

Vaccination with LAG-3Ig (IMP321) and Peptides Induces Specific CD4 and CD8 T-Cell Responses in Metastatic Melanoma Patients—Report of a Phase I/IIa Clinical Trial

Amandine Legat¹, H el ene Maby-El Hajjami¹, Petra Baumgaertner¹, Laur ene Cagnon^{1,2}, Samia Abed Maillard^{1,2}, Christine Geldhof¹, Emanuela M. Iancu², Luc Lebon¹, Philippe Guillaume³, Danijel Dojcinovic³, Olivier Michielin², Emanuela Romano², Gr egoire Berthod², Donata Rimoldi¹, Fr ed eric Triebel⁴, Immanuel Luescher³, Nathalie Rufer^{1,2}, and Daniel E. Speiser^{1,2}

Abstract

Purpose: Cancer vaccines aim to generate and maintain antitumor immune responses. We designed a phase I/IIa clinical trial to test a vaccine formulation composed of Montanide ISA-51 (Incomplete Freund's Adjuvant), LAG-3Ig (IMP321, a non-Toll like Receptor agonist with adjuvant properties), and five synthetic peptides derived from tumor-associated antigens (four short 9/10-mers targeting CD8 T-cells, and one longer 15-mer targeting CD4 T-cells). Primary endpoints were safety and T-cell responses.

Experimental Design: Sixteen metastatic melanoma patients received serial vaccinations. Up to nine injections were subcutaneously administered in three cycles, each with three vaccinations every 3 weeks, with 6 to 14 weeks interval between cycles. Blood samples were collected at baseline, 1-week after the third, sixth and ninth vaccination, and 6 months after the last vaccination.

Circulating T-cells were monitored by tetramer staining directly *ex vivo*, and by combinatorial tetramer and cytokine staining on *in vitro* stimulated cells.

Results: Side effects were mild to moderate, comparable to vaccines with Montanide alone. Specific CD8 T-cell responses to at least one peptide formulated in the vaccine preparation were found in 13 of 16 patients. However, two of the four short peptides of the vaccine formulation did not elicit CD8 T-cell responses. Specific CD4 T-cell responses were found in all 16 patients.

Conclusions: We conclude that vaccination with IMP321 is a promising and safe strategy for inducing sustained immune responses, encouraging further development for cancer vaccines as components of combination therapies. *Clin Cancer Res*; 22(6):1330–40.  2015 AACR.

Introduction

Melanoma is a highly aggressive cancer, with increasing incidence and mortality rates. Although surgery can cure melanoma detected at early stages, the prognosis of most patients with metastatic disease is unfavorable. Chemotherapy, irradiation and therapy with IFN  are of limited efficacy (1, 2). Recently, much more effective therapies have been developed, with the introduction of BRAF inhibitors active for patients whose tumor expresses

V600 mutations in the *BRAF* gene (3). High-dose interleukin 2 (IL2) was showing marked successes but with considerable side effects and costs, and only for selected patients (4). More recently, a novel and more widely applicable form of immunotherapy has been introduced: a "checkpoint blockade," consisting of a monoclonal antibody specific for cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), named Ipilimumab. It was approved in 2011 for the treatment of metastatic melanoma (5). Subsequently, antibodies that block PD-1/PD-L1 (Programmed Death 1/Programmed Death-Ligand 1) pathway have been introduced and shown great progress in the treatment of metastatic melanoma (6) leading to the first market approvals in 2014. Of high interest, positive clinical studies are also reported in patients with carcinomas of lung, kidney, bladder, and head and neck, and large efforts with current studies are also ongoing in patients with other cancers. Besides CTLA-4 and PD-1/PD-L1, the targeting of further inhibitory lymphocyte receptors ("checkpoints") is explored in an increasing number of preclinical studies (7, 8). One of these is lymphocyte activation gene-3 (LAG-3) or CD223, an inhibitory receptor that modulates T-cell homeostasis, proliferation, and activation (9). The LAG-3-specific antibody BMS986016 is already in clinical development, with two clinical trials recruiting patients with

¹Ludwig Cancer Research Center, University of Lausanne, Lausanne, Switzerland. ²Department of Oncology, Lausanne University Hospital Center (CHUV) and University of Lausanne, Lausanne, Switzerland. ³TCMetrix, Epalinges, Switzerland. ⁴Immutep, SA, Orsay, France.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

A. Legat and H. Maby-El Hajjami contributed equally to this article.

Corresponding Author: Daniel E. Speiser, Ludwig Center for Cancer Research, University of Lausanne, Chemin des Boveresses 155, Biopole 3 - 02DB92, CH-1066 Epalinges, Switzerland. Phone: 41-21-314-0182; Fax: 41-21-692-5995; E-mail: doc@dspeiser.ch

doi: 10.1158/1078-0432.CCR-15-1212

 2015 American Association for Cancer Research.

Translational Relevance

Active immunotherapy represents a promising anticancer strategy aiming to trigger specific T-cell responses against tumor cells to avoid disease relapse or progression. However, the enhancement of clinical efficacy depends on strong *in vivo* T-cell activation, which requires optimization of vaccine formulations with powerful antigens and adjuvants. In this clinical trial, we tested a vaccine formulation consisting of Montanide, IMP321/LAG-3Ig, and five tumor-associated antigens/peptides. Montanide is clinically graded Incomplete Freund's Adjuvant with very good depot effects. IMP321 is a non-TLR agonist with interesting adjuvant properties. The five peptides were chosen to elicit a broad spectrum of cytotoxic CD8 T-cells and a helper CD4 T-cell response. All components have a favorable safety profile. Serial vaccinations induced tumor-specific T-cell responses in all 16 vaccinated melanoma patients, encouraging further development of this approach, for example, in combination with checkpoint blockade.

solid tumors or hematologic neoplasms, respectively (www.clinicaltrials.gov).

Therapeutic vaccination is a promising strategy against malignant diseases. Similar to other immunotherapies, a major challenge is to break immune tolerance, to induce a powerful and targeted immune response, and to avoid autoimmune side effects. Over the last decades major efforts were made to develop immunomodulatory molecules that act as vaccine adjuvants. An important class of innate immune receptors, the Toll-like receptors (TLR) are targeted and studied in animals and in humans, uncovering novel means of inducing strong immune responses (10). Beside TLRs, there are additional receptors for activating innate immune pathways, such as RIG-I-like receptors, NOD-like receptors, AIM2-like receptors, and STING (11). Although these receptors are interesting for future vaccine development, this field is less advanced than targeting TLRs.

Since several years, the non-TLR agonist, soluble LAG-3Ig (IMP321, Immutep S.A.) is in clinical development (12, 13). The rationale is at least two-fold. First, this molecule can block inhibitory signals in T-cells similar to the "checkpoint blockade" principle. Second, it can stimulate antigen-presenting cells (APC), which makes LAG-3Ig an attractive vaccine adjuvant. LAG-3 is a CD4-like protein able to bind to major histocompatibility complex (MHC) class II molecules with a 100-fold higher affinity than CD4. IMP321 is one of a few clinical-grade non-TLR adjuvants for T-cell vaccination. LAG-3Ig stimulates the innate immune system by inducing activation and migration of APCs, particularly dendritic cells. Consequently, it enhances specific immune responses, by stimulation of antigen cross presentation to CD8 T-cells, and by decreasing regulatory T-cell frequency and function (9). Clinical trials with IMP321 showed already promising results when used as adjuvant for vaccination against hepatitis B and influenza viruses in healthy individuals (14, 15). Several trials have provided evidence for clinical activity in patients with advanced renal cell carcinoma, metastatic breast carcinoma, and metastatic melanoma (16–18).

Antigens used in cancer vaccines should be exclusively expressed by the tumor cells, or should be lineage specific in case

of tumors arising from nonvital tissues/cells such as melanoma. The melanocyte differentiation antigen melanoma antigen recognized by T-cells-1 (MART-1)/Melan-A is one of the rare antigens that are expressed by a vast majority of patient's tumors (19, 20). Often, this antigen triggers T-cell responses upon tumor progression, which can be amplified by immunotherapy (21–24). Furthermore, cancer-testis antigens such as melanoma-associated antigen 3 (Mage-A3) and NY-ESO-1 are excellent antigens, due to their high degree of tumor specificity, the relatively high affinity of peptides derived from these antigens that bind to human leukocyte antigen (HLA)-A*0201 and presentation to specific CD8 T-cells. NA-17 represents also a promising target antigen for the development of melanoma immunotherapy as it triggers tumor-specific CD8 T-cells and showed promising clinical responses in a dendritic cell-based vaccine trial (25). Importantly, simultaneous targeting of multiple antigens likely reduces the risk of tumor outgrowth by escape variants.

Besides components that trigger innate immune cells and antigen specific ones, vaccines may also be enhanced by delivery systems with depot effect. One of the best adjuvants for T-cell vaccines is incomplete Freund's adjuvant (IFA), produced as Montanide ISA-51. Because of its nonresorbable mineral oil component, it persists at subcutaneous injection sites for several weeks up to multiple months and contributes to continuous or repetitive T-cell stimulation.

Based on the above outlined evidence and on our own previous experience (18), we designed a vaccine formulation composed of IMP321 (LAG-3Ig), Montanide ISA-51 (IFA), and tumor antigen-specific peptides. We combined four short HLA-A2-restricted peptides (Melan-A, NY-ESO-1, Mage-A3, and NA-17) to target CD8 T-cells, and one longer 15-mer peptide (Mage-A3), containing HLA class II epitopes to target CD4 T-cells. Sixteen metastatic melanoma patients were included in this study and vaccinated up to nine times.

For comprehensive immune monitoring, we developed a combinatorial tetramer staining allowing parallel quantification of 13 specific CD8 T-cell populations in T-cell cultures after one stimulation *in vitro*. In addition, Melan-A-specific CD8 T-cells were quantified directly *ex vivo*, without any culture steps. Finally, we quantified the frequency and evaluated the functional potential of Mage-A3-specific CD4 T-cells induced by vaccination.

The vaccinations were well tolerated, showing comparable (mostly local) adverse events like vaccination with Montanide alone. Remarkably, the vaccines induced specific CD4 T-cell responses in all 16 vaccinated melanoma patients (100%), and specific CD8 T-cells to at least one antigen formulated in the vaccine in 13 patients (81%).

Patients, Materials, and Methods

More details are given in the Supplementary Materials and Methods section.

Patients, vaccination, and blood samples

HLA-A2⁺ patients with histologically confirmed metastatic melanoma of stage III to IV, expressing Melan-A/MART-1 [determined by reverse transcription (RT)-PCR or immunohistochemistry] were included upon informed consent, in this phase I/IIa, single center, open, noncomparative study. The primary objective was measurement of antigen-specific immune responses besides safety and tolerability assessments. Local ethic review committees and

responsible health authorities approved the study, which was carried out according to the Good Clinical Practice guidelines and the Declaration of Helsinki and was registered at www.clinicaltrials.gov (NCT01308294).

Eligible patients were immunized subcutaneously with the synthetic peptides and 250 µg IMP321/LAG-3Ig (Immutep S.A.). The first six vaccines were formulated with 1 mL Montanide ISA-51 (Seppic Inc.) and the last three vaccines without Montanide. Patients with an expected survival of at least 3 months (status at entry is described in Supplementary Table S1A) received nine vaccines scheduled in three cycles with three vaccinations (3 weeks intervals between vaccines, 6–14 weeks between cycles), based on observations that multiple boosters can not only induce but also maintain T-cell responses during many months or even years (26, 27). The clinical-grade peptides used in the vaccines were: Melan-A₂₆₋₃₅ native EAAGIGILTV or analogue (A27L) ELAGIGILTV, NY-ESO-1₁₅₇₋₁₆₅ SLLMWITQC, Mage-A3₁₁₂₋₁₂₀ KVAELVHFL, NA17 VLPDVFIRC, all representing known class I HLA-A2 peptides; and Mage-A3₂₄₃₋₂₅₈ KKLLTQHFVQENYLEY, containing a class II HLA-DP4 epitope. Blood samples were collected before the first vaccine (C0), 1 week after the third vaccination of each cycle (C1, C2, C3), and 6 months after the end of the third cycle (follow up: FU) for some patients. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient using Lymphoprep (Axis-Shield) and immediately cryopreserved in RPMI1640 supplemented with 40% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO).

***In vitro* stimulation (IVS) of T-cells with specific peptides**

CD8⁺ T-cells or CD4⁺ T-cells were enriched using magnetic activated cell sorting (MACS) technology (Miltenyi Biotec). The negative fraction of cells was loaded with laboratory-grade peptides (listed in Supplementary Table S2), irradiated (30 Gray), and used as APCs to stimulate peptide-specific T-cells during 10 or 12 days in presence of IL2.

Flow cytometry: quantification of specific T-cells by tetramer staining

Freshly thawed cells or cultured cells were stained using similar protocols. As a first step, single tetramer staining or combinatorial tetramer staining (TCMetrix) was performed. Cells were incubated 45 minutes at room temperature with class I tetramers (combinatorial tetramers or tetramer Melan-A-PE) or 60 minutes at 37°C with class II tetramer (tetramer Mage-A3/DP4-PE). The combinatorial tetramers are listed in Supplementary Table S2. In addition, surface antibodies and dead cell exclusion marker were used to identify live CD4 and CD8 T-cells. Data were acquired on a Gallios Flow Cytometer (Beckman Coulter) and analyzed using FlowJo 7.6.5 software (TreeStar).

Mage-A3-specific CD4 T-cell clones, cell sorting, and stimulation

A blood sample from patient Lau1187 (collected after three vaccines; C1) was stimulated with Mage-A3₂₄₃₋₂₅₈ peptide for 16 days, as described above in IVS section. CD4⁺ tetramer-low and CD4⁺ tetramer-high cells were separately cloned. The specificity of each clone was checked by tetramer staining analyzed by flow cytometry (BD FACS Array). Twelve days after expansion, clones were phenotyped in separate tubes for their expression level of CD4, T-cell receptor (TCR) and for their tetramer brightness.

In parallel, clones were stimulated with 0.5 and 5 µg/mL Mage-A3₂₄₃₋₂₅₈ peptide in presence of Brefeldin A 10 µg/mL for 6 hours. Intracellular cytokine staining (ICS) was performed as described in the Supplementary Materials and Methods section. Analysis of cytokine coexpression was done with SPICE software version 5.2. Finally, clones were clonotyped as previously described (28).

Statistical analysis

The Student *t* test (two-sample two-tailed comparison) or paired *t* test was used for statistical calculations. *P* < 0.05 was considered significant (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001; ns, not significant).

Results

Favorable safety profile

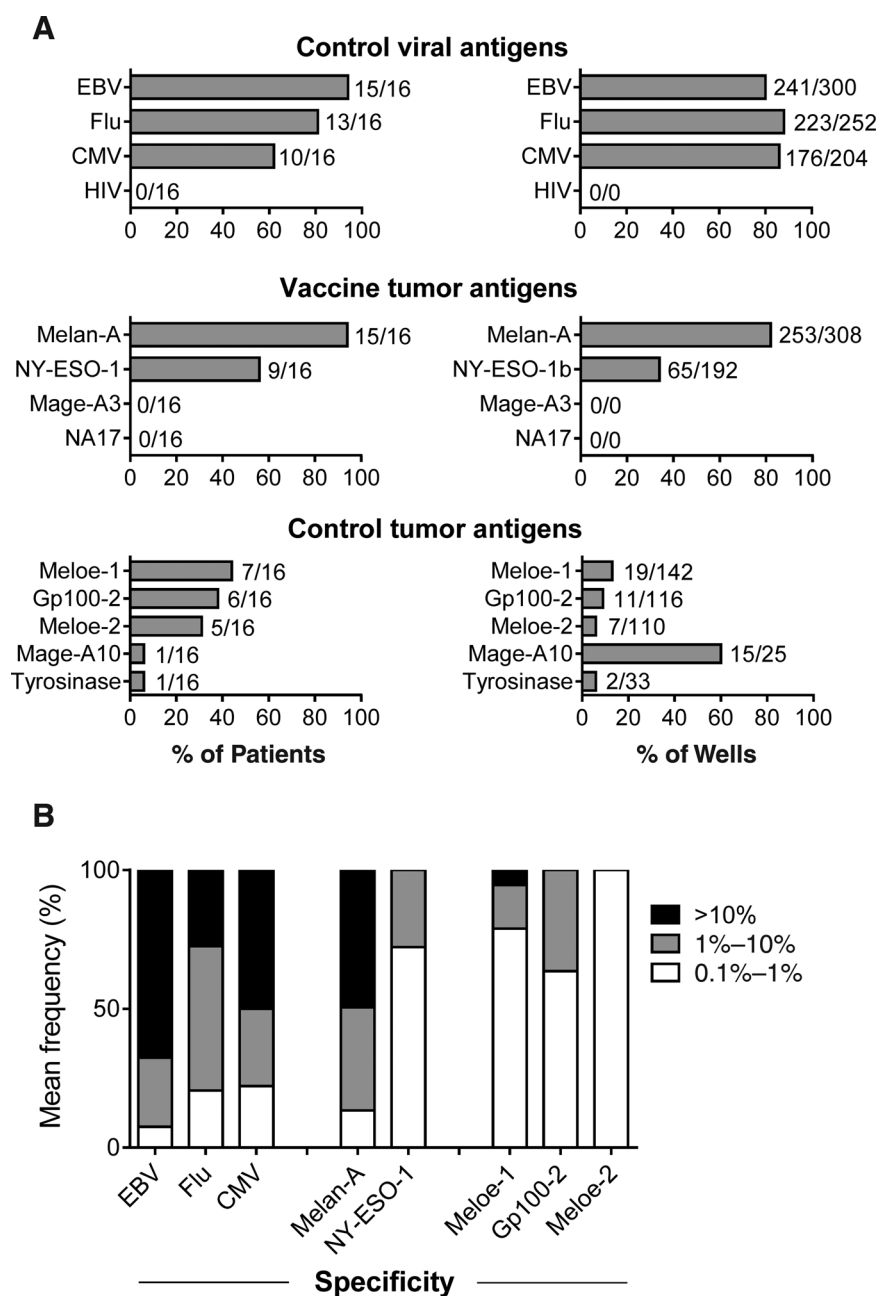
In this study, 16 HLA-A2⁺ patients with metastatic melanoma received serial vaccines (up to nine vaccines; see Supplementary Table S1A). The vaccines contained IMP321, Montanide and four short peptides representing class I epitopes presented by HLA-A2, and one long 15-mer peptide containing a class II epitope presented by HLA-DP4.

The patient's baseline characteristics are shown in Supplementary Table S1A. There were eight women and eight men, with an age ranging from 21 to 84 years. Ten patients had stage III melanoma and six patients with stage IV melanoma at study entry. Fourteen patients finished the entire clinical trial protocol and two patients discontinued the study earlier due to disease progression.

The safety analysis of the treatment showed a comparable severity of side-effects commonly reported for cancer vaccines. The 16 patients experienced in total 588 adverse events. One event was of life-threatening severity and was qualified as serious adverse event as it involved hospitalization of the patient, but it was not related to the study treatment. Twelve events were of severe intensity, all caused by melanoma metastases and considered as not related to the study treatment. The majority (534 events, i.e. 91%) of all adverse events were of mild intensity. Supplementary Table S1B shows all adverse events with an incidence of more than 5, according to their severity and their relationship to the study treatment. A total of 135 injections were applied to 16 patients, reported adverse events were mainly expected/prelisted local reactions at vaccine injection sites (313 events, i.e. 53.2%), consisting of induration (100 events), erythema (83 events), pain (72 events), warmth (38 events), and injection site reactivation (20 events). They were mostly mild and were resolved rapidly except indurations that could last for several weeks. Indeed, Montanide ISA-51 is well known to form a depot allowing long-term local vaccine persistence and causing local inflammation. The systemic reactions reported during study corresponded to 113 events of all adverse events (19.2%). The ones that were considered as possibly or probably related to the study treatment were mainly of mild severity. The most frequent systemic events were headache, myalgia, chills, asthenia, and arthralgia. Tumor progression was the only type of grade 3 adverse event. No grade 4 adverse events related to the study treatment were observed.

All together, the combination of IMP321, Montanide, and five peptides was safe and well tolerated, with mild severity local

Figure 1. Overview of specific T-cell frequencies. CD8 purified cells from vaccinated melanoma patients were stimulated with peptide pools for 12 days, harvested, and stained with combinatorial tetramers, as described in Materials and Methods. The detection limit was set at 0.1%. The left part of A shows numbers of patients with detectable specific cells, considering patients that had at least one culture well with detectable cells. The right part shows the culture wells with detectable specific cells of the numbers of stimulated wells, in patients with positive cultures. Percentages are indicated with bars, accompanied by the numbers of patients and wells, respectively. B, detected specific T-cells were divided in three categories depending on frequency: >10%, between 1% and 10%, and between 0.1% and 1%. Graph shows the distribution of frequency found in positive wells for each specificity in at least two patients. The numbers of patients and wells analyzed in this graph are listed in A.



adverse events, no cases of ulcerations at the vaccine injection sites, and no treatment related systemic adverse events higher than grade 2.

Quantification of specific CD8 T-cells by combinatorial tetramer staining after IVS

To obtain maximal information on specific CD8 T-cells with a minimum of blood volume, we chose to use combinatorial tetramer staining. This technique is based on the combination of multiple tetramers labeled with a combination of multiple dyes (29, 30), allowing the detection of multiple specific T-cell populations in the same sample (up to 15 specificities with four dyes). We set up a panel detecting 13 specificities allowing the quanti-

fication of 13 populations of virus and tumor-specific CD8 T-cells in 10 million PBMCs (see the list of specificities in Supplementary Table S2).

As a first step, we expanded the T-cells by IVS with peptides in two conditions, one with viral antigens [Epstein-Barr virus (EBV), influenza (Flu), cytomegalovirus (CMV), human immunodeficiency virus (HIV)] and the other one with tumor antigens; some of which corresponded to the antigens included in the vaccinations (Melan-A, NY-ESO-1, Mage-A3, NA17), and others not ["control" tumor antigens, namely melanoma-overexpressed antigen-1 (Meloe-1), glycoprotein 100-2 (Gp100-2), Meloe-2, Mage-A10, Tyrosinase]. In addition, to avoid competition of the well-growing specificities versus the less frequent

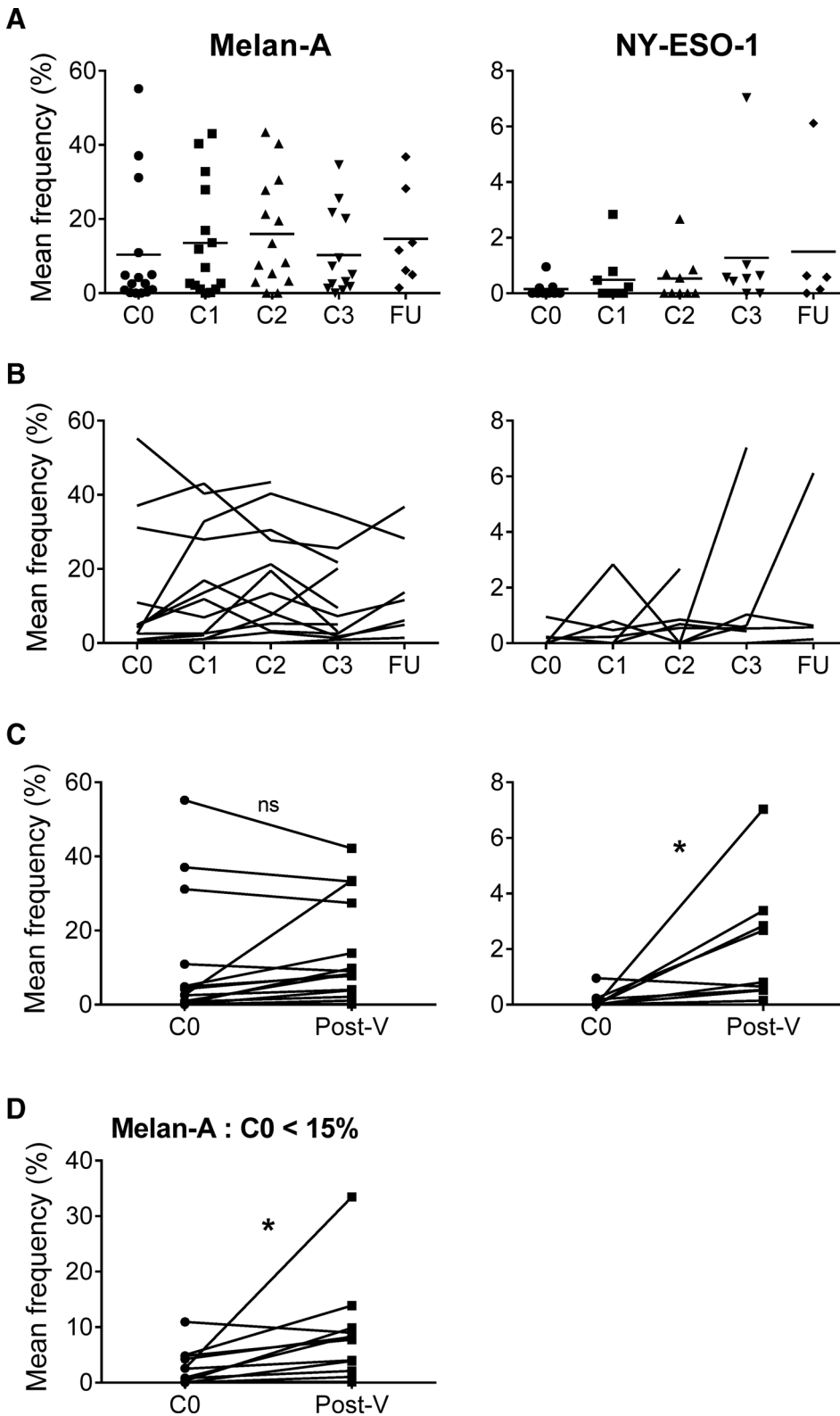


Figure 2.

Frequencies of Melan-A and NY-ESO-1-specific CD8 T-cells after IVS, before and after vaccination. Blood samples of vaccinated melanoma patients were collected before vaccination (C0), after one, two, and three cycles of vaccination (C1, C2, and C3 respectively), and for some patients, 6 months after the last vaccine (FU: follow up). PBMC were isolated, CD8 T-cells purified and stimulated with peptide pools for 12 days (IVS), harvested, and stained with combinatorial tetramers, as described in Materials and Methods. A and B, graphs show the mean of the positive wells in positive patients, corresponding to a frequency above detection limit (>0.1%). B, lines link samples from the same patient. C and D, post-V corresponds to the mean of all positive wells analyzed after vaccination, from C1 to FU. D, analysis of the frequency changes after excluding the 3 patients who showed high frequencies already at baseline (C0 > 15%; patients Lau 1366, Lau 616, and Lau 1438). The numbers of patients and wells analyzed are listed in Fig. 1A.

Downloaded from <http://aacrjournals.org/clinccancerres/article-pdf/22/6/1330/2035759/1330.pdf> by guest on 06 August 2024

(weaker-growing) ones, we split the cells in 2 to 12 independent cultures (96 wells) depending on the quantity of CD8 T cells we purified. After 12 days of culture, the frequencies of

specific CD8 T-cells were quantified by combinatorial tetramer staining, determined using the gating strategy shown in Supplementary Fig. S1.

The majority of patients had antigen-specific T-cells for EBV, Flu, and/or Melan-A (13–15 patients of 16; detection limit at 0.1%; Fig. 1A; Supplementary Table S3). Furthermore, two thirds of the patients had detectable amounts of CMV and/or NY-ESO-1-specific CD8 T-cells; 1/3 Meloe-1, Gp100-2, and/or Meloe-2-specific cells. Mage-A10 or Tyrosinase-specific T-cells were only detected in one patient (Lau 616 and Lau 1366, respectively). None of them had HIV, Mage-A3, or NA17-specific CD8 T-cells above the detection limit, despite that the patients had been vaccinated with the two latter peptides. HIV was a reliable negative control, based on the negative HIV serology result available for each patient.

In addition, EBV, Flu, CMV, and Melan-A-specific CD8 T-cells were found in more than 80% of the cultures (Fig. 1A and B), and the frequencies of specific cells were mainly above 10% of the cells (Fig. 1B). NY-ESO-1-specific CD8 T-cells were found in 34% of culture wells, and Meloe-1, Gp100-2, and Meloe-2-specific CD8 T-cells maximally in 13% of cultures. For these specificities, the frequencies mainly ranged between 0.1% and 1%. Mage-A10-specific CD8 T-cells were at high frequency in one patient (Lau 616) who had previously been vaccinated with this peptide, with different adjuvants (31).

For further analysis of the impact of vaccination on antigen-specific CD8 T-cells, we focused on the specificities found in more than one patient, and calculated mean frequencies in positive culture wells, excluding wells with frequencies below the detection limit of 0.1%. Thus, we focused on EBV, Flu, and CMV for the control viral antigens; on Melan-A and NY-ESO-1 for the tumor antigens against which the patients were vaccinated; and on Meloe-1, Gp100-2, and Meloe-2 for the remaining tumor antigens.

We were able to detect Melan-A and/or NY-ESO-1-specific CD8 T-cells in 15 and 9 patients, respectively. Overall, we found no significant increase of the frequencies of Melan-A-specific CD8 T-cells after vaccination when analyzed after IVS, but a significant increase was found for NY-ESO-1-specific CD8 T-cells (Fig. 2A–C). However, it is well known that IVS with peptide efficiently amplifies previously primed and thus highly frequent Melan-A-specific T-cells, leading to the saturation of this kind of proliferation system. Therefore, we analyzed the frequency changes with vaccination on Melan-A-specific cells after excluding the three patients who showed high frequencies already at baseline, due to tumor priming or previous treatment ($CO > 15\%$). In the remaining 12 patients, we found a significant increase of Melan-A-specific CD8 T-cells following vaccination (Fig. 2D).

Concerning the tumor antigen specificities not included in the vaccine (Meloe-1, Meloe-2, and Gp100-2) and the viral antigens (EBV, Flu, and CMV), we did not observe any significant frequency changes (Supplementary Fig. S2).

Together, the IVS-combinatorial tetramer technique showed increased frequencies of circulating CD8 T-cells (corresponding to an increase of 2-fold or more as compared to baseline) to at least one of the four class I peptides administered, in 13 of 16 metastatic melanoma patients.

Direct *ex vivo* quantification of circulating Melan-A-specific CD8 T-cells

In the majority of vaccine studies in cancer patients, IVS is required to allow the detection of tumor antigen-specific T-cells, as their *in vivo* frequencies are below the detection limits (which is approximately 0.01% of CD8 T-cells for flow cytometry-based

techniques). Detection and analysis of tumor-specific T-cells directly *ex vivo* is still a major challenge. Yet, we and others were able to detect, in blood samples, Melan-A-specific CD8 T-cells, directly *ex vivo*, without any culture step, as these cells are unusually frequent. Moreover, some vaccine components such as CpG-B are capable of inducing extraordinarily strong expansion of human CD8 T-cells (32). We monitored the frequencies of Melan-A-specific CD8 T-cells before and after each vaccination cycle, directly *ex vivo* (Fig. 3 and Supplementary Table S4). Six patients showed an increase of 2-fold or more in their frequencies of Melan-A-specific CD8 T-cells after vaccination (Supplementary Table S4). Overall, this approach allowed revealing that the frequencies of Melan-A-specific T-cells increased significantly after vaccination (Fig. 3A). Remarkably, data obtained from frequencies of Melan-A-specific CD8 T-cells as determined by the IVS-combinatorial tetramer technique strongly correlated ($P < 0.0001$) to those collected after direct *ex vivo* analysis with standard tetramers (Fig. 3B).

All together, our data demonstrate that vaccination with IMP321, Montanide, and short peptides induced significant expansion of tumor-specific CD8 T-cells *in vivo*.

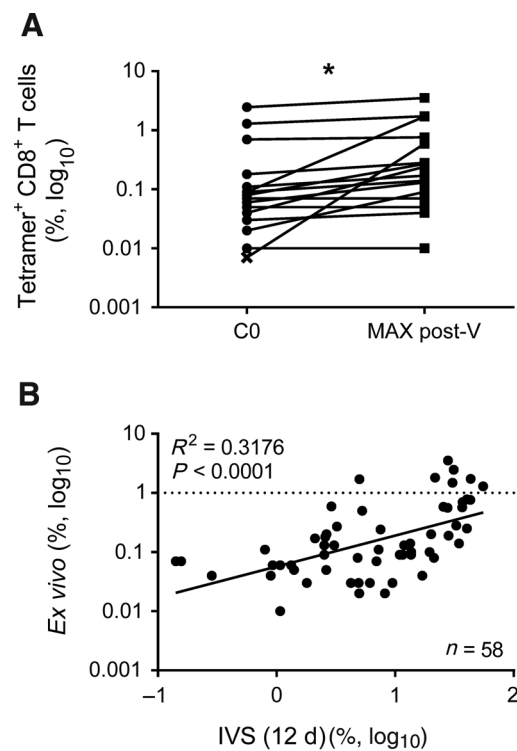


Figure 3. Frequencies of circulating Melan-A-specific CD8 T-cells. Melan-A-specific CD8 T-cells were measured directly, without any culture step, after CD8 enrichment, in blood samples of vaccinated melanoma patients. A, frequency of tetramer positive cells among the CD8⁺ T-cells, in Log₁₀ scale, before vaccination (C0), and the maximum frequency measured after vaccination (in C1, C2, C3, or FU; MAX post-V). The cross represents a frequency below the detection limit of 0.01%, drawn arbitrarily at 0.005%. The detailed data are shown in Supplementary Table S3. B, correlation between direct *ex vivo* measurements (A) and those after IVS (Fig. 2), from the same blood sample, when both measures were above the detection limits. Axes show calculated Log₁₀ of the frequency. The curve shows linear regression.

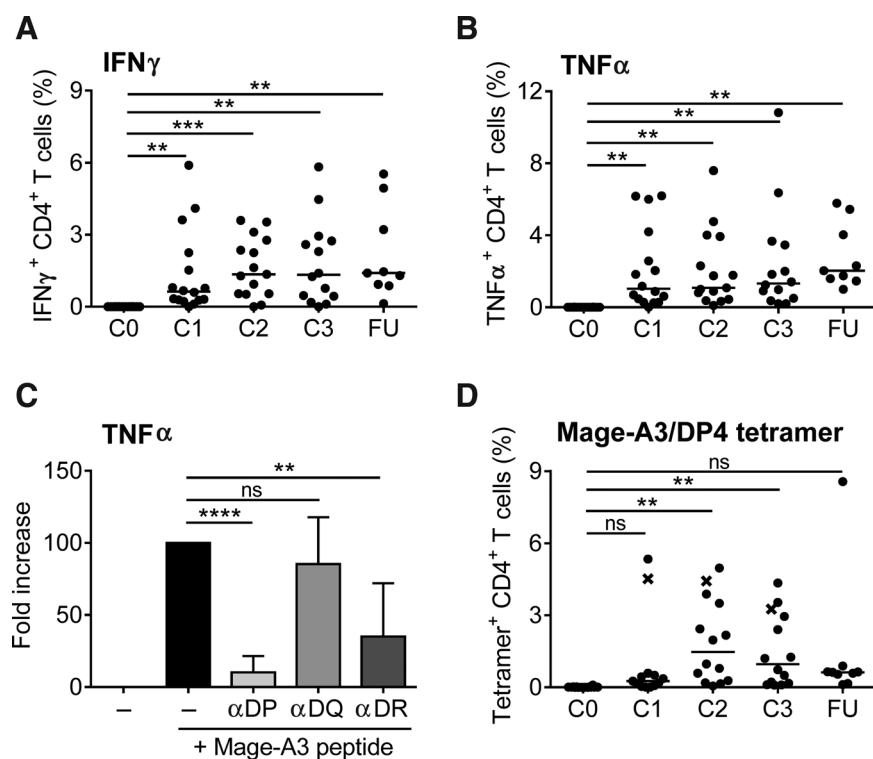


Figure 4. Quantification of Mage-A3₂₄₃₋₂₅₈-specific CD4 T-cells. CD4 T-cells from vaccinated melanoma patients were purified and stimulated with Mage-A3₂₄₃₋₂₅₈ peptide for 10 days (IVS), as described in Materials and Methods. C0, C1, C2, C3, and FU correspond to the number of vaccination cycles administrated. A and B, frequency of intracellular IFN γ and TNF α producing T-cells after 4 hours of peptide challenge. C, cultures from 6 patients ($n = 6$; Lau 1187, Lau 1268, Lau 1314, Lau 1342, Lau 1366, and Lau 1409) were challenged with the Mage-A3₂₄₃₋₂₅₈ peptide for 5 hours, in presence of blocking anti-HLA-DP, anti-HLA-DQ, or anti-HLA-DR antibodies. "Fold increase" was determined by background subtraction and normalization in reference to the condition stimulated with peptide alone. D, quantification of Mage-A3/DP4 tetramer positive cells among total CD4⁺ T-cells in the HLA-DP4 positive patients and patient Lau616 (cross symbol; $n = 14$).

Induction of Mage-A3-specific CD4 T-cells in all 16 vaccinated patients

In addition to the short peptides, one longer peptide (15-mer) derived of the Mage-A3 protein was used for vaccination, with the aim to activate tumor-specific CD4 T-cells. For immunologic monitoring, we stimulated PBMC *in vitro* with the peptide for 10 days, followed by challenging the cells with the Mage-A3₂₄₃₋₂₅₈ peptide for 4 hours, allowing the quantification of IFN γ - and TNF α -positive cells upon ICS (Fig. 4A and B). Remarkably, the frequencies of IFN γ and TNF α producing cells were always increased after vaccination, demonstrating that all 16 patients uniformly generated CD4 T-cell responses with at least 2-fold higher frequencies as compared to baseline (C0).

Patients had been included in the study irrespective of their HLA class II genes. Previous publications reported that the Mage-A3₂₄₃₋₂₅₈ peptide can be presented by HLA-DP4 and HLA-DQ6 (33, 34). Using blocking antibodies specific for HLA-DP, HLA-DQ, or HLA-DR, we found a dominant usage of HLA-DP (Fig. 4C). As 13 of the patients were HLA-DP*0401 and/or HLA-DP*0402 (Supplementary Table S1A), we synthesized a class II tetramer to quantify Mage-A3/DP4-specific CD4 T-cells (Fig. 4D). Indeed, the frequencies of Mage-A3/DP4-specific CD4 T-cells were increased in all HLA-DP4+ melanoma patients, with an expansion of 2-fold or higher, including patient Lau 616 who was not typed for HLA class II.

In contrast to the first six vaccines, Montanide ISA-51 was omitted for the last three vaccines, with the aim to reduce local toxicities, and to determine whether this strategy may nevertheless achieve the desired booster effects. Despite slight trends toward reduced frequencies of some of the T-cell populations analyzed after cycle 3 as opposed to the previous two cycles, there were no statistically significant differences, suggesting

that booster vaccinations without Montanide are a valuable option.

Characterization of two distinct Mage-A3-specific CD4 T-cell populations identified in patient Lau 1187

While analyzing the Mage-A3-specific CD4 T-cells, we observed two populations of specific cells with different tetramer staining intensities in patient Lau 1187 (Fig. 5A), which we sorted and cloned separately. Each clone showed a tetramer-low (described thereafter as Low) or tetramer-high (High) staining, corresponding to the tetramer staining intensity before sorting (Fig. 5A).

First, we checked the tetramer staining characteristics after expansion of the two clonal families and confirmed that the tetramer-high and tetramer-low profiles (Fig. 5B) were stable over time (data not shown). Theoretically, higher intensity tetramer staining could be associated to a higher expression at the surface level of the TCR and/or the co-receptor (CD4), and/or to a higher TCR avidity (35, 36). We next measured the expression level of TCR and CD4 separately, in order to avoid competition of the staining antibodies. As shown in Fig. 5B, a higher tetramer staining was not associated with a higher TCR or CD4 staining. We further tested the functionality of the clones in a 6-hour peptide stimulation assay, and quantified cytokines by ICS (Fig. 5C). We found that the two families of clones differed in their cytokine profile (Fig. 5C), with tetramer-high clones producing more TNF α , IL2, and also the Th2 cytokine IL13, but the same amounts or less of the Th1 cytokine IFN γ than tetramer-low clones (Fig. 5C). Tetramer-high clones were also more polyfunctional than tetramer-low clones (Fig. 5D).

Sequencing of the complementarity determining region 3 β (CDR3 β) of the TCR revealed that all the tetramer-low clones were of the same clonotype (VB6, CDR3 β SIGLAGGTDITQ, JB2.3),

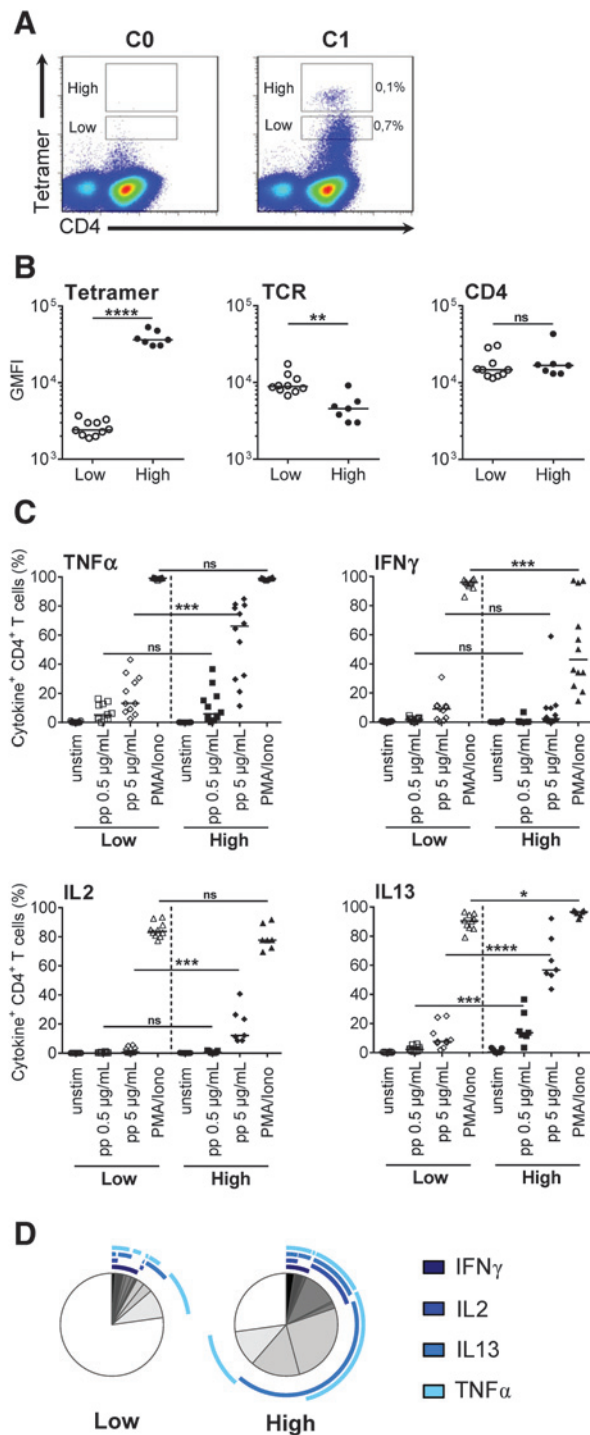


Figure 5.

Mage-A3/DP4-specific CD4 T-cells from patient Lau 1187 showing two phenotypes with tetramer-high and tetramer-low staining. CD4 T-cells from Lau 1187 were purified and stimulated with Mage-A3₂₄₃₋₂₅₈ peptide (IVS), as described in Materials and Methods. Blood sample collected after three vaccinations showed two distinct tetramer positive populations. Tetramer-high (High) and tetramer-low (Low) stained cells were sorted separately, and cloned. A, FACS dot plots of the tetramer staining at the time points C0 (considered as background) and C1 (before sorting). B, the clones were phenotyped to determine the expression levels of tetramer, TCR and CD4, in separate tubes. C, intracellular cytokine production of tetramer-high and

tetramer-low clones after 6 hours of stimulation. D, polyfunctionality (coexpression of cytokines: TNF α , IFN γ , IL2, and IL13) of tetramer-high and tetramer-low clones in response to 5 μ g/mL Mage-A4₂₄₃₋₂₅₈ peptide. Colors of the pie arcs depict the expression of individual cytokines, whereas the patterns of the pie depict the number of coexpressed cytokines from zero (white) to four cytokines (black).

Clinical Results

Although it was not in the focus of the study, we documented the clinical results. Twelve of the 16 patients entered the study without detectable tumor, due to previous surgical resection. Ten of these 12 patients remained tumor-free throughout the entire study period, whereas 2 patients developed new metastases, causing 1 patient to stop after the second cycle of vaccination. The remaining 4 patients entered the study with detectable metastases and all had disease progression. Of those, 1 patient stopped study participation after the first vaccination cycle. At the end of the vaccination period, all patients were alive. Subsequently, two of the 16 patients died after 8 and 14 months, respectively. The median follow up time was 47.2 months (with a range from 35.9 to 57.3 months) at the time of analyses (July 8, 2015). After the study, 9 of the 16 patients received one or more additional antimelanoma treatments, which were surgery (7 patients), chemotherapy (3 patients), irradiation (3 patients), BRAF inhibitors (3 patients, 2 of which were the 2 deceased patients), and anti-CTLA-4 antibody followed by anti-PD-1 antibody (1 patient). Obviously, poststudy treatments may impact on the clinical outcome in an uncontrolled manner, a caveat inherent to most clinical trials.

Discussion

In this phase I/IIa study, 16 metastatic melanoma patients were vaccinated with IMP321 (LAG-3Ig), Montanide ISA-51 (IFA), and five synthetic peptides, resulting in CD4 and CD8 T-cell responses that were antigen specific, as no effect was seen on T-cells specific for other antigens than those used for vaccination (summarized in Table 1).

The treatment was well tolerated; none of the patients discontinued the study due to treatment-related adverse events. Montanide ISA-51 has been used in a large number of cancer patients, and in cumulative doses up to 16 mL (37). Despite the overall favorable safety profile of Montanide ISA-51, the local side effects can be strong and may require surgical removal of the nonresorbable material when causing persistent local inflammation with ulceration leading to bacterial infections (38). This was, however, not encountered in this study; the maximal local toxicity was grade 2.

The safety and toxicity profile of IMP321 has been established in two randomized phase I trials of subcutaneous vaccination against Influenza virus (Flu, $n = 60$) and the Hepatitis virus (HBsAg, $n = 48$) in healthy volunteers (14, 15). Both studies revealed very good clinical tolerability with a low toxicity profile for the four dose levels of IMP321: 3, 10, 30, and 100 μ g.

tetramer-low clones after 6 hours of stimulation. D, polyfunctionality (coexpression of cytokines: TNF α , IFN γ , IL2, and IL13) of tetramer-high and tetramer-low clones in response to 5 μ g/mL Mage-A4₂₄₃₋₂₅₈ peptide. Colors of the pie arcs depict the expression of individual cytokines, whereas the patterns of the pie depict the number of coexpressed cytokines from zero (white) to four cytokines (black).

Table 1. Summary of the immune responses induced by vaccination in melanoma patients

Study no.	Patient	Specific CD8 T-cell responses			Mage-A3-specific CD4 T-cell responses		
		Melan-A after IVS	NY-ESO-1 after IVS	Melan-A <i>ex vivo</i>	IFN γ	TNF α	DP4 tetramer
1	Lau 1268	+	nd	+	+	+	+
2	Lau 1366	-	+	-	+	+	+
3	Lau 465	+	nd	+	+	+	+
4	Lau 1187	-	nd	-	+	+	+
5	Lau 1342	+	+	-	+	+	+
6	Lau 1409	-	+	-	+	+	+
7	Lau 1171	+	+	-	+	+	+
8	Lau 1142	+	nd	-	+	+	+
9	Lau 1314	+	+	+	+	+	+
10	Lau 1456	+	nd	-	+	+	HLA-DP4 neg
11	Lau 1438	-	+	-	+	+	HLA-DP4 neg
12	Lau 1477	+	+	+	+	+	+
13	Lau 1499	+	nd	+	+	+	+
14	Lau 1486	+	+	+	+	+	+
15	Lau 1523	nd	nd	nd	+	+	+
16	Lau 616	-	-	-	+	+	+

NOTE: The table summarizes specific CD8 T-cell responses obtained after IVS (Fig. 2), Melan-A-specific CD8 T-cell responses quantified *ex vivo* (Fig. 3A and Supplementary Table S3) and Mage-A3-specific CD4 T-cell responses (Fig. 4) with an increase of 2-fold or more (+) compared to baseline (CO). +, increase of 2-fold or more in specific T-cell frequencies after vaccination compared to baseline (CO). -, frequency of specific T cells not changed by vaccination. nd, specific T-cells not detected.

The vaccinations induced specific CD8 T-cell responses to at least one of the injected antigens in 13 of 16 melanoma patients (81%), and specific CD4 T-cell responses in all patients (16/16, 100%). Thus, the vaccine fulfilled the intended purpose to simultaneously induce CD8 and CD4 T-cell responses, according to the principle that CD8 cytotoxic T-cells are key players in the control and the killing of tumor cells, and CD4 T-cell help supports the CD8 T-cell responses (39, 40). We detected Melan-A- and NY-ESO-1-specific CD8 T-cells in the majority of patients (15/16 and 9/16 patients, respectively). Among them, 10 patients showed an increase in the frequency of Melan-A-specific cells and 8 patients in the frequency of NY-ESO-1-specific cells (Fig. 2 and Table 1). Unfortunately, we did not find CD8 T-cell responses to Mage-A3 and NA17. Although difficult to explain, it may be possible that the applied vaccine formulation has weakness for activating T-cells with low precursor frequencies, a problem that is also observed with other types of vaccines (26, 27, 41).

Remarkably, all 16 patients developed Mage-A3-specific CD4 T-cell responses, independently of their HLA class II genotypes. The Mage-A3₂₄₃₋₂₅₈ peptide is presented by HLA-DP4 and HLA-DQ6 (33, 34). Among the 16 patients, 13 were HLA-DP4 positive, in line with the reported high frequency of this allele (33). Therefore, it was not surprising that we could frequently detect HLA-DP-restricted cells (Supplementary Table S1A).

Besides studying cytokine production by the Mage-A3₂₄₃₋₂₅₈ peptide-specific CD4 T-cells, we used highly purified tetramers (42) produced with this peptide and recombinant HLA-DP*0401 protein, allowing to identify two CD4 T-cell populations with different tetramer staining intensities. Interestingly, they represented two dominant clonotypes with different cytokine profiles. It is worthwhile to note that the differences in tetramer staining intensity remained stable over time, and did not reflect different levels of TCR downregulation. On the contrary, the low tetramer staining cells expressed even higher TCR levels, which could be due to lower triggering and thus less TCR downregulation. This may possibly be associated with lower TCR affinity, and/or different fine specificity. Indeed, it has been shown that a given peptide can make different configurations on a particular MHC class II protein, resulting in different epitopes recognized by

different TCR (fine) specificities (43–45). In this regard, our observations are based solely on a single patient. Generalization would require more extensive studies which are beyond the scope of this clinical trial.

In conclusion, the vaccinations of this study induced tumor-specific T-cells in the majority of patients. CD4 T-cell responses were very satisfactory. CD8 T-cell responses were less frequent, but still comparable to vaccines formulated with TLR2 ligands (46) or TLR4 ligands (24), and more frequent than with protein vaccines (20). The CD8 T-cell responses were however less frequent and less strong as compared to vaccines with short peptides, IFA and CpG-B, representing the currently most potent synthetic vaccine formulation for the induction of human CD8 T-cell responses (32). Future vaccine strategies may profit from combinations with multiple TLR/innate immune stimulators, potentially capable of mimicking immune responses to viruses that can generate more robust and long lasting T-cell responses (ref. 47 and manuscript in preparation). Thus, besides multiple antigens (possibly also including mutated antigens; ref. 48), future vaccines may also require multiple immune "adjuvants." In such scenarios, the role of IMP321 and its activatory effects on APCs (9) remains to be determined.

Although the clinical results were relatively favorable, they cannot be firmly interpreted as this is a phase I study and has not been designed to determine clinical efficacy. Before designing larger phase III studies with clinical endpoints, it will be useful to further define the clinical role of IMP321, particularly with respect to vaccine component combinations as mentioned above. Carefully performed phase I/II studies may evaluate candidate treatment combinations, based on their capabilities of sound systemic T-cell activation and also overcoming immune suppression and T-cell exhaustion in the tumor microenvironment (49). Triggering HLA class II with IMP321 may have effects that are complementary or even synergistic to TLR stimulation (9). Furthermore, although we did not combine with checkpoint blockade (e.g., anti-CTLA-4 or anti-PD1/PD-L1 antibodies), there is a sound rationale for doing so, as the antigen-specific nature of vaccines enhances treatment specificity and thus may increase the efficacy/toxicity ratio of checkpoint blockade (8, 49).

Disclosure of Potential Conflicts of Interest

O. Michielin is a consultant/advisory board member for Bristol-Myers Squibb, GlaxoSmithKline, Novartis, and Roche. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: A. Legat, P. Baumgaertner, D.E. Speiser
Development of methodology: A. Legat, F. Triebel, D.E. Speiser
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Legat, P. Baumgaertner, S.A. Maillard, E.M. Iancu, L. Lebon, D. Dojcinovic, O. Michielin, E. Romano, G. Berthod, D. Rimoldi, D.E. Speiser, L. Cagnon
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Legat, P. Baumgaertner, L. Cagnon, E.M. Iancu, L. Lebon, E. Romano, D. Rimoldi, D.E. Speiser, H. Maby-El Hajjami
Writing, review, and/or revision of the manuscript: A. Legat, H. Maby-El Hajjami, P. Baumgaertner, L. Cagnon, O. Michielin, E. Romano, F. Triebel, I. Luescher, N. Rufer, D.E. Speiser
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Maby-El Hajjami, L. Cagnon, S.A. Maillard, C. Geldhof, P. Guillaume, D. Dojcinovic, I. Luescher, D.E. Speiser
Study supervision: O. Michielin, D.E. Speiser
Other (clinical trial coordination): H. Maby-El Hajjami
Other (clinical trial management and coordination): S.A. Maillard, C. Geldhof, L. Cagnon, H. Maby-El Hajjami

Acknowledgments

The authors thank the patients for their dedicated collaboration, and Immunet and Ludwig Cancer Research for providing IMP321 and clinical-grade

References

1. Tsao H, Atkins MB, Sober AJ. Management of cutaneous melanoma. *N Engl J Med* 2004;351:998–1012.
2. Sznol M. Molecular markers of response to treatment for melanoma. *Cancer J* 2011;17:127–33.
3. Bollag G, Hirth P, Tsai J, Zhang J, Ibrahim PN, Cho H, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature* 2010;467:596–9.
4. Petrella T, Quirt I, Verma S, Haynes AE, Charette M, Bak K, et al. Single-agent interleukin-2 in the treatment of metastatic melanoma. *Curr Oncol* 2007;14:21–6.
5. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010;363:711–23.
6. Curti BD, Urba WJ. Clinical deployment of antibodies for treatment of melanoma. *Mol Immunol* 2015;67:18–27.
7. Naidoo J, Page DB, Wolchok JD. Immune modulation for cancer therapy. *Br J Cancer* 2014;111:2214–9.
8. Shin DS, Ribas A. The evolution of checkpoint blockade as a cancer therapy: what's here, what's next? *Curr Opin Immunol* 2015;33C:23–35.
9. Sierro S, Romero P, Speiser DE. The CD4-like molecule LAG-3, biology and therapeutic applications. *Expert Opin Ther Targets* 2011;15:91–101.
10. Makkouk A, Weiner GJ. Cancer immunotherapy and breaking immune tolerance: new approaches to an old challenge. *Cancer Res* 2015;75:5–10.
11. Brubaker SW, Bonham KS, Zanoni I, Kagan JC. Innate immune pattern recognition: a cell biological perspective. *Annu Rev Immunol* 2015;33:257–90.
12. Fougeray S, Brignone C, Triebel F. A soluble LAG-3 protein as an immunopotentiator for therapeutic vaccines: Preclinical evaluation of IMP321. *Vaccine* 2006;24:5426–33.
13. Brignone C, Grygar C, Marcu M, Schakel K, Triebel F. A soluble form of lymphocyte activation gene-3 (IMP321) induces activation of a large range of human effector cytotoxic cells. *J Immunol* 2007;179:4202–11.
14. Brignone C, Grygar C, Marcu M, Perrin G, Triebel F. IMP321 (sLAG-3), an immunopotentiator for T cell responses against a HBsAg antigen in healthy adults: a single blind randomised controlled phase I study. *J Imm Based Ther Vacc* 2007;5:5–20.
15. Brignone C, Grygar C, Marcu M, Perrin G, Triebel F. IMP321 (sLAG-3) safety and T cell response potentiation using an influenza vaccine as a model antigen: a single-blind phase I study. *Vaccine* 2007;25:4641–50.
16. Brignone C, Escudier B, Grygar C, Marcu M, Triebel F. A phase I pharmacokinetic and biological correlative study of IMP321, a novel MHC class II agonist, in patients with advanced renal cell carcinoma. *Clin Cancer Res* 2009;15:6225–31.
17. Brignone C, Gutierrez M, Mefti F, Brain E, Jarcau R, Cvitkovic F, et al. First-line chemoimmunotherapy in metastatic breast carcinoma: combination of paclitaxel and IMP321 (LAG-3Ig) enhances immune responses and antitumor activity. *J Translat Med* 2010;8:71.
18. Romano E, Michielin O, Voelter V, Laurent J, Bichat H, Stravodimou A, et al. MART-1 peptide vaccination plus IMP321 (LAG-3Ig fusion protein) in patients receiving autologous PBMCs after lymphodepletion: results of a Phase I trial. *J Translat Med* 2014;12:97.
19. Pittet MJ, Zippelius A, Speiser DE, Assenmacher M, Guillaume P, Valmori D, et al. Ex vivo IFN-gamma secretion by circulating CD8 T lymphocytes: implications of a novel approach for T cell monitoring in infectious and malignant diseases. *J Immunol* 2001;166:7634–40.
20. Valmori D, Souleimanian NE, Tosello V, Bhardwaj N, Adams S, O'Neill D, et al. Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. *Proc Natl Acad Sci U S A* 2007;104:8947–52.
21. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P. Human T cell responses against melanoma. *Annu Rev Immunol* 2006;24:175–208.
22. Ayyoub M, Zippelius A, Pittet MJ, Rimoldi D, Valmori D, Cerottini JC, et al. Activation of human melanoma reactive CD8+ T cells by vaccination with an immunogenic peptide analog derived from Melan-A/melanoma antigen recognized by T cells-1. *Clin Cancer Res* 2003;9:669–77.
23. Goldinger SM, Dummer R, Baumgaertner P, Mihic-Probst D, Schwarz K, Hammann-Haenni A, et al. Nano-particle vaccination combined with TLR-7 and -9 ligands triggers memory and effector CD8(+) T-cell responses in melanoma patients. *Eur J Immunol* 2012;42:3049–61.
24. Lienard D, Rimoldi D, Marchand M, Dietrich PY, van Baren N, Geldhof C, et al. Ex vivo detectable activation of Melan-A-specific T cells correlating with inflammatory skin reactions in melanoma patients vaccinated with peptides in IFA. *Cancer Immun* 2004;4:4–23.

Grant Support

This work was supported by the Cancer Research Institute (USA), Ludwig Cancer Research (USA), the Cancer Vaccine Collaborative (USA), Atlantic Philanthropies (USA), the Wilhelm Sander-Foundation (Germany), a Swiss Cancer Research grant (3507-08-2014), a Swiss National Science Foundation grant (Sinergia CRSII3_141879), and a SwissTransMed grant (KIP 18).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 22, 2015; revised August 27, 2015; accepted September 1, 2015; published OnlineFirst October 23, 2015.

25. Lesimple T, Neidhard EM, Vignard V, Lefeuvre C, Adamski H, Labarriere N, et al. Immunologic and clinical effects of injecting mature peptide-loaded dendritic cells by intralymphatic and intranodal routes in metastatic melanoma patients. *Clin Cancer Res* 2006;12:7380–8.
26. Slingluff CLJ. The present and future of peptide vaccines for cancer: single or multiple, long or short, alone or in combination? *Cancer J* 2011;17:343–50.
27. Speiser DE, Romero P. Molecularly defined vaccines for cancer immunotherapy, and protective T cell immunity. *Semin Immunol* 2010;22:144–54.
28. Iancu EM, Gannon PO, Laurent J, Gupta B, Romero P, Michielin O, et al. Persistence of EBV antigen-specific CD8 T cell clonotypes during homeostatic immune reconstitution in cancer patients. *PLoS One* 2013;8:e78686.
29. Hadrup SR, Bakker AH, Shu CJ, Andersen RS, van Veluw J, Hombrink P, et al. Parallel detection of antigen-specific T-cell responses by multi-dimensional encoding of MHC multimers. *Nat Methods* 2009;6:520–6.
30. Newell EW, Klein LO, Yu W, Davis MM. Simultaneous detection of many T-cell specificities using combinatorial tetramer staining. *Nat Methods* 2009;6:497–9.
31. Bordry N, Costa-Nunes CM, Cagnon L, Gannon PO, Abed-Maillard S, Baumgaertner P, et al. Pulmonary sarcoid-like granulomatosis after multiple vaccinations of a long-term surviving patient with metastatic melanoma. *Cancer Immunol Res* 2014;2:1148–53.
32. Speiser DE, Lienard D, Rufer N, Rubio-Godoy V, Rimoldi D, Lejeune F, et al. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. *J Clin Invest* 2005;115:739–46.
33. Schultz ES, Lethé B, Cambiaso CL, VanSnick J, Chau P, Corthals J, et al. A MAGE-A3 peptide presented by HLA-DP4 is recognized on tumor cells by CD4+cytolytic T lymphocytes. *Cancer Res* 2000;60:6272–5.
34. Schultz ES, Schuler-Thurner B, Stroobant V, Jenne L, Berger TG, Thielemanns K, et al. Functional analysis of tumor-specific Th cell responses detected in melanoma patients after dendritic cell-based immunotherapy. *J Immunol* 2004;172:1304–10.
35. Hebeisen M, Oberle SG, Presotto D, Speiser DE, Zehn D, Rufer N. Molecular insights for optimizing T cell receptor specificity against cancer. *Front Immunol* 2013;4:154.
36. Stone JD, Chervin AS, Kranz DM. T-cell receptor binding affinities and kinetics: impact on T-cell activity and specificity. *Immunology* 2009;126:165–76.
37. Powell DJ, Jr., Rosenberg SA. Phenotypic and functional maturation of tumor antigen-reactive CD8+ T lymphocytes in patients undergoing multiple course peptide vaccination. *J Immunother* 2004;27:36–47.
38. Slingluff CL, Petroni GR, Smolkin ME, Chianese-Bullock KA, Smith K, Murphy C, et al. Immunogenicity for CD8+ and CD4+ T cells of 2 formulations of an incomplete Freund's adjuvant for multi-peptide melanoma vaccines. *J Immunother* 2010;33:630–8.
39. Shedlock DJ, Shen H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* 2003;300:337–9.
40. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 2003;421:852–6.
41. Rizzuto GA, Merghoub T, Hirschhorn-Cymerman D, Liu C, Lesokhin AM, Sahawneh D, et al. Self-antigen-specific CD8+ T cell precursor frequency determines the quality of the antitumor immune response. *J Exp Med* 2009;206:849–66.
42. Ayyoub M, Dojcinovic D, Pignon P, Raimbaud I, Schmidt J, Luescher I, et al. Monitoring of NY-ESO-1 specific CD4+ T cells using molecularly defined MHC class II/His-tag-peptide tetramers. *Proc Natl Acad Sci USA* 2010;107:7437–42.
43. Bankovich AJ, Girvin AT, Moesta AK, Garcia KC. Peptide register shifting within the MHC groove: theory becomes reality. *Mol Immunol* 2004;40:1033–9.
44. Cecconi V, Moro M, Del Mare S, Sidney J, Bachi A, Longhi R, et al. The CD4+T-cell epitope-binding register is a critical parameter when generating functional HLA-DR tetramers with promiscuous peptides. *Eur J Immunol* 2010;40:1603–16.
45. Landais E, Romagnoli PA, Corper AL, Shires J, Altman JD, Wilson IA, et al. New design of MHC class II tetramers to accommodate fundamental principles of antigen presentation. *J Immunol* 2009;183:7949–57.
46. Lienard D, Avril MF, Le Gal FA, Baumgaertner P, Vermeulen W, Blom A, et al. Vaccination of melanoma patients with Melan-A/Mart-1 peptide and Klebsiella outer membrane protein p40 as an adjuvant. *J Immunother* 2009;32:875–83.
47. Pulendran B, Oh JZ, Nakaya HI, Ravindran R, Kazmin DA. Immunity to viruses: learning from successful human vaccines. *Immunol Rev* 2013;255:243–55.
48. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Science* 2015;348:69–74.
49. Verdeil G, Fuertes Marraco SA, Murray T, Speiser DE. From T cell "exhaustion" to anti-cancer immunity. *Biochim Biophys Acta* 2015. doi: 10.1016/j.bbcan.2015.06.007.