 T cell responses in 17/18 vaccinated patients (4). The majority of vaccine-induced CD4 T cells were directed against two immunodominant regions of ESO, corresponding to peptides 81-100 and 119-143. ESO₁₁₉₋₁₄₃ has been previously reported to bind to multiple MHC class II molecules (21, 22) and epitopes located in the ESO₁₁₉₋₁₄₃ region and recognized by specific CD4 T cells in the context of several HLA-DR molecules have been identified (21, 23, 24). We have recently generated tetramers of ESO₁₁₉₋₁₄₃ presented in the context of DR52b (DRB3*0202), an alternate DR molecule frequently expressed by Caucasians (25). Whereas the use of DR52b $\alpha\beta$ chains containing leucine zipper motifs was not sufficient, alone, for the successful generation of DR52b/ESO tetramers, we have shown that the use of ESO peptides bearing an amino-terminal His-tag, that allows the isolation of folded monomers by affinity purification, allows the generation of efficient tetramers (25).

In this study, we generated tetramers of DRB1*0101 (DR1) incorporating peptide ESO₁₁₉₋₁₄₃, using this strategy. We initially validated the DR1/ESO₁₁₉₋₁₄₃ tetramers on specific and control clones. We then assessed peptide-stimulated cultures from vaccinated patients expressing DR1, isolated DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ cells by cell sorting and further characterized them functionally. Finally, we used the DR1/ESO₁₁₉₋₁₄₃ tetramers to assess vaccine-induced CD4 T cells *ex vivo* and characterize them phenotypically.

Material and Methods

Generation of fluorescent HLA-DR1/ESO peptide tetramers. Soluble DR1 molecules were produced in *D. mel-2* cells, purified by anti-HLA-DR (clone L243) immuno-affinity chromatography, loaded with peptide and biotinylated as previously described (25). When DR1 molecules were loaded with untagged ESO peptides, complexes were directly purified by gel filtration in PBS pH 7.4, 100 mM NaCl on a Superdex S200 column (GE Healthcare Life Sciences) and the fractions corresponding to the monomeric pMHC complexes were pooled and concentrated. Alternatively, ESO peptides were extended at the N-terminus by a sequence containing 6 His residues and a linker (Ser-Gly-Ser-Gly). DR1/His-tag-ESO peptide complexes were purified using a HisTrap HP 1 ml column (GE Healthcare Life Sciences) prior to purification by gel filtration. Biotinylation and purity, as assessed by SDS-PAGE in an avidin shift assay, were >90%. Biotinylated DR1/peptide monomers were multimerized by mixing with small aliquots of streptavidin-PE (Invitrogen) up to the calculated 4:1 stoichiometry.

Patients samples, cells and tissue culture. Peripheral blood samples were collected from cancer patients enrolled in a clinical trial of vaccination with rESO, Montanide ISA-51 and CpG 7909 (4) upon informed consent and approval by the Institutional Review Boards. MHC class II alleles were determined by high resolution molecular typing (24). L.DR1, DR1-transfected mouse cells kindly provided by Dr. Hassane M. Zarour (Department of Medicine and Melanoma Center, University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA), were maintained in complete RPMI medium and periodically typed for HLA-DR1 expression. ESO₁₁₉₋₁₄₃-specific DR1-restricted CD4 T cell clones were obtained from post-vaccine samples from DR1⁺ patients as previously described (24). Clones were expanded by periodic

(every 3-4 wk) stimulation with phytohemagglutinin (PHA, OXOID) and allogeneic irradiated PBMC and cultured in complete IMDM medium in the presence of rhIL-2 (100 IU/mL).

Assessment of ESO-specific CD4 T cells, tetramer staining and flow cytometric analysis and sorting. For assessment of specific CD4 T cell responses following *in vitro* stimulation, CD4⁺ cells were enriched from PBMC by magnetic cell sorting (Miltenyi Biotec Inc.), stimulated with irradiated autologous APC in the presence of ESO peptides, as indicated, rhIL-2 and rhIL-7 as previously described (24) and maintained in culture during 10-15 days prior to tetramer staining. Peptide stimulated cultures and specific monoclonal and polyclonal populations were incubated with tetramers at a final concentration of 3 µg/ml for 1 hr at 37°C, unless otherwise indicated, in complete IMDM medium, washed and then stained with CD4 (BD Biosciences) or TCR Vβ (Beckman Coulter) specific mAb in PBS, 5% FCS for 15 minutes at 4°C and analyzed by flow cytometry (FACS Aria, BD Biosciences). In order to generate specific polyclonal T cell populations, tetramer⁺ cells within peptide-stimulated cultures were sorted by flow cytometry (FACS Aria, BD Biosciences) and expanded by stimulation with PHA and irradiated allogeneic PBMC in the presence of rhIL-2 (26). For *ex vivo* enumeration and phenotyping of specific cells, CD4⁺ cells enriched from PBMC were rested overnight, incubated with tetramers (3 µg/ml) for 2 hrs at 37°C and then stained with CD4-, CD45RA- (BD Biosciences) and CCR7- (Miltenyi Biotec Inc.) specific mAb and analyzed by flow cytometry. Tetramers used throughout the study contained His-tagged ESO peptides, unless otherwise indicated.

Antigen recognition assays. DR1⁺ ESO-specific monoclonal or polyclonal CD4 T cell populations were stimulated in the absence or presence of ESO peptides (2 µM) or PMA (100

ng/ml) and ionomycin (1 μ g/ml), as indicated, and cytokine production was assessed in a standard 4 hr intracellular cytokine staining assay using mAb specific for IFN- γ , TNF- α , IL-2, IL-4, IL-10 (BD Biosciences) and IL-17 (eBiosciences) and flow cytometric analysis, as previously described (24, 27). In order to assess DR1 restriction of monoclonal and polyclonal ESO-specific populations, they were incubated for 4 hrs with L.DR1 cells or with untransfected mouse fibroblasts that have been pulsed with peptide ESO₁₁₉₋₁₄₃ for 1 hr at 37°C and washed 3 times, and IFN- γ production was assessed by intracellular staining and flow cytometric analysis. In other experiments, specific polyclonal cultures were incubated for 24 hrs with either L.DR1 cells and serial dilutions of ESO peptides or monocyte derived dendritic cells pre-incubated overnight with serial dilutions of rESO, and IFN- γ was measured by ELISA in 24 hr culture supernatants, as previously described (4, 24).

Results and Discussion

Generation and validation of DRB1*0101/ESO₁₁₉₋₁₄₃ tetramers. Direct assessment with fluorescent MHC class II tetramers incorporating immunodominant peptides from frequently expressed tumor antigens is an attractive approach for the monitoring of anti-tumor CD4 T cells. At variance with MHC class I/peptide tetramers, originally developed in 1996 (11) that have since been generated for a large number of alleles incorporating a variety of peptides, including ones from tumor antigens, the development of MHC class II/peptide tetramers has proven significantly more difficult (12, 15-17). Among limiting factors are the high polymorphism of MHC class II molecules, the often low binding affinity of peptides from tumor/self antigens, and the structural characteristics of MHC class II molecules. Namely, because MHC class II $\alpha\beta$ chain monomers are unstable in solution, one strategy to improve tetramer generation has consisted in adding leucine zippers to facilitate $\alpha\beta$ pairing (28). MHC class II $\alpha\beta$ chains incorporating leucine zippers, however, can form stable complexes also in the absence of bound peptides, which can lead to the generation of tetramers formed by “empty” MHC class II molecules. While attempting to generate tetramers of the alternate DR molecule DR52b incorporating peptide ESO₁₁₉₋₁₄₃, we found that the use of leucine zipper-containing DR52b molecules alone was insufficient for the generation of tetramers able to significantly stain specific CD4 T cells. We therefore implemented the approach by using His-tagged peptides, allowing the isolation of folded MHC class II/peptide monomers by affinity purification, which resulted in the generation of efficient DR52b/ESO tetramers (25). In this study, we used the same strategy to generate tetramers of DRB1*0101 (DR1) incorporating ESO₁₁₉₋₁₄₃. To validate the DR1/ESO₁₁₉₋₁₄₃ tetramers, we initially assessed them on a specific clone (Figure 1A) obtained from a DR1⁺ patient who had been immunized with the rESO

vaccine (4). As shown in Figure 1B, the tetramers efficiently stained the specific clone but not an irrelevant clone used as control. To optimize the tetramer staining conditions, we assessed the effect of tetramer concentration, incubation time and temperature on specific and control clones. We obtained significant staining of specific clones with relatively low doses of tetramer (1 $\mu\text{g/ml}$). The staining intensity increased with the dose of tetramer, up to 30 $\mu\text{g/ml}$, without reaching a plateau (Figure 1B). Staining of specific clones was more efficient at high temperature (37°C) and after prolonged incubation times (Figure 1C). Thus, the use of leucine zipper-containing DR1 molecules and His-tagged ESO peptides resulted in the generation of efficient tetramers. Because the loading efficiency of MHC class II/peptide complexes, and therefore the need for using His-tagged peptides, could significantly vary for different MHC class II molecules and peptides, we also prepared DR1/ESO tetramers using untagged peptides. As shown in Figure 1D, DR1/ESO tetramers generated with the untagged peptide ESO₁₁₉₋₁₄₃ also stained ESO specific clones, although with slightly lower efficiency as compared to DR1/ESO tetramers prepared using His-tagged peptides. Thus, in contrast to DR52b/ESO tetramers, the use of His-tagged peptides was helpful but not indispensable for the generation of DR1/ESO tetramers.

Assessment of peptide-stimulated cultures from vaccinated patients using DRB1*0101/ESO₁₁₉₋₁₄₃ tetramers. To evaluate vaccine-induced CD4 T cells in DR1⁺ immunized patients, we initially stained post-vaccine CD4 T cells from patient N03 (a high responder to the vaccine, expressing DRB1*0101) previously stimulated *in vitro* for 12 days with a pool of long overlapping peptides spanning the entire ESO sequence (4), with DR1/ESO₁₁₉₋₁₄₃ tetramers during 1 hr at 37°C. As illustrated in Figure 2A, this analysis identified a significant proportion of DR1/ESO₁₁₉₋₁₄₃ tetramers⁺ CD4 T cells in the culture.

DR1 tetramers incorporating peptide ESO₉₅₋₁₀₆, used as an internal control, failed to identify significant proportions of tetramer⁺ cells. In a separate experiment, we stimulated post-vaccine samples from patient N03 and from 3 additional vaccinated patients expressing DR1 alleles (N11 and C04 also expressing DRB1*0101 and C03 expressing DRB1*0103) with peptide ESO₁₁₉₋₁₄₃ alone and assessed them 12 days later with the DR1/ESO₁₁₉₋₁₄₃ tetramers. As illustrated in Figure 2B, we detected significant proportions of tetramer⁺ cells in cultures from all patients. DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ cells had clearly been induced by vaccination, as they were not detectable at significant levels in pre-vaccine samples stimulated in the same conditions.

Isolation and characterization of vaccine-induced DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ cells. To assess vaccine-induced DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ cells, we isolated them by flow cytometry cell sorting and expanded them *in vitro*, as polyclonal monospecific cultures (Figure 3A). Isolated tetramer⁺ cells specifically recognized peptide ESO₁₁₉₋₁₄₃ but not a control ESO peptide (Figure 3A). Antigen recognition by polyclonal monospecific tetramer⁺ cells was restricted by DR1, as efficient antigen presentation was obtained using DR1-transfected mouse cells pre-incubated with peptide ESO₁₁₉₋₁₄₃ (Figure 3B). To further characterize vaccine-induced DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ cells, we assessed their capacity to efficiently recognize the full-length recombinant ESO protein (rESO) processed and presented by autologous APC. To this purpose we generated monocyte-derived dendritic cells (moDC) by culturing autologous CD14⁺ cells with GM-CSF and IL-4 as described (4), incubated them with serial dilutions of rESO and tetramer⁺ cells and assessed IFN- γ secretion in the culture supernatant. As shown in Figure 3C, tetramer⁺ cells recognized rESO processed and presented by autologous moDC with high efficiency as half-maximal recognition was obtained at a concentration of rESO

similar to that of ESO₁₁₉₋₁₄₃ peptide presented by DR1-expressing APC. To assess the type of vaccine-induced DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ cells with respect to cytokine secretion, we stimulated them with PMA and ionomycin, permeabilized and stained them with mAb specific for signature cytokines produced by different T_H cell subsets. As illustrated in Figure 3D, tetramer⁺ cells displayed a typical T_H1 profile as they secreted IFN- γ , IL-2 and TNF- α , but not IL-4, IL-10 or IL-17.

DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ cells use a conserved TCR repertoire. T cells recognizing defined MHC/peptide complexes often exhibit conserved features including the use of defined variable regions of the TCR α and β chains (V α and V β). To address if DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ cells exhibited such conserved features, we assessed the polyclonal monospecific tetramer⁺ populations from vaccinated patients with a panel of anti-V β mAb covering about 50% of the human TCR repertoire. Examples of co-staining with anti-V β mAb and tetramers are shown in Figure 4A and a summary of the data obtained is reported in Figure 4B. We found a frequent usage of several V β segments, including V β 1, V β 2 and V β 3. V β 1 tetramer⁺ cells were prevalent in the culture of patient N03, representing half of the total population. The large majority of tetramer⁺ cells in the culture of patient C03 and a significant proportion of tetramer⁺ cells in the cultures of two other patients, N11 and C04, used V β 2. Finally, about half of tetramer⁺ cells in the culture of patient N11 used V β 3. Thus, DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ cells frequently used few selected V β regions, indicating the presence of a conserved TCR repertoire.

Assessment of the minimal ESO peptide optimally recognized by DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ cells. In a previous study assessing ESO₁₁₉₋₁₄₃ binding to several MHC class II alleles,

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including DR1, the 15-mer ESO₁₂₃₋₁₃₇ showed a binding affinity for DR1 similar to that of ESO₁₁₉₋₁₄₃ (21). To better define the DR1 epitope with respect to recognition by specific T cells, we assessed the recognition of truncated peptides within the ESO₁₁₉₋₁₄₃ region by tetramer⁺ T cells. NH₂-terminal truncations up to amino acid 123 did not significantly affect recognition by tetramer⁺ T cells (Figure 5A). Further truncation, however, significantly reduced recognition. Similarly, COOH-terminal truncations up to amino acid 137 did not significantly affect recognition, whereas further truncation reduced it. This analysis identified ESO₁₂₃₋₁₃₇ as the minimal peptide optimally recognized by DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ CD4 T cells. In line with these results, DR1 tetramers incorporating peptide ESO₁₂₃₋₁₃₇ stained specific clones with the same efficiency as compared to DR1/ESO₁₁₉₋₁₄₃ tetramers (Figure 5B) and identified similar proportions of CD4 tetramer⁺ cells in peptide-stimulated cultures from post-vaccine samples (Figure 5C).

Ex vivo assessment of the frequency and phenotype of vaccine-induced ESO-specific CD4 T cell responses with DR1/ESO₁₁₉₋₁₄₃ tetramers. The relatively high frequency of DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ CD4 T cells detected in peptide-stimulated cultures from vaccinated patients encouraged us to attempt assessing the frequency and phenotype of vaccine-induced CD4 T cells in DR1 expressing patients *ex vivo*. To this end, for each patient, we isolated CD4 T cells by magnetic cell sorting from samples taken prior to and at different time points after vaccination, when available, and stained them with DR1/ESO tetramers together with antibodies directed against markers that distinguish CD4 T cells according to their differentiation stage (29). For 3 of the 4 patients, samples taken prior to vaccination were available. The frequency of DR1/ESO tetramer⁺ cells among memory (CD45RA⁻) CD4 T cells in pre-vaccine samples was below detection limits (< 1:100 000) (Figure 6A and 6B). In

contrast, in post-vaccine samples from all patients taken after 3 vaccine injections (PV 3) DR1/ESO tetramer⁺ cells were detectable at a frequency that was variable among different patients and was in average of about 1:10 000 memory CD4 T cells. For 3 patients for whom additional samples taken after 4 vaccine injections (PV 4) were available, DR1/ESO tetramer⁺ cells were detectable at a frequency that was, for each patient, comparable to that detected after 3 injections. For 2 patients, C03 and C04, additional samples taken 4 and 5 months respectively after the 4th and last injection (post-treatment, PT) were also available. In these samples, DR1/ESO tetramer⁺ cells were still detectable at a frequency similar, for each patient, to that detected one week after the last injection (PV 4). Vaccine-induced DR1/ESO tetramer⁺ cells included both central memory (CCR7⁺) representing “reservoir” memory populations (30, 31) and effector memory populations (CCR7⁻) (Figure 6C).

In conclusion, assessment of vaccine-induced CD4 T cells using DR1/ESO tetramers confirmed the ability of the ESO vaccine to induce strong and long lasting CD4 T cell memory responses of T_H1 type, that are generally associated with efficient anti-tumor responses. The high efficiency and specificity of the staining obtained with the DR1/ESO tetramers allowed the direct *ex vivo* detection of specific cells among total CD4 T cells, without the need for enrichment steps used in previous studies (28, 32). It is noteworthy that the frequency of vaccine-induced ESO-specific CD4 T cells detected *ex vivo* (in average 1:10 000 memory cells) is in the same range of *ex vivo* frequencies of previously reported DR1-restricted CD4 T cells specific for viral epitopes (28, 32). The generation and validation of DR1/ESO tetramers reported in this study encourage their further use for the evaluation of CD4 T cells specific for this important tumor antigen in the context of spontaneous or vaccine-induced immune responses in DR1 expressing patients.

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Figure legends

Fig. 1. DR1/ESO₁₁₉₋₁₄₃ tetramers stain ESO₁₁₉₋₁₄₃-specific DR1-restricted CD4 T cell clones. *A*, ESO₁₁₉₋₁₄₃-specific clonal populations from DR1⁺ patient N03 were incubated with untransfected or DR1-expressing mouse fibroblasts that had been pulsed or not with peptide ESO₁₁₉₋₁₄₃ and IFN- γ production was assessed in a 4 hr intracellular cytokine staining assay. *B*, ESO-specific DR1-restricted and control clonal populations were stained with serial dilutions of DR1/His-ESO₁₁₉₋₁₄₃ tetramers for 1 hr at 37°C followed by staining with anti-CD4 mAb and flow cytometry analysis. Examples of dot plots for the ESO-specific clone and the mean fluorescence intensity (MFI) of tetramer staining for both clones at all concentrations are shown. *C*, ESO-specific DR1-restricted and control clonal populations were stained with DR1/His-ESO₁₁₉₋₁₄₃ tetramers (3 μ g/ml) at 4°C, 23°C or 37°C for the indicated periods and analyzed as in *B*. *D*, ESO-specific DR1-restricted and control clonal populations were stained with DR1 tetramers containing untagged or His-tagged ESO₁₁₉₋₁₄₃ peptides and analyzed as in *B*. Examples of dot plots for staining of ESO-specific cells with both tetramers at 10 μ g/ml and MFI of tetramer staining for all conditions are shown.

Fig. 2. DR1/ESO₁₁₉₋₁₄₃ tetramers stain peptide-stimulated CD4 T cells from post-vaccine but not from pre-vaccine samples of DR1⁺ patients. *A*, Post-vaccine CD4 T cells from DR1⁺ patient N03, stimulated *in vitro* with a pool of overlapping long ESO peptides spanning the full-length ESO sequence, were stained with DR1/ESO₁₁₉₋₁₄₃ or control DR1/ESO₉₅₋₁₀₆ tetramers (3 μ g/ml) for 1 hr at 37°C and anti-CD4 mAb and analyzed by flow cytometry. *B*, Pre- and post-vaccine CD4 T cells from DR1⁺ patients, stimulated *in vitro* with peptide ESO₁₁₉₋₁₄₃, were stained with DR1/ESO₁₁₉₋₁₄₃ tetramers and anti-CD4 mAb and analyzed by flow cytometry. Dot plots for patient N11 and data for all patients are shown.

Fig. 3. Isolation and functional characterization of vaccine-induced DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ CD4 T cells. *A*, Post-vaccine CD4 T cells were stimulated *in vitro* with peptide ESO₁₁₉₋₁₄₃, stained with DR1/ESO₁₁₉₋₁₄₃ tetramers (left dot plot) and tetramer⁺ and tetramer⁻ cells were isolated by flow cytometry cell sorting. Aliquots of sorted cells were directly re-analyzed by flow cytometry (middle dot plots). Tetramer⁺ cells were expanded *in vitro* and the purity of the resulting polyclonal populations was assessed by flow cytometry analysis following tetramer staining (right dot plot). Polyclonal populations were also incubated with L.DR1 cells and serial dilutions of ESO₁₁₉₋₁₄₃ or control peptide and IFN- γ was measured by ELISA in 24 hrs culture supernatants. Results are shown for one patient, N03, representative of four. *B*, Tetramer⁺ polyclonal populations were incubated with L.DR1 cells, that have been pulsed or not with peptide ESO₁₁₉₋₁₄₃, and IFN- γ production was assessed in a 4 hr intracellular cytokine staining assay. *C*, Tetramer⁺ polyclonal populations were incubated either with L.DR1 cells and serial dilutions of ESO₁₁₉₋₁₄₃ or control peptide (left panel) or with DR1⁺ monocyte-derived dendritic cells pre-incubated with serial dilutions of rESO or control protein (middle panel) and IFN- γ was measured by ELISA in 24 hrs culture supernatants. Examples of peptide and protein recognition are shown for patient N11 and the concentration of peptide and protein resulting in half maximal IFN- γ secretion (EC₅₀) is shown for all patients. *D*, Polyclonal cultures were stimulated with PMA and ionomycin and cytokine production was assessed in a 4 hr intracellular cytokine assay. Examples of dot plots for patient N03 and data obtained for all patients and all cytokines tested are shown.

Fig. 4. Assessment of TCR V β usage by vaccine-induced ESO₁₁₉₋₁₄₃-specific DR1-restricted CD4 T cells. Polyclonal monospecific tetramer⁺ populations from vaccinated patients were

first stained with DR1/ESO₁₁₉₋₁₄₃ tetramers and then with a panel of anti-TCR V β mAb and analyzed by flow cytometry. Examples of dot plots obtained with anti-V β 1 and anti-V β 2 mAb staining for patient N03 are shown in *A*. Numbers correspond to the percentage of V β ⁺ cells among tetramer⁺ cells in the culture. Results corresponding to the percentage of V β ⁺ cells, for all V β tested, among tetramer⁺ cells for all patients are summarized in *B*.

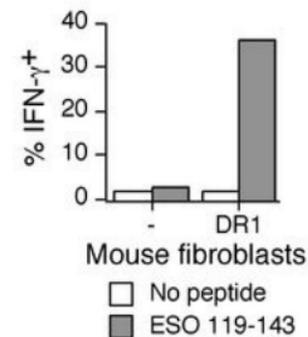
Fig. 5. Assessment of the minimal peptide optimally recognized by vaccine-induced ESO-specific DR1-restricted CD4 T cells. *A*, Polyclonal DR1/ESO₁₁₉₋₁₄₃ tetramer⁺ cultures, obtained as in Figure 3A, from patient N03 were incubated with L.DR1 cells and serial dilutions of truncated peptides within the 119-143 region or ESO₁₋₂₀ control peptide and IFN- γ was measured by ELISA in 24 hrs culture supernatants (examples are shown in the left panel). The activity of each peptide (EC₅₀) was calculated relative to that of peptide ESO₁₁₉₋₁₄₃ (right panel). *B*, ESO-specific DR1-restricted or control clonal populations were stained with serial dilutions of DR1 tetramers containing peptides ESO₁₁₉₋₁₄₃ or ESO₁₂₃₋₁₃₇ and analyzed by flow cytometry as in Figure 1B. *C*, Post-vaccine CD4 T cells from DR1⁺ patients were stimulated *in vitro* with peptide ESO₁₁₉₋₁₄₃, stained with DR1/ESO₁₁₉₋₁₄₃ or DR1/ESO₁₂₃₋₁₃₇ tetramers and anti-CD4 mAb and analyzed by flow cytometry. Dot plots for patient C04 and data for all patients are shown.

Fig. 6. *Ex vivo* assessment of vaccine-induced ESO-specific DR1-restricted CD4 T cells. CD4 T cells purified from PBMC from pre- and post-vaccine samples of DR1⁺ patients were stained *ex vivo* with DR1/ESO₁₁₉₋₁₄₃ tetramers (3 μ g/ml) during 2 hrs at 37°C and were then stained with anti-CD4, -CD45RA and -CCR7 mAb and analyzed by flow cytometry. *A*, Examples of dot plots for pre- and post-vaccine samples. Numbers in dot plots correspond to

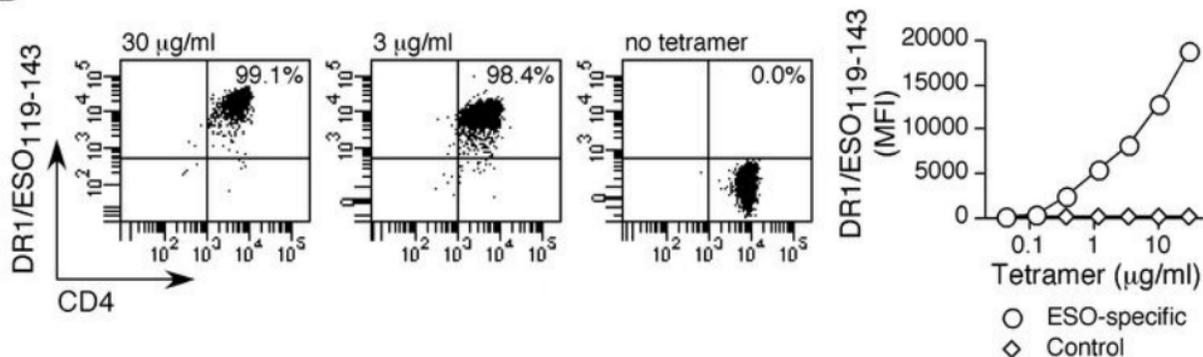
the percentage of tetramer⁺ cells among memory CD45RA⁻ CD4 T cells. *B*, Percentage of tetramer⁺ cells among memory CD45RA⁻ CD4 T cells in pre-vaccine and post-vaccine samples (PV 3, one week following the 3rd vaccine injection; PV 4, one week following the 4th vaccine injection; PT, 4 to 5 months following the 4th and last vaccine injection). *C*, Phenotype of tetramer⁺ cells in PV 3 samples based on CD45RA and CCR7 staining (CM, central memory CD45RA⁻CCR7⁺; EM, effector memory CD45RA⁻CCR7⁻).

Figure 1

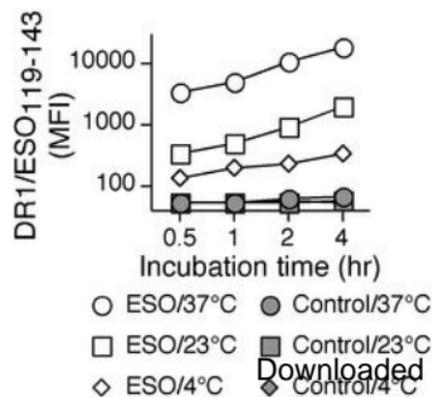
A



B



C



D

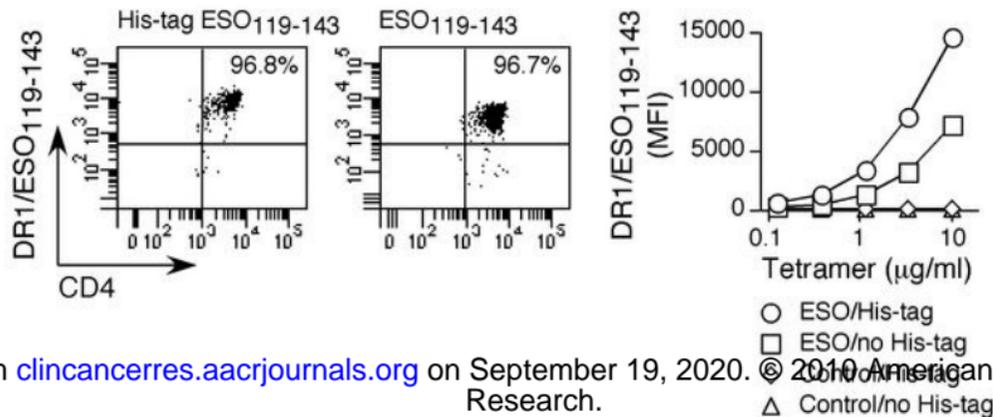
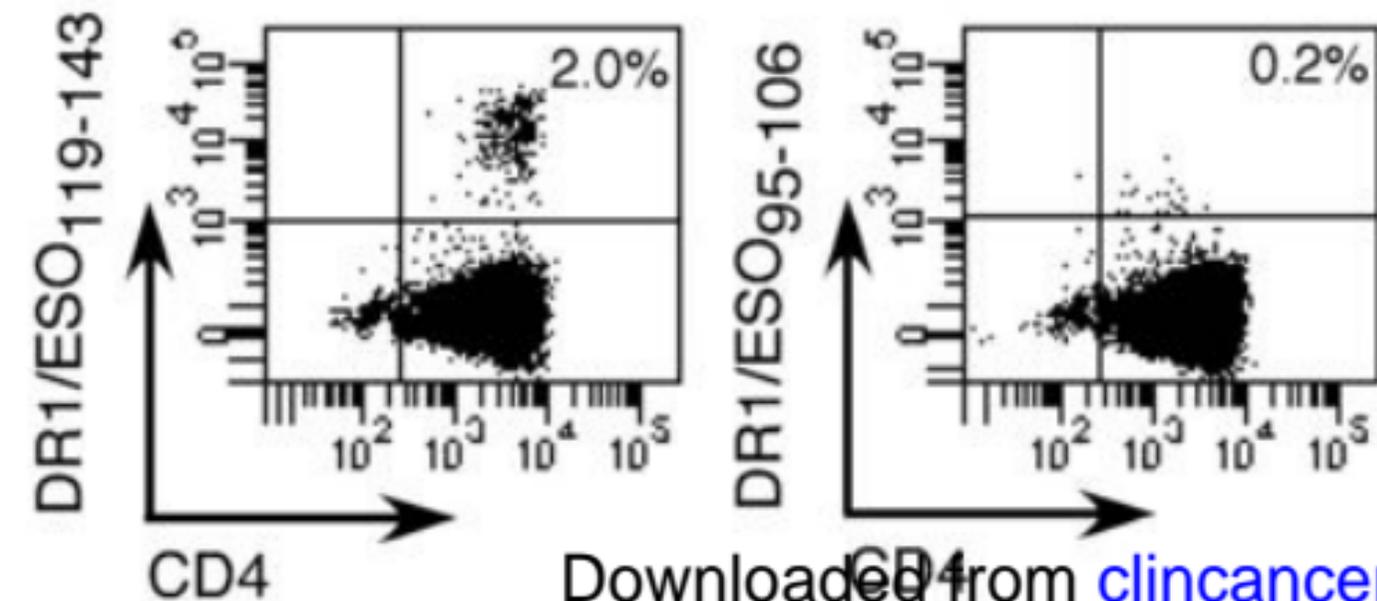


Figure 2

A



B

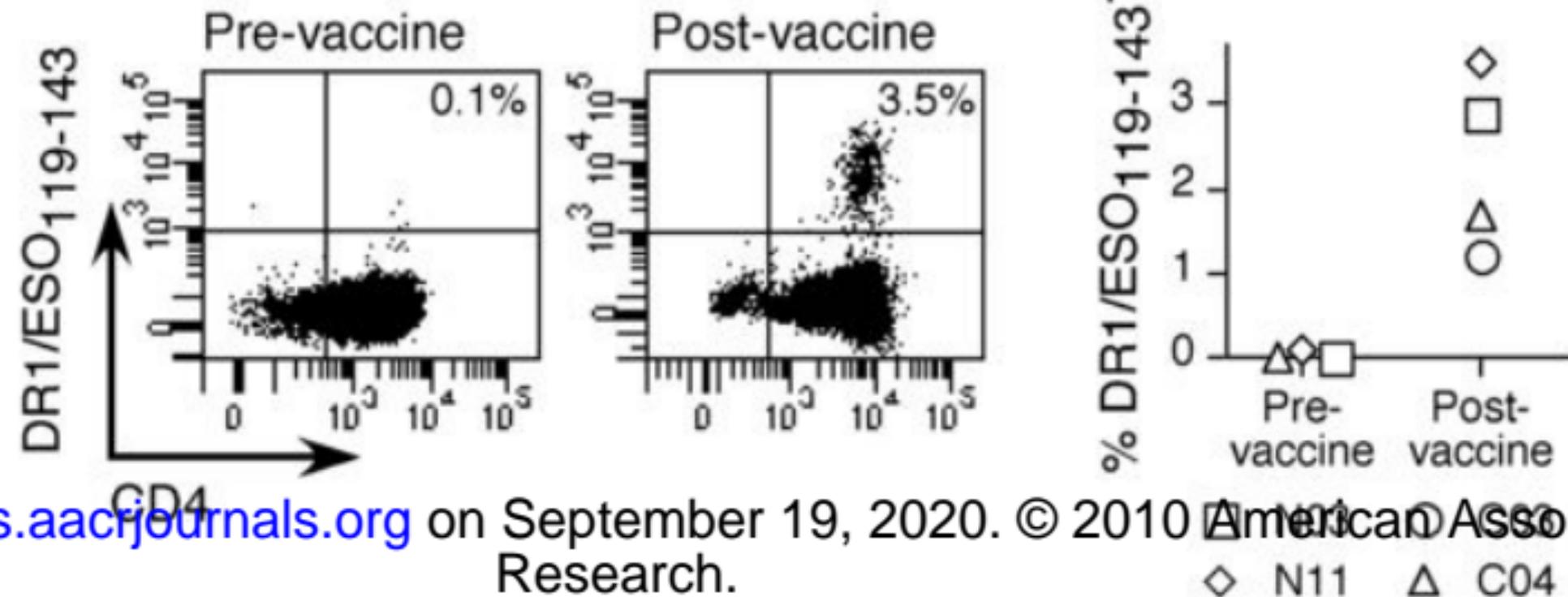
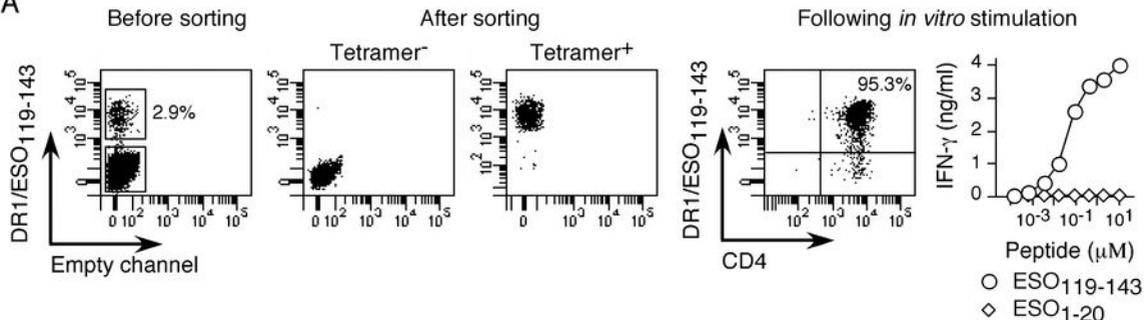
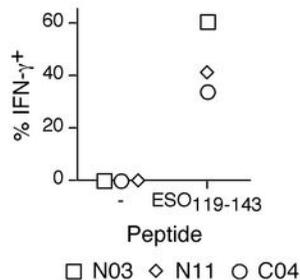


Figure 3

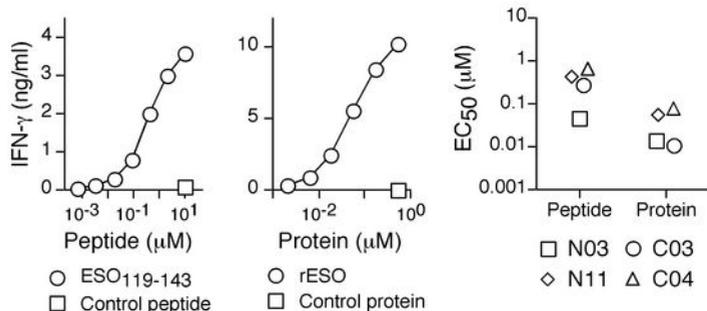
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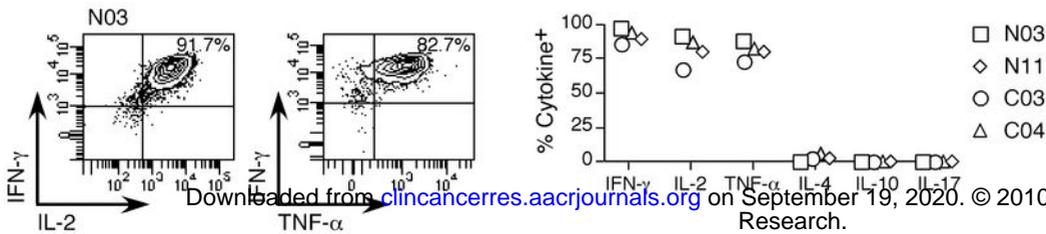


Figure 4

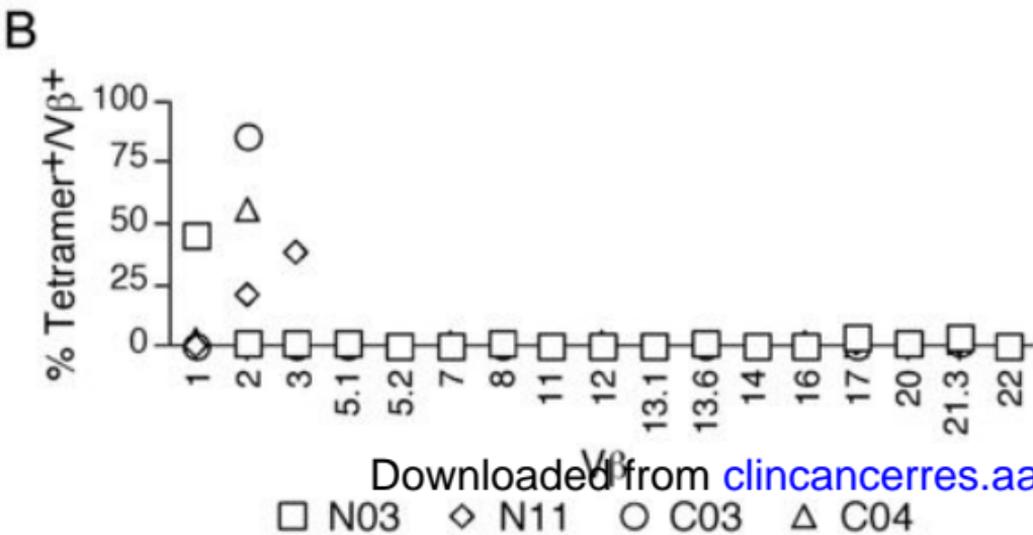
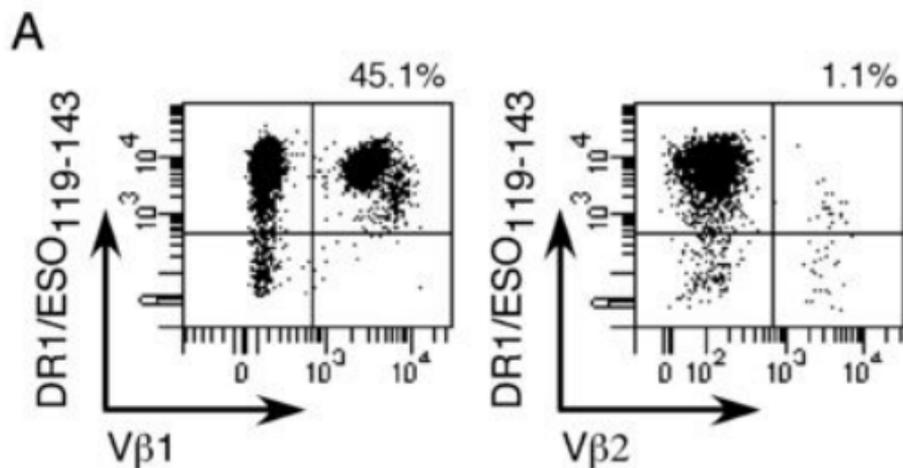
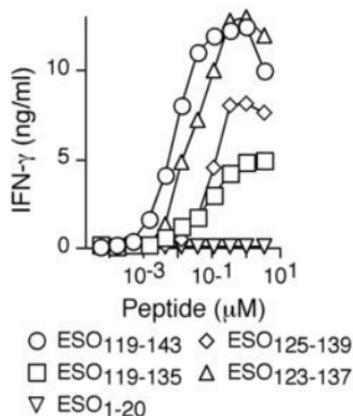
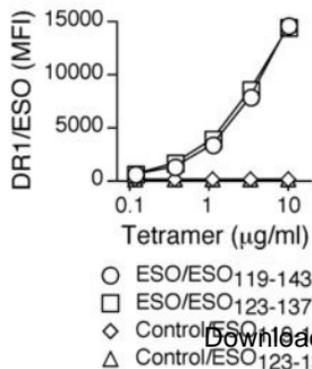


Figure 5

A



B



C

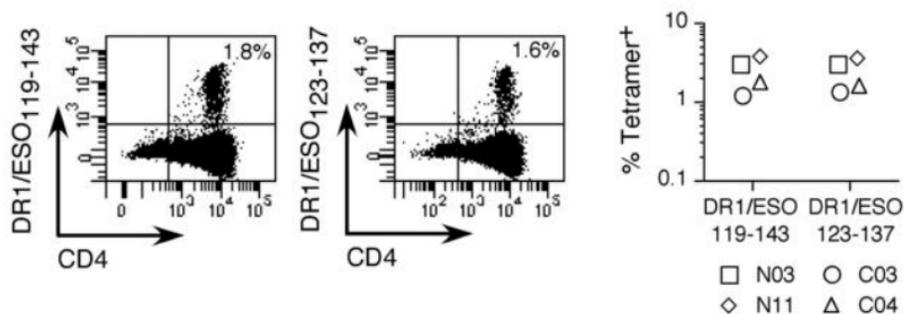
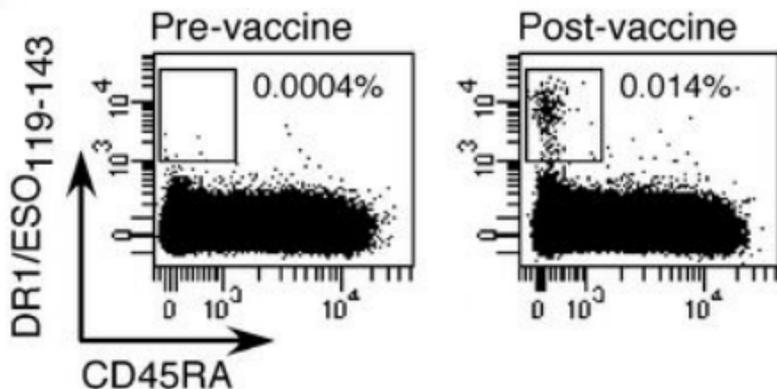
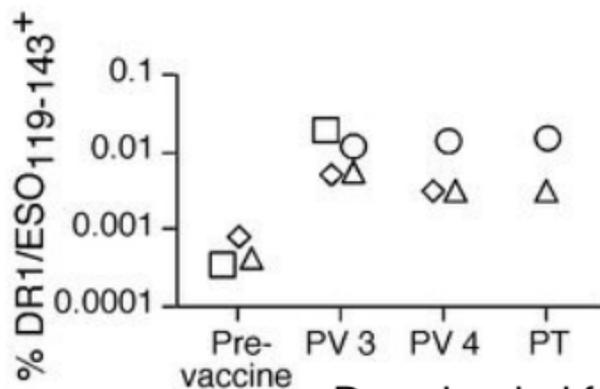


Figure 6

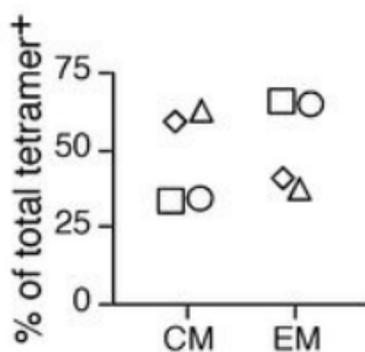
A



B



C



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Clinical Cancer Research

Assessment of vaccine-induced CD4 T cell responses to the 119-143 immunodominant region of the tumor-specific antigen NY-ESO-1 using DRB1*0101 tetramers

Maha Ayyoub, Pascale Pignon, Danijel Dojcinovic, et al.

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