

Imaging, Diagnosis, Prognosis

## 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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### 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 anticancer vaccines. The ESO<sub>119-143</sub> region is immunodominant following immunization with a recombinant ESO vaccine. In this study, we generated DRB1\*0101/ESO<sub>119-143</sub> tetramers and used them to assess CD4 T-cell responses in vaccinated patients expressing DRB1\*0101 (DR1).

**Experimental Design:** We generated tetramers of DRB1\*0101 incorporating peptide ESO<sub>119-143</sub> using a previously described strategy. We assessed ESO<sub>119-143</sub>-specific CD4 T cells in peptide-stimulated post-vaccine cultures using the tetramers. We isolated DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> cells by cell sorting and characterized them functionally. We assessed vaccine-induced CD4<sup>+</sup> DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> T cells *ex vivo* and characterized them phenotypically.

**Results:** Staining of cultures from vaccinated patients with DR1/ESO<sub>119-143</sub> tetramers identified vaccine-induced CD4 T cells. Tetramer<sup>+</sup> cells isolated by cell sorting were of T<sub>H</sub>1 type and efficiently recognized full-length ESO. We identified ESO<sub>123-137</sub> as the minimal optimal epitope recognized by DR1-restricted ESO-specific CD4 T cells. By assessing DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> cells using T cell receptor (TCR)  $\beta$  chain variable region (V $\beta$ )-specific antibodies, we identified several frequently used V $\beta$ . 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/ESO<sub>119-143</sub> 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. *Clin Cancer Res*; 16(18); 4607–15. ©2010 AACR.

Active elicitation of immune responses to tumor-specific antigens through vaccination is currently being 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 antitumor effector cells, CD4 T-cell responses are key to the development of efficient antitumor 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 anticancer 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

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### Translational Relevance

The development of reagents allowing rapid and efficient immunologic monitoring of vaccine-induced CD4 T-cell responses to human tumor antigens is instrumental for the development of anticancer vaccines. Whereas MHC class I fluorescent tetramers incorporating tumor antigen peptides, allowing direct analysis of specific CD8 T cells, are widely used, only a 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 NY-ESO-1 (ESO), presently viewed as an important model antigen for the development of generic anticancer vaccines, and used them to characterize vaccine-induced CD4 T-cell responses in vaccinated patients expressing DRB1\*0101 (DR1). DR1/ESO<sub>119-143</sub> 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/ESO<sub>119-143</sub> tetramers will be instrumental for the assessment of spontaneous and vaccine-induced immune responses to ESO in DR1<sup>+</sup> patients.

used in recent years (12–14). However, the development of MHC class II-peptide tetramers 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 histologic types but not in normal somatic tissues, is an important candidate for the development of generic anticancer vaccines (18, 19). Several candidate anticancer 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 obtained induction of CD4 T-cell responses in 17 of 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. ESO<sub>119-143</sub> has been previously reported to bind to multiple MHC class II molecules (21, 22), and epitopes located in the ESO<sub>119-143</sub> region and recognized by specific CD4 T cells in the context of several Human Leukocyte Antigen (HLA)-DR molecules have been identified (21, 23, 24). We have recently generated tetramers of ESO<sub>119-143</sub> 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, which

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 ESO<sub>119-143</sub>, using this strategy. We initially validated the DR1/ESO<sub>119-143</sub> tetramers on specific and control clones. We then assessed peptide-stimulated cultures from vaccinated patients expressing DR1, isolated DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> cells by cell sorting, and further characterized them functionally. Finally, we used the DR1/ESO<sub>119-143</sub> tetramers to assess vaccine-induced CD4 T cells *ex vivo* and characterize them phenotypically.

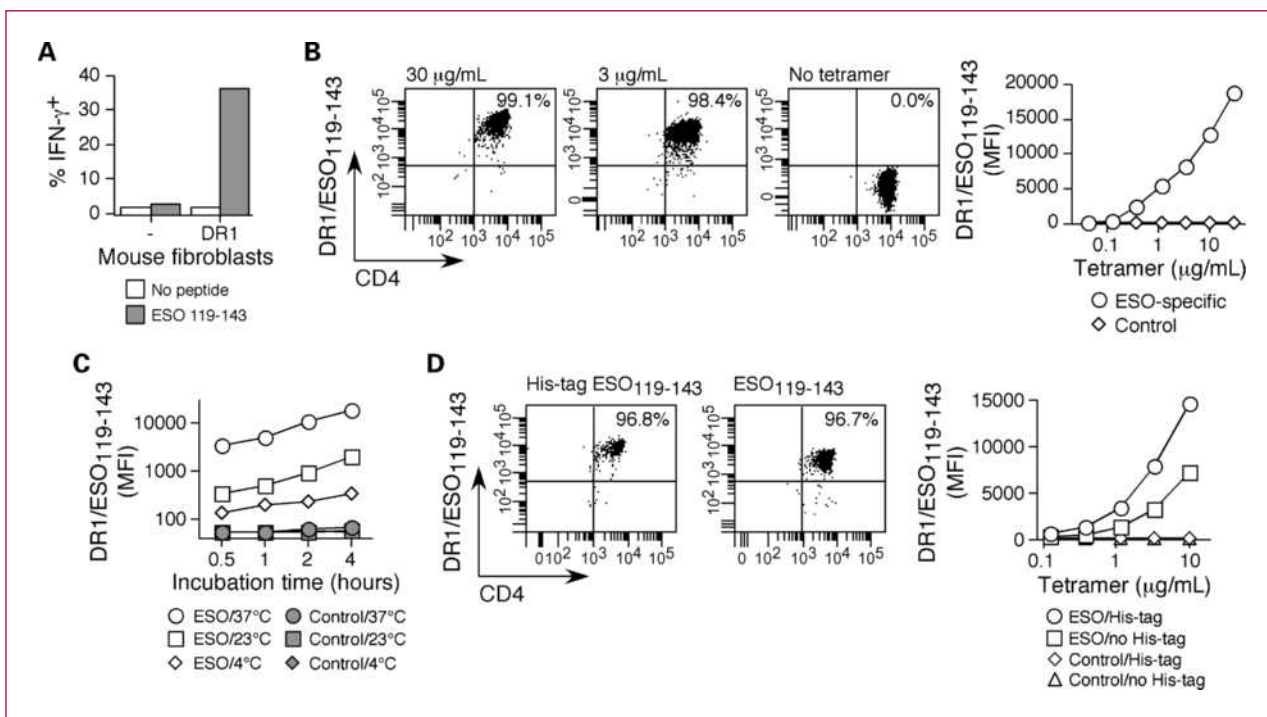
### Materials and Methods

#### 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 mmol/L 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 NH<sub>2</sub>-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-phycoerythrin (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). LDR1, 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. ESO<sub>119-143</sub>-specific DR1-restricted CD4 T cell clones were obtained from postvaccine samples from DR1<sup>+</sup> patients as previously described (24). Clones were expanded by periodic (every 3-4 weeks) stimulation with phytohemagglutinin (PHA; Oxoid) and allogeneic irradiated peripheral blood mononuclear cells (PBMC), and were cultured in complete Iscove's modified Dulbecco's medium (IMDM) in the presence of recombinant human (rh) interleukin (IL)-2 (100 IU/mL).

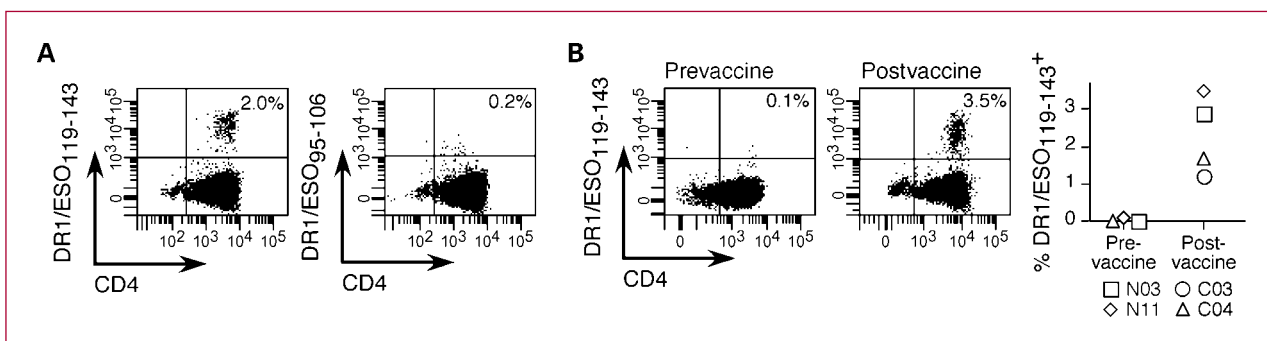


**Fig. 1.** DR1/ESO<sub>119-143</sub> tetramers stain ESO<sub>119-143</sub>-specific DR1-restricted CD4 T-cell clones. **A**, ESO<sub>119-143</sub>-specific clonal populations from DR1<sup>+</sup> patient N03 were incubated with untransfected or DR1-expressing mouse fibroblasts that had been pulsed or not with peptide ESO<sub>119-143</sub>, and IFN- $\gamma$  production was assessed in a 4-hour intracellular cytokine staining assay. **B**, ESO-specific DR1-restricted and control clonal populations were stained with serial dilutions of DR1/His-ESO<sub>119-143</sub> tetramers for 1 hour 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-ESO<sub>119-143</sub> tetramers (3  $\mu$ 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 ESO<sub>119-143</sub> peptides and analyzed as in **B**. Examples of dot plots for staining of ESO-specific cells with both tetramers at 10  $\mu$ g/mL and MFI of tetramer staining for all conditions are shown.

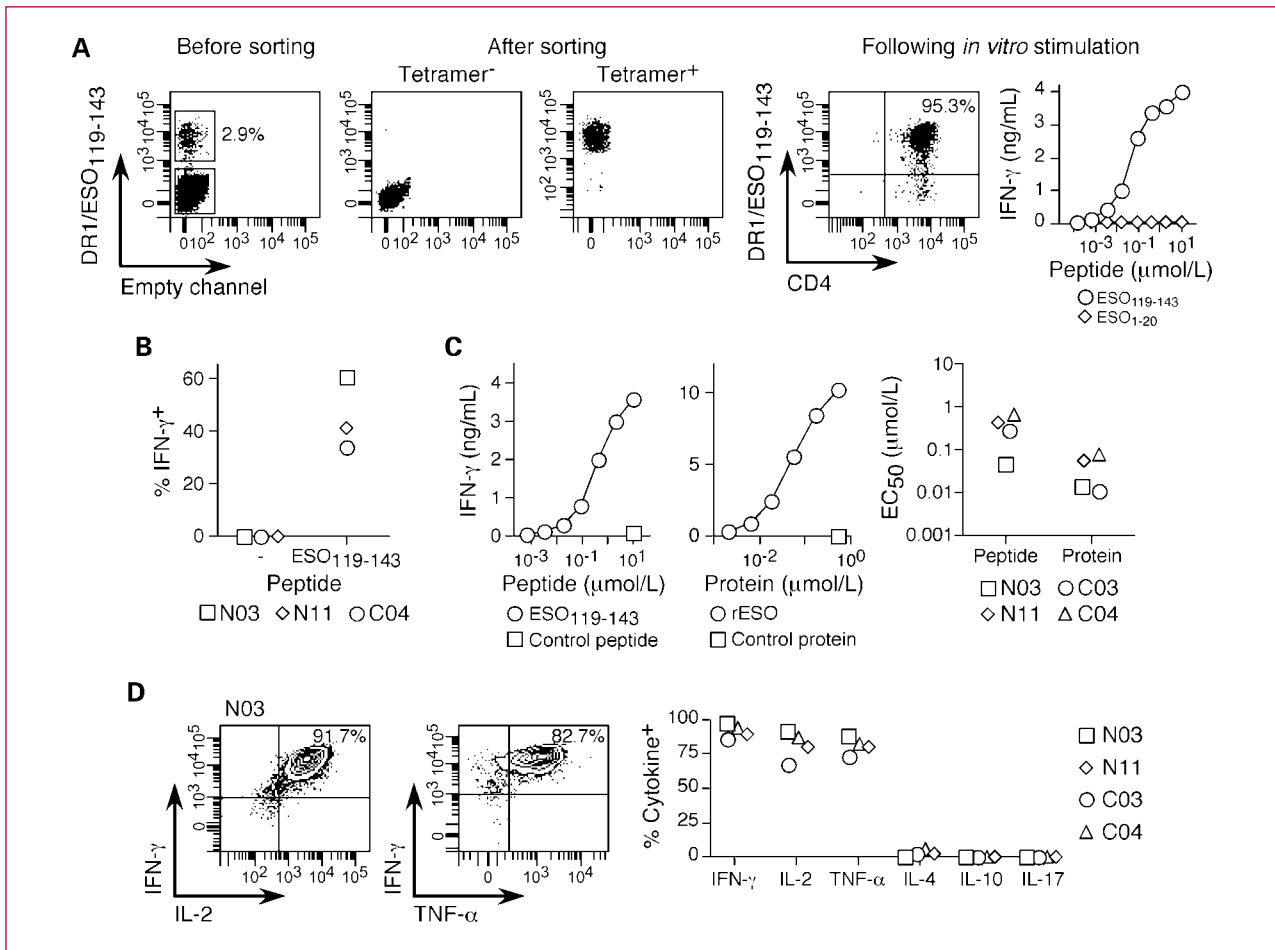
### Assessment of ESO-specific CD4 T cells, tetramer staining, and flow cytometric analysis and sorting

For assessment of specific CD4 T-cell responses following *in vitro* stimulation, CD4<sup>+</sup> cells were enriched from PBMC by magnetic cell sorting (Miltenyi Biotec Inc.), stimulated with irradiated autologous antigen-presenting cells

(APC) in the presence of ESO peptides, as indicated, rhIL-2 and rhIL-7 as previously described (24), and maintained in culture for 10 to 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  $\mu$ g/mL for 1 hour at 37°C,



**Fig. 2.** DR1/ESO<sub>119-143</sub> tetramers stain peptide-stimulated CD4 T cells from postvaccine but not from prevaccine samples of DR1<sup>+</sup> patients. **A**, postvaccine CD4 T cells from DR1<sup>+</sup> patient N03, stimulated *in vitro* with a pool of overlapping long ESO peptides spanning the full-length ESO sequence, were stained with DR1/ESO<sub>119-143</sub> or control DR1/ESO<sub>95-106</sub> tetramers (3  $\mu$ g/mL) for 1 hour at 37°C and anti-CD4 mAb and analyzed by flow cytometry. **B**, prevaccine and postvaccine CD4 T cells from DR1<sup>+</sup> patients, stimulated *in vitro* with peptide ESO<sub>119-143</sub>, were stained with DR1/ESO<sub>119-143</sub> 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/ESO<sub>119-143</sub> tetramer<sup>+</sup> CD4 T cells. **A**, postvaccine CD4 T cells were stimulated *in vitro* with peptide ESO<sub>119-143</sub>, stained with DR1/ESO<sub>119-143</sub> tetramers (left dot plot), and tetramer<sup>+</sup> and tetramer<sup>-</sup> cells were isolated by flow cytometry cell sorting. Aliquots of sorted cells were directly reanalyzed by flow cytometry (middle dot plots). Tetramer<sup>+</sup> 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 ESO<sub>119-143</sub> or control peptide, and IFN- $\gamma$  was measured by enzyme-linked immunosorbent assay (ELISA) in 24-hour culture supernatants. Results are shown for one patient, N03, representative of four. **B**, tetramer<sup>+</sup> polyclonal populations were incubated with L.DR1 cells that have been pulsed or not with peptide ESO<sub>119-143</sub>, and IFN- $\gamma$  production was assessed in a 4-hour intracellular cytokine staining assay. **C**, tetramer<sup>+</sup> polyclonal populations were incubated with L.DR1 cells and serial dilutions of ESO<sub>119-143</sub> or control peptide (left) or with DR1<sup>+</sup> monocyte-derived dendritic cells preincubated with serial dilutions of ESO or control protein (middle), and IFN- $\gamma$  was measured by ELISA in 24-hour 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- $\gamma$  secretion (EC<sub>50</sub>) is shown for all patients. **D**, polyclonal cultures were stimulated with PMA and ionomycin, and cytokine production was assessed in a 4-hour intracellular cytokine staining assay. Examples of dot plots for patient N03 and data obtained for all patients and all cytokines tested are shown.

unless otherwise indicated, in complete IMDM, washed, and then stained with CD4 (BD Biosciences) or T cell receptor (TCR)  $\beta$  chain variable region (V $\beta$ ) (Beckman Coulter) specific monoclonal antibody (mAb) in PBS, 5% FCS for 15 minutes at 4°C, and analyzed by flow cytometry (FACSaria, BD Biosciences). To generate specific polyclonal T-cell populations, tetramer<sup>+</sup> cells within peptide-stimulated cultures were sorted by flow cytometry (FACSaria, 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<sup>+</sup> cells enriched from PBMC were

rested overnight, incubated with tetramers (3  $\mu$ g/mL) for 2 hours at 37°C, and then stained with CD4-, CD45RA- (BD Biosciences), and CCR7-specific (Miltenyi Biotec Inc.) mAb and analyzed by flow cytometry. Tetramers used throughout the study contained His-tagged ESO peptides, unless otherwise indicated.

#### Antigen recognition assays

DR1<sup>+</sup> ESO-specific monoclonal or polyclonal CD4 T-cell populations were stimulated in the absence or presence of ESO peptides (2  $\mu$ mol/L) or phorbol 12-myristate 13-acetate (PMA; 100 ng/mL) and ionomycin

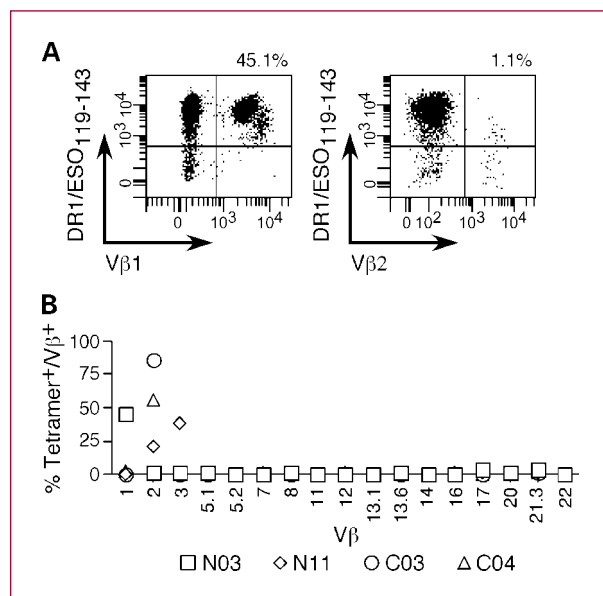


(1  $\mu\text{g}/\text{mL}$ ), as indicated, and cytokine production was assessed in a standard 4-hour intracellular cytokine staining assay using mAb specific for IFN- $\gamma$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-2, IL-4, IL-10 (BD Biosciences), and IL-17 (eBiosciences), and flow cytometric analysis, as previously described (24, 27). To assess DR1 restriction of monoclonal and polyclonal ESO-specific populations, they were incubated for 4 hours with L.DR1 cells or with untransfected mouse fibroblasts that had been pulsed with peptide ESO<sub>119-143</sub> for 1 hour at 37°C and washed three times, and IFN- $\gamma$  production was assessed by intracellular staining and flow cytometric analysis. In other experiments, specific polyclonal cultures were incubated for 24 hours with either L.DR1 cells and serial dilutions of ESO peptides or monocyte-derived dendritic cells preincubated overnight with serial dilutions of rESO, and IFN- $\gamma$  was measured by enzyme-linked immunosorbent assay in 24-hour culture supernatants, as previously described (4, 24).

## Results and Discussion

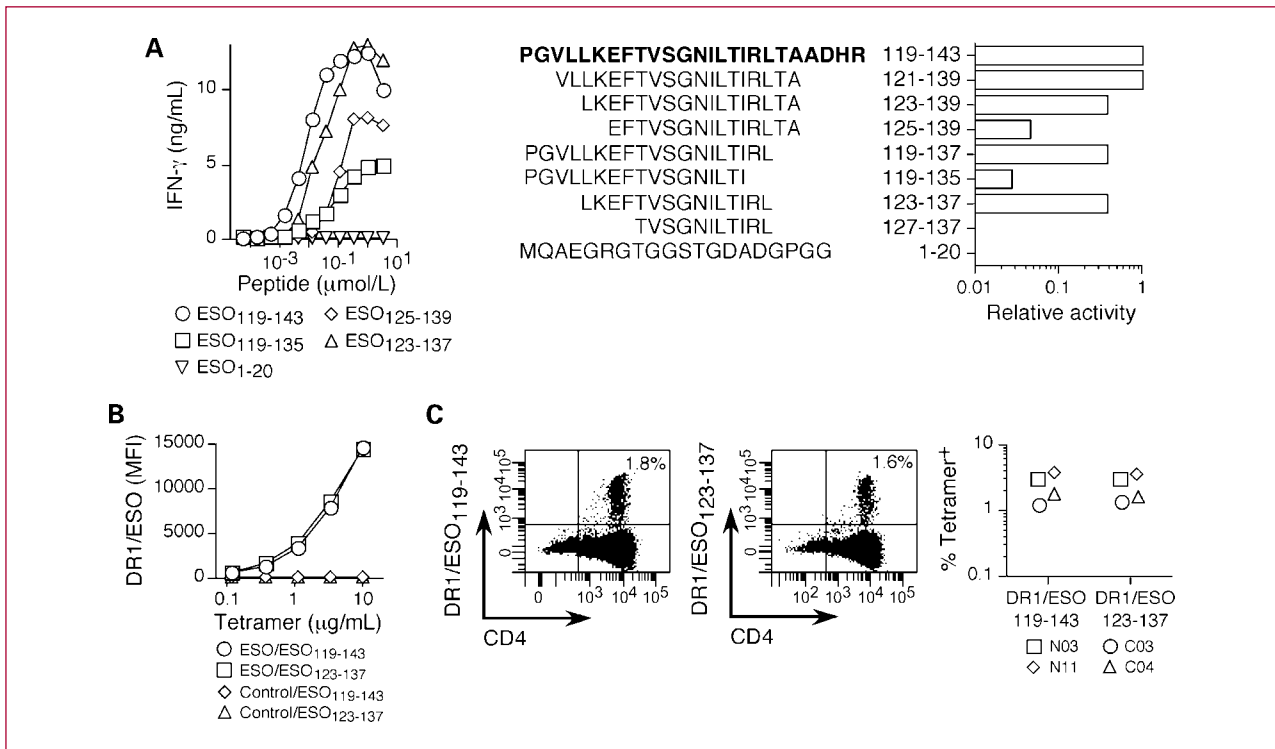
### Generation and validation of DRB1\*0101/ESO<sub>119-143</sub> tetramers

Direct assessment with fluorescent MHC class II tetramers incorporating immunodominant peptides from frequently expressed tumor antigens is an attractive



**Fig. 4.** Assessment of TCR V $\beta$  usage by vaccine-induced ESO<sub>119-143</sub>-specific DR1-restricted CD4 T cells. Polyclonal monospecific tetramer<sup>+</sup> populations from vaccinated patients were first stained with DR1/ESO<sub>119-143</sub> tetramers and then with a panel of anti-TCR V $\beta$  mAb and analyzed by flow cytometry. A, examples of dot plots obtained with anti-V $\beta$ 1 and anti-V $\beta$ 2 mAb staining for patient N03. Numbers correspond to the percentage of V $\beta$ <sup>+</sup> cells among tetramer<sup>+</sup> cells in the culture. B, summary of results corresponding to the percentage of V $\beta$ <sup>+</sup> cells, for all V $\beta$  tested, among tetramer<sup>+</sup> cells for all patients.

approach for the monitoring of antitumor CD4 T cells. At variance with MHC class I/peptide tetramers, originally developed in 1996 (11) and 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. Specifically, 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). However, MHC class II  $\alpha\beta$  chains incorporating leucine zippers 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 ESO<sub>119-143</sub>, 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 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 ESO<sub>119-143</sub>. To validate the DR1/ESO<sub>119-143</sub> tetramers, we initially assessed them on a specific clone (Fig. 1A) obtained from a DR1<sup>+</sup> patient who had been immunized with the rESO vaccine (4). As shown in Fig. 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  $\mu\text{g}/\text{mL}$ ). The staining intensity increased with the dose of tetramer, up to 30  $\mu\text{g}/\text{mL}$ , without reaching a plateau (Fig. 1B). Staining of specific clones was more efficient at high temperature (37°C) and after prolonged incubation times (Fig. 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 Fig. 1D, DR1/ESO tetramers generated with the untagged peptide ESO<sub>119-143</sub> also stained ESO-specific clones, although with slightly lower efficiency as compared with 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.



**Fig. 5.** Assessment of the minimal peptide optimally recognized by vaccine-induced ESO-specific DR1-restricted CD4 T cells. A, polyclonal DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> cultures, obtained as in Fig. 3A, from patient N03 were incubated with L.DR1 cells and serial dilutions of truncated peptides within the 119-143 region or ESO<sub>1-20</sub> control peptide, and IFN- $\gamma$  was measured by ELISA in 24-hour culture supernatants (examples in left). The activity of each peptide (EC<sub>50</sub>) was calculated relative to that of peptide ESO<sub>119-143</sub> (right). B, ESO-specific DR1-restricted or control clonal populations were stained with serial dilutions of DR1 tetramers containing peptides ESO<sub>119-143</sub> or ESO<sub>123-137</sub> and analyzed by flow cytometry as in Fig. 1B. C, postvaccine CD4 T cells from DR1<sup>+</sup> patients were stimulated *in vitro* with peptide ESO<sub>119-143</sub>, stained with DR1/ESO<sub>119-143</sub> or DR1/ESO<sub>123-137</sub> tetramers and anti-CD4 mAb, and analyzed by flow cytometry. Dot plots for patient C04 and data for all patients are shown.

### Assessment of peptide-stimulated cultures from vaccinated patients using DRB1\*0101/ESO<sub>119-143</sub> tetramers

To evaluate vaccine-induced CD4 T cells in DR1<sup>+</sup> immunized patients, we initially stained postvaccine 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/ESO<sub>119-143</sub> tetramers for 1 hour at 37°C. As illustrated in Fig. 2A, this analysis identified a significant proportion of DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> CD4 T cells in the culture. DR1 tetramers incorporating peptide ESO<sub>95-106</sub>, used as an internal control, failed to identify significant proportions of tetramer<sup>+</sup> cells. In a separate experiment, we stimulated postvaccine samples from patient N03 and from three additional vaccinated patients expressing DR1 alleles (N11 and C04 also expressing DRB1\*0101, and C03 expressing DRB1\*0103) with peptide ESO<sub>119-143</sub> alone and assessed them 12 days later with the DR1/ESO<sub>119-143</sub> tetramers. As illustrated in Fig. 2B, we detected significant proportions of tetramer<sup>+</sup> cells in cultures from all patients. DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> cells had clearly been induced by vaccination, as they were not detectable at

significant levels in prevaccine samples stimulated in the same conditions.

### Isolation and characterization of vaccine-induced DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> cells

To assess vaccine-induced DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> cells, we isolated them by flow cytometry cell sorting and expanded them *in vitro*, as polyclonal monospecific cultures (Fig. 3A). Isolated tetramer<sup>+</sup> cells specifically recognized peptide ESO<sub>119-143</sub> but not a control ESO peptide (Fig. 3A). Antigen recognition by polyclonal monospecific tetramer<sup>+</sup> cells was restricted by DR1, as efficient antigen presentation was obtained using DR1-transfected mouse cells preincubated with peptide ESO<sub>119-143</sub> (Fig. 3B). To further characterize vaccine-induced DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> cells, we assessed their capacity to efficiently recognize the full-length rESO processed and presented by autologous APC. To this purpose we generated monocyte-derived dendritic cells (moDC) by culturing autologous CD14<sup>+</sup> cells with granulocyte macrophage colony-stimulating factor and IL-4 as described (4), incubated them with serial dilutions of rESO and tetramer<sup>+</sup> cells, and assessed IFN- $\gamma$  secretion in the culture supernatant. As

shown in Fig. 3C, tetramer<sup>+</sup> 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 ESO<sub>119-143</sub> peptide presented by DR1-expressing APC. To assess the type of vaccine-induced DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> cells with respect to cytokine secretion, we stimulated them with PMA and ionomycin, and permeabilized and stained them with mAb specific for signature cytokines produced by different T<sub>H</sub>-cell subsets. As illustrated in Fig. 3D, tetramer<sup>+</sup> cells displayed a typical T<sub>H</sub>1 profile as they secreted IFN- $\gamma$ , IL-2, and TNF- $\alpha$ , but not IL-4, IL-10, or IL-17.

### DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> 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  $\alpha$  and  $\beta$  chains (V $\alpha$  and V $\beta$ ). To address if DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> cells exhibited such conserved features, we assessed the polyclonal monospecific tetramer<sup>+</sup> populations from vaccinated patients with a panel of anti-V $\beta$  mAb covering about 50% of the human TCR repertoire. Examples of

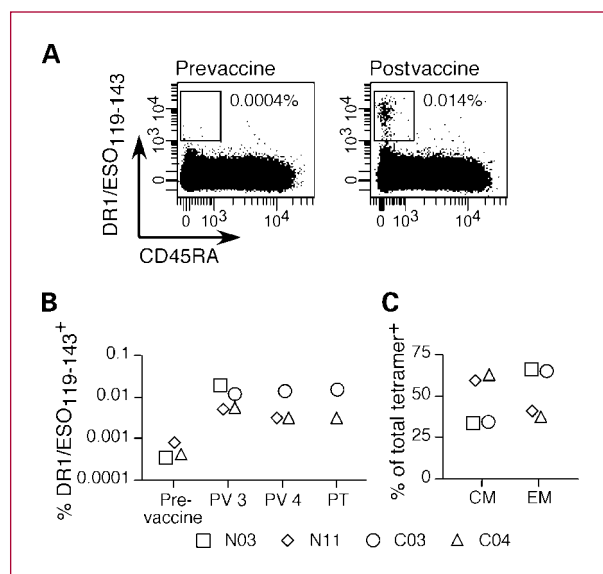
costaining with anti-V $\beta$  mAb and tetramers are shown in Fig. 4A, and a summary of the data obtained is reported in Fig. 4B. We found a frequent usage of several V $\beta$  segments, including V $\beta$ 1, V $\beta$ 2, and V $\beta$ 3. V $\beta$ 1 tetramer<sup>+</sup> cells were prevalent in the culture of patient N03, representing half of the total population. The large majority of tetramer<sup>+</sup> cells in the culture of patient C03 and a significant proportion of tetramer<sup>+</sup> cells in the cultures of two other patients, N11 and C04, used V $\beta$ 2. Finally, about half of tetramer<sup>+</sup> cells in the culture of patient N11 used V $\beta$ 3. Thus, DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> cells frequently used few selected V $\beta$  regions, indicating the presence of a conserved TCR repertoire.

### Assessment of the minimal ESO peptide optimally recognized by DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> cells

In a previous study assessing ESO<sub>119-143</sub> binding to several MHC class II alleles, including DR1, the 15-mer ESO<sub>123-137</sub> showed a binding affinity for DR1 similar to that of ESO<sub>119-143</sub> (21). To better define the DR1 epitope with respect to recognition by specific T cells, we assessed the recognition of truncated peptides within the ESO<sub>119-143</sub> region by tetramer<sup>+</sup> T cells. NH<sub>2</sub>-terminal truncations up to amino acid 123 did not significantly affect recognition by tetramer<sup>+</sup> T cells (Fig. 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 ESO<sub>123-137</sub> as the minimal peptide optimally recognized by DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> CD4 T cells. In line with these results, DR1 tetramers incorporating peptide ESO<sub>123-137</sub> stained specific clones with the same efficiency as compared with DR1/ESO<sub>119-143</sub> tetramers (Fig. 5B) and identified similar proportions of CD4 tetramer<sup>+</sup> cells in peptide-stimulated cultures from postvaccine samples (Fig. 5C).

### Ex vivo assessment of the frequency and phenotype of vaccine-induced ESO-specific CD4 T-cell responses with DR1/ESO<sub>119-143</sub> tetramers

The relatively high frequency of DR1/ESO<sub>119-143</sub> tetramer<sup>+</sup> 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 three of the four patients, samples taken prior to vaccination were available. The frequency of DR1/ESO tetramer<sup>+</sup> cells among memory (CD45RA<sup>-</sup>) CD4 T cells in prevaccine samples was below detection limits (<1:100,000; Fig. 6A and B). In contrast, in postvaccine samples from all patients taken after three vaccine injections (PV 3) DR1/ESO tetramer<sup>+</sup> cells were detectable at a frequency that was variable



**Fig. 6.** *Ex vivo* assessment of vaccine-induced ESO-specific DR1-restricted CD4 T cells. CD4 T cells purified from PBMC from prevaccine and postvaccine samples of DR1<sup>+</sup> patients were stained *ex vivo* with DR1/ESO<sub>119-143</sub> tetramers (3  $\mu$ g/mL) for 2 hours 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 prevaccine and postvaccine samples. Numbers in dot plots correspond to the percentage of tetramer<sup>+</sup> cells among memory CD45RA<sup>-</sup> CD4 T cells. B, percentage of tetramer<sup>+</sup> cells among memory CD45RA<sup>-</sup> CD4 T cells in prevaccine and postvaccine 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<sup>+</sup> cells in PV 3 samples based on CD45RA and CCR7 staining (CM, central memory CD45RA<sup>-</sup>CCR7<sup>+</sup>; EM, effector memory CD45RA<sup>+</sup>CCR7<sup>-</sup>).

among different patients and was on average of about 1:10,000 memory CD4 T cells. For three patients for whom additional samples taken after four vaccine injections (PV 4) were available, DR1/ESO tetramer<sup>+</sup> cells were detectable at a frequency that was, for each patient, comparable with that detected after three injections. For two patients, C03 and C04, additional samples taken four and five months respectively after the 4th and last injection (PT) were also available. In these samples, DR1/ESO tetramer<sup>+</sup> 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<sup>+</sup> cells included both central memory (CCR7<sup>+</sup>), representing "reservoir" memory populations (30, 31), and effector memory populations (CCR7<sup>-</sup>; Fig. 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 Th1 type, that are generally associated with efficient antitumor 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 worth noting that the frequency of vaccine-induced ESO-

specific CD4 T cells detected *ex vivo* (on 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.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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