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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

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Running title: LAG-3Ig as adjuvant for a cancer vaccine

Keywords: LAG-3Ig (IMP321), metastatic melanoma, peptide vaccination, CD4 and CD8 T-cells, immunotherapy

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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-cells responses in all 16 vaccinated melanoma patients, encouraging further development of this approach, e.g. in combination with checkpoint blockade.

Abstract

Purpose: Cancer vaccines aim to generate and maintain anti-tumor 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 9 injections were subcutaneously administered in 3 cycles, each with 3 vaccinations every 3 weeks, with 6-14 weeks interval between cycles. Blood samples were collected at baseline, one week after the 3^{rd}, 6^{th} and 9^{th} 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.

Data interpretation: 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.

Introduction

Melanoma is a highly aggressive cancer, with increasing incidence and mortality rates. While surgery can cure melanoma detected at early stages, the prognosis of most patients with metastatic disease is unfavorable. Chemotherapy, irradiation and therapy with IFN-α 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 (IL-2) 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 CTLA-4 (Cytotoxic T-Lymphocyte-Associated protein 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 & 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 pre-clinical studies (7, 8). One of these is LAG-3 (Lymphocyte Activation Gene-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 solid tumors or hematologic neoplasms, respectively (www.clinicaltrials.gov).

Therapeutic vaccination is a promising strategy against malignant diseases. Similar as for 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 (TLRs) 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). While 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., France) is in clinical development (12, 13). The rational 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 (APCs), which makes LAG-3Ig an attractive vaccine adjuvant. LAG-3 is a CD4-like protein able to bind to MHC (Major Histocompatibility Complex) 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-cells 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 non-vital tissues/cells such as melanoma. The melanocyte differentiation antigen MART-1 (Melanoma Antigen Recognized by T-cells-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 Mage-A3 (Melanoma-associated antigen 3) 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 HLA (Human Leukocyte Antigen)-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 (Seppic, France). Due to its non-resorbable 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 9 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 shown 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, non comparative 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., France). The first 6 vaccines were formulated with 1 mL Montanide ISA-51 (Seppic Inc, France), the last 3 vaccines without Montanide. Patients with an expected survival of at least 3 months (status at entry is described in Supplementary Table S1A) received 9 vaccines scheduled in 3 cycles with 3 vaccinations (3 weeks intervals between vaccines, 6 to 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-A_{26-35} native EAAGIGILTV or analog (A27L) ELAGIGILTV, NY-ESO-$1_{157-165}$ SLLMWITQC, Mage-$A3_{112-120}$ KVAELVHFL, NA17 VLPDVFIRC, all representing known class I HLA-A2 peptides; and Mage-$A3_{243-258}$ KKLLTQHFVQENYLEY, containing a class II HLA-DP4 epitope. Blood samples were collected before the first vaccine (C0), one 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 (PBMCs) were isolated by density gradient using Lymphoprep (Axis-Shieldy) and immediately cryopreserved in RPMI1640 supplemented with 40% FCS (Fetal Calf Serum) and 10% DMSO (dimethyl sulfoxide).

In Vitro Stimulation (IVS) of T-cells with specific peptides

$CD8^+$ T-cells or $CD4^+$ T-cells were enriched using MACS (Magnetic Activated Cell Sorting) 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 IL-2.

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, Lausanne, Switzerland) 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 3 vaccines; C1) was stimulated with Mage-$A3_{243-258}$ 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). 12 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-$A3_{243-258}$ 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 co-expression was done with SPICE software version 5.2. Finally, clones were clonotyped as previously described (28).

Statistics

Student's 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, sixteen HLA-A2^{+} patients with metastatic melanoma received serial vaccines (up to 9 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 8 women and 8 men, with an age ranging from 21 to 84 years. Ten patients had stage III melanoma and 6 patients with stage IV melanoma at study entry. 14 patients finished the entire clinical trial protocol and 2 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 5 peptides was safe and well tolerated, with mild severity local 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 4 dyes). We set up a panel detecting 13 specificities allowing the quantification 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 2 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 Meloe-1 (Melanoma-overexpressed antigen-1), Gp100-2 (Glycoprotein 100-2), Meloe-2, Mage-A10, Tyrosinase). In addition, to avoid competition of the well-growing specificities versus the less frequent (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 to 15 patients of 16; detection limit at 0.1%; Fig. 1A and Supplementary Table S3). Furthermore, 2/3 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 1 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 1B), 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 to 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 3 patients who showed high frequencies already at baseline, due to tumor priming or previous treatment (C0 > 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 4 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.

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

In addition to the short peptides, one longer peptide (a 15-mer) derived of the Mage-A3 protein was used for vaccination, with the aim to activate tumor-specific CD4 T-cells. For immunological monitoring, we stimulated PBMC in vitro with the peptide for 10 days, followed by challenging the cells with the Mage-$A3_{243-258}$ peptide for 4 hours, allowing the quantification of IFN-γ and TNF-α positive cells upon ICS (Fig. 4A and 4B). 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-$A3_{243-258}$ 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 6 vaccines, Montanide ISA-51 was omitted for the last 3 vaccines, with the aim to reduce local toxicities, and to determine whether this strategy may nevertheless achieve the desired booster effects. Despite slight trends towards 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 2 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 2 families of clones differed in their cytokine

363 profile (Fig. 5C), with tetramer-high clones producing more TNF-α, IL-2, and also the Th2
364 cytokine IL-13, but the same amounts or less of the Th1 cytokine IFN-γ than tetramer-low
365 clones (Fig. 5C). Tetramer-high clones were also more polyfunctional than tetramer-low clones
366 (Fig. 5D).

367 Sequencing of the CDR3β (Complementarity Determining Region 3β) region of the TCR
368 revealed that all the tetramer-low clones were of the same clonotype (VB6, CDR3β
369 SIGLAGGTDTQ, JB2.3), whereas all the tetramer-high clones came from a second clonotype
370 (VB7, CDR3β SRGTLPPMNTEA, JB1.1). These data suggest that each of the two clonal
371 populations originated from a single precursor.

372

373 *Clinical Results*

374 Although it was not in the focus of the study, we documented the clinical results. Twelve of the
375 16 patients entered the study without detectable tumor, due to previous surgical resection. Ten
376 of these 12 patients remained tumor-free throughout the entire study period, whereas 2 patients
377 developed new metastases, causing 1 patient to stop after the second cycle of vaccination. The
378 remaining 4 patients entered the study with detectable metastases and all had disease
379 progression. Of those, 1 patient stopped study participation after the first vaccination cycle. At
380 the end of the vaccination period, all patients were alive. Subsequently, two of the 16 patients
381 died after 8 and 14 months, respectively. The median follow up time was 47.2 months (with a
382 range from 35.9 months to 57.3 months) at the time of analyses (July 8th, 2015). After the study,
383 9 of the 16 patients received one or more additional anti-melanoma treatments, which were
384 surgery (7 patients), chemotherapy (3 patients), irradiation (3 patients), BRAF inhibitors (3
385 patients, 2 of which were the 2 deceased patients) and anti-CTLA-4 antibody followed by anti-

PD-1 antibody (1 patient). Obviously, post-study 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 non-resorbed 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 µg.

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-$A3_{243-258}$ 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-$A3_{243-258}$ 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 (47) (and manuscript in preparation). Thus, beside multiple antigens (possibly also including mutated antigens; (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 rational 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).

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Figure and Table legends

Fig. 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 panel (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 3 categories depending on frequency : > 10%, between 1% to 10%, and between 0.1% to 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 the panel A.

Fig. 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 1, 2, and 3 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.

Fig. 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 (in panel 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.

Fig. 4: Quantification of Mage-A3$_{243-258}$-specific CD4 T-cells

CD4 T-cells from vaccinated melanoma patients were purified and stimulated with Mage-A3$_{243-258}$ 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-A3$_{243-258}$ 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).

Fig. 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-$A3_{243-258}$ peptide (IVS), as described in Materials and Methods. Blood sample collected after 3 vaccinations showed 2 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 (co-expression of cytokines: TNF-α, IFN-γ, IL-2 and IL-13) of tetramer-high and tetramer-low clones in response to 5 µg/mL Mage-$A4_{243-258}$ peptide. Colors of the pie arcs depict the expression of individual cytokines, while the patterns of the pie depict the number of co-expressed cytokines from zero (white) to four cytokines (black)

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

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 (C0).

Supplementary Fig. S1: Gating strategy used to determine the T-cell frequencies with the combinatorial tetramer staining.

Supplementary Fig. S2: Specific CD8 T-cell frequencies before and after each cycle of vaccination

675 To complete the results depicted in Fig. 2, we show here the frequencies found for (A) virus and
676 (B) tumor-specific T-cells in PBMC collected before the first vaccine (C0), and one week after
677 the third vaccination of each cycle (C1, C2, C3). Graphs show the mean of the positive wells in
678 positive patients, corresponding to frequencies above the detection limit (> 0.1%). Lines link
679 samples from the same patient. Post-V corresponds to the mean of all positive wells analyzed
680 after vaccination, from C1 to FU. The numbers of patients and wells analyzed are listed in Fig.
681 1A.

682 *Supplementary Table S1: Patients' characteristics and adverse events list*

683 (A) Detailed information on melanoma patients. (B) List of adverse events encountered during
684 the study with an incidence of > 5 according to their CTCAE severity scale and their relationship
685 to the study treatment.

686 *Supplementary Table S2: Combinatorial tetramer staining for HLA-A2 restricted CD8 T-*
687 *cells*

688 List of tetramers used in combinatorial tetramer staining. All these tetramers were HLA-A2
689 tetramers, produced with 9-mers or 10-mers, and conjugated with a single or combination of
690 different indicated fluorochromes.

691 *Supplementary Table S3: Maximum frequency of the antigen-specific T-cells identified*
692 *in the IVS cultures*

693 The figure lists the detailed data that is summarized in Fig. 1A, showing all individual maximum
694 specific T-cell frequencies found for each patient in Fig. 2A, 2B and Supplementary Fig. S2,
695 upper panel (detected at a frequency of > 0.1%; -: not detected in the patient).

Supplementary Table S4: Direct ex vivo analysis of circulating Melan-A-specific CD8 T-cell frequencies

The data show the detailed results that were summarized for the generation of Fig. 3. The frequencies indicate the percentage of A2/Melan-A tetramer^{+} cells of total CD8^{+} T-cells.

Fig. 1 :

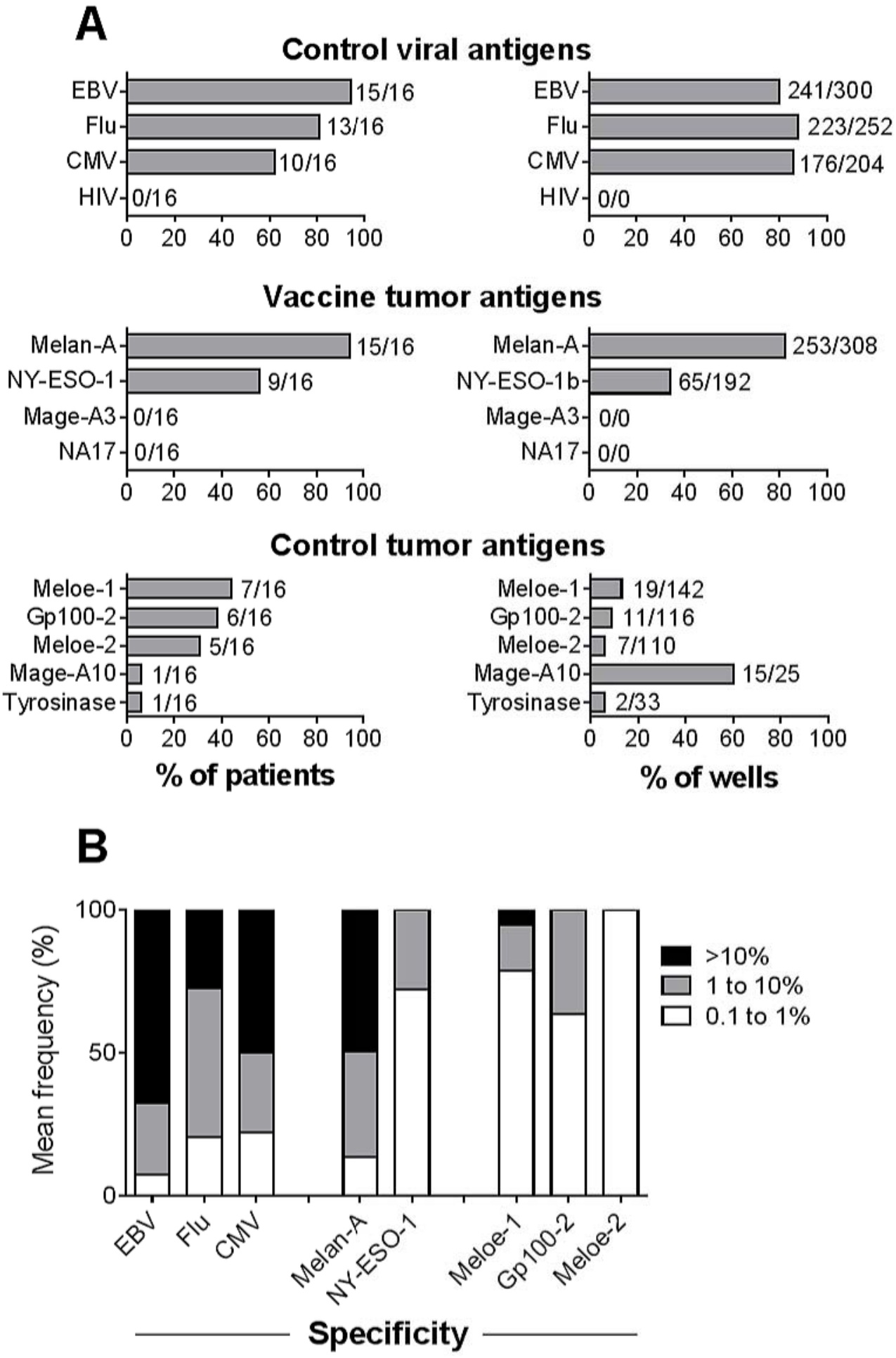

A
Control viral antigens
EBV
Flu
CMV
HIV
15/16
13/16
10/16
0/16
241/300
223/252
176/204
0/0
0 20 40 60 80 100
Vaccine tumor antigens
Melan-A
NY-ESO-1
Mage-A3
NA17
15/16
9/16
0/16
0/16
Melan-A
NY-ESO-1b
Mage-A3
NA17
253/308
65/192
0/0
0/0
Control tumor antigens
Meloe-1
Gp100-2
Meloe-2
Mage-A10
Tyrosinase
7/16
6/16
5/16
1/16
1/16
19/142
11/116
7/110
15/25
2/33
% of patients
% of wells
B
Mean frequency (%)
100
50
0
>10%
1 to 10%
0.1 to 1%
EBV
Flu
CMV
Melan-A
NY-ESO-1
Meloe-1
Gp100-2
Meloe-2
Specificity

Fig. 2 :

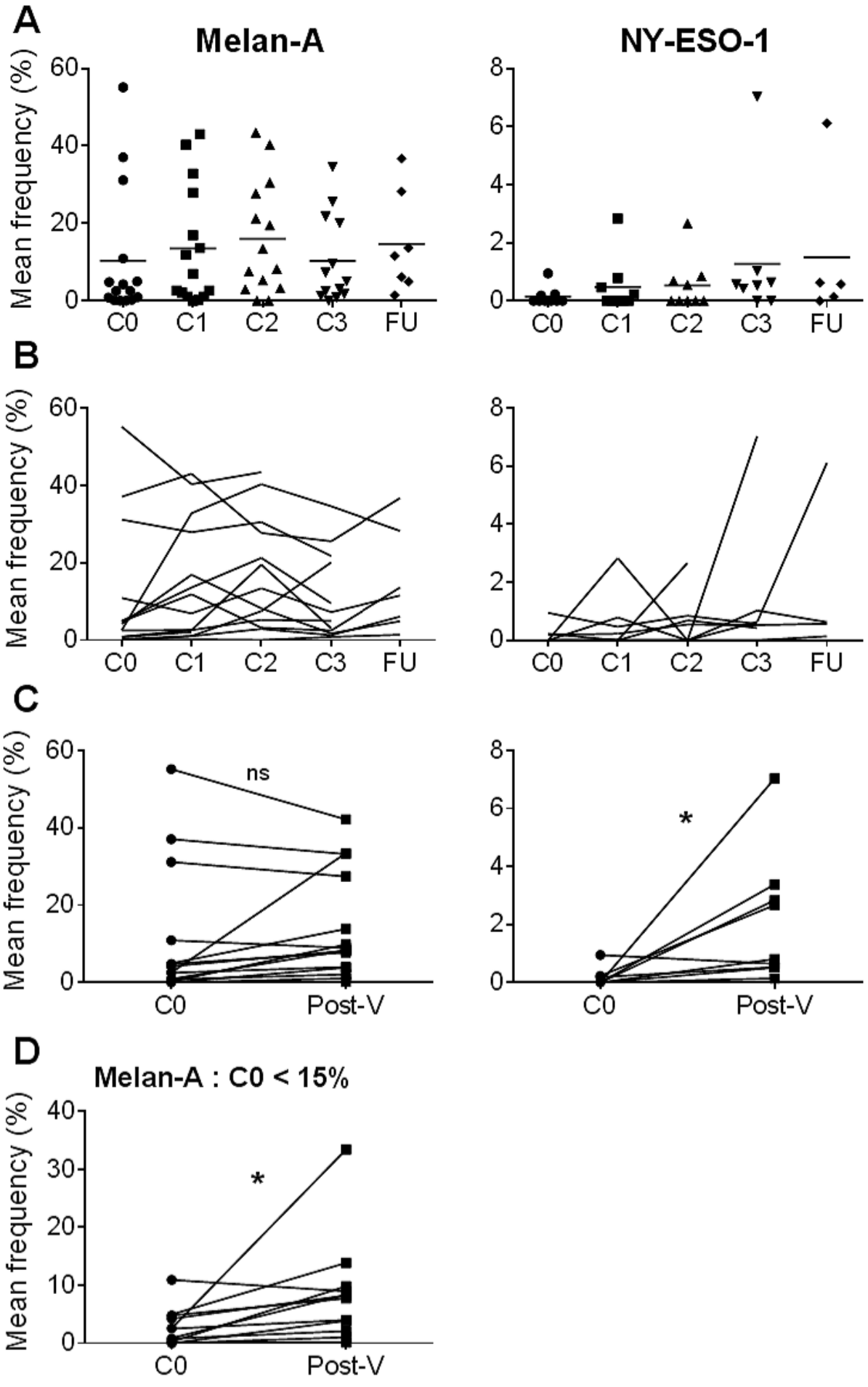

A
Melan-A
NY-ESO-1
Mean frequency (%)
C0
C1
C2
C3
FU
B
C
ns
*
Post-V
D
Melan-A : C0 < 15%

Fig. 3 :

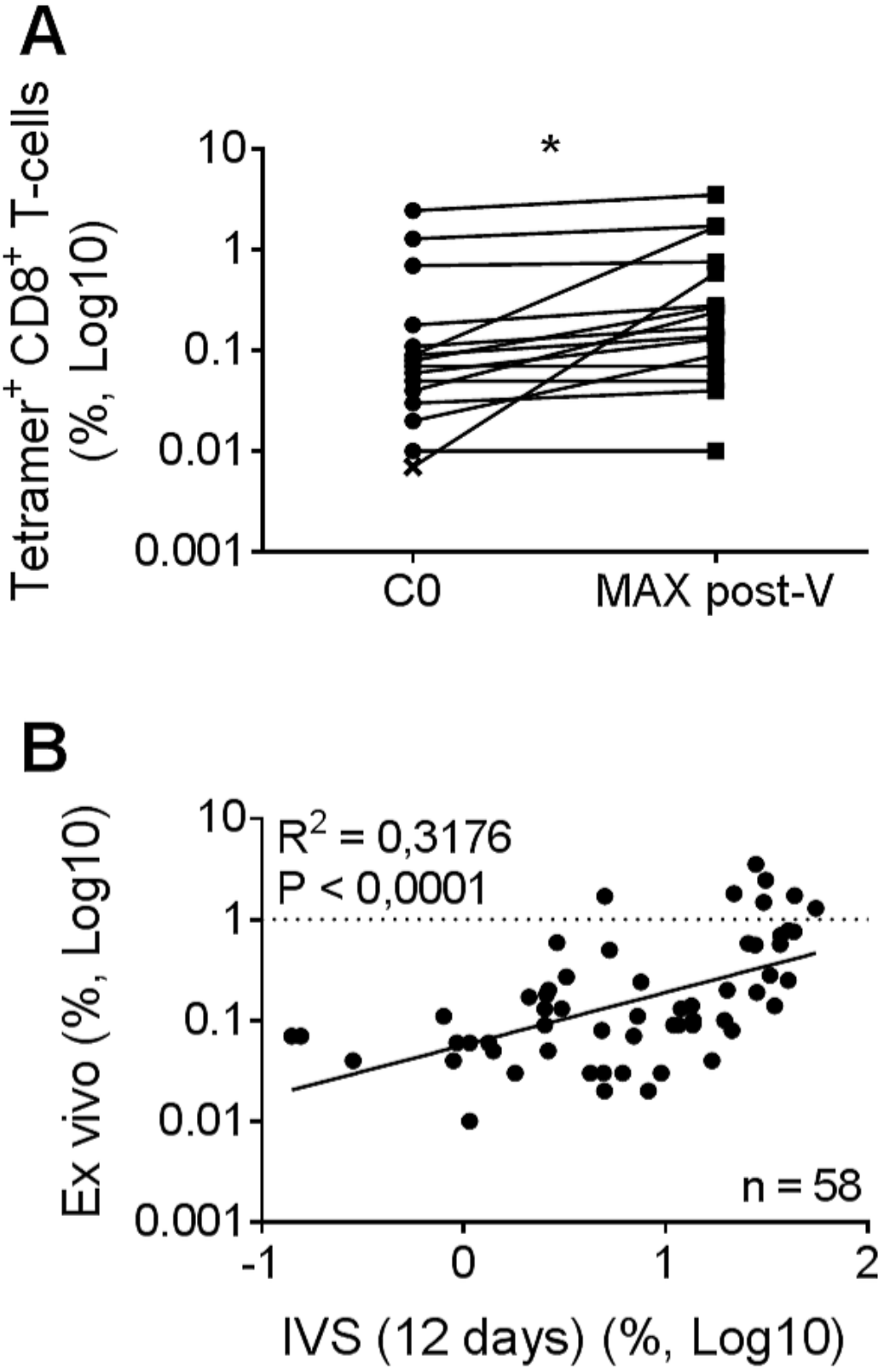

A
Tetramer+ CD8+ T-cells (%, Log10)
10
1
0.1
0.01
0.001
*
C0
MAX post-V
B
Ex vivo (%, Log10)
R2 = 0,3176
P < 0,0001
n = 58
-1
0
1
2
IVS (12 days) (%, Log10)

Fig. 4 :

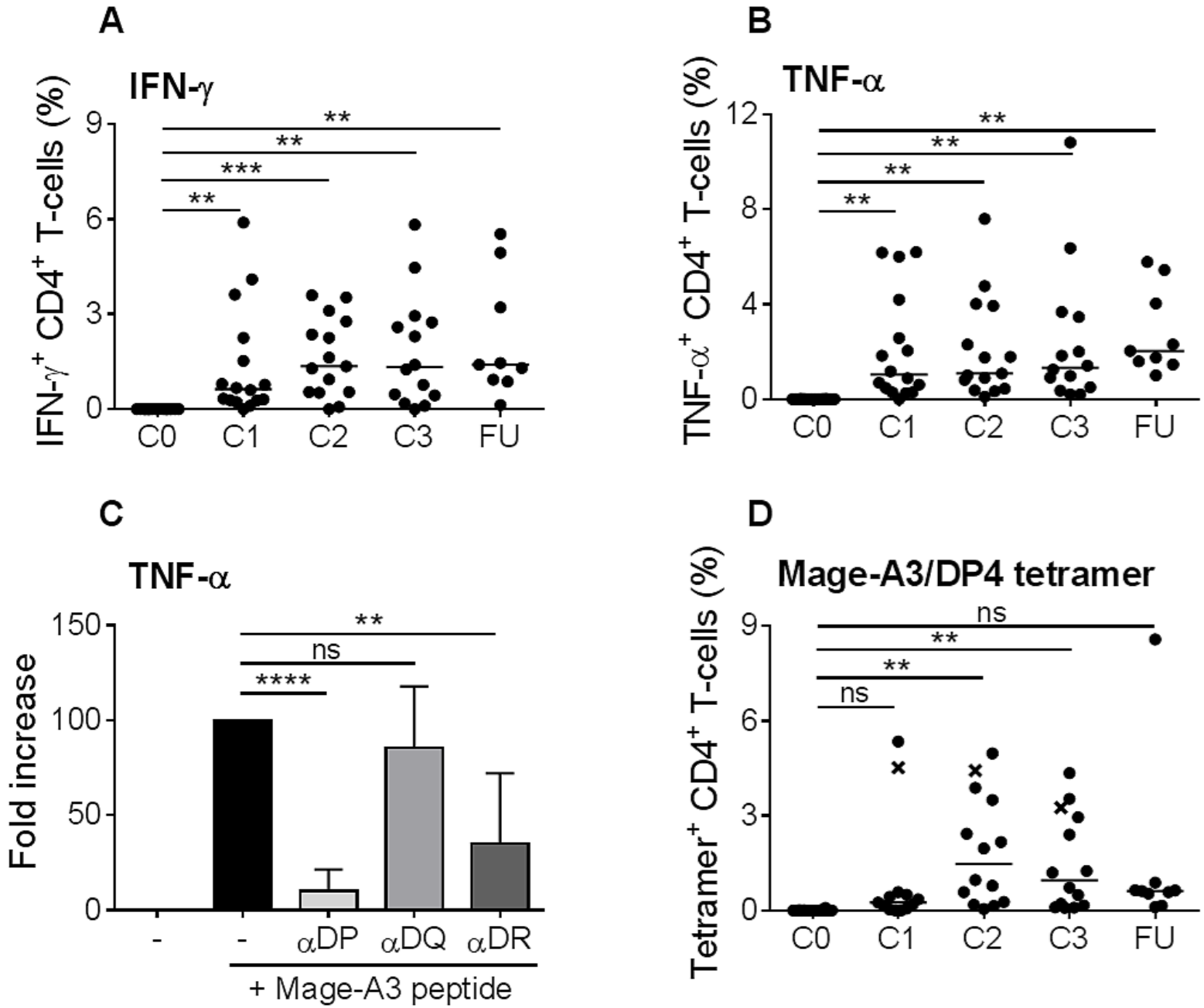

A
IFN-γ
IFN-γ+ CD4+ T-cells (%)
9
6
3
0
**
**
***
**
C0
C1
C2
C3
FU
B
TNF-α
TNF-α+ CD4+ T-cells (%)
12
8
4
0
**
**
**
**
C0
C1
C2
C3
FU
C
TNF-α
Fold increase
150
100
50
0
**
ns
****
-
-
αDP
αDQ
αDR
+ Mage-A3 peptide
D
Mage-A3/DP4 tetramer
Tetramer+ CD4+ T-cells (%)
9
6
3
0
ns
**
**
ns
C0
C1
C2
C3
FU

Fig. 5 :

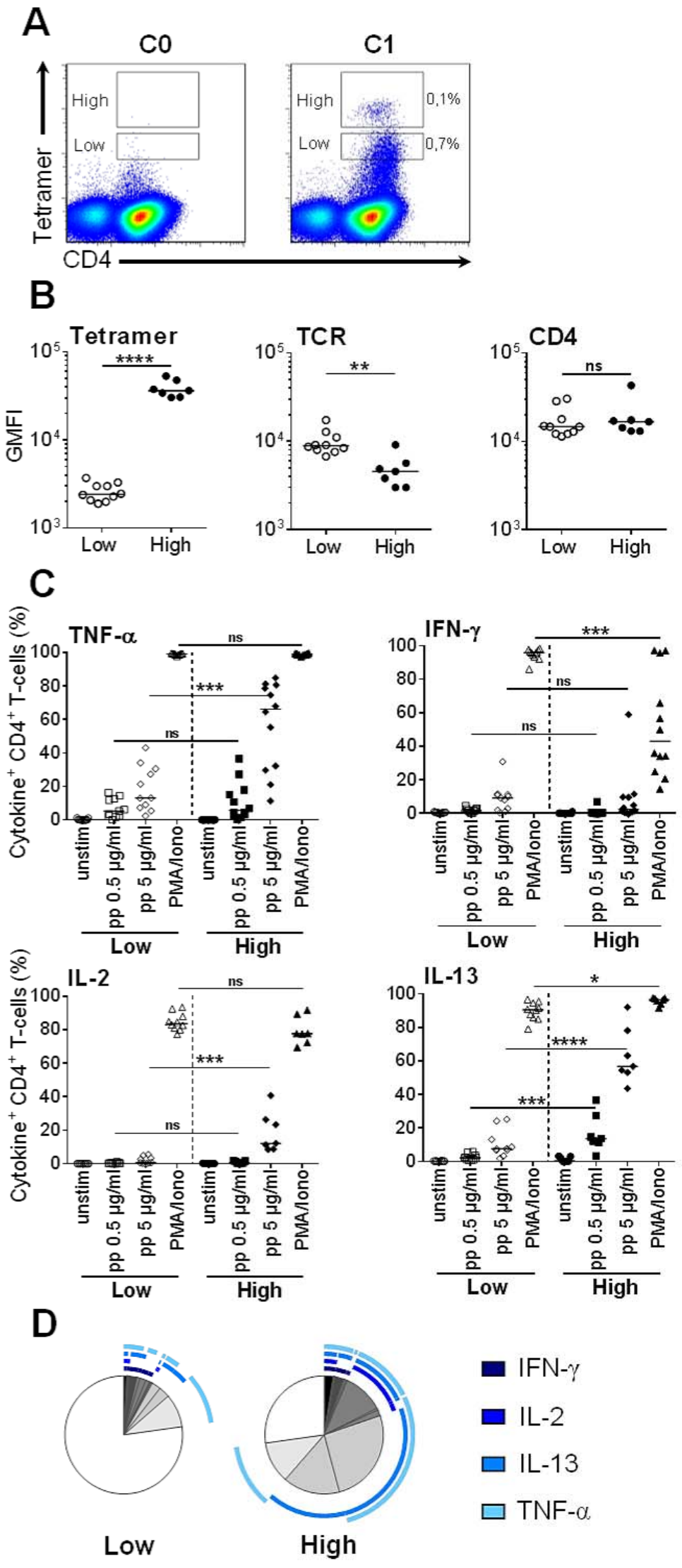

A
C0
C1
High
Low
0,1%
0,7%
Tetramer
CD4
B
Tetramer
TCR
CD4
GMFI
****
**
ns
Low
High
C
TNF-α
IFN-γ
IL-2
IL-13
Cytokine+ CD4+ T-cells (%)
unstim
pp 0.5 µg/ml
pp 5 µg/ml
PMA/Iono
Low
High
ns
***
****
*
D
IFN-γ
IL-2
IL-13
TNF-α
Low
High

Table 1 :

Summary of the immune responses induced by vaccination in melanoma patients

Study n°	Patient	Specific CD8 T-cell responses			Mage-A3 specific CD4 T-cell responses		
		Melan-A after IVS	NY-ESO-1 after IVS	Melan-A ex vivo	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	-	-	-	+	+	+

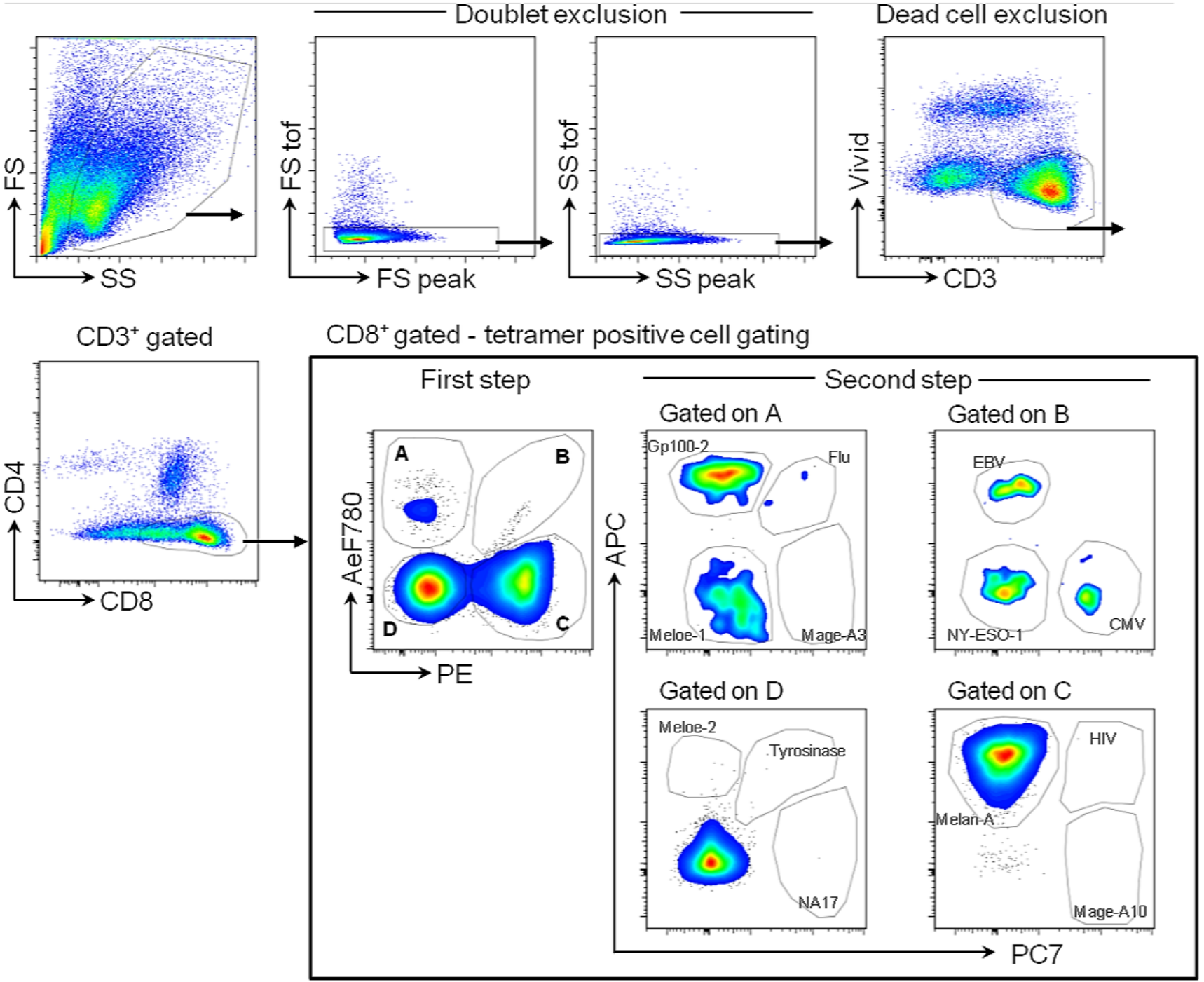
+ : increase of 2-fold or more in specific T-cell frequencies after vaccination compared to baseline (C0)

- : frequency of specific T-cells not changed by vaccination

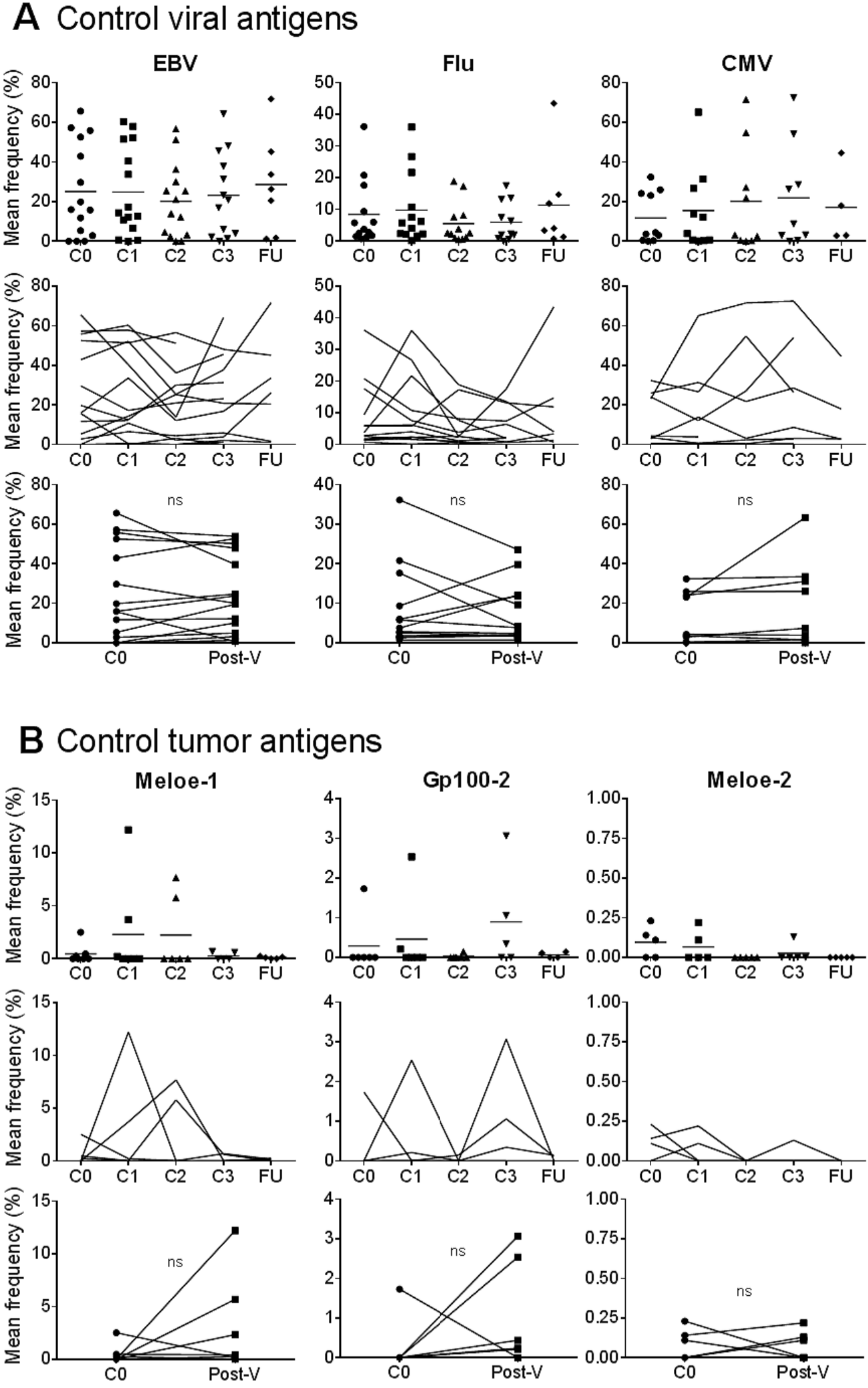
nd : specific T-cells not detected

Supplementary Fig. S1 :

Gating strategy of combinatorial tetramer staining



Supplementary Fig. S2 :



Supplementary Table S1 :

A Patients' characteristics

Study n°	Patient	Gender	Age	Disease at study entry			Previous treatment	Completed in vaccination		Status at study stop	HLA typing			
				TNM	Stage	Status		Cycles	Vaccines		HLA-A	HLA-DPB1	HLA-DQB1	HLA-DRB1
1	Lau 1268	M	84	pT3N3M0	IIIC	ED	surgery, chemotherapy, immunotherapy (a)	3	9	PD	02:01	04:01, 14:01	03:01/24, 06:01	11:04, 15:02
2	Lau 1366	M	59	pT1bN2M1c	IV	NED	surgery, chemotherapy	3	9	NED	02, 25	01:01, 04:01	02:01, 03:02	03:01/50, 04:01
3	Lau 465	M	58	pT3N2bM0	II	NED	surgery, immunotherapy (b)	3	9	NED	02:01/02:22, 68:08/69:01	01:01, 04:01	02, 03:01/09	03:01, 04:01
4	Lau 1187	M	65	pT2bN1aM0	IIIA	NED	surgery, immunotherapy (c)	3	9	NED	02:01	04:01, 14:01	03:01, 06:02	04:01, 15:01
5	Lau 1342	F	69	pTxN3M0	IIIC	ED	surgery	3	9	PD	02, 03	01:01, 04:02	02:02, 03:01	07:01, 11:01
6	Lau 1409	F	61	cTxN2bM0	IIIB	NED	surgery, radiotherapy	3	9	NED	02:01, 32	02:01, 04:01	02:01, 06	03, 15
7	Lau 1171	F	64	pT4aN3M1a	IV	NED	surgery, immunotherapy (a)	3	9	NED	02:01, 03	04:01, 04:02	03:01, 05:01	01:01, 11:01
8	Lau 1142	M	74	pT1aN3M0	IIIC	NED	surgery, radiotherapy, immunotherapy (a)	3	9	NED	02:01	03:01, 04:02	04:02, 05:01	01:01, 08:01/39
9	Lau 1314	M	81	pT4bN3M1a	IV	NED	surgery	3	9	NED	02, 03	03:01, 04:02	03:02, 06:02	04:05, 15:01
10	Lau 1456	F	47	pT3N2aM0	IIIB	ED	surgery	1	3	PD*	02:01, 26	01:01, 05:01	02:01, 06	03, 13
11	Lau 1438	F	21	pT2NxM1c	IV	NED	surgery	2	6	PD*	02:01, 66	02:01, 06:01	03	11, 13
12	Lau 1477	F	52	pT3aN1aM1a	IV	NED	surgery	3	9	NED	02:01	04:01	05, 06	01:01, 15:01
13	Lau 1499	M	63	pT4bN1M0	IIIB	NED	surgery	3	9	NED	02:01	04:01, 04:01/39:0	02:01, 04:02	03, 08:01
14	Lau 1486	F	67	pT3bN0M1b	IV	NED	surgery, radiotherapy	3	9	NED	02:01	03:01, 04:02	03, 06	04:01, 13
15	Lau 1523	F	48	pT4pN3M0	IIIC	ED	surgery	3	9	NED	02:05, 11	03:01, 04:01	02:02, 03	07:01
16	Lau 616	M	53	pT3N1bM0	IIIB	NED	surgery, radiotherapy, immunotherapy (d, e)	3	9	NED	02:05, 32(19)	nd	nd	nd

ED : evidence of disease

NED : no evidence of disease

PD : progressive disease

Previous immunotherapies were all done by cancer vaccines as follows:

a : CYT004-MelQbG10 04 : Virus-like particules MelQbG10 (long Melan-A 16-35 (A27L) peptide) + Montanide ISA-51 (23)

b : LUDWIG 96-010 : Melan-A 26-35 (A26L) peptide + FluMa 58-66 peptide + Montanide ISA-51 + low dose rhIL-2 (24)

c : LUDWIG 01-003 : Melan-A 26-35 (A27L) peptide + Montanide ISA-51 (manuscript in preparation)

d : LUDWIG 98/009 : Melan-A 26-35 (A26L) peptide + Mage-A10 254-262 peptide + SB AS-2 adjuvant (22)

e : LUDWIG 01/003 : Melan-A 26-35 (A27L) peptide + Mage-A10 254-262 peptide + NY-ESO-1b 157-165 (C165A) peptide +/- CpG-7909/PF-3512676 +/- Montanide ISA-51 (31)

* Progressive disease is the reason of vaccination protocol interruption

nd : not done

B Adverse events with an incidence of >5

Adverse events	N° events	CTCAE severity scale				Relationship to the study treatment					N° patients	% of patients
		1	2	3	4	1	2	3	4	5		
Injection site induration	100	99	1	0	0	0	0	1	0	99	15	94
Injection site erythema	83	82	1	0	0	0	0	1	0	82	15	94
Injection site pain	72	71	1	0	0	0	0	1	0	71	15	94
Injection site warmth	38	38	0	0	0	0	0	0	0	38	8	50
Headache	31	27	4	0	0	0	0	9	21	1	4	25
Injection site recall reaction	20	20	0	0	0	0	0	0	0	20	10	63
Myalgia	19	18	1	0	0	0	0	5	14	0	8	50
Chills	16	16	0	0	0	0	0	5	11	0	4	25
Asthenia	14	12	2	0	0	0	0	7	6	1	6	38
Arthralgia	13	12	1	0	0	0	0	3	10	0	5	31
Metastasis	12	0	1	11	0	12	0	0	0	0	4	25
Nausea	9	8	1	0	0	0	0	3	6	0	4	25
Cough	9	8	1	0	0	5	2	2	0	0	5	31
Injection site pruritus	8	8	0	0	0	0	0	1	1	6	3	19
Malaise	7	6	1	0	0	0	0	6	1	0	2	13
Dizziness	5	3	2	0	0	0	2	2	1	0	3	19

CTCAE (Common Terminology Criteria for Adverse Events) severity scale: 1=mild, 2=moderate, 3=severe and 4=life threatening

Relationship to the study treatment: 1=not related, 2=unlikely related, 3=possibly related, 4=probably related and 5=definitely related

Supplementary Table S2 :

Peptide and tetramer list

	Peptide	Location	Sequence	Fluorochromes used for HLA-A2 tetramer
Control viral antigens	EBV BMLF1	259-267	GLC TLV AML	PE + APC + AeF
	Flu MA	58-66	GIL GFV FTL	PC7 + APC + AeF
	CMV pp65	495-503	NLV PMV ATV	PE + PC7 + AeF
	HIV pol	476-484	ILK EPV HGV	PE + PC7 + APC
Vaccine tumor antigens	Melan-A	26-35	ELA GIG ILT V	PE + APC
	NY-ESO-1	157-165	SLL MWI TQA	PE + AeF
	Mage-A3	112-120	KVA ELV HFL	PC7 + AeF
	NA17	1-10	VLP DVF IRC V	PC7
Control tumor antigens	Meloe-1	36-44	TLN DEC WPA	AeF
	Gp100-2	209-217	IMD QVP FSV	APC + AeF
	Meloe-2	27–35	RLP PKP PLA	APC
	Mage-A10	254-262	GLY DGM EHL	PE + PC7
	Tyrosinase	369-377	YMD GTM SQV	PC7 + APC

PE : r-phycoerythrin
APC : allophycocyanin
PC7 : PE-Cy7
AeF : APC-eFluor® 780

Supplementary Table S3 :

Maximum frequency of the antigen-specific T-cells identified in the in vitro stimulated cultures

Study n°	Patient	EBV	Flu	CMV	HIV	Melan-A	NY-ESO-1	Mage-A3	NA17	Meloe-1	Gp100-2	Meloe-2	Mage-A10	Tyrosinase
1	Lau 1268	15.8	2.4	3.6	-	5.3	-	-	-	-	-	-	-	-
2	Lau 1366	56.6	1.3	31.4	-	43.0	0.6	-	-	0.7	1.7	0.2	-	1.0
3	Lau 465	33.7	17.3	-	-	13.7	-	-	-	7.7	0.3	0.1	-	-
4	Lau 1187	29.7	-	0.5	-	0.2	-	-	-	-	-	-	-	-
5	Lau 1342	2.1	20.8	3.2	-	1.4	6.1	-	-	-	-	-	-	-
6	Lau 1409	25.3	43.5	-	-	13.4	1.0	-	-	2.5	1.1	0.2	-	-
7	Lau 1171	26.3	3.4	72.4	-	40.3	2.8	-	-	5.8	3.1	0.1	-	-
8	Lau 1142	6.6	36.0	13.8	-	16.9	-	-	-	-	-	-	-	-
9	Lau 1314	71.8	17.6	-	-	6.1	0.1	-	-	-	-	0.1	-	-
10	Lau 1456	12.2	2.1	4.4	-	2.1	-	-	-	0.2	-	-	-	-
11	Lau 1438	57.9	6.2	-	-	55.2	2.7	-	-	12.2	0.2	-	-	-
12	Lau 1477	60.4	2.6	54.8	-	21.3	0.7	-	-	-	-	-	-	-
13	Lau 1499	65.7	21.7	0.2	-	19.5	-	-	-	-	2.5	-	-	-
14	Lau 1486	31.4	4.1	54.1	-	20.1	7.0	-	-	-	-	-	-	-
15	Lau 1523	-	-	-	-	-	-	-	-	-	-	-	-	-
16	Lau 616	3.2	-	-	-	31.2	1.0	-	-	0.2	-	-	0.4	-

- : specific T-cells not detected with a detection limit > 0.1%

Supplementary Table S4 :

Ex vivo Melan-A-specific CD8 T-cell frequencies

Study n°	Patient	C0	C1	C2	C3	FU
1	Lau 1268	0.09	**0.20**	**0.50**	**1.71**	-
2	Lau 1366	0.70	0.76	0.56	0.58	0.57
3	Lau 465	0.08	0.13	**0.27**	0.13	0.10
4	Lau 1187	0.07	0.07	0.03	0.03	-
5	Lau 1342	0.05	0.03	0.02	0.04	0.05
6	Lau 1409	0.09	0.07	0.14	0.11	0.09
7	Lau 1171	0.18	0.28	0.25	0.14	0.19
8	Lau 1142	0.03	0.04	0.02	0.03	0.03
9	Lau 1314	<0.01	0.01	**0.59**	**0.06**	**0.03**
10	Lau 1456	0.11	0.17	-	-	-
11	Lau 1438	1.29	0.78	1.73	-	-
12	Lau 1477	0.02	**0.09**	**0.08**	0.03	-
13	Lau 1499	0.06	0.05	0.10	**0.13**	-
14	Lau 1486	0.04	0.06	**0.24**	**0.20**	0.05
15	Lau 1523	0.01	0.01	0.01	0.01	<0.01
16	Lau 616	2.47	3.53	1.48	1.81	-

- : no sample

Supplementary text :

Supplementary Materials and Methods

Vaccination

The clinical-grade peptides used in the vaccines were: Melan-A_{26-35} native EAAGIGILTV or analog (A27L) ELAGIGILTV, NY-ESO-$1_{157-165}$ SLLMWITQC, Mage-$A3_{112-120}$ KVAELVHFL, NA17 VLPDVFIRC, all representing known class I HLA-A2 peptides; and Mage-$A3_{243-258}$ KKLLTQHFVQENYLEY, containing a class II HLA-DP4 epitope. In the first cycle, patients received 10µg Mage-A3/A2, NA17 and NY-ESO-1 peptides, and 100µg Melan-A native peptide. In the second and the third cycles, the peptide dose was increased to 100µg each, and the Melan-A analog peptide was used in place of the native. Mage-A3/DP4 peptide was injected at the dose of 200µg during all cycles. Vaccines of the third cycle were prepared without Montanide. Blood samples were collected before the first vaccine (C0), one 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.

In Vitro Stimulation (IVS) of T-cells with specific peptides

Cryopreserved PBMCs were thawed, and CD8+ T-cells or CD4+ T-cells were enriched using MACS technology (Miltenyi Biotec). The negative fraction of cells were loaded with laboratory-grade peptides (listed in Supplementary Table S2A), irradiated (30 Gray) and used as antigen-presenting cells to stimulate peptide-specific T-cells. 100.000 cells per well of each fraction were plated in 96 well U-bottom plates, or 1 million per well of 24 well plates. Cultured cells were maintained 10 or 12 days at 37°C in RPMI1640 medium supplemented with 8% human serum. CD4+ T-cells were stimulated in presence of 20 U/ml IL-2 from day 0; CD8+ T-cells were supplemented with IL-2 (100 U/ml) starting after one day of culture.

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, Lausanne, Switzerland) 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 S2A. One or three washing steps (for single or combinatorial staining, respectively) were performed in FACS buffer (PBS supplemented with 5mM EDTA, 0.2% azide and 0.2% BSA). Surface staining was performed to identify CD4 and CD8 T-cells at 4°C for 20 minutes using CD4-FITC (clone 13B8.2), CD8-PerCP-Cy5.5 (clone SK1), CD3-APC (clone UCHT1), CD4-APC-H7 (clone SK3) and/or CD3-Alexa Fluor 700 (clone HIT3a). Finally, LIVE/DEAD-Fixable-Aqua (Invitrogen) or DAPI was used as a dead cell exclusion marker. 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 3 vaccines; C1) was stimulated with Mage-$A3_{243-258}$ peptide for 16 days, as described above in IVS section. Cells were harvested and stained with Mage-A3/DP4 tetramer (TCMetrix, Lausanne, Switzerland) and CD4-APC-H7 antibody as described in tetramer staining section. CD4+ tetramer-low and CD4+ tetramer-high cells were separately sorted with a BD FACS Aria cell sorter, and cultured in Terasaki plates at 0.5 cell per well with 10.000 feeder cells (mix of irradiated PBMC from 2 healthy donors) per well, 150 U/mL IL-2, 1 µg/mL PHA. After at least 1 week in culture, growing wells were transferred in 96 well U-bottom plates for expansion. The specificity of each clone was checked by tetramer staining analyzed by flow cytometry (BD FACS Array). Clones were restimulated, when necessary, with feeder cells, IL-2 and PHA. 12 days after stimulation, clones were stained with tetramer Mage-A3/DP4-PE, or CD4-PE (clone 13B8.2), or TCR pan alpha/beta-PE (clone IP26A) antibodies to phenotype the cells. DAPI was used to exclude dead cells. In parallel, clones were stimulated with 0,5 and 5 µg/mL Mage-$A3_{243-258}$ peptide in presence of Brefeldin A 10µg/mL for 6 hours. Intracellular Cytokine Staining (ICS) was done as described below.

ICS, assessed by flow cytometry

The quantification of cytokine amounts specifically produced by T-cells in response to a peptide was assessed by flow cytometry using intracellular staining. T-cells were stimulated for 4 to 6 hours with or without 1 or several peptides at 1 µM each, in RPMI1640 medium supplemented with 10% FCS, plus 10µg/ml Brefeldin A, at 37°C. PMA/Ionomycin stimulation was used as positive control. After stimulation, surface and dead cell staining were performed as described in previous section, using the following antibodies: CD3-PE (clone UCHT1), CD8-PerCP-Cy5.5 (clone SK1), and CD4-Pacific Blue (clone 13B8.2). Stained cells were then fixed with fixation buffer (PBS, 1% formaldehyde, 2% glucose, 5mM azide), permeabilised with 0.1% saponin in FACS buffer, and incubated with anti-cytokine antibodies (IL-2-FITC, clone MQ1-17H12 ; IFN-γ-PE-Cy7, clone 4S.B3 ; IL-13-APC, clone JES10-5A2 ; and TNF-α-Alexa Fluor 700, clone MAb11), for 20 minutes at room temperature, in the dark. Analysis of cytokine co-expression was done with SPICE software version 5.2.