Effect of Montanide and poly-ICLC adjuvant on human self/tumor antigen-specific CD4+ T cells in Phase I overlapping long peptide vaccine trial

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ABSTRACT

Vaccination of ovarian cancer patients with overlapping long peptides (OLP) from cancer-testis antigen NY-ESO-1 and poly-ICLC in Montanide-ISA-51 (Montanide) was found to consistently induce integrated immune responses (antibody, CD4+ and CD8+ T cells). Using detailed methods, we investigated the respective effects of poly-ICLC and Montanide adjuvant on pre- and post-vaccine NY-ESO-1–specific CD4+ T cells, because of their central function for induction and maintenance of both antibody and CD8+ T cells. Polyclonal NY-ESO-1-specific CD4+ T cell lines were generated from 12 patients using CD154-based selection of precursors before and after vaccination with either (I) OLP alone, (II) OLP in Montanide, or (III) OLP and poly-ICLC in Montanide. Kinetics, quantification, fine specificity, avidity, and cytokine-producing pattern were analyzed in-depth and compared between vaccine cohorts. Vaccination with OLP alone did not elicit CD4+ T cell responses; it suppressed high-avidity CD4+ T cell precursors that recognized naturally-processed NY-ESO-1 protein before vaccination. Emulsification of OLP in Montanide was required for the expansion of high-avidity NY-ESO-1-specific CD4+ T cell precursors. Poly-ICLC significantly enhanced CD4+ Th1 responses while suppressing the induction of IL-4-producing Th2 and IL-9-producing Th9 cells. In summary, Montanide and poly-ICLC had distinct and cooperative effects for the induction of NY-ESO-1-specific Th1 cells and integrated immune responses by OLP vaccination. These results support the use of admixing poly-ICLC in Montanide adjuvant to rapidly induce anti-tumor type-1 immune responses by OLP from self/tumor antigens in human cancer vaccines.
Introduction

Most human cancer vaccine trials target self-antigens that are overexpressed in malignant cells but have limited expression in normal tissues, such as cancer-testis antigens, oncofetal antigens, and melanosomal antigens (1). Although spontaneous immune responses can develop against certain self/tumor antigens and lead to anti-tumor effects \textit{in vitro} and \textit{in vivo}, effectors mediating these immune responses are still regulated by central and peripheral tolerance (2, 3). Furthermore, cancer cells are known to utilize multiple mechanisms to escape immunosurveillance (4-6). As a consequence, achieving consistent polyclonal and high-avidity antibody, CD8\textsuperscript{+} and CD4\textsuperscript{+} T cell responses by cancer vaccination has been challenging.

In animal models, toll-like receptor (TLR) signals were shown to improve the efficacy of cancer vaccines targeting self-antigens (7). Activation of innate immune cells and production of inflammatory cytokines also play a central role in the induction of self/tumor antigen-specific immune responses (8, 9). Based on such experimental evidence, several TLR agonists have been developed for use as vaccine immunostimulatory adjuvants in human immunotherapy (10). However, in clinical trials, the specific effect of TLR ligands has rarely been analyzed primarily due to a lack of comparative control groups. Because the expression pattern of TLR is different between mice and humans, the effect of TLR ligands as adjuvants in humans may differ from experimental animal model predictions (11). Therefore, it is critical to understand the \textit{in vivo} effect of TLR ligands in humans to further improve the composition of cancer vaccines.

TLR3 is an endosomal molecule that recognizes viral double-stranded RNA molecules and plays an important role in anti-viral immunity (12). Synthetic mimic of double-stranded RNA, polyinosinic-polycytidylic acid (poly-IC) effectively stimulates TLR3 to induce inflammatory responses. In addition, poly-IC stabilized with polylysine and carboxymethylcellulose (poly-ICLC) can enhance the efficacy of self/tumor antigen-targeting vaccine in mice (13). Poly-ICLC has been tested in human clinical trials, and demonstrated safety as well as induction of inflammatory responses (14). However, its effect as a vaccine adjuvant on the quantity and quality of vaccine-induced immune...
responses has not been characterized in detail.

Recently, we reported safety data and immune responses in ovarian cancer patients who received immunizations with overlapping long peptides (OLP) from cancer-testis antigen NY-ESO-1 (15). To investigate the immunogenicity of OLP and the comparative effect of adjuvants, patients received either OLP alone, OLP emulsified in Montanide-ISA-51 (Montanide) or OLP and poly-ICLC in Montanide. Cohort 1 received OLP alone to establish safety. Cohort 2 received OLP emulsified in Montanide as a water-in-oil adjuvant historically used for multiple other NY-ESO-1-based trials. For Cohort 3, poly-ICLC was added to the emulsion to assess if it could further improve vaccination. From immunomonitoring of NY-ESO-1-specific antibody and T cells, we found that vaccination with OLP alone did not induce measurable humoral or cellular immune responses, while OLP in Montanide induced NY-ESO-1-specific CD4⁺ T cells but inconsistent or transient antibody and CD8⁺ T cell responses. Inclusion of poly-ICLC significantly accelerated the induction of NY-ESO-1-specific antibodies with increased titers and polyclonality, accelerated the induction of CD4⁺ T cell responses, and induced persistent CD8⁺ T cell responses. In summary, the combination of OLP/poly-ICLC/Montanide induced integrated immune responses consisting of OLP-specific CD4⁺ T cell, CD8⁺ T cell, and antibody responses in nearly all patients. Importantly, though not a primary endpoint, OLP and poly-ICLC in Montanide significantly prolonged progression-free survival in patients who had NY-ESO-1-expressing tumors as compared with those who had tumors with no NY-ESO-1 expression. However, due to the limited number of T cells used for standard immunomonitoring, mechanisms for the activities of Montanide and poly-ICLC as adjuvant were not explored in that original study (15).

We hypothesized that Montanide was important for protecting and slowly releasing the antigen, thereby increasing immunogenicity, while poly-ICLC help prime APC at the injection site and lead to better quality and quantity of effector cells. It has been known that antigen-specific CD4⁺ T cells are required for the induction and maintenance of cognate antigen-specific antibody and CD8⁺ T cell responses through direct cell-cell interaction and indirect cytokine production (16, 17). To investigate the effects of Montanide and poly-ICLC in the differential immunogenicity of OLP, we addressed the quantitative and
qualitative changes in vaccine-induced NY-ESO-1-specific CD4⁺ T cells depending on vaccine compositions using an in-depth sensitive approach. As we reported previously, upregulation of CD154 (CD40-ligand) on CD4⁺ T cells restimulated with tumor antigen peptides after a single in vitro peptide sensitization was used successfully to isolate tumor antigen-specific CD4⁺ T cells (18). CD154 upregulation is sufficiently sensitive to allow the isolation of low-frequency tumor antigen-specific CD4⁺ T cell precursors even from healthy donors or from patients before vaccination. By sorting CD154-expressing CD4⁺ T cells followed by polyclonal expansion, we generated NY-ESO-1-specific CD4⁺ T cell lines before and after OLP vaccination in four representative patients from each vaccine cohort, i.e., OLP alone (Cohort 1), OLP in Montanide (Cohort 2), or OLP and poly-ICLC in Montanide (Cohort 3). Frequency, epitopes, avidity, and cytokine-producing pattern of vaccine-induced CD4⁺ T cells were compared to characterize the respective effects of Montanide and poly-ICLC.
Materials and Methods

Patients and vaccination. All patients provided written informed consent for this institutional review-approved protocol (trial identifier: NCT00616941). Safety, immune responses, and characteristics of each patient were reported previously (15). Patients had no detectable tumor at the start of vaccination. In the present study, 4 patients from each group were selected for detailed analyses based on availability of samples. From immunohistochemical analyses of pre-vaccination specimens, patients M01 in Cohort 1, M05 in Cohort 2, and M21 in Cohort 3 had NY-ESO-1-expressing tumors, while tumors from all other patients showed no NY-ESO-1-expression except for M20 in Cohort 3 whose tumor specimen was not available. Vaccine OLP were a mixture of NY-ESO-179-108, NY-ESO-1100-129, NY-ESO-1121-150, and NY-ESO-1142-173 peptides and were manufactured by Multiple Peptide Systems and Phares. Another set of overlapping long peptides (assay OLP) was prepared independently for in vitro experiments (Bio-Synthesis or Genscript). Patients in Cohort 1 received 1.0 mg NY-ESO-1 OLP in 0.5 mL diluent; Cohort 2 received 1.0 mg NY-ESO-1 OLP in 0.5 mL diluent + 0.5 mL Montanide-ISA-51 VG (total of 1.0 mL); and Cohort 3 received 1.0 mg NY-ESO-1 OLP in 0.3 mL diluent + 0.7 mL (1.4 mg) poly-ICLC + 1.0 mL Montanide-ISA-51 VG (total of 2.0 mL). Vaccines were administered s.c. on weeks 1, 4, 7, 10, and 13 with final study assessment on week 16. Samples at three time points (pre-vaccination, week 4 or 7, and week 13 or 16) were used for each patient based on the availability of samples. In Cohort 1, only 4 patients were originally enrolled and therefore all patients were analyzed in this study. For patient M02 in Cohort 1, the vaccine was discontinued after week 7 because of disease progression. Therefore, only two time points (pre-vaccine and week 7) were analyzed for M02. Other patients who were analyzed in the present study received all 5 vaccines. In total, the 12 patients selected in the present report represent 60% of patients who completed the original clinical trial. About $1 \times 10^7$ cryopreserved PBMCs were used for the analyses.

Generation of CD4+ T cell lines. CD4+ and CD8+ T cells were isolated by magnetic beads (Dynal-Invitrogen). CD4+CD8- cells were pulsed overnight with a 20-mer overlapping
peptide pool (assay OLP), extensively washed and irradiated. CD4⁺ T cells (5×10⁵) were stimulated with peptide-pulsed CD4⁺CD8⁻ cells (1×10⁶) and cultured for 19-21 days in the presence of 10 U/ml IL-2 (Roche) and 20 ng/ml IL-7 (R&D systems). This expansion was required to ensure sufficient numbers of antigen-specific CD4⁺ T cells. Remaining CD4⁺ T cells were polyclonally expanded using phytohemagglutinin (PHA) in the presence of IL-2 and IL-7 and were used as antigen presenting cells (T-APC) in a CD154-expression assay (18, 19). Remaining CD4⁺CD8⁻ cells were cultured in supernatant from B95-8 cell line to generate Epstein-Barr virus-transformed B cell lines (EBV-B). NY-ESO-1 peptide-presensitized CD4⁺ T cells were restimulated with peptide-pulsed and CFSE-labeled T-APC for 6 hours in the presence of PE-labeled anti-CD154 mAb (BD Biosciences) and monensin. CFSE⁺PE⁺ cells were sorted by a FACSaria instrument using FACSDiva software (BD Biosciences). Data were analyzed using FlowJo software. Sorted CD4⁺ T cells were expanded by PHA in the presence of irradiated allogeneic PBMCs for 20 days. Typically, more than 2×10⁷ NY-ESO-1-specific CD4⁺ T cell lines were available after expansion for detailed characterization.

**Analyses of NY-ESO-1-specific CD4⁺ T cells.** NY-ESO-1-specific cytokine production was evaluated by ELISA. Expanded NY-ESO-1-specific CD4⁺ T cell lines were stimulated by peptide- or recombinant NY-ESO-1 protein-pulsed autologous EBV-B cells for 16-20 hours. Culture supernatant was harvested and stored at -20°C until the measurement. All ELISA kits were purchased from eBioscience. In some experiments, cytokine production was confirmed by intracellular cytokine staining. NY-ESO-1-specific CD4⁺ T cell lines were cocultured for 6 hours with peptide-pulsed and CFSE-labeled EBV-B cells in the presence of monensin. Cells were fixed and permeabilized using BD cytofix/cytoperm kit (BD Biosciences) according to the manufacturer’s instruction and stained by fluorochrome-conjugated anti-cytokine mAbs. All anti-cytokine mAbs were purchased from BD Biosciences except for PE-labeled anti-IL-4 mAb from eBioscience.

**Statistical Analyses.** Data were analyzed using Prism 5 (GraphPad Software, Inc.) by unpaired two-tailed t-test. P values less than 0.05 were considered significant. Responses
for NY-ESO-1 protein-specific CD4+ T cell lines were considered significant if > 0.5 ng/ml GM-CSF and if > 2× the level of GM-CSF with unpulsed control targets.
RESULTS

Detection of NY-ESO-1-specific CD4+ T cells by CD154 expression assay

We previously reported that tumor antigen-specific CD4+ T cells with different functions such as IFN-γ-producing Th1 and IL-4-producing Th2 are sensitively detected by the expression of CD154, also known as CD40-ligand, which is transiently expressed on antigen-specific CD4+ T cells after antigenic stimulation (18, 20). To assess NY-ESO-1-specific cellular immune response, CD4+ T cells isolated from PBMCs of OLP-vaccinated patients were presensitized once with an assay NY-ESO-1 peptide pool (assay OLP) and after a 20-day culture period, CD154-expressing cells were enumerated after restimulation with peptide-pulsed or -unpulsed target cells by flow cytometry. In preliminary analyses, we found that in vitro expansion of antigen-specific CD4+ T cells by presensitization was required to consistently detect CD154-expressing cells (data not shown). As shown in Fig 1A, CD154 was significantly upregulated on CD4+ T cells after peptide restimulation not only at week 7 or 13 after vaccination, but also in some pre-study samples, even though the three patients shown in Fig. 1A had no detectable IFN-γ-producing T cells at baseline by ELISPOT assays (data not shown) nor NY-ESO-1 expression in their resected tumor. Fig. 1B summarizes the frequency of NY-ESO-1-specific CD4+ T cells by CD154-expression assays in all patients tested. As expected, before vaccination, baseline seropositive patient M01 showed the highest frequency of CD154-expressing CD4+ T cells, while the baseline seronegative patients showed variable frequencies (Fig. 1B). The frequency of CD154-expressing cells significantly increased in most patients after vaccinations with OLP in Montanide in the presence or absence of poly-ICLC. In contrast, OLP alone vaccination induced no significant increase in 2/4 patients. It is clear that vaccination with OLP and poly-ICLC in Montanide (Cohort 3: M18-M21) significantly accelerates the induction of CD4+ T cells compared to OLP alone or OLP in Montanide vaccination, resulting in higher frequency of CD154-expressing cells at week 4 or 7 (Fig. 1C).

Although CD154-based detection following presensitization was a very sensitive assay that allowed the detection of low frequency NY-ESO-1-specific CD4+ T cells as naïve precursors from all samples tested, the number of antigen-specific cells was not sufficient...
for functional characterization such as cytokine production. To enable further analyses, CD154-expressing CD4+ T cells were isolated by flow cytometric cell sorting, and polyclonally expanded with PHA. After expansion for 14-20 days, at least $1 \times 10^7$ polyclonal CD4+ T cell lines became available from all samples except for pre-vaccine sample from M19, which showed the lowest frequency of CD154-expressing cells after re-stimulation (0.2% increase compared to unstimulated CD4+ T cells).

**Epitopes recognized by vaccine-induced NY-ESO-1-specific CD4+ T cells.**

Because most NY-ESO-1 epitopes that were previously reported to be recognized by CD4+ T cells (21) were included in the present vaccine OLP, the vaccine is able to induce multiple epitope-specific CD4+ T cells. The number and region of epitopes recognized by NY-ESO-1-specific CD4+ T cells before and after vaccination were determined by testing reactivity of NY-ESO-1-specific CD4+ T cell lines against individual assay OLP (22). Peptide recognition was determined by measuring GM-CSF because GM-CSF was reported to be produced by multiple CD4+ T cell subsets including Th1 and Th2 cells (23). Fig. 2 summarizes GM-CSF production from NY-ESO-1-specific CD4+ T cell lines from samples at week 13 or week 16 except for M02 at week 7. In most patients, the NY-ESO-1119-143 peptide, which was reported to bind to multiple MHC class II molecules (24), was the most strongly recognized. In addition, the NY-ESO-181-100 peptide was recognized by all CD4+ T cells induced after vaccination with either OLP in Montanide or OLP and poly-ICLC in Montanide. These NY-ESO-1119-143 and NY-ESO-181-100 peptides align with the hydrophobic regions of the NY-ESO-1 protein (Fig. 2, red line) (25). All 20-mer assay peptides in vaccinated OLP region were recognized by at least one CD4+ T cell line, which supports using overlapping peptides spanning an immunogenic (hydrophobic) region to simultaneously induce multiple epitope-specific CD4+ T cells in patients with different HLAs. Unexpectedly, NY-ESO-1-specific CD4+ T cell lines from three patients (M01, M05 and M21) showed relatively weak but significant ($\geq 1$ ng/ml GM-CSF) reactivity against a region not included in the vaccine OLP (NY-ESO-151-70 and/or NY-ESO-161-80). These non-vaccinated region-specific reactivities were not observed before vaccination and at week 7 for patients M05 and M21, while baseline seropositive patient M01 showed strong
preexisting immunity against NY-ESO-161-80 (data not shown). The potential mechanism is epitope-spreading, i.e., vaccine-induced T cells destroyed NY-ESO-1-expressing cancer cells and NY-ESO-1 protein was cross-presented to CD4+ T cells. Although this unexpected reactivity was restricted to three patients (M01, M05 and M21) who had NY-ESO-1-expressing cancer cells, the patients had no detectable tumor during the entire vaccination period.

**Recognition of naturally-processed NY-ESO-1 protein by OLP-induced NY-ESO-1-specific CD4+ T cells.**

Because vaccination with peptides may induce peptide-reactive T cells that are unable to respond to naturally-processed antigens, it is important to test the recognition of naturally-processed NY-ESO-1 protein by peptide vaccine-induced T cells. Significant NY-ESO-1 protein recognition was detectable before vaccination in 5/12 patients, indicating the presence of high-avidity T cell precursors in PBMC (Fig. 3A). Interestingly, NY-ESO-1 protein-recognizing CD4+ T cell precursors in patients M02 and M03, who received OLP alone vaccination, rapidly became undetectable after vaccination. In addition, NY-ESO-1 protein-recognizing CD4+ T cells were not detected before and after vaccination with OLP alone in M04. Patient M01, who showed spontaneous NY-ESO-1-specific antibody before vaccination, had high frequency preexisting NY-ESO-1-specific CD4+ T cells that efficiently recognized NY-ESO-1 protein before vaccination. In this patient, NY-ESO-1 protein-recognizing capability of CD4+ T cells continued to be detectable following vaccination, suggesting that in vivo-primed NY-ESO-1-specific T cells are expanded by OLP alone vaccination. In sharp contrast, independent of the presence of poly-ICLC adjuvant, OLP emulsified in Montanide consistently induced NY-ESO-1 protein-recognizing CD4+ T cells, even in patients who did not have detectable protein-recognizing CD4+ T cell precursors before vaccination. All eight patients who received Montanide-containing OLP injections had significant increases in CD4+ T cell responses to NY-ESO-1 protein after vaccination compared to baseline (even patient M17, whose responses were weaker at week 13 compared to week 7, but still positive compared to the absence of significant response at baseline).
To assess differences in the functional avidity of NY-ESO-1-specific vaccine-induced CD4⁺ T cells, reactivity against titrated amounts of individual 20-mer assay OLP was determined. To facilitate the comparison of apparent avidity for each peptide, an EC₅₀ value was interpolated from titration curves as the peptide concentration required to induce 50% of the GM-CSF produced from saturating amounts of peptide (10 μM). As shown in Fig. 3B for M05 after vaccination, there was a wide range of avidities elicited by each peptide within the polyclonal CD4⁺ T cell line. Consistent with a decrease in NY-ESO-1 protein-recognition after vaccination with OLP alone, apparent avidities of CD4⁺ T cell lines generated from Cohort 1 patients were low (EC₅₀ > 100 ng/ml) except for M01 who had preexisting responses (Fig. 3C and Fig. S1A). In contrast, NY-ESO-1-specific vaccine-induced CD4⁺ T cell lines from Cohort 2 (OLP in Montanide) and Cohort 3 patients (OLP and poly-ICLC in Montanide) recognized at least 1 peptide with high avidity (EC₅₀ < 100 nM) (Fig. 3C). Consistent with the change in protein recognition, the avidity generally decreased after OLP alone vaccination but increased after OLP vaccination in the presence of Montanide (Fig. S1A-C). Although multiple HLA-binding epitopes may be included within the 20-mer peptides tested, and knowing that minimal concentration of 20-mer peptide triggering T cell recognition is only a rough estimation of the avidity of T cells for minimal epitopes, vaccine-induced CD4⁺ T cells with high EC₅₀ apparent avidity were only observed in patients receiving OLPs emulsified in Montanide (median of 3 peptides with high-avidity recognition in Cohorts 2 and 3 vs. 0 peptide in Cohort 1; Fig. 3C).

**Cytokine-producing pattern of vaccine-induced CD4⁺ T cells**

To investigate the effect of Montanide and poly-ICLC on the differentiation and polarization of CD4⁺ T cells, cytokines in the supernatant of CD4⁺ T cell lines stimulated with the assay OLP pool were evaluated by ELISA. The amount of GM-CSF, which is produced by multiple CD4⁺ T cell subsets, was significantly different between CD4⁺ T cell lines, most likely reflecting the difference in the purity of NY-ESO-1-specific CD4⁺ T cells in each CD4⁺ T cell line (Fig. 4A). The difference in purity of NY-ESO-1-specific CD4⁺ T cells made it difficult to compare absolute cytokine levels characterizing CD4⁺ T cell differentiation. To compensate for the different percentages of NY-ESO-1-specific CD4⁺ T
cells within T cell lines, cytokine production was normalized against GM-CSF (Fig. 4B). Fig. 5 compares the normalized cytokine production by vaccine-induced NY-ESO-1-specific CD4+ T cell lines following vaccination. There was a trend for more IFN-γ production from CD4+ T cells induced by OLP and poly-ICLC in Montanide as compared with those induced by OLP in Montanide, although the difference was not statistically significant because of the large variations within groups. Moreover, poly-ICLC adjuvant significantly reduced the production of IL-4 and IL-13, cytokines produced by Th2 cells. It appeared that emulsification in Montanide increased IL-4 and IL-13 production as compared with OLP alone, indicating a Th2-differentiating effect of Montanide. The IFN-γ/IL-4 ratio, as an indication of the Th1/Th2 ratio, was increased by adding poly-ICLC adjuvant to Montanide. Differentiation to IL-10-producing Th2 or Tr1 cells was also suppressed by poly-ICLC. Recently, various CD4+ T cell subsets, other than Th1 and Th2 cells, were identified in both mice and humans including IL-17-producing Th17, IL-9-producing Th9, and TGF-β-producing regulatory T cells. As shown in Fig. 4B, significant NY-ESO-1-specific IL-17 production was detected only in patient M21 before vaccination (IL-17/GM-CSF = 0.31) but it disappeared after vaccination while all other CD4+ T cell lines showed low IL-17/GM-CSF ratio (below 0.02), indicating that the present vaccine compositions did not induce Th17 cells. In addition, TGF-β production was low (below 1,000 pg/ml) and transient. Interestingly, significant IL-9 production was detected in all NY-ESO-1-specific CD4+ T cells induced after vaccination with OLP in Montanide and it showed a gradual increase following vaccinations (Fig. 4B). IL-9-producing CD4+ T cells were also detectable by intracellular cytokine staining (Fig. 6). The staining demonstrated the presence of both IL-9 single positive cells and IL-9 and IL-4 co-producing cells. In contrast, IFN-γ-producing cells rarely co-produced IL-9. Vaccine-induced differentiation to IL-9-producing cells was completely inhibited by inclusion of poly-ICLC in the adjuvant (Figs. 5 and 6). In contrast, no significant difference in cytokine-production was found between the 3 cohorts before vaccination, except for the IFN-γ/IL-4 ratio which was slightly higher in Cohort 1 compared with Cohort 2 (Fig. S2). This suggested that differences in cytokine profiles post-vaccination reflected the effect of vaccine adjuvants, and not preexisting variability in precursors.
Discussion

Montanide and poly-ICLC were previously found to cooperatively enhance the induction of both humoral and cellular responses to NY-ESO-1 OLP vaccination, resulting in an integrated NY-ESO-1-specific antibody, CD4⁺ and CD8⁺ T cell immune response in nearly all patients (15). In this study, we used a sensitive CD154-expression-based assay to characterize vaccine-induced CD4⁺ T cell responses in detail and assess the effect of adjuvants on these responses. We generated 12 pre-vaccine and 23 post-vaccine NY-ESO-1-specific CD4⁺ T cell lines from 4 representative patients of each cohort (corresponding to 60% of patients who completed the original study). Consistent with our previous report where IFN-γ-ELISPOT assays were used as the primary assay to detect NY-ESO-1-specific CD4⁺ T cells (15), CD154-expression-based detection indicated enhancement of OLP vaccine-induced CD4⁺ T cell responses by Montanide. Inclusion of poly-ICLC significantly accelerated the induction of CD4⁺ T cells. Increased expression of costimulatory molecules on APC by TLR3-signaling and induction of a type I IFN milieu could explain the accelerated induction of CD4⁺ T cell responses (26). Consequently, CD4⁺ T cell frequency at week 13 or 16 after 4-5 injections did not increase from that at week 7 after 2 injections, indicating the response reached a plateau early after vaccination, while rapidly induced CD4⁺ T cells were maintained throughout vaccination. Because of the high sensitivity of the assay, we were able to detect and isolate NY-ESO-1-specific CD4⁺ T cells in most samples before vaccination, even from patients who did not show responses to the vaccine by IFN-γ ELISPOT assays. After polyclonal expansion of isolated NY-ESO-1-specific CD4⁺ T cells, they were characterized for peptide specificity, avidity, and cytokine-producing profiles. It is possible that these characteristics are affected by in vitro culture, which contains IL-2 and IL-7 that could skew repertoires and function of T cells. This same cytokine cocktail was also used in our original paper, following T cell stimulation with CD4⁻CD8⁻ APC pooled from pre- and post-vaccine to minimize APC-related qualitative differences. In both previous and current studies, we found statistically significant differences in vaccine-induced CD4⁺ T cell responses depending on adjuvant used, suggesting that our experimental protocol did not introduce important in vitro distortions.
and was still able to significantly distinguish characteristics and functions of CD4⁺ T cells established *in vivo* by vaccine components. Likely, the differences in CD4⁺ T cell frequency and quality reflect the effect of adjuvants on APC and their capacity to prime effectors. Further studies on activation and cytokine-producing profiles for APC subsets in the various cohorts are warranted.

As we reported previously, circulating NY-ESO-1-specific CD4⁺ T cell precursors capable of recognizing naturally processed NY-ESO-1 protein were detected in many patients before vaccination although at very low frequency (6). Interestingly, these high-avidity precursors became undetectable after vaccination with OLP alone, strongly suggesting that OLP should be administered in a proper carrier system such as Montanide. Preferential expansion of low-avidity CD4⁺ T cells by OLP alone vaccination is unlikely because total NY-ESO-1 peptide-reactive CD4⁺ T cell frequency did not significantly increase following OLP alone vaccination. These results indicated that OLP alone vaccination selectively deleted or anergized high-avidity precursors. A similar observation was made in patients vaccinated with MAGE-A3 protein alone in comparison with patients vaccinated with MAGE-A3 in the adjuvant system AS02B, indicating that this phenomenon is not just limited to peptide vaccination (18). Interestingly, patients who received MAGE-A3 protein alone did not respond to booster vaccinations with MAGE-A3+AS02B adjuvant, indicating that deletion or persistent anergy of high-avidity CD4⁺ T cells led to long-term tolerance (27, 28). In contrast, OLP in Montanide with or without poly-ICLC increased the protein-recognizing capability of NY-ESO-1-specific CD4⁺ T cells. Taken together, emulsification in Montanide appears essential to expand high-avidity CD4⁺ T cells by OLP vaccine, possibly due to involvement of local inflammation, facilitated antigen uptake, or slow release of antigens. The use of Montanide was recently called into question because it was found to act as a trap for T cells after short peptide vaccination, reducing circulating high-avidity effectors by attracting cells to the vaccine site itself instead of the tumor (29). Yet, this trapping effect in Montanide depots was not observed with long peptide vaccination (30). Beside peptide length, species difference, *i.e.*, humans and mice, may contribute to the favorable effect of Montanide observed in the current study. In mice, it was shown that long peptides are selectively presented by professional antigen presenting
cells (31). However, we found that in humans, many cell types including activated T cells and B cells efficiently process and present long peptides on both HLA class I and class II in vitro (32). Although it is possible that presentation of OLP by non-professional APC contributes to the deletion or unresponsiveness of high-avidity CD4+ T cells in the absence of Montanide, the emulsification of OLP appears to overcome this limitation, possibly by routing antigen presentation to professional antigen presenting cells for adequate priming of naïve T cells (33).

Characterization of cytokine-producing pattern of OLP vaccine-induced CD4+ T cells revealed a significant effect of Montanide and poly-ICLC on CD4+ T cell differentiation. As known for incomplete Freund's adjuvant (IFA) in mice, Montanide appeared to promote Th2 differentiation of vaccine-induced NY-ESO-1-specific CD4+ T cells (34). Inclusion of poly-ICLC with Montanide significantly suppressed Th2-associated cytokines such as IL-4, IL-13 and IL-10, which resulted in Th1 over Th2 polarization of vaccine-induced NY-ESO-1-specific CD4+ T cells. It has been proposed that Th1 immunity is more favorable for anti-tumor responses (35). In addition, differentiation to IL-9-producing Th9 cells was completely suppressed by poly-ICLC. In vitro culture experiments have shown that IL-9 production is regulated by IL-4 and TGF-β in mice and humans (36, 37). Therefore, Th9 suppression after vaccination with OLP and poly-ICLC in Montanide may be due to the strong suppressing effect of poly-ICLC on IL-4 production. Th9 cells have only recently been characterized as a novel CD4+ T cell subset and their in vivo differentiation, especially in humans, is largely unknown (36, 37). Our results suggest that similar to in vitro cultures, in vivo Th9-differentiation is regulated by Th2-derived cytokine(s), presumably IL-4. As reported for in vitro-differentiated Th9 cells, in patients vaccinated with OLP in Montanide, IL-9-production can be found in CD4+ T cells whether or not they produce IL-4. However IL-9 production is rarely found in IFN-γ-producing cells. Recently, an anti-tumor effect of Th9 cells was demonstrated in mouse models in vivo via direct induction of tumor apoptosis and indirect mechanism through mast cells (38). Because the NY-ESO-1-specific IL-9-producing cells were only about 1% of total NY-ESO-1-specific CD4+ T cells (Fig. 6), the role of tumor antigen-specific Th9 cells in humans would require a new vaccine strategy that preferentially induces antigen-specific
Th9 cells, but not Th2 cells.

CD4⁺ T cells help the induction of antibody production by secreting cytokines and activating B cells. From the immunomonitoring of humoral immune responses, it was found that poly-ICLC significantly accelerated and enhanced humoral immune responses, probably by modulating and enhancing CD4⁺ T cell responses. Additionally, CD8⁺ T cell induction was higher and more sustained if poly-ICLC was present in the vaccine formulation; this observation may be explained by the interplay with CD4⁺ T cells and adequately activated APCs at priming. As both antigen-specific CD4⁺ and CD8⁺ T cell populations could be detected after a single vaccine injection in many patients receiving OLP and poly-ICLC in Montanide (15), a direct role of adjuvant on CD8⁺ T cell responses may need to be explored in future studies. Nevertheless, the current study identified priming of high-avidity polyclonal Th1 responses with poly-ICLC and Montanide adjuvants as critical in achieving integrated immune response by helping the induction and maintenance of antibody production and CD8⁺ T cell responses. Our study did not evaluate poly-ICLC as adjuvant without Montanide, and clinical trials to address this question are ongoing. These and future studies testing the effect of checkpoint blockade on vaccine-induced responses should be evaluated using the detailed quantitative and qualitative analyses described here.
References


Figure Legends

**Fig. 1** Detection of NY-ESO-1-specific CD4⁺ T cells by CD154 expression before and after vaccination with OLP with or without adjuvant. CD4⁺ T cells were isolated from PBMC and stimulated with NY-ESO-1 assay OLP. After about 20 days, cells were restimulated with assay OLP-pulsed autologous T-APC and CD154 expression was evaluated by flow cytometry. (A) CD154 expression on CD4⁺ T cells in a typical patient in each vaccination group. (B) Induction of NY-ESO-1-specific CD4⁺ T cells in each time point in patients vaccinated with OLP alone (open symbols and dotted lines), OLP in Montanide (grey symbols and thin lines), or OLP and poly-ICLC in Montanide (closed symbols and thick lines). M01 was a baseline seropositive patient. (C) Comparison of the frequency of NY-ESO-1-specific CD4⁺ T cells at week 4 or 7. Bars indicate mean ± SD. *: p=0.024 by Student’s t-test.

**Fig. 2.** Peptide specificity of NY-ESO-1-specific CD4⁺ T cell lines after vaccination with overlapping peptides. CD154-expressing NY-ESO-1-specific CD4⁺ T cells after stimulation with NY-ESO-1 OLP were isolated and expanded. CD4⁺ T cell lines were stimulated for 16-20 hours with autologous EBV-B cells pulsed with a NY-ESO-1 peptide and the GM-CSF level in the culture supernatant was measured by ELISA. Bars indicate GM-CSF production at week 13 or week 16 except for M02 at week 7. A red line indicates predicted hydrophobicity determined from the NY-ESO-1 protein sequence. Location of NY-ESO-1 assay and vaccine OLP is shown by lines with amino acid numbers.

**Fig. 3.** Avidity of vaccine-induced NY-ESO-1-specific CD4⁺ T cells. (A) Recognition of naturally-processed NY-ESO-1 protein by CD4⁺ T cell lines from samples before and after vaccination. NY-ESO-1-specific CD4⁺ T cell lines were stimulated for 20 hours with autologous EBV-B cells pulsed with 20 μg/ml recombinant NY-ESO-1 protein. GM-CSF level in the supernatant was measured by ELISA. (B) Determination of apparent avidity of CD4⁺ T cell lines from M05 at week 13. The NY-ESO-1-specific CD4⁺ T cell line was stimulated with EBV-B cells pulsed overnight with the indicated concentrations of
respective NY-ESO-1 assay peptide for 20 hours. GM-CSF levels in the supernatant were measured by ELISA. GM-CSF levels normalized to those produced against 10 μM peptide are shown. EC50 was defined as the peptide concentration able to induce 50% of GM-CSF levels elicited by 10 μM peptide and was calculated by interpolation of a fitting curve. (C) Comparison of apparent avidity. EC50 values for all recognized epitopes at week 13 or 16 except for week 7 for M02 are shown, and responses were considered as high-avidity for EC50 < 10^2 nM (above grey area), correlating with the ability to recognize naturally processed NY-ESO-1 protein.

**Fig. 4.** Cytokine production of NY-ESO-1-specific CD4+ T cell lines obtained before and after vaccination. (A) GM-CSF production from NY-ESO-1-specific CD4+ T cell lines after co-culture with autologous EBV-B cells pulsed (filled) or unpulsed (open) with a NY-ESO-1 assay OLP pool. (B) Normalized cytokine production. Measured cytokine levels were normalized against the GM-CSF level.

**Fig. 5.** Comparison of cytokine production. Normalized cytokine production from vaccine-induced NY-ESO-1-specific CD4+ T cells is shown for statistical consideration. The shape of symbols indicates a single patient as shown in Fig. 1B. Open and closed symbols indicate CD4+ T cell lines at week 4 or 7 and week 13 or 16, respectively. Bars indicate mean ± SD. *: p<0.05; **: p<0.01; and ***: p<0.001 by Student’s t-test.

**Fig. 6.** Intracellular cytokine staining of NY-ESO-1-specific CD4+ T cell lines. CD4+ T cells were co-cultured for 6 hours with autologous CFSE-labeled EBV-B cells pulsed with or without a NY-ESO-1 assay OLP pool in the presence of monensin. After fixation and permeabilization, cells were stained with fluorochrome-labeled mAbs against IFN-γ, IL-4 and IL-9. Staining patterns of CD4+ T cells stimulated with peptide-pulsed EBV-B cells are shown. Numbers in each quadrant indicate the percentages of cytokine-producing cells. Parenthesized numbers indicate the background cytokine production after co-culture with unpulsed EBV-B cells.
Figure 3

A

<table>
<thead>
<tr>
<th>OLP alone</th>
<th>OLP in Montanide</th>
<th>OLP+ polyIC in Montanide</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF (ng/ml)</td>
<td>NY-ESO-1 protein</td>
<td>Unpulsed</td>
</tr>
<tr>
<td>Pre</td>
<td>Wk7</td>
<td>Wk13</td>
</tr>
<tr>
<td>M01</td>
<td>M02</td>
<td>M03</td>
</tr>
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B

M05/Wk13

Relative GM-CSF (%)

Peptide concentration (nM)

C

$EC_{50}$ (nM)

OLP alone | OLP in Montanide | OLP+ polyIC in Montanide

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Figure 5

The figure shows the expression levels of various cytokines and their ratios in different treatments. The treatments include OLP alone, OLP in Montanide, and OLP+ polyIC in Montanide. The cytokines measured are IFN-γ/GM-CSF, IL-4/GM-CSF, IL-13/GM-CSF, IL-10/GM-CSF, IFN-γ/IL-4, and IL-9/GM-CSF. The results are presented as box plots, where the median, interquartile range, and outliers are indicated.

For IFN-γ/GM-CSF, OLP alone showed a lower median compared to OLP and OLP+ polyIC in Montanide. For IL-4/GM-CSF, a similar trend was observed, with OLP alone having a lower median than the other treatments.

IL-13/GM-CSF showed a significant increase in OLP+ polyIC in Montanide compared to OLP and OLP alone. IL-10/GM-CSF also showed a significant increase in OLP+ polyIC in Montanide.

IFN-γ/IL-4 showed a significant increase in OLP plus polyIC in Montanide. IL-9/GM-CSF did not show a significant difference between the treatments.
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Effect of Montanide and poly-ICLC adjuvant on human self/tumor antigen-specific CD4+ T cells in Phase I overlapping long peptide vaccine trial


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