MHC class II/ESO tetramer-based generation of in vitro primed anti-tumor T-helper lines for adoptive cell therapy of cancer

Caroline Poli,[1] Caroline Raffin,[1] Danijel Dojcinovic,[2] Immanuel Luescher,[2] Maha Ayyoub,[1] and Danila Valmori[1,3]

[1]Institut National de la Santé et de la Recherche Médicale, Unité 1102, Institut de Cancérologie de l'Ouest, Nantes-Saint Herblain, France; [2]Ludwig Center for Cancer Research, University of Lausanne, Epalinges, Switzerland; and [3]L'UNAM Université, Faculty of Medicine, University of Nantes, Nantes, France

ABSTRACT

Generation of tumor-antigen specific CD4+ T-helper (T_H) lines through *in vitro* priming is of interest for adoptive cell therapy of cancer, but the development of this approach has been limited by the lack of appropriate tools to identify and isolate low frequency tumor antigen-specific CD4+ T cells. Here, we have used recently developed MHC class II/peptide tetramers incorporating an immunodominant peptide from NY-ESO-1 (ESO), a tumor antigen frequently expressed in different human solid and hematologic cancers, to implement an *in vitro* priming platform allowing the generation of ESO-specific T_H lines. We isolated phenotypically defined CD4+ T-cell subpopulations from circulating lymphocytes of DR52b+ healthy donors by flow cytometry cell sorting and stimulated them *in vitro* with peptide $ESO_{119-143}$, autologous APC and IL-2. We assessed the frequency of ESO-specific cells in the cultures by staining with DR52b/$ESO_{119-143}$ tetramers (ESO-tetramers) and TCR repertoire of ESO-tetramer+ cells by co-staining with TCR variable β chain (BV) specific antibodies. We isolated ESO-tetramer+ cells by flow cytometry cell sorting and expanded them with PHA, APC and IL-2 to generate ESO-specific T_H lines. We characterized the lines for antigen recognition, by stimulation with ESO peptide or recombinant protein, cytokine production, by intracellular staining using specific antibodies, and alloreactivity, by stimulation with allo-APC. Using this approach, we could consistently generate ESO-tetramer+ T_H lines from conventional CD4+CD25- naïve and central memory populations, but not from effector memory populations or CD4+CD25+ Treg. *In vitro* primed T_H lines recognized ESO with affinities comparable to ESO-tetramer+ cells from patients immunized with an ESO vaccine and used a similar TCR repertoire. In this study, using MHC class II/ESO tetramers, we have implemented an *in vitro* priming platform allowing the generation of ESO-monospecific polyclonal T_H lines from non-immune individuals. This is an approach that is of potential interest for adoptive cell therapy of patients bearing ESO-expressing cancers.

Introduction

Analysis of spontaneous immune responses to tumor antigens in cancer patients has led to the identification of those most relevant for immune-based therapies. One of the most immunogenic of them, called NY-ESO-1 (ESO), belongs to the cancer/testis antigen (CTA) group, including antigens that, in adults, have an expression pattern restricted to testis and tumor cells.[1,2] ESO is frequently expressed in various solid (e.g. melanoma, ovarian cancer)[3,4] and hematologic (e.g. multiple myeloma (MM) adult T-cell leukemia/lymphoma (ATLL))[5-9] tumors, and represents an attractive target for cancer immunotherapy. Different ESO-based immunotherapeutic approaches are under development, including vaccines[10,11] and passive adoptive cell transfer (ACT) therapy using adoptively transferred ESO-specific T cells, which is particularly attractive for the treatment of patients with recurrent disease.

ACT using tumor-infiltrating lymphocytes (TIL) amplified *in vitro*, in association with lymphodepleting chemotherapy and IL-2, has indeed been shown to mediate, in patients with refractory metastatic melanoma, the regression of large established tumors.[12] Trials using defined tumor antigen-specific CD8+ T-cell (CTL) populations have shown a more limited efficacy, together with a short *in vivo* persistence of transferred populations, in the absence of specific CD4+ T-cell help.[13,14] A recent study, however, has reported a long-term complete remission in a patient with metastatic melanoma adoptively transferred with an *in vitro* expanded autologous ESO-specific CD4+ T-cell clone that persisted *in vivo* and appeared to induce endogenous responses to additional tumor antigens.[15] The potential of adoptive transfer of tumor antigen-specific CD4+ T cells for the eradication of established tumors has been further supported by recent studies in murine models.[16-18] Together, these results encourage the implementation of further studies assessing the clinical efficacy of ESO-specific CD4+ T cells administered to cancer patients bearing antigen-expressing tumors, alone or in association with ESO-specific CTL.

Whereas the generation of tumor antigen-specific CD4+ T_H cell clones for ACT from patients with spontaneous immune responses to the antigen, as currently performed, is labor intensive, not economically advantageous and is not applicable to all patients,[19] an alternative approach is to generate tumor-specific T_H populations of defined antigen specificity and HLA-restriction through *in vitro* priming of CD4+ T cells from non-immune individuals, including histocompatible donors. Because an important element for a successful therapy based on the adoptive transfer of tumor-specific T cells is their ability to persist and expand *in vivo*, populations derived from naïve CD4+ T cells may be, in this respect, superior in their ability to maintain a high replicative potential and resist terminal differentia-

 doi:10.3324/haematol.2012.071712
The online version of this article has a Supplementary Appendix.
Manuscript received June 6, 2012. Manuscript accepted July 26, 2012.
Correspondence: anila.valmori@univ-nantes.fr

tion.[16,20,21] In addition, *ex vivo* removal of CD25$^+$ regulatory T cells (Treg) from CD4$^+$ T-cell populations may be suitable, to avoid their presence in the transferred T_H lines.[22] The development of this approach, however, has been limited, to date, by the lack of appropriate tools to specifically identify and separate low frequency tumor antigen-specific CD4$^+$ T cells, particularly from non-immune individuals.

Soluble fluorescent MHC-peptide tetramers that allow the direct identification and separation of antigen-specific T cells, have in recent years become essential tools for T-cell analysis. MHC class I/peptide tetramers have been generated for a large number of murine and human alleles and peptides, and are widely used to assess antigen-specific CD8$^+$ T cells.[23,24] The development of MHC class II tetramers, and particularly of those incorporating peptides from tumor and self-antigens, has been more complex and only a few of them have been successfully developed.[25-27] Through a strategy that combines the use of Histidine (His)-tagged peptides with isolation of MHC/peptide monomers by affinity purification, we have recently generated MHC class II tetramers incorporating an ESO immunodominant peptide (ESO-tetramers).[28,29] We have shown that MHC class II/ESO tetramers specifically and avidly bind to ESO-specific CD4$^+$ T cells, allowing their highly sensitive detection and isolation from polyspecific populations. In this study, we have used ESO-tetramers to implement an *in vitro* priming platform, allowing the generation of ESO-monospecific polyclonal T_H lines from CD4$^+$ T cells of non-immune individuals. This approach can potentially be applied to the treatment of large numbers of patients bearing ESO-expressing cancers.

Design and Methods

Healthy donor samples, cells and ex vivo cell sorting

Peripheral blood samples were collected from healthy donors after obtaining written informed consent and approval from the Institutional Review Board (Etablissement Français du Sang Pays de la Loire, Nantes, France). Peripheral blood mononuclear cells (PBMC) were obtained from peripheral blood by density gradient centrifugation (LSM 1077 lymphocyte separation medium, PAA laboratories GmbH). CD4$^+$ T cells were enriched by positive selection from PBMC by magnetic cell sorting (Miltenyi Biotec). For *ex vivo* flow cytometry cell sorting, CD4$^+$ T cells were stained with fluorochrome-labeled monoclonal antibodies (mAb) specific for CD45RA (BD Biosciences), CD25 (Beckman Coulter), CD127 (eBioscience) and CCR7 (BD Biosciences) and were separated into conventional naïve (N; CD45RA$^+$CCR7$^+$CD25$^-$), natural naïve Treg (NnTreg; CD45RA$^+$CCR7$^+$CD25$^+$CD127^{low}), conventional memory (M; CD45RA$^-$CCR7$^{+/-}$CD25$^-$) and memory Treg (MTreg; CD45RA$^-$CCR7$^{+/-}$CD25$^+$CD127^{low}) populations to high purity (>97%) using a FACSAria (BD Biosciences). In some experiments conventional memory cells were sorted into central memory (CM; CD45RA$^-$CCR7$^+$CD25$^-$) and effector memory (EM; CD45RA$^-$CCR7$^-$CD25$^-$) populations. Monocyte-derived dendritic cells (DC) were generated from enriched CD14$^+$ cells, isolated from PBMC by magnetic sorting (Miltenyi Biotec), by culture in the presence of rhGM-CSF and rhIL-4 (1000 U/ml each; R&D Systems) for five days. The DR52b$^+$ EBV-transformed B-cell line, JBUSH (9035), was obtained from the National Marrow Donor Program/American Society for Histocompatibility and Immunogenetics (NMDP/ASHI) Cell Repository. Healthy donors were functionally typed for DR52b expression based on the capacity of their antigen presenting cells (APC) to present peptide $ESO_{119-143}$ to a specific DR52b-restricted CD4$^+$ T-cell clone, as previously described.[30]

Priming of ESO-specific CD4$^+$ T cells, tetramer staining, TCR BV analysis and generation of specific T_H lines

Fluorescent HLA-DR52b/$ESO_{119-143}$ (DR52b/ESO) and HLA-DR52b/Influenza matrix $protein_{73-92}$ (DR52b/Flu) tetramers were generated using His-tagged antigenic peptides, as previously described.[28] *Ex vivo* sorted CD4$^+$ T-cell subpopulations (3-5x10^6) were stimulated *in vitro* with peptide ESO119-143 (2 mM) in the presence of irradiated autologous CD4$^-$ cells (3-5x10^6) and were cultured in the presence of rhIL-2 (100 U/mlL, Chiron). Day 12 cultures were incubated with tetramers at a final concentration of 3 g/mL for 1 h at 37°C and then stained with anti-CD4 mAb and analyzed by flow cytometry. TCR variable β chain (BV) usage by ESO-specific cells was determined by flow cytometry analysis following staining of Day 12 cultures with ESO-tetramers and anti-BV2 mAb (Immunotech), as previously described.[28] ESO-specific T cells were isolated from peptide-stimulated cultures by tetramer-guided flow cytometry cell sorting, and expanded by stimulation with PHA and irradiated allogeneic PBMC in the presence of IL-2, as previously described.[28] The specificity of the obtained polyclonal T_H lines was assessed by tetramer staining and flow cytometry analysis.

Antigen recognition, cytokine production and alloreactivity

For the assessment of their functional avidity, ESO-specific polyclonal T_H lines (10^4) were incubated with JBUSH cells (10^4) in the presence of serial dilutions of peptide $ESO_{119-143}$ (1 mM) or a control irrelevant peptide (ESO^{1-20}), and IFN-γ was assessed in 24-h culture supernatants by ELISA (Invitrogen). For the assessment of the reactivity of ESO-specific lines to naturally processed full-length ESO, 10^4 cells were co-cultured with DR52b$^+$ DC (5x10^4), pre-incubated for 16 h with serial dilutions of recombinant ESO or Melan-A proteins, and IFN-γ was assessed in 24-h culture supernatants by ELISA. The capacity of ESO-specific T_H lines to produce cytokines was assessed in a standard 4-h intracellular cytokine staining assay using specific mAb (IL-2, TNF-α, IFN-γ, IL-4 and IL-10, BD Biosciences; IL-17, eBioscience) following stimulation with peptide $ESO_{119-143}$ (1 mM). To assess the extent of alloreactivity of ESO-specific lines, 104 cells were incubated with DC (5x10^4) derived from a panel of 8 DR52b$^+$ healthy donors, in the presence or absence of peptide $ESO_{119-143}$ (1 mM), and IFN-γ was assessed in 24-h culture supernatants by ELISA.

Results

In vitro priming of conventional naïve CD4$^+$CD25$^-$ T cells from DR52b$^+$ donors by stimulation with peptide $ESO_{119-143}$ and detection with DR52b/$ESO_{119-143}$ tetramers

We have recently described the generation and validation of tetrameric complexes of DR52b, DRB3*0202, an alternate MHC class II molecule that is frequently expressed in Caucasians, incorporating the immunodominant peptide $ESO_{119-143}$.[28] In CD4$^+$ T cells of patients immunized with an ESO vaccine, $ESO_{119-143}$-specific DR52b-restricted cells were detected by DR52b/$ESO_{119-143}$ tetramers *ex vivo* at an average frequency of 1/5,000 memory CD4$^+$ T cells. In contrast, in DR52b$^+$ healthy donors, DR52b/$ESO_{119-143}$ tetramer$^+$ cells were not detectable *ex vivo*, indicating that they are present in the pre-immune repertoire at frequencies close or below tetramer detection limit (in the range of 1/100,000 cells *ex vivo*). To assess the feasibility of generating ESO-specific lines from CD4$^+$ T cells of healthy donors through *in vitro* priming, we enriched CD4$^+$ T cells from DR52b$^+$ donors by magnetic cell sorting and isolated conventional naïve (CD45RA$^+$CCR7$^+$CD25$^-$) populations by flow cytometry cell sorting (*Online Supplementary Figure S1*). We stimulated

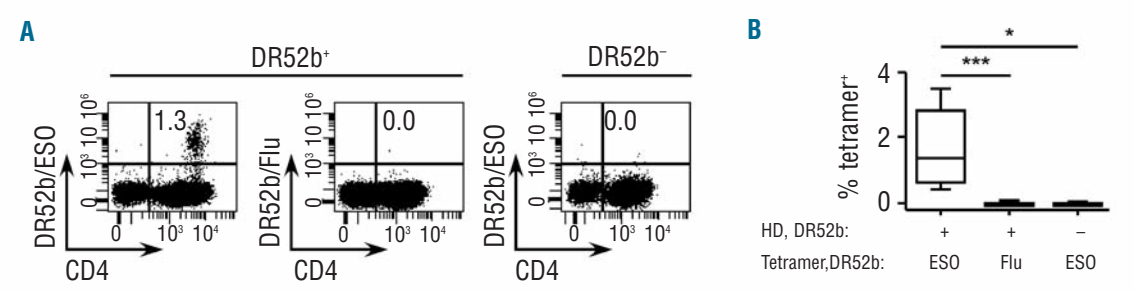

Figure 1. *In vitro* priming of $ESO_{119-143}$-specific $CD4^+$ T cells from conventional naïve $CD4^+CD25^-$ T cells of $DR52b^+$ healthy donors. Conventional naïve (N, $CD45RA^+CCR7^+CD25^-$) cells isolated *ex vivo* by flow cytometry cell sorting from $CD4^+$ T cells of $DR52b^+$ (n=11) and DR52b- (n=3) healthy donors (HD), as shown in *Online Supplementary Figure S1*, were stimulated *in vitro* with peptide ESO119-143 in the presence of autologous APC and Day 12 cultures were stained with DR52b/ESO or control DR52b/Flu tetramers and anti-CD4 mAb, and analyzed by flow cytometry. (A) Dot plots for one $DR52b^+$ and one $DR52b^-$ donor. (B) Percentages of $tetramer^+$ cells in all cultures. Statistical analyses were performed using the Mann-Whitney test. *P<0.05; ***P<0.001.

isolated conventional naïve $CD4^+$ T cells with peptide $ESO_{119-143}$ in the presence of autologous APC and IL-2, cultured them for 12 days, and then assessed the cultures by staining with DR52b/$ESO_{119-143}$ (DR52b/ESO thereafter) tetramers and anti-CD4 mAb. As illustrated in Figure 1A and summarized in Figure 1B, ESO-$tetramer^+$ cells were readily detectable in the cultures from all $DR52b^+$ donors assessed at an average frequency of 1.8 ± 1.1% of $CD4^+$ T cells. No significant staining was obtained using DR52b tetramers incorporating an unrelated peptide from influenza matrix protein (DR52b/Flu). In addition, no significant ESO-tetramer staining was detected by assessing control $CD4^+$ T-cell cultures from $DR52b^-$ donors. Together, these results demonstrate that *in vitro* priming of ESO-$tetramer^+$ cells is consistently and efficiently obtained by antigen stimulation of $CD4^+$ T cells from non-immune individuals.

ESO-$tetramer^+$-T_H can be efficiently generated through in vitro priming of conventional central memory but not effector memory or Treg populations

In some experiments, in parallel to conventional naïve $CD4^+$ T-cell populations, we stimulated conventional memory ($CD45RA^-CCR7^{+/-}CD25^-$) populations simultaneously isolated by cell sorting (*Online Supplementary Figure S1*). To our surprise, we obtained efficient *in vitro* priming of ESO-$tetramer^+$ cells from conventional memory populations, although at lower frequencies (0.4±0.4%) as compared to those obtained with conventional naïve populations (Figure 2A). To further define the subpopulation containing ESO-specific T_H precursors in the memory compartment, we separated conventional memory $CD4^+$ T cells into $CCR7^+$ central memory (CM) and $CCR7^-$ effector memory (EM) populations and stimulated them under the described conditions. We consistently obtained *in vitro* priming of ESO-specific T_H from CM, at frequencies on average approximately 3-fold lower than those obtained with naïve populations (0.7±0.6%) (Figure 2B). In contrast, we obtained no significant *in vitro* priming from EM populations (Figure 2B) or from naïve or memory Treg populations ($CD25^+CD127^{low}$) simultaneously isolated and stimulated under the same conditions (Figure 2C)

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In vitro primed ESO-$tetramer^+$-T_H lines display a functional avidity similar to ESO-$tetramer^+$-T_H from patients immunized with an ESO vaccine and recognize full-length ESO antigen processed and presented by dendritic cells

To characterize $ESO_{119-143}$-specific T_H cells generated through *in vitro* priming, we isolated ESO-$tetramer^+$ populations from Day 12 cultures by flow cytometry cell sorting, expanded them by mitogen stimulation in the presence of feeder cells, and assessed the resulting populations. The ESO-$tetramer^+$-T_H lines obtained through this procedure typically contained more than 90% of $tetramer^+$ cells (Figure 3A). To assess the functional avidity of antigen recognition of *in vitro* primed ESO-$tetramer^+$-T_H lines, we incubated $DR52b^+$ JBUSH cells with serial dilutions of peptide $ESO_{119-143}$ and the *in vitro* primed lines and assessed IFN-γ secretion in the culture supernatant, by ELISA. As illustrated in Figure 3B, *in vitro* primed ESO-$tetramer^+$-T_H lines recognized peptide $ESO_{119-143}$ with a similar functional avidity as $ESO_{119-143}$ $tetramer^+$-T_H from a cancer patient immunized with a recombinant ESO vaccine. To assess the ability of *in vitro* primed lines to recognize, in addition to peptide $ESO_{119-143}$, also the native ESO antigen, we incubated overnight $DR52b^+$ DC, generated from isolated circulating $CD14^+$ monocytes as described,[31] with a recombinant full-length ESO protein, and assessed antigen recognition by the lines by measuring IFN-γ secretion.[28] Similar to vaccine-induced ESO-T_H, *in vitro* primed ESO-$tetramer^+$-T_H lines efficiently recognized the recombinant ESO protein processed and presented by DC, whereas they failed to recognize DC incubated with a control protein (Figure 3C).

In vitro primed ESO-$tetramer^+$-T_H lines frequently use TCR BV2 similar to ESO-$tetramer^+$-T_H from patients immunized with an ESO vaccine

We have previously shown that $ESO_{119-143}$-specific DR52b-restricted $CD4^+$ T cells induced *in vivo* by vaccination with a recombinant ESO vaccine exhibit a significant conservation of TCR usage among different individuals, with frequent usage of the TCR BV2.[28,30] To assess if this restricted BV usage was a shared feature of DR52b/ $ESO_{119-143}$ $tetramer^+$ populations generated through *in vitro* priming, we co-stained Day 12 cultures with tetramers and anti-BV2 antibodies. We found a high enrichment of BV2 usage in ESO-$tetramer^+$ cells in *in vitro* primed cultures (Figure 4A and B). Indeed, whereas $BV2^+$ cells represented, on average, 9.2% (range 6-11.8%) of total $CD4^+$ T cells in the cultures, they represented on average 45% (range 10.9-82%) of *in vitro* primed ESO-$tetramer^+$ cells. This was comparable to 40.6% of $BV2^+$ among ESO-$tetramer^+$ cells in patients vaccinated with the ESO protein.[28] Therefore, $ESO_{119-143}$-specific DR52b-restricted $CD4^+$ T cells obtained through *in vitro* priming exhibited a TCR repertoire that frequently used BV2 similar to that found in patients immunized with the recombinant ESO vaccine.

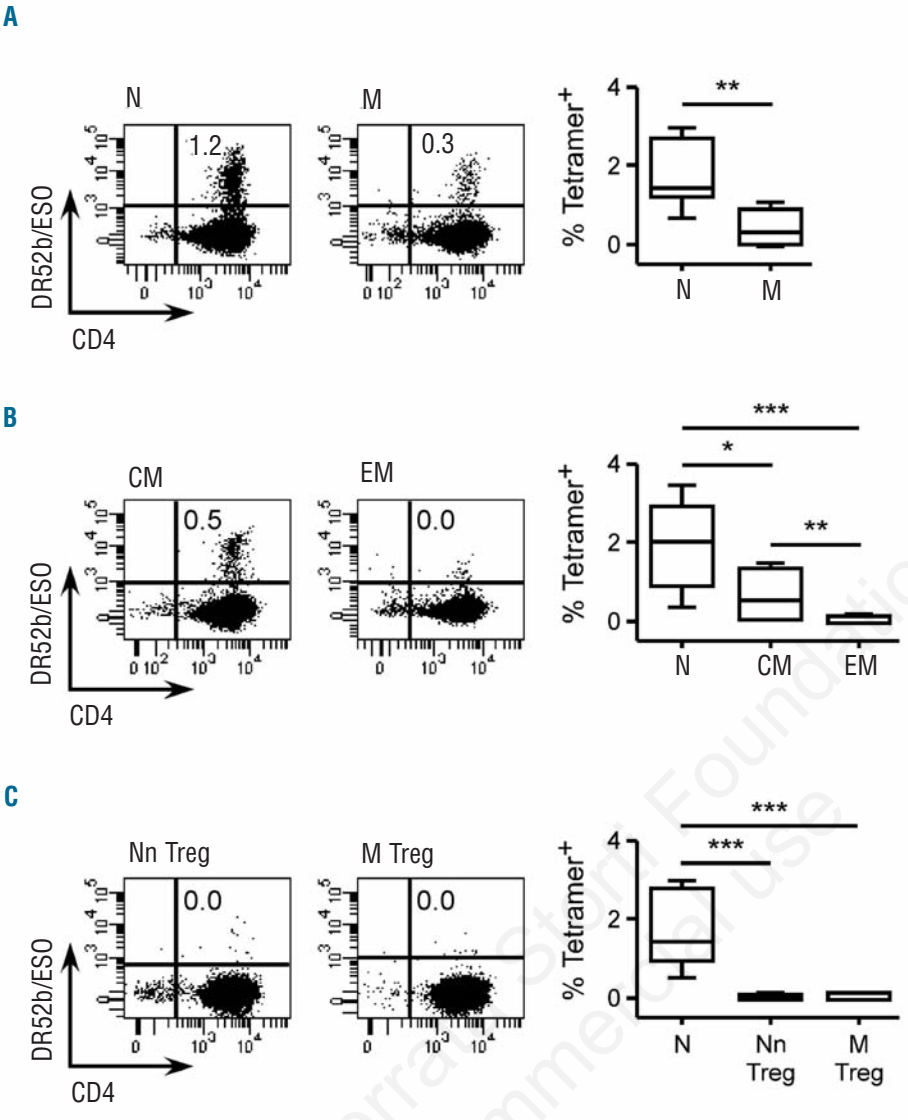

Figure 2. $ESO_{119-143}$-specific $CD4^+$ T cells can be generated from conventional central memory but not effector memory $CD4^+$ T cells or Treg of $DR52b^+$ healthy donors. (**A**) Conventional naïve (N, $CD45RA^+CCR7^+CD25^-$) and memory (M, $CD45RA^-CCR7^{+/-}CD25^-$) cells isolated *ex vivo* from $CD4^+$ T cells of $DR52b^+$ healthy donors (n=7), as shown in *Online Supplementary Figure S1*, were stimulated as in Figure 1 and stained with DR52b/ESO tetramers and anti-CD4 and analyzed by flow cytometry. Dot plots for one donor and data for all donors assessed are shown. (**B**) Conventional naïve (N, $CD45RA^+CCR7^+CD25^-$) as well as central memory (CM, $CD45RA^-CCR7^+CD25^-$) and effector memory (EM, $CD45RA^-CCR7^-CD25^-$) cells, isolated *ex vivo* by cell sorting from $CD4^+$ T cells of $DR52b^+$ donors (n=9) as shown in *Online Supplementary Figure S1*, were assessed as in **A**. Dot plots for one donor and data for all donors assessed are shown. (**C**) NnTreg ($CD45RA^+CCR7^+CD25^+CD127^{low}$) and Mtreg ($CD45RA^-CCR7^{+/-}CD25^+CD127^{low}$) populations were isolated *ex vivo* by cell sorting from $CD4^+$ T cells of $DR52b^+$ donors (n=9), as shown in *Online Supplementary Figure S1*, and assessed as in **A**. Dot plots for one donor and data for all donors assessed are shown. Statistical analyses were performed using the Wilcoxon's matched pairs test. **P*<0.05; ***P*<0.01; ****P*< 0.001.

In vitro *primed ESO-tetramer$^+$-T_H lines secrete IL-2 and effector cytokines and do not recognize allogeneic dendritic cells*

To further characterize the ESO-tetramer$^+$-T_H lines generated by *in vitro* priming functionally, we assessed their cytokine secretion profile, by intracellular staining using cytokine-specific mAb, following stimulation with the antigen. Importantly, we found that large proportions of cells in the ESO-tetramer$^+$-T_H lines were able to produce IL-2 in response to stimulation with the antigen, suggesting that the lines could support their own growth (Figure 5A). Slightly lower, but significant, proportions of them produced TNF-α and IFN-γ (Figure 5A). IL-4, but not IL-17 or IL-10, was also produced by smaller proportions of cells in the ESO-tetramer$^+$-T_H lines (Figure 5A). Because the *in vitro* primed lines were strictly selected by a single MHC class II allele/peptide complex, their potential alloreactivity was expected to be low. To provide evidence in support of this, we stimulated the lines in the presence of DC from a panel of 8 DR52b-expressing donors that had been pre-incubated or not with peptide $ESO_{119-143}$, and assessed IFN-γ secretion in the culture supernatants. As shown in Figure 5B, we observed significant recognition of allo-DC only in the presence of the ESO peptide.

Discussion

Recent data encourage us to explore the therapeutic potential of ACT using tumor-specific $CD4^+$ T cells.[15-17] There are, however, many problems in the development of this approach, including that of generating tumor-specific $CD4^+$ T clones for all patients. To overcome this limitation, in this study, we have explored the possibility of generating ESO-specific T_H lines for ACT through *in vitro* priming of defined $CD4^+$ T-cell populations from non-immune individuals and isolation with DR52b/$ESO_{119-143}$ tetramers that we have recently developed.[28] We have shown that a single stimulation of circulating conventional naïve $CD4^+$ T cells from $DR52b^+$ donors with peptide $ESO_{119-143}$, that is highly immunodominant,[30] results in the consistent and efficient *in vitro* priming of ESO-tetramer$^+$ populations, comprising on average more than 1% of $CD4^+$ T cells in Day 12 cultures. Based on this frequency, it can be estimated that $ESO_{119-143}$-specific DR52b-restricted precursors represent on average approximately $1x10^{-5}$ of circulating conventional naïve $CD4^+$ T cells in DR52b-expressing individuals. This frequency, in the range of the limit of detection by the tetramers, explains our inability to detect $ESO_{119-143}$-specific DR52b-restricted precursors *ex vivo*. The high specificity of our stimulation and detection methods was confirmed by

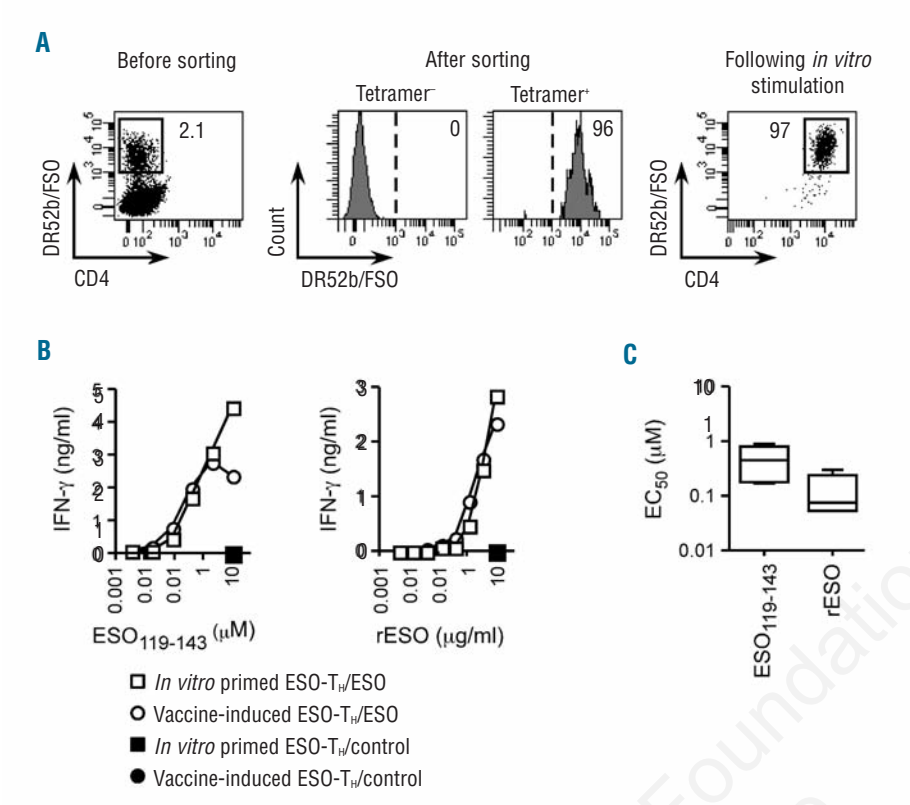

Figure 3. Isolation and functional assessment of ESO-tetramer⁺-T_H lines generated from *in vitro* primed conventional naïve CD4⁺ T cells. (A) *In vitro* stimulated conventional naïve CD4⁺ T-cell cultures were stained with DR52b/ESO tetramers (left dot plot) and ESO-tetramer⁺ cells were isolated by flow cytometry cell sorting and expanded *in vitro* by mitogen stimulation. Aliquots of sorted tetramer⁺ and control tetramer⁻ populations were analyzed by flow cytometry (middle histograms). Aliquots of T_H lines obtained following *in vitro* expansion of sorted ESO-tetramer T_H cells were stained with DR52b/ESO tetramers and anti-CD4 mAb and analyzed by flow cytometry (right dot plot). (B and C) ESO-tetramer⁺-T_H lines obtained following *in vitro* priming and $ESO_{119-143}$-specific DR52b-restricted TH obtained from cancer patients vaccinated with rESO were assessed functionally by incubation with either DR52b⁺ JBUSH cells and serial dilutions of $ESO_{119-143}$ or control peptide or with DR52b⁺ DC pre-incubated with serial dilutions of full length recombinant ESO or control protein. IFN-γ was measured in 24-h culture supernatants by ELISA. (B) Examples of peptide and protein recognition. (C) Concentration of peptide and protein resulting in half maximal IFN-γ secretion (EC_{50}) for all *in vitro* primed ESO-tetramer⁺- T_H lines assessed (n=6).

the lack of detectable responses when stimulating CD4⁺ T cells from DR52b⁻ donors, along with the lack of significant staining obtained with control tetramers.

After isolation of ESO-tetramer⁺ populations by flow cytometry cell sorting and expansion by mitogen stimulation, polyclonal monospecific ESO-tetramer⁺-T_H lines were consistently obtained. In support of their physiological relevance, the ESO-tetramer⁺-T_H lines generated through *in vitro* priming displayed a functional avidity of antigen recognition similar to that of ESO T_H from patients immunized with a recombinant ESO vaccine, showed, similar to the latter, a preferential use of TCR BV2, and were able to recognize the full-length ESO antigen processed and presented by DC. In addition, ESO-tetramer⁺-T_H lines produced IL-2, which is in line with their origin from naïve cells, as well as effector cytokine, but did not produce IL-10. Together, these functional characteristics suggest that these populations may be particularly efficient, upon *in vivo* transfer, at inducing anti-tumor responses.

A surprising finding of our study was the efficient *in vitro* priming obtained with conventional memory populations from healthy donors, who have no spontaneous immune responses to ESO, as supported by the lack of detectable levels of ESO-specific serum antibodies in this population.[32] A comparative assessment of conventional memory subpopulations separated according to their differentiation stage, namely CCR7⁺ (CM) *versus* CCR7⁻ (EM), revealed that *in vitro* priming was consistently obtained with CM cells, a population at an early differentiation stage, but not from EM cells. Interestingly, ESO-tetramer⁺ lines derived from CM cells exhibited a functional avidity of antigen recognition similar to those derived from naïve populations (*data not shown*). Human CD4⁺ CM cells are heterogeneous, including pre-T_H1, pre-T_H2 and non-polarized populations,[33] and it has been shown that DC activated by thymic stromal lymphopoietin stimulate the homeostatic expansion of autologous naïve CD4⁺ T cells that adopt and maintain a CM phenotype and a polyclonal repertoire.[34] Thus, our interpretation of these data is that ESO-specific CD4⁺ precursors in CM cells could be part of a population that is phenotypically memory but is generated through homeostatic expansion of naïve cells and maintain a similar TCR repertoire composition. This concept, however, still has to be confirmed in further studies, including comparative studies in self- and pathogen-derived antigenic systems. We obtained no significant *in vitro* priming of ESO-specific CD4⁺ T cells with CD25⁺ Treg, both naïve and memory. Treg have been shown by others and us to be enriched in self-reactive cells,[35,36] but their antigen specificity is still largely unknown. Thus, although CD4⁺ T-cell precursors specific for other tumor associated self-antigens may be present at high frequency among Treg, this does not appear to be the case for ESO, which further supports its relevance for use in immunotherapy. However, because Treg have a more limited capacity of expansion *in vitro* as compared to conventional CD4⁺ T cells, this result should be considered with caution, and confirmed by assessment through additional approaches.

Because ESO is specifically and frequently expressed in many cancers, both solid and hematologic, ACT with *in vitro* primed ESO-specific T_H lines could be of use in many different clinical settings. For example, their use in the autologous setting, as currently used for melanoma, will extend this application to patients with ESO-expressing tumors but no detectable spontaneous immune response to the antigen. However, we suggest that a major application could be in hematologic tumors such as MM or ATLL, where ESO is frequently expressed, particularly in patients

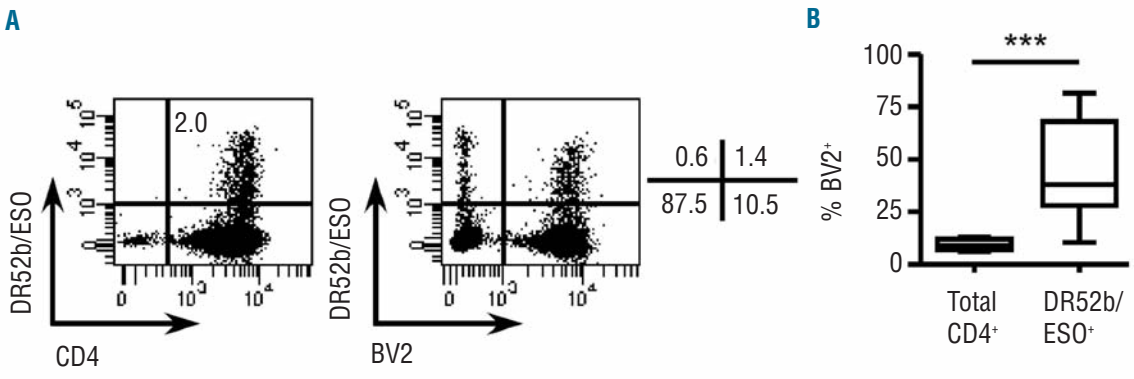

Figure 4. Analysis of TCR BV usage by *in vitro* primed ESO-tetramer$^+$-T_H. Conventional naïve CD4$^+$ T cells from healthy donors were stimulated with peptide $ESO_{119-143}$ as in Figure 1A and Day 12 cultures were stained with DR52b/ESO tetramers and anti-CD4 and anti-BV2 mAb and analyzed by flow cytometry. (A) Examples of dot plots showing tetramer staining *versus* anti-CD4 staining in the culture and anti-BV2 staining *versus* tetramer staining gated on CD4$^+$ cells. (B) The proportions of BV2$^+$ cells in total CD4$^+$ cells in the cultures and in ESO-tetramer$^+$ cells are summarized for all donors assessed (n=11). Statistical analyses were performed using Wilcoxon's matched pairs test. ***P<0.001.

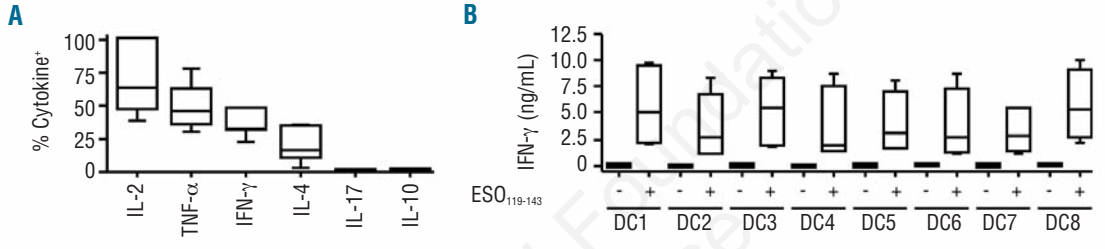

Figure 5. Assessment of cytokine production and alloreactivity of ESO-tetramer$^+$-T_H lines generated from *in vitro* primed conventional naïve CD4$^+$ T cells. (A) ESO-tetramer$^+$-T_H lines (n=7) obtained as in Figure 3A from *in vitro* primed conventional naïve CD4$^+$ T cells were stimulated with peptide $ESO_{119-143}$ and cytokine production was assessed in a 4-h intracellular cytokine staining assay and analyzed by flow cytometry. The proportions of cells producing the indicated cytokines are summarized for all ESO-tetramer$^+$-T_H lines tested. (B) ESO-tetramer$^+$-T_H lines (n=6) were incubated with DC from 8 DR52b$^+$ allogeneic healthy donors in the absence or presence of peptide $ESO_{119-143}$ and IFN-γ was measured in 24-h culture supernatants by ELISA.

with poor prognosis.[6] MM patients who relapse following chemotherapy receive allogeneic stem cell transplantation (alloSCT), and, if they experience further relapse, transfer of additional T cells from the original donor, a therapy known as donor leukocyte infusion. Interestingly, it has been shown that some MM patients who had received alloSCT develop strong antibody as well as CD4$^+$ T-cell responses to CTA, including ESO, suggesting that CTA might represent natural targets for graft-*versus*-myeloma (GVM) effects.[7] Thus, the use of *in vitro*-primed allogeneic ESO-specific T_H lines could represent a very efficient way to guarantee a strong GVM effect in this patient population. Interestingly, with the exception of melanoma, most solid and hematologic malignancies, including MM, do not express MHC class II molecules and cannot, therefore, be directly recognized by tumor antigen-specific CD4$^+$ T cells. The latter, however, have been shown to be able to mediate anti-tumor immune responses through different mechanisms[37] including by providing help for the initiation and maintenance of CD8$^+$ T-cell and antibody responses,[38] as well as through direct mechanisms in the effector phase of tumor rejection mediated by effector cytokines such as IFN-γ.[39,40] This, together with recent studies in cancer patients and in mouse models that have reported tumor regression following ACT therapy with tumor antigen-specific CD4$^+$ T cells, encourage the implementation of studies assessing the efficacy of ACT of tumor antigen-specific T_H, alone or in combination with CTL, for the treatment of patients bearing antigen-expressing tumors of different histological types.

Because the specificity of the lines is strictly selected on a single MHC class II allele/peptide complex, their alloreactivity was expected to be limited, although this would need to be confirmed in each case. In support of the limited potential alloreactivity of the *in vitro* primed ESO-specific lines, we failed to detect significant recognition of DC from DR52b$^+$ donors in the absence of antigen. Interestingly, a similar approach using allogeneic EBV-specific CTL from donors is currently used to treat patients with EBV$^+$ post-transplantation lymphoproliferative disease and has been found to be safe, with very rare occurrence of allo-responses.[41] Even in cases in which a degree of alloreactivity is detected, they could still be used through the development of appropriate pre-conditioning regimens, as recently shown in the case of "T-bodies", re-directed T cells bearing fusion receptor proteins composed of single chain tumor-specific antibodies linked to the signaling part of the TCR.[42]

Together, the results of this study indicate that an ACT approach based on the use of ESO-specific CD4$^+$ T_H generated through *in vitro* priming is feasible and could be applicable to the treatment of large numbers of patients bearing ESO-expressing cancers. In addition, we expect that the implementation of this versatile *in vitro* platform, allowing the efficient and consistent priming of tumor-specific CD4$^+$ T cells, will allow further pre-clinical studies to explore the possibility of generating tumor-specific T_H lines of different types (e.g. T_H1, T_H17) and it would be very interesting to

compare these in terms of clinical efficacy.[43] They could be used in the pre-clinical evaluation of the effects of different vaccine adjuvants (such as TLR ligands)[11,44] or immunomodulatory molecules[45] that are increasingly used in clinical cancer immunotherapy trials.

Acknowledgments

The authors would like to thank Mrs. P. Pignon and I. Raimbaud for their technical assistance.

Funding

The study was supported by the Ludwig Institute for Cancer Research and the Cancer Research Institute (USA). CP was supported through a fellowship from the Fondation pour la Recherche Médicale (France) and CR from the Ligue Contre le Cancer, France.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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