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Vaccination of stage III/IV melanoma patients with long NY-ESO-1 peptide and CpG-B elicits robust CD8 and CD4 T-cell responses with multiple specificities including a novel DR7-restricted epitope

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ABSTRACT

Long synthetic peptides and CpG-containing oligodeoxynucleotides are promising components for cancer vaccines. In this phase I trial, 19 patients received a mean of 8 (range 1-12) monthly vaccines s.c. composed of the long synthetic NY-ESO$_{79-108}$ peptide and CpG-B (PF-3512676), emulsified in Montanide ISA-51. In 18/18 evaluable patients, vaccination induced antigen-specific CD8 and CD4 T-cell and antibody responses, starting early after initiation of immunotherapy and lasting at least one year. The T-cells responded antigen-specifically, with strong secretion of IFNγ and TNFα, irrespective of patients' HLAs. The most immunogenic regions of the vaccine peptide were NY-ESO-1$_{89-102}$ for CD8 and NY-ESO-1$_{83-99}$ for CD4 T-cells. We discovered a novel and highly immunogenic epitope (HLA-DR7/NY-ESO-1$_{87-99}$); 7/7 HLA-DR7^{+} patients generated strong CD4 T-cell responses, as detected directly *ex vivo* with fluorescent multimers. Thus, vaccination with the long synthetic NY-ESO-1$_{79-108}$ peptide combined with the strong immune adjuvant CpG-B induced integrated, robust and functional CD8 and CD4 T-cell responses in melanoma patients, supporting the further development of this immunotherapeutic approach.

INTRODUCTION

Over the past years, several vaccines consisting of exactly fitting MHC class-I binding peptides have been evaluated for therapeutic efficacy in different cancer types[1]. Although induction of specific CD8 T-cell responses was observed[2-4], absence of concomitant CD4 T-cell activation may have been a reason that clinical benefit remained minimal. In agreement with this notion, inclusion of MHC class II peptides in the vaccine formulation showed superior CD8 T-cell responses in both preclinical models and clinical trials[5]. More recently, the use of long synthetic peptides (LSPs) harboring both CTL and T helper epitopes has demonstrated induction of strong immune responses[6, 7]. The additional advantages of LSPs are that they need professional APCs for efficient MHC I epitope presentation, and that their use is not limited to patients with defined HLA molecules[8]. In various Phase I/II clinical trials using LSPs, we and others showed potent CD8 and CD4 T-cell responses in patients with different solid cancers, as well as in pre-malignant lesions as vulvar intraepithelial neoplasia[9-14].

The cancer germ line antigen New York esophageal squamous cell carcinoma-1 (NY-ESO-1) was discovered in 1997[15], and the NY-ESO-1 protein is aberrantly overexpressed in malignant transformed cells of different histological types[15]. During the past years, it has emerged as a potential target for cancer immunotherapy since it is highly immunogenic and includes both humoral and T-cell epitopes[16]. Interestingly, among all currently known NY-ESO-1 T-cell epitopes, approximately half of them are present within the region 80-111[17-20], making it an attractive protein stretch to be used for patient's immunization using LSPs.

Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG (CpG–ODNs) are TLR-9 agonists. Class B CpG-ODNs directly induce activation and maturation of

plasmacytoid dendritic cells and promote B cells differentiation[21]. Various results in mice demonstrated the improvement of therapeutic responses of DC-based vaccines, short and long peptide immunizations and protein vaccines with this adjuvant[22]. Similarly, short peptide-based clinical trials that included CpG-B in melanoma patients showed the generation of a stronger and more rapid Melan-A-specific CD8 T-cell response compared to the vaccine alone[3]. In another vaccination trial with recombinant NY-ESO-1 protein supplemented with CpG-B and Montanide, results showed a significant augmentation of tumor-specific antibodies as well as the detection of NY-ESO-1-specific CD8 T-cells[23].

To date, no clinical trial evaluated vaccination with CpG-B in combination with LSPs. Some recent studies assessed the safety and *in vivo* immunogenicity of synthetic overlapping long NY-ESO-1 peptides in combination with diverse adjuvants. In an initial study, 91% of patients in the cohort receiving the vaccine supplemented with the TLR-3 agonist Poly-ICLC showed T-cell responses, as compared to the modest specific T-cell induction in the absence of Poly-ICLC. The cellular response correlated in these patients with an acceleration of seroconversion and a significant increase in specific antibody titers[14]. Similar results were obtained by Tsuji et al., who characterized NY-ESO-1–specific vaccine-induced CD4 T-cell lines to investigate the effect of both Montanide and Poly-ICLC adjuvants[24]. While Montanide promoted a Th2 polarization and an expansion of high avidity vaccine induced CD4 T-cells through a better protein recognition, the addition of Poly-ICLC abrogated IL-4, IL-13, and IL-10 secretion, resulting in a more prominent Th1 polarization. As comparison, only half of the patients had CD8 T-cell responses when vaccinated with full NY-ESO-1 protein supplemented with Montanide and CpG-B (CpG-7909/ PF-3512676)[23].

In this study we evaluated safety and immunogenicity of the combination of the 30 amino

109 acid LSP NY-ESO-$1_{79\text{-}108}$, administered in combination with CpG-B (CpG-7909/ PF-
110 3512676) and Montanide ISA-51 subcutaneously, accompanied or not by low dose
111 interleukin-2, in patients with advanced malignant melanoma.

RESULTS

Patients' characteristics

In this clinical trial, 19 patients with resected cutaneous melanoma of stage III or IV were enrolled in 2 groups, as summarized in Table I and supplemental Tables I-II [1.1; number labeling based on the MIATA checklist is highlighted in green throughout the manuscript]. Ten patients were in group A (without IL-2) and 9 patients in group B (with IL-2). Six (60%) and 7 (78%) patients from groups A and B, respectively, discontinued the study treatment prior to completion of the 3 vaccination cycles (Table I and Supplemental Tables I-II), mainly due to disease progression. Patient LAU 1408 received only 1 vaccine and was thus not evaluable for immune response.

Expression of Melan-A, and NY-ESO-1/LAGE-1, and MAGE-A/MAGE-A10 was assessed for each patient. Table I shows TAA expression as assessed either by immunohistochemical analysis or qPCR depending on material availability [1.1]. For IHC detection of MAGE-A expression, we used clone 6C1 specific for MAGE-A1/A2/A3/A4/A6/A10 and A12[25]. Unfortunately, there is no MAGE-A10 mono-specific antibody available.

Safety and tolerability

The vaccine was generally well tolerated. Amongst all patients enrolled in groups A and B there were a total of 2 events of Grade 3 (10%) for those who were definitely, probably and possibly related to the study treatment, while no Grade 4 (life-threatening) or Grade 5 (death) adverse events were observed during the study (Supplemental Table III).

The most commonly reported adverse events were general disorders and conditions of mild intensity mainly represented by injection site reactions (rash/erythema, skin induration, pain and warmth) and systemic reactions (chills, myalgia, arthralgia, asthenia and headache). There were no severe adverse events related to the study drugs.

As expected, the low dose IL-2 treatment (group B) induced frequent side effects, with inflammatory reactions at s.c. injection sites, and systemic effects (chills, fever, asthenia, headache, arthralgia, myalgia, nausea, diarrhea and insomnia). Many patients required IL-2 dose reductions and/or stopped IL-2 treatment prematurely. Instead of the 45 intended injections for each patient on average, patients received 33 injections on average, resulting in an average of 73.8% of the intended cumulated dose (Supplemental Table IV).

Monitoring of NY-ESO-1-specific CD8 T-cell responses

A total of 18 patients were analyzed for immune responses by intracellular cytokine staining, separately for CD8 and CD4 T-cells. As shown in representative flow cytometry examples in Figures 2A and 3A, significant responses were observed both in CD8 and CD4 T-cells. Baseline frequencies of NY-ESO-1-specific CD8 T-cells were undetectable or very low in the majority of the patients (Figure 2B-C, Supplemental Figure 2A-B). However, 6 patients showed significant NY-ESO-1-specific natural responses, reaching frequencies of IFNγ and TNFα positive cells of 1.23 % of total CD8 T-cells before vaccination (Figure 2B-D and Figure 4). All patients mounted significant responses upon vaccination, as shown by the patient's individual longitudinal curves (Figure 2B, summary in Figures 2C-D and 4). Frequencies of cytokine^{+} CD8 T-cells readily increased after 2-4 immunizations in the

majority of the patients. Total cytokine$^+$ cells reached high levels, accounting for one third of total CD8 T-cells (total cytokine$^+$ CD8 T-cells ranging from 0.05 to 33.1%). Some patients displayed a more delayed kinetics and showed initial responses only during cycle 2. Specific CD8 T-cell responses were sustained, as assessed by cytokine measurement during the third cycle of immunization (Figure 2D, Supplemental Figure 2C). No significant differences in CD8 T-cell responses were observed when Group A and Group B patients were compared.

Monitoring of NY-ESO-1-specific CD4 T-cell responses and specific antibody responses

Similar to CD8 T-cell responses (Figure 2), 4 out of 18 patients already showed baseline detectable frequencies of IVS specific CD4 T-cells (ranging from 0.1 - 5.77%). Nevertheless, in all patients NY-ESO-1-specific CD4 T-cells were highly significantly increased by immunization (Figure 3B-D, Supplemental Figure 3A-B). Responses were observed as early as after 2 vaccinations, and reached 70% of specific cells after IVS. They were long-lasting, since high frequencies of cytokine$^+$ cells were detectable one year after the initiation of the trial (Figure 3D, Supplemental Figure 3C). In general, CD4 T-cell expansions occurred earlier than the ones of CD8 T-cells, suggesting that CD4 T-cell help might be necessary for specific CD8 T-cell induction (Figure 4). Responses with higher magnitude were detected at earlier time points in Group B, however long-term no difference was observed between the two groups. Importantly, not only Type I, but also Type II cytokines were detectable, arguing for the capacity of the vaccine to also impact on T-cell polarization. Type II cytokines might also have contributed to the generation of specific antibodies. We measured a significant increase in NY-ESO-1-specific antibodies in the majority of the patients, with also a discrete increase of antibodies specific for other tumor antigens, in particular Melan-A, arguing for induction of antigen spreading (Figure 4 and Supplemental Figure 4).

Determination of immunodominant regions for CD8 and CD4 T-cells, and identification of a novel HLA-DR7 specific epitope

Results using individual overlapping peptides showed that the a.a. regions 90-102 and 87-99 were the most immunogenic sequences for CD8 and CD4 T-cells, respectively (Figure 5A). However, the analysis of fine specificity of recognition using the set of overlapping nonapeptides revealed individual patterns (Supplemental Figure 5A), and suggests that each patient focuses CD8 T-cell responses on distinct portions of the LSP. EC50 of peptide recognition for specific CD8 T-cells ranged from micro- to nanomolar concentrations (Supplemental Figure 5B). Using HLA-B35 multimers loaded with NY-ESO-$1_{94\text{-}104}$, we identified specific CD8 T-cells in HLA-B35 patients not only after IVS, but also directly *ex vivo* (Figure 5B). For CD4 T-cells, the contribution of individual MHC class II was evaluated using blocking antibodies and HLA class II typing (Supplemental Table I). In 8/9 patients that could be included in these analyses, we observed a partial or complete abrogation of NY-ESO-1-specific CD4 T-cell responses in the presence of pan-HLA-DR blocking antibodies (Figure 5C). In *in vitro* peptide competition assays, we identified the peptide NY-ESO-$1_{87\text{-}99}$ as a strong binder to HLA-DR7 (data not shown). We generated DR7/NY-ESO-$1_{87\text{-}99}$ multimers and stained IVS cultures from the 7 HLA-$DR7^+$ patients included in our study. We identified specific cells in 7/7 HLA-$DR7^+$ patients. As shown in a representative example in Figure 5D and as summarized in Table II multimer$^+$ cells accounted for a large proportion of the overall response induced by vaccination. Interestingly, in all 7 HLA-$DR7^+$ patients, multimer$^+$ cells could be detected in samples collected before immunization. Their frequency significantly increased during time and was maintained until completion of the trial. Notably, in one patient that was previously recruited in another vaccination trial consisting of MAGE-

A1 immunizations, high baseline DR7/NY-ESO-$1_{87\text{-}99}$ multimer^{+} cells were observed (e.g. 19.6%). This data suggest that natural CD4 T-cell responses to the novel NY-ESO-1 epitope might have been induced in this patient by antigen spreading upon vaccination with MAGE-A1 peptide.

Polyfunctionality and cytolytic activity of HLA-DR7/NY-ESO-$1_{87\text{-}99}$-specific CD4 T-cell clones

We generated HLA-DR7/NY-ESO-$1_{87\text{-}99}$-specific CD4 T-cell clones and lines from 4 HLA-DR7^{+} patients (Figure 6A, upper panel). By functional characterization we defined the peptide 87-99 as the minimal epitope inducing maximal responses in 15/19 of the clones (data not shown). Clones responded by secreting both Th1 and Th2-prototypic cytokines, albeit with a different EC50 (Figure 6A, lower panel). Then, we assessed whether DR7/NY-ESO-$1_{87\text{-}99}$-specific cells are able to recognize the newly identified NY-ESO-1 epitope when presented by tumor cells. We used HLA-DR7$^{+/-}$ melanoma cell lines, pre-treated or not with IFNγ, and co-cultured them with specific CD4 T-cell clones. We observed that specific clones secreted significant amounts of cytokines in response to NY-ESO-1/DR7 peptide presented by tumor cells (Figure 6B, left panel). We also assessed whether NY-ESO-$1_{87\text{-}99}$ CD4 T-cell clones could directly kill target cells. When co-cultured with 3 different HLA-DR7^{+} melanoma cell lines, displaying either endogenous or IFNγ-induced MHC II expression, the clones induced significant tumor cell lysis of the HLA-DR7^{+} tumor cells, pulsed with the specific peptide (Figure 6B, right panel). As expected, HLA-DR7^{-} tumor cells were not susceptible to killing.

Direct *ex vivo* visualization of HLA-DR7/NY-ESO-$1_{87\text{-}99}$-specific CD4 T-cells

Finally, we performed multicolor flow cytometry analyses directly *ex vivo* (without prior *in vitro* T-cell expansion) from HLA-DR7^{+} patients. Remarkably, in 7/7 patients we were able to detect multimer^{+} cells without prior *in vitro* stimulation (representative examples in Figure 6C, summary in Figure 6D). Their frequencies varied between 0.01 and 0.18% of total CD4 T-cells, and their phenotype corresponded to antigen-experienced, memory cells (data not shown).

Follow-up and clinical observations

The median follow up time was 63.8 months for group A (ranging from 8.5 to 80.5 months) and 55.9 months for group B (ranging from 2.2 to 68.4 months) at the time of analysis (December 8th, 2015). Overall, the median follow up time was 56.8 months (with a range from 2.2 to 80.5 months). At the last follow-up, twelve patients were alive (5 of group A and 7 of group B), whereas 7 patients died (5 of group A and 2 of group B) due to progressive disease. Eight patients (4 patients of each of the two groups) remained without evidence of disease (Table I). Patients with above median levels of IFNγ^{+} NY-ESO-1-specific CD4 T-cells showed tendencies for longer overall and progression-free survival than patients with below median levels, but the differences were not statistically significant (Figure 7). These clinical results are relatively favorable, but not conclusive as expected for a phase I study.

DISCUSSION

Montanide in combination with TLR3 agonists and LSPs has been shown to elicit both humoral and cellular responses in cancer patients[14, 24]. Here, we assessed for the first time the combination of Montanide, CpG-B and LSP in advanced melanoma patients. We report on the induction of strong and long-lasting polyspecific CD8 and CD4 T-cell responses, with T-cells able to recognize and kill tumor cells, and the generation of specific antibodies. By in-depth characterization of the induced CD4 T-cell responses, we identified a novel, immunodominant HLA-DR7 restricted NY-ESO-1 epitope, that triggers CD4 T-cells detectable directly *ex vivo* in all evaluable patients. Hence, combination of LSP and CpG-B represents an attractive immunotherapy strategy in cancer patients, beyond virus-driven tumors.

DNA containing CG repeats, mimicked by CpG-ODN, ligate TLR9 and induce production of TNFα (CpG-B) or IFNα (CpG-A). Thus, CpG-ODN are considered the most advanced danger signals for the development of adjuvants for immunotherapy. CpG-A ODNs spontaneously assembly in nanoparticles[26] and have been shown to induce tumor specific CD8 T-cell responses in VLP based vaccines[27]. Yet, current evidence suggests that CpG-B might be superior, as previously reported by us and others in adjuvanted short peptide- and protein-based trials[2, 3, 23, 28-30].

Here, we show that the combination of CpG-B and a 30-amino acid long peptide is safe and well tolerated by the majority of the patients. Short peptide-based vaccines have the disadvantage to be limited for use in selected cohorts of HLA-compatible patients, and harbor the potential hazard to directly bind to MHC molecules and induce tolerance[31]. In contrast, full protein-based vaccines depend on processing for epitope generation, lead to a broad

spectrum of T-cell epitopes and induce antibody responses; however, they are expensive, might lead to the generation of poorly immunogenic epitopes, and are often suboptimal in inducing CD8 T-cell responses[32]. Therefore, selection of strong and immunogenic LSP has proven interesting. Hence, patients with HPV-driven pre-malignant and malignant tumors treated with E6/E7 LSP show immunological[10, 11, 33], preclinical and clinical benefits[12, 34], and combination of a TLR3 agonist with overlapping LSP from NY-ESO-1 also resulted in significant T-cell induction in ovarian cancer patients[14, 24]. Here, we observe early onset, highly significant, sustained NY-ESO-1-specific CD4 T-cell responses, followed by very high frequencies of specific CD8 T-cells, in all patients. Importantly, kinetics of CD8 T-cell responses are delayed compared to CD4 T-cells, suggesting that helper CD4 T-cells[35] and CpG induced DC-activation might play a critical role in activating and sustaining the effector phase of CD8 T-cells. Moreover, by deconvoluting the specific T-cell responses using individual overlapping peptides, we show that CD8 T-cells recognize different regions of the LSP, while CD4 T-cell responses are confined to a common stretch of the LSP (aa 83-97). These data suggest that in contrast to short peptide and protein vaccination, the use of a LSP allows generation of multiple MHC class I epitopes[36], likely favored by the presence of CpG. Immunodominant regions for CD8 T-cells in the LSP sequence are heterogeneous and probably linked to the MHC class I of the patients. HLA-B35^{+} patients showed directly *ex vivo* detectable CD8 T-cell responses to a known NY-ESO-1-B35 epitope, while HLA-B35^{-} patients mounted responses to other regions of the LSP, including putative novel MHC class I epitopes. In contrast, different MHC class II molecules might efficiently bind peptides processed from the region 83-97 and promiscuously present the same peptide, as previously shown by us and others for other epitopes from tumor-associated antigens[17, 37]. In that regard, numerous publications have reported on the immunogenicity of NY-ESO-1, and in particular of the protein region 87-111[17, 18, 20, 38]. Mandic et al. previously reported on the

306 characterization of a CD4 T-cell clone recognizing the epitope 87-101 presented by HLA-
307 DR7 transfected cells[17,] By fine mapping of specific CD4 T-cell responses in our cohort of
308 HLA-DR7^{+} patients, we identified NY-ESO-1 87-99 as a minimal, immunogenic epitope
309 presented by HLA-DR7. Importantly, the presence of directly *ex vivo* detectable DR7-
310 restricted NY-ESO-1-specific CD4 T-cells in patients even before immunization convincingly
311 show the endogenous generation of this epitope. Given the abundance of this HLA in the
312 Caucasian population (25%) further evaluation of directly *ex vivo* and *in vitro* expanded
313 DR7/NY-ESO-1-specific CD4 T-cells is warranted. By comparing frequencies of total
314 cytokine^{+} CD4 T-cells to those of multimer^{+} CD4 T-cells after IVS, our initial quantification
315 points towards a dominant contribution of the DR7-restricted response to the total specific T-
316 cell response. Furthermore, phenotypic and functional characterization of DR7/NY-ESO-1 T-
317 cells indicates a predominant Th1 polarization. Yet, by stimulating specific clones with high
318 peptide doses we observed secretion of Type-2 cytokines, arguing for plasticity and
319 polyfunctionality of these cells. In that regard, it was previously reported both in mice and
320 primates that TLR9 agonists induce potent antitumor effects, through induction of adaptive
321 Th1 cellular responses. Inversely, Montanide seems to favor Th2 differentiation of vaccine-
322 induced TAA-specific CD4 T-cells[39], suggesting that a careful evaluation of adjuvant and
323 peptide doses are needed to optimize vaccinations based on LSP with CpG-B/Montanide
324 combination, as compared to other adjuvants. In addition, caution will be needed when LSP
325 and potent molecularly-defined immune adjuvants are used, in order to avoid life threatening
326 immune responses and vaccine toxicity, as reported in a murine study using HY-LSP
327 combined with CpG[40]. Nevertheless, an advantage in the utilization of TLR9 agonists, but not
328 TLR4, TLR5 or TLR7-agonists[41], is the ability of CpG to safely tilt the immunologic balance
329 towards effector rather than regulatory T-cells [29, 42], thus favoring the overcoming of immune
330 tolerance. The direct *ex vivo* phenotypic characterization of DR7/NY-ESO-1 CD4 T-cells

showed very low levels of regulatory T-cells upon immunization (data not shown), in line with our previous results[29].

Finally, beside Type 1 cytokine secretion upon co-culture with HLA-matched tumor cells, DR7/NY-ESO-1 CD4 T-cells were also able to directly kill targets. Importantly, beside implications of killer specific CD4 T-cells against viruses, recent reports on killer CD4 T-cells in solid tumors have emerged[43]. It was recently reported on the potent rejection of melanoma in lymphopenic mice after transfer of small numbers of naive CD4 T-cells in combination with CTLA-4 blockade[44]. Similarly, co-culture of antigen-specific CD4 T-cells obtained from patients treated with anti-CTLA-4 antibodies specifically recognized and killed tumors[45]. In parallel, an increased killing capacity of specific CD4 T-cells was triggered via OX40/OX40L in combination with chemotherapy[46]. Additional work is needed to define the exact contribution of these cells to tumor eradication and the potential involvement of CpG-B in their generation. Moreover, it will be of interest to determine antigen recognition of endogenously processed antigens by tumor cells as compared to professional APCs. In this context, it has recently been shown that in addition to endosomal/lysosomal proteases that are typically involved in MHC class II antigen processing, other pathways usually used for MHC class I presentation, could also be involved in the presentation of intracellular NY-ESO-1 on MHC class II by ovarian tumor cells[47]. Thus, these observations suggest that inclusion of multiple LSP from different TAA in optimized vaccine formulations might exploit these novel pathways, favor the induction of epitope spreading and promote the generation of robust and combined CD8 and CD4 T-cell, and humoral responses.

We did not observe significant differences in the results from patients without vs. with IL-2 treatment, even though a trend for stronger CD4 T-cell responses was observed in the patients receiving IL-2, at the early immunomonitoring time points. This contradicts previous studies

showing that frequencies of tumor-antigen specific T-cells are reduced in the blood of patients treated with low dose IL-2, likely due to T-cell emigration into peripheral tissues[48, 49]. However, the IL-2 doses delivered in our study were very low, and therefore perhaps without significant consequences for T-cell functions. Despite the very low doses, patients experienced many adverse events typical for IL-2 treatment. The maximally tolerated dose was as low as 1 Mio UI/m^2/day, thus significantly lower than what is conventionally regarded as low dose IL-2 therapy. We suspect that the concomitant treatment with CpG-B may have contributed to this relatively high toxicity, because previous peptide/Montanide vaccination studies without CpG showed lower toxicity despite higher IL-2 doses[50]. However, a direct comparison is required to determine a potential role of CpG-B in IL-2 toxicity.

Finally, we observed a trend for longer overall and progression-free survival in patients with above median levels of IFNγ^+ NY-ESO-1-specific CD4 T-cells. However, beside the 8 patients that remained without evidence of disease throughout the study, the others experienced progressive disease. The discrepancy between strong immunological and only modest clinical responses might be due to the fact our trial was performed in advanced melanoma patients (stage III/IV). In addition, we monitored T-cell responses in the circulation, but not at tumor site. We and others previously showed that local tumor-derived factors might block efficient immune responses in tumors[2]. Future studies on tumor-infiltrating lymphocytes in patients receiving LSP combined with CpG will provide additional information on T-cell fitness directly at tumor site.

In conclusion, the high immunogenicity power of NY-ESO-$1_{79\text{-}108}$ LSP combined with CpG-B, the relatively low synthesis costs and the relative ease of production defines this vaccine formulation as a great candidate to be explored for cancer immunotherapy.

MATERIALS AND METHODS

Study Design, Patients and Treatment

This is a phase I vaccination study of stage III and IV (American Joint Committee on Cancer-AJCC) malignant melanoma patients [1.1]. The vaccines were composed of clinical-grade antigenic peptides, CpG-B 7909/PF-3512676 (Pfizer Inc) and Montanide ISA-51 (Seppic SA) and were administered subcutaneously (s.c.). Antigenic peptides were the 30-amino acid long NY-ESO-$1_{79\text{-}108}$ peptide (for all patients), and the short HLA-A2 restricted peptides Melan-$A_{26\text{-}35}$ (native EAAGIGILTV), Melan-$A_{26\text{-}35(A27L)}$ (analog ELAGIGILTV) and MAGE-$A10_{254\text{-}262}$ (GLYDGMEHL) (only for HLA-A2 positive patients). Vaccinations were administered in cycles of 4 monthly vaccines with intervals of 2 months between the cycles (Figure 1).

The primary objectives of the study were safety and specific cellular immune responses to NY-ESO-1, Melan-A and MAGE-A10. The secondary endpoints were tumor responses and disease status. Nineteen patients were enrolled in this study and first assigned to group A (without IL-2), followed by group B with supplementary daily low dose IL-2 (Novartis) s.c. for 10 days after each vaccination, starting the day of the second vaccination up to the end of cycle 3. IL-2 was administered following a dose escalation scheme (3 doses injected: 0.5, 1 or 2 Mio UI/m^2/day). For inclusion, tumors had to express either NY-ESO-1 (LAGE-2) or LAGE-1, and Melan-A in HLA-A2 positive patients (HLA haplotype analysis and immunohistochemistry/PCR for TAA expression was performed on tumor biopsies, after given written informed consent [1.1]).

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Administered vaccines were composed of 0.5 mg NY-ESO-1_{79-108} peptide, 1 mg CpG-B 7909/PF-3512676 and 0.5 ml Montanide (syringe 1). HLA-$A2^{+}$ patients received a second injection (syringe 2), with the short Melan-A_{26-35} and MAGE-$A10_{254-262}$ peptides, 1 mg CpG-B and 0.5 ml Montanide. The three vaccines of the cycle 3 were formulated without Montanide. The short peptides were given in a "prime-boost" approach, as follows: for the first cycle 0.1 mg Melan-A_{26-35} natural peptide and 0.02 mg MAGE-$A10_{254-262}$ peptide, and for the following cycles 0.1 mg Melan-$A_{26-35(A27L)}$ analog peptide and 0.1 mg MAGE-$A10_{254-262}$ peptide. A total of 19 immunocompetent patients (5 female and 14 male) with median age of 59 years old were vaccinated [1.1].

Disease status was assessed every 3 months for patients with measurable disease and every 6 months for patients with no measurable disease. The study (NCT00112242) sponsored by the Ludwig Center for Cancer Research was approved by the Lausanne University Hospital Ethics Committee and written informed consent was obtained from patients prior to enrolment. Safety was evaluated according to the National Cancer Institute CTC Scale (Version 2.0; April 30, 1999).

This study was performed under GLP conditions [5.1], following SOP developed in the laboratory [5.4] and using investigative assays [5.5].

Blood collection and PBMCs isolation

Heparinized blood samples were withdrawn by venipuncture [1.2, 1.3, 1.4] at baseline, after 2 and 4 vaccinations during the first cycle and at the end of each of the following cycles. Peripheral blood mononuclear cells were isolated by Lymphoprep centrifugation gradient [1.6] from blood kept at room temperature [1.5] no longer than 4 hours following blood drawn [1.7]. Isolated PBMCs were immediately stored in cryovials at 10 x 10^6 cells in 1ml of

cold 50% RPMI (Gibco), 40% FCS (PAA laboratories) and 10% DMSO freezing medium (Sigma Aldrich) [1.9, 1.12] at -80°C, and further into liquid nitrogen until use [1.10, 1.11]. Before freezing cells were counted using trypan blue [1.20] and viability was >95% [1.15, 1.16].

Cryopreserved cells were thawed at 37°C, washed once and resuspended at the desired concentration in RPMI supplemented with 10% FCS, 1.15% nonessential amino acids (Sigma), 1% penicillin□streptomycin (Sigma), 1% Hepes buffer (Gibco, Life Tchnologies) [2.3]. Cells were resuspended in RPMI (Gibco), 8% Human Serum (pooled human sera from healthy donors' blood from the local blood bank), 1% nonessential amino acids, 1% penicillin-streptomycin, 1% L. glutamine (Gibco, Life Technologies), 1% sodium pyruvate (Gibco, Life Technologies), and 0.1% 2-mercaptoethanol (Sigma) [2.1] and counted using trypan blue [1.20] with viability >80% [1.15, 1.17].

Cell media supplemented with serum were negative from previous tests for extracellular contamination sources [2.2].

Peptide/MHC Multimers

Fluorescent mulitmers were: HLA-B35/NY-ESO-1_{94-104}, HLA-DR*0701/NY-ESO-1_{87-99}. All multimers were provided by TCMetrix [2.4].

***In vitro* peptide stimulation (IVS) of CD8 and CD4 T-cells**

Patients' CD8 T-cells were purified by positive selection using MACS isolation microbeads (Miltenyi), followed by CD4 T-cell positive selection starting from the CD8 negative fraction. Positive T-cells were stimulated *in vitro* (IVS) and CD4/CD8 depleted PBMCs were irradiated (30 Gy) and used as feeders for stimulation of the cultures [2.4]. T-cells and autologous APCs were mixed at 1:1 ratio and co-cultured for 14 days with three 18-mers (NY-ESO-1_{79-96}, NY-ESO-1_{85-102}, NY-ESO-1_{91-108}) spanning the entire vaccine NY-ESO-1_{79-108} sequence, with 12 amino acids overlaps, at 2 μM, in RPMI 1640 medium supplemented

with 8% heat inactivated, pooled human serum. In parallel, a CD4 T-cell blast culture was set up using 1 μg/ml PHA. At day 2, 100 U/ml IL-2 was added and cultured until day 14 [2.1, 2.4].

NY-ESO-$1_{79\text{-}108}$-specific T-cell responses were evaluated after IVS in a 6-hour re-challenge experiment using overlapping peptide pools (2 μM final concentration) in the presence of Brefeldin A (10 μg/ml): for evaluation of CD4 T-cell responses 10 13-mer peptides overlapping by 11 amino acids were used, while for CD8 T-cell responses 22 9-mer peptides were pulsed on autologous PHA CD4 T-cell blasts for 1 hour, before addition to the CD8 T-cells [2.4]. As negative control, cells from the same cultures were left unchallenged and as positive control, 2 wells for CD8 and CD4 T-cells were stimulated with PMA/Ionomycin in the presence of Brefeldin A (Figure 2) [2.5].

NY-ESO-1 restimulated T-cells were evaluated for IFNγ, TNFα, and IL-2 production and analyzed by flow cytometry. Additionally, CD4 T-cells were concomitantly analyzed for IL-5 and IL-13 production. Cells were first stained for CD3-APC AF750 (BD Biosciences), CD4-PB (BD Pharmingen) and CD8-ECD (Beckman Coulter) and Live/Dead Aqua (Invitrogen), followed by a fixation step. Cells were washed with buffer (PBS, 0.2% BSA, 0.2% azide, 5 μM EDTA) and permeabilized with 0.1% saponin for staining for IL-2-FITC (BD Pharmingen), IFNγ-PECy7 (BD Pharmingen), TNFα-AF700 (BD Pharmingen) for read out of CD8, while CD4 T-cells were additionally stained for IL-5 and IL-13-APC (BD Biosciences) [2.4]. Samples were aquired on a Gallios flow cytometer (Beckman Coulter) and data were analyzed using FlowJo software (TreeStar) [3.1, 3.2]. The PMT voltages were adjusted for each fluorescence channel using unstained PBMCs and compensations were set using PBMCs stained with single antibodies according to a local SOP [3.2]. The analysis was performed on living, singlets, $CD3^+CD4^+$ or $CD3^+CD8^+$ lymphocytes [3.3]. A representative example of the

full gating strategy is shown on Supplementary Figure 6 [3.4]. Dot plots can be provided per request [4.3]. Stainings were considered as positive for each measured cytokine if the stimulated responses were at least three times higher than the unstimulated control [4.4] as defined during the study design [4.6].

Generation of HLA-DR7-restricted NY-ESO-1$_{87-99}$-specific CD4 T-cell clones and lines

Polyclonal cultures from HLA-DR7 patients containing NY-ESO-1$_{87-99}$-specific CD4 T-cells were sorted using NY-ESO-1$_{87-99}$/HLA-DR7 multimers by fluorescence activated cell sorting following the staining panel: anti-CD4 and anti-CD3 antibodies, DAPI and PE-conjugated NY-ESO-1$_{87-99}$/HLA-DR7 multimer [4.6]. Clones were obtained by limiting dilution (0.5 cell/well) in Terasaki plates and cultured in RPMI medium with 8% HS and 100 U/ml IL-2, 10000 irradiated allogenic feeder cells per well and 1 μg/ml PHA [2.4]. Unstained cultures from the same patients were used as controls [2.5].

***Direct ex vivo* enumeration of HLA-DR7-restricted NY-ESO-1$_{87-99}$-specific CD4 T-cells**

PBMCs from HLA-DR7^{+} patients were stained directly *ex vivo* using a combination of PE-conjugated NY-ESO-1$_{87-99}$/HLA-DR7 multimer, followed by staining using PerCP-Cy5.5-conjugated anti-CD3 (Biolegend), AF700-conjugated anti-CD45RA (Biolegend), APC-H7 conjugated anti-CD4 (BD Bioscience) and Vivid Aqua (Invitrogen).

Killing Assay

The specific lytic activity of the NY-ESO-1_{87-99} CD4 T-cell lines was assessed against HLA-DR7^{+} (T331A; GEF I; GEF II) or HLA-DR7^{-} (T1415A) melanoma cell lines, pre-treated or not with hrIFNγ (50 U/ml, Peprotech) for 48 hours [2.4]. Cells were labeled with ^{51}Chromium (Amersham Biosciences), loaded or not with peptides, and washed [2.4]. Labeled target cells were incubated with effectors at the indicated ratio for 4 hours at 37°C [2.4]. The supernatants were harvested and radioactivity was counted in an automatic gamma-counter [3.1]. The percentage of specific lysis was determined using the formula: (experimental-spontaneous release)/(maximum-spontaneous) x 100. Internal controls were included in each assay to measure the spontaneous release (target cells alone) and the total release (target cells with 1 M HCl) [2.5].

IFNγ ELISA

NY-ESO-1_{87-99} CD4 T-cells were co-cultured at 1:1 ratio with HLA-DR7^{+} (T331A; GEF I; GEF II) or HLA-DR7^{-} (T1415A) melanoma cell lines, pre-treated or not with hrIFNγ (50 U/ml, Peptrotech) for 48 hours, pulsed or not with peptides [2.4]. Supernatants from stimulated conditions and unstimulated controls [2.5] were harvested after 24 hours and IFNγ ELISA was performed using the Human BD OptEIA ELISA set (BD Biosciences) [2.4, 3.1].

IFNγ ELISPOT

The Elispot was performed using the ELISpot^{PRO} kit for Human IFN-γ from MABTECH (3420-2APT-10), following the standard supplier instructions. CD8 T-cells after IVS were thawed and rested in presence of 100 U/ml IL-2 for two days before use. The cell number per well was adjusted to have maximum 3% of cytokine producing cells per well to avoid

saturation of the membrane (values taken from the previous immune monitoring). For the analysis the values had been normalized to equal percentages of spot forming units (SFU) [2.4].

Serology

Recombinant NY-ESO-1 protein and control dihydrofolate reductase (DHFR) proteins were used to coat plates and measure specific serum antibody levels in ELISA as previously described[14] [2.4, 2.5]. A reciprocal titer was estimated from optical density readings of serially diluted plasma samples. Negative control sera from healthy individual and positive control sera for each antigen from patients with cancer were always included [2.5]. The anti-human immunoglobulin antibodies used as secondary reagents were: alkaline phosphatase (AP)-labeled goat-anti-human IgG (polyclonal antisera; Southern Biotech), biotinylated mouse-anti-human IgG1 (Clone JDC-1; BD Pharmingen), AP-labeled mouse anti-human IgG2 (clone HP6002; Southern Biotech), AP-labeled mouse anti-human IgG3 (clone HP6050; Southern Biotech), AP-labeled mouse anti-human IgG4 (clone HP6023; Southern Biotech), AP-labeled goat-anti-human IgA (polyclonal antisera; Southern Biotech), AP-labeled mouse-anti-human IgD-AP (clone IADB6; Southern Biotech), AP-labeled goat-anti-human IgE (Clone HP6029; Southern Biotech) [2.4], and AP-labeled goat-anti-human IgM (polyclonal antisera; Southern Biotech). To be considered significant, reciprocal titers had to be more than 100 [4.4].

Tumor responses

In patients with measurable disease, tumor responses were classified as follows: complete

response as disappearance of all the tumor signs for at least 4 weeks, partial response as decrease of at least 50% of all tumor lesions for at least 4 weeks, minor response as decrease of all the lesions by at least 25% for the same minimum period of time, stable disease as no more than 25% changes in size of previous lesions for the same minimum period of time, progressive disease as appearance of new lesions or increased lesions by at least 25% in size, and major progressive disease as tumor progression requiring other standard therapy comprising chemotherapy and /or radiotherapy [4.6].

Statistical Analyses

In IVS, baseline values from the same unstimulated CD8 and CD4 T-cell cultures were used as negative control and excluded from the peptide challenged responses [2.5]. For each patient and each time point at least 6 individual cultures were analyzed [2.8]. Vaccination effects on tumor specific CD8 and CD4 T-cell responses were analyzed according to the vaccination schedule and relative to the same results at study entry. For statistical analysis unpaired Kruskal-Wallis test was used. For all analyses, a p value less than 0.05 was considered as statistically significant and labeled with *, very significant less than 0.01 with **, strongly significant less than 0.001 with *** and less than 0.0001 with **** [4.4]. Not significant differences were labeled with ns.

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FIGURE LEGENDS

Figure 1: Study design. Vaccinations (V) consisted of three cycles (C1-C3) of four monthly subcutaneous (s.c.) injections of 0.5 mg of NY-ESO-$1_{79\text{-}108}$ long peptide. HLA-A2+ patients were also vaccinated with 0.1 mg of Melan-$A_{26\text{-}35}$ native peptide and 20µg of Mage-$A10_{254\text{-}262}$ peptide in the first cycle, followed by 0.1 mg Melan-$A_{26\text{-}35(A27L)}$ analog peptide and 0.1 mg of Mage-$A10_{254\text{-}262}$ peptide in the following cycles. In addition, Group B patients were treated with low dose rh-IL-2. Peptides for HLA-$A2^+$ patients were emulsified in 1 ml Montanide® ISA-51 and 2 mg CpG-7909/PF-3512676, peptides for HLA-$A2^-$ patients were emulsified in 0.5 ml Montanide® ISA-51 and 1 mg CpG-7909/PF-3512676. The 3 vaccines of the cycle 3 were formulated without Montanide. Blood samples were withdrawn and PBMC were prepared at baseline (100 ml), after 2 vaccinations (2 samples at 7 days interval: 30 and 100 ml) and after 4 vaccinations (2 samples at 7 days interval: 30 and 100 ml) for the assessment of immune responses.

Figure 2: Specific CD8 T-cell responses before and after vaccination with NY-ESO-1 LSP. **A.** Representative example of a NY-ESO-1-specific CD8 T-cell response 14 days after IVS. Cytokine secreting cells are enumerated after 6-hour challenging of the expanded cells with the NY-ESO-1 pool of overlapping peptides, or without any peptide as control. **B.** Details of longitudinal NY-ESO-1-specific CD8 T-cell responses (IFNγ, TNFα, and IL-2) measured individually in each patient before and during vaccination. **C.** Polyfunctionality of NY-ESO-1-specific CD8 T-cell responses assessed as $IFN\gamma^+TNF\alpha^+$ or $IFN\gamma^+TNF\alpha^+IL\text{-}2^+$ cells, measured individually in each patient before and during vaccination. **D.** Quantification of the contribution of each individual cytokine (IFNγ, TNFα and IL-2) to the NY-ESO-1-specific CD8 T-cell response, before and during vaccination. The mean of the response for

each cytokine is shown for all patients grouped as % of the total response (that is defined as 100%). The magnitude (mean for all patients grouped) of the total response at each time point is indicated on the bottom of each pie.

Figure 3: Specific CD4 T-cell responses before and after vaccination with NY-ESO-1 LSP. A. Representative example of a NY-ESO-1-specific CD4 T-cell response 14 days after IVS. Cytokine secreting cells are enumerated after 6-hour challenging of the expanded cells with the NY-ESO-1 pool of overlapping peptides, or without any peptide as control. **B.** Details of longitudinal NY-ESO-1-specific CD4 T-cell responses (IFNγ, TNFα, IL2 and IL-13) measured individually in each patient before and during vaccination. **C.** Polyfunctionality of NY-ESO-1-specific CD4 T-cell responses assessed as $IFN\gamma^{+}TNF\alpha^{+}$, or $IFN\gamma^{+}TNF\alpha^{+}IL\text{-}2^{+}$, or $IFN\gamma^{+}TNF\alpha^{+}IL\text{-}2^{+}IL\text{-}13^{+}$ cells, measured individually in each patient before and during vaccination. **D.** Quantification of the contribution of each individual cytokine (IFNγ, TNFα, IL-13 and IL-2) to the NY-ESO-1-specific CD4 T-cell responses, before and during vaccination. The mean of the response for each cytokine is shown for all patients grouped as % of the total response (that is defined as 100%). The magnitude (mean for all patients grouped) of the total response at each time point is indicated on the bottom of each pie.

Figure 4: Summary of NY-ESO-1-specific CD8 and CD4 T-cell responses, and antibody responses. Cellular responses were measured 14 days after IVS and humoral responses were analysed by ELISA against the NY-ESO-1 protein in plasma collected from enrolled patients' pre- treatment and during treatment as indicated.

Figure 5: Mapping of NY-ESO-1-specific CD8 and CD4 T-cell responses. A. Using individual overlapping peptides covering the entire NY-ESO-1 LSP sequence, NY-ESO-1-

838 specific CD8 T-cell responses (n=5 patients) and CD4 T-cell responses (n=9 patients) were
839 mapped, by monitoring IFNγ+TNFα (CD8 T-cells) and IFNγ (CD4 T-cells) production after
840 6-hour peptide challenge. **B.** Representative example of NY-ESO-$1_{94\text{-}104}$/B35 multimer
841 staining directly *ex vivo* and after IVS of CD8 T-cells from HLA-B35^{+} patients. **C.** MHC
842 class II restriction of NY-ESO-1-specific CD4 T-cell responses was assessed in a 6-hour
843 peptide challenge in the absence or presence of blocking anti-DR, -DP, or DQ antibodies.
844 Specific responses were measured by quantification of IFNγ production. **D.** Representative
845 example of NY-ESO-$1_{87\text{-}99}$/DR7 multimer staining of IVS CD4 T-cells obtained from HLA-
846 DR7^{+} patients, before and during immunization.
847
848 **Figure 6: NY-ESO-$1_{87\text{-}99}$ peptide represents a novel MHC II epitope. A.** NY-ESO-$1_{87\text{-}99}$-
849 specific CD4 T-cell clones were generated and stained with NY-ESO-1/DR7 multimers
850 (upper panels). Reactivity to specific peptide was tested and EC50 was calculated for both
851 Type1 and Type2 cytokines (lower panels). **B.** NY-ESO-$1_{87\text{-}99}$-specific-CD4 T-cell clones
852 were assessed for their capacity to secrete IFNγ or kill HLA-DR7^{+} targeT-cells, in the
853 presence or absence of specific peptide. **C.** Representative example of direct *ex vivo* multimer
854 staining of NY-ESO-$1_{87\text{-}99}$-specific-CD4 T-cells in HLA-DR7^{+} patients. **D.** Summary of
855 frequencies of direct *ex vivo* detectable NY-ESO-$1_{87\text{-}99}$-specific-CD4 T-cells in HLA-DR7^{+}
856 patients.
857
858 **Figure 7: Overall survival and progression-free survival** depending on the maximal level
859 of IFNγ^{+} NY-ESO-1-specific CD8 T-cell (**A**) and CD4 T-cell (**B**) frequencies reached during
860 the study after IVS. **A**. Overall survival (left panel) and progression-free survival (right panel)
861 in patients with low frequencies of IFNγ^{+} NY-ESO-1-specific CD8 T-cells (lower than the
862 median, n=9) and in patients with high frequencies of IFNγ^{+} NY-ESO-1-specific CD8 T-cells

(higher than the median, n=9). **B**. Overall survival (left panel) and progression-free survival (right panel) in patients with low frequencies of IFNγ^+ NY-ESO-1-specific CD4 T-cells (lower than the median, n=9) and in patients with high frequencies of IFNγ^+ NY-ESO-1-specific CD4 T-cells (higher than the median, n=9).

867 **Table I: Patients' characteristics**

Study vaccination groups	Patient ID	Sex (F/M)	Age at study entry	AJCC tumor staging at study entry	Vaccines received	Tumor outcome		Best response	Disease Free Survival (months)	Overal Survival (months)	Discontinuation reason
						Study entry	Study end				
Group A (no IL-2)											
	LAU 986	M	35	IIIA	12	NED	NED	NED	63.8	63.8	
	LAU 205	M	36	IV	2	ED	PD	PD	73.6	8.5	PD
	LAU 331	M	45	IV	18	NED	NED	NED	80.5	80.5	
	LAU 518	M	65	IV	9	ED	PD	PD	5.8	33.1	PD
	LAU 1280	M	59	IIIB	8	NED	PD	NED	9.4	11.4	PD
	LAU 1330	M	61	IIIC	8	NED	PD	PD	3.5	11.3	PD
	LAU 1293	F	65	IV	12	NED	NED	NED	49.9	49.9	
	LAU 1286	F	31	IIIC	10	NED	NED	NED	71.6	71.6	Patient's choice
	LAU 1352	M	65	IIIC	12	NED	PD	NED	14.0	24.5	
	LAU 1350	M	61	IV	6	ED	PD	PD	3.9	34.6	PD
	all patients	*2/8*	*60 (31-65)*		*10 (2-18)*				*32.0*	*33.8*	
Group B (IL-2)											
	LAU 1357	M	58	IIIB	12 + IL-2	NED	NED*	NED	6.7	68.4	
	LAU 1397	F	36	IIIC	8 + IL-2	NED	PD	NED	8.7	61.2	PD
	LAU 1408	M	64	IIIB	1	NED	PD	PD	1.1	2.2	Degraded health status
	LAU 1415	M	46	IV	5 + IL-2	NED	PD	NED	5.1	12.4	PD
	LAU 1417	M	67	IIIA	8 + IL-2	NED	PD	NED	8.0	55.9	PD
	LAU 466	F	65	IV	2 + IL-2	NED	NED	NED	41.3	41.3	Patient's choice
	LAU 1394	M	64	IIIC	4 + IL-2	NED	PD	PD	8.8	56.8	PD
	LAU 1402	F	59	IIIA	4 + IL-2	NED	NED	NED	49.1	49.1	Patient's choice
	LAU 1504	M	37	IV	12	NED	NED	NED	20.2	20.2	
	all patients	*3/6*	*59 (36-67)*		*6 (1-12)*				*8.7*	*49.1*	
All groups											
	all patients	5/14	59 (31-67)	3 - IIIA 3 - IIIB 5 - IIIC 8 - IV	8 (2-18)	16 - NED 3 - ED 0 - PD	8 - NED 11 - PD	13-NED 6-PD	9.4	41.3	

AJCC: American Joint Committee on Cancer
NED: no evidence of disease
ED: evidence of disease
PD: progressive disease
* The patient was rendered tumor-free by resection of a left axillary metastase, after V6

868
869

Table II: Summary of frequencies of NY-ESO-1/DR7-specific CD4 T-cells, detected after 1 round of IVS in HLA-DR7^{+} patients

Patient ID	Before	2vacc.	4vacc.	6vacc.	8vacc.	12vacc.
LAU 331	19.60	ND	ND	6.50	1.38	6.74
LAU 1293	2.94	2.52	5.00	ND	ND	ND
LAU 1352	1.72	ND	1.54	ND	4.91	8.62
LAU 1350	0.13	0.26	ND	6.80	ND	ND
LAU 1357	0.18	0.55	ND	1.41	1.44	ND
LAU 1397	0.08	9.67	9.94	ND	3.35	ND
LAU 466	0.10	3.69	ND	ND	ND	ND

ND: not done

Figure 1

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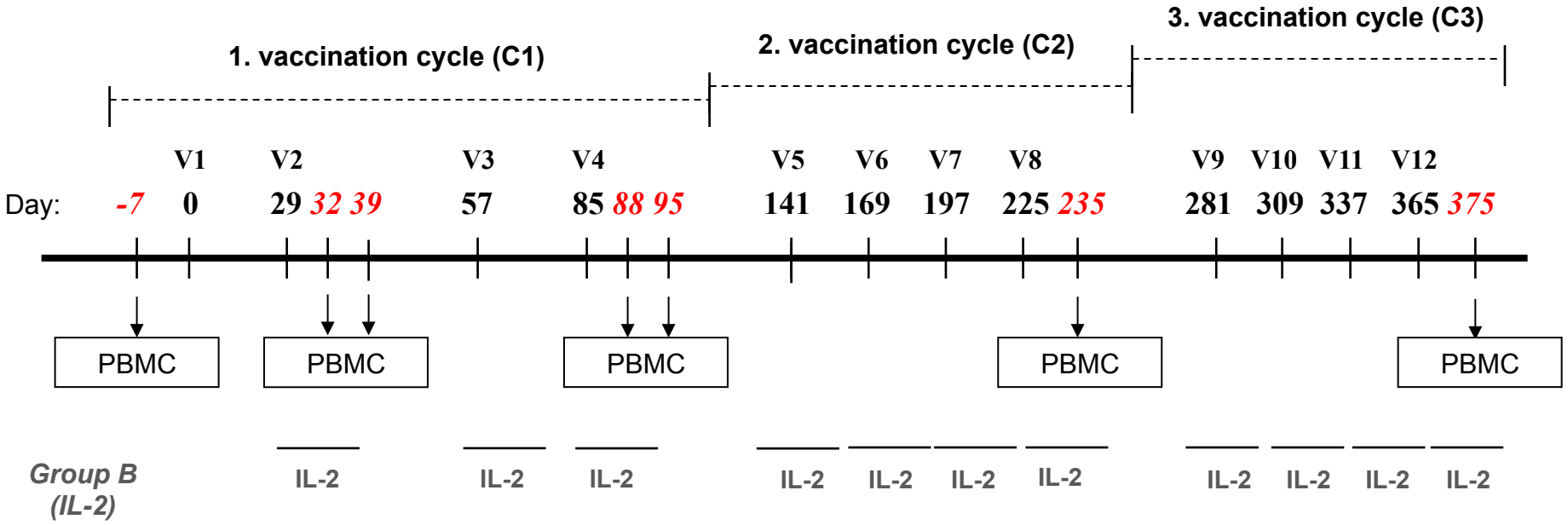

Figure 2

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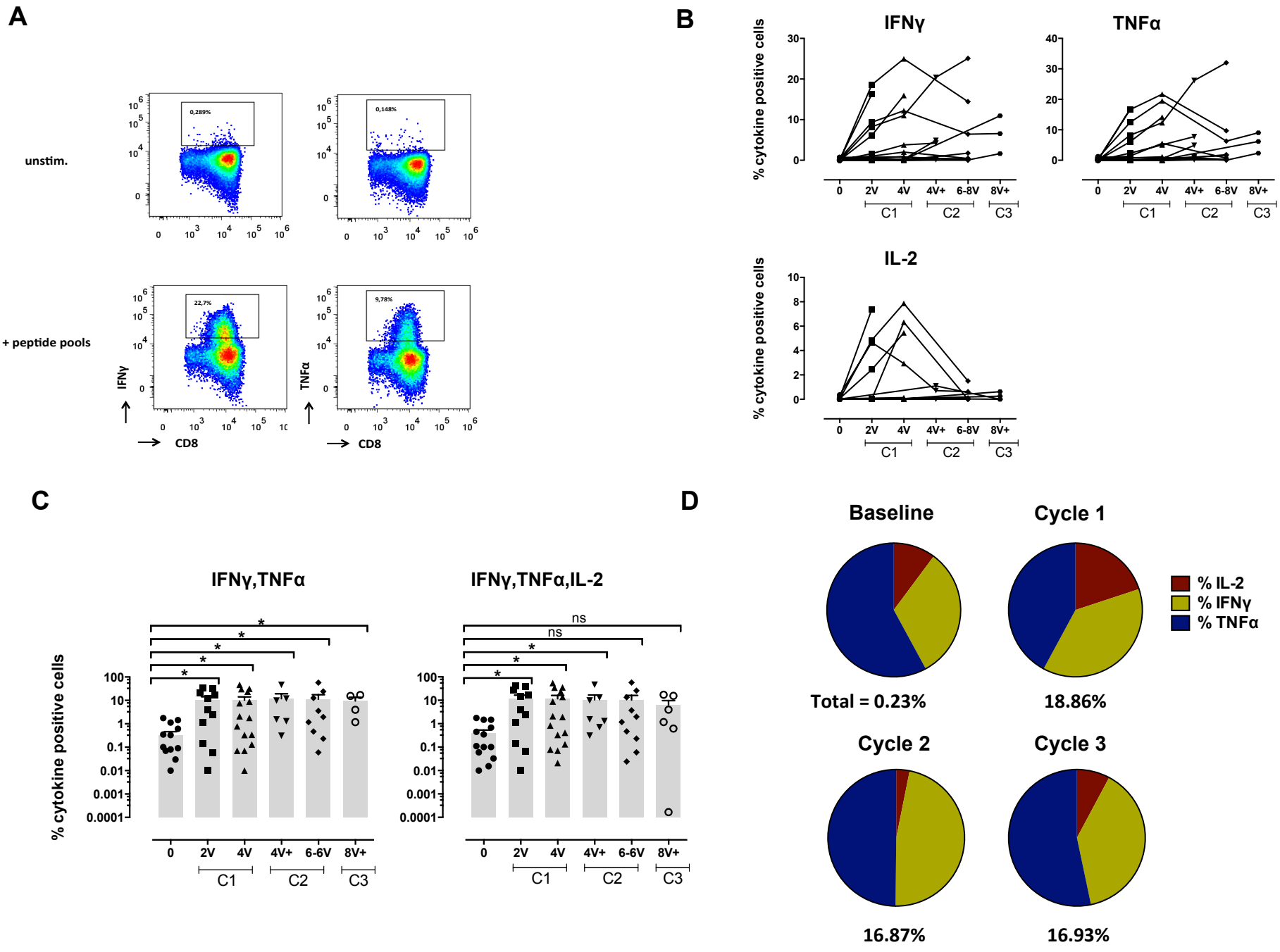

Figure 3

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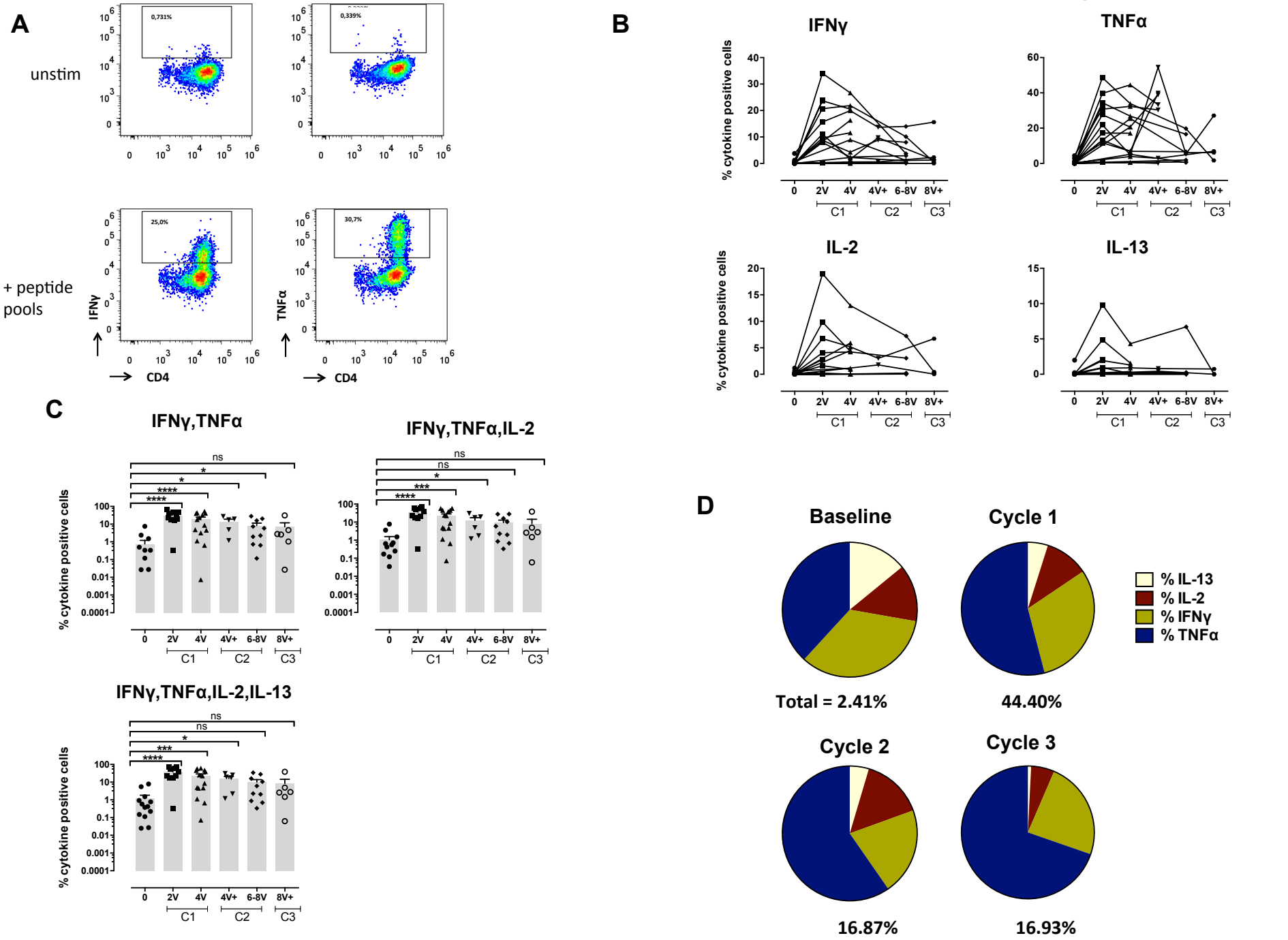

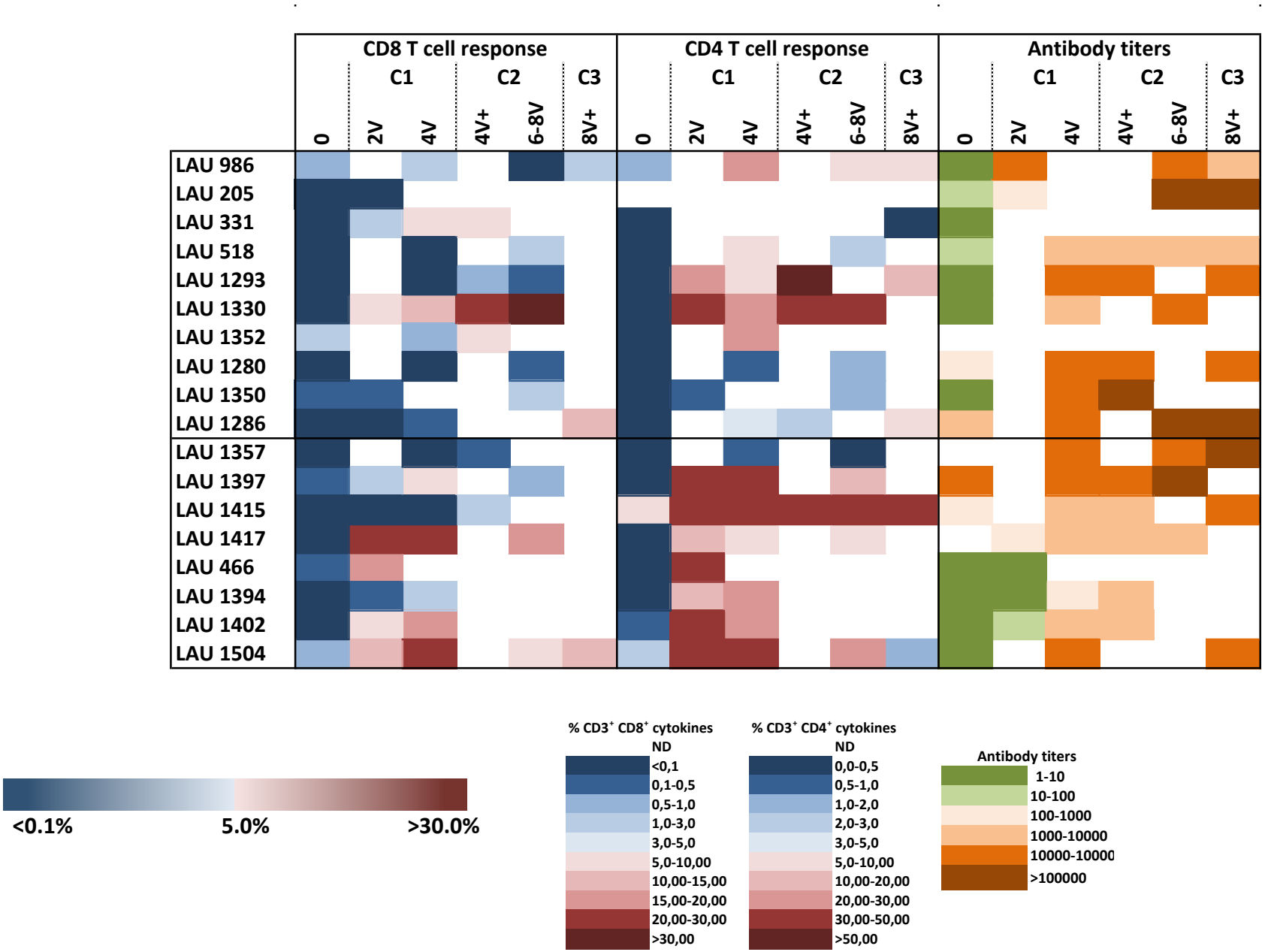
CD8 T cell response
CD4 T cell response
Antibody titers
C1
C2
C3
0
2V
4V
4V+
6-8V
8V+
LAU 986
LAU 205
LAU 331
LAU 518
LAU 1293
LAU 1330
LAU 1352
LAU 1280
LAU 1350
LAU 1286
LAU 1357
LAU 1397
LAU 1415
LAU 1417
LAU 466
LAU 1394
LAU 1402
LAU 1504
<0.1%
5.0%
>30.0%
% CD3+ CD8+ cytokines
ND
<0,1
0,1-0,5
0,5-1,0
1,0-3,0
3,0-5,0
5,0-10,00
10,00-15,00
15,00-20,00
20,00-30,00
>30,00
% CD3+ CD4+ cytokines
ND
0,0-0,5
0,5-1,0
1,0-2,0
2,0-3,0
3,0-5,0
5,0-10,00
10,00-20,00
20,00-30,00
30,00-50,00
>50,00
Antibody titers
1-10
10-100
100-1000
1000-10000
10000-10000
>100000

Figure 5

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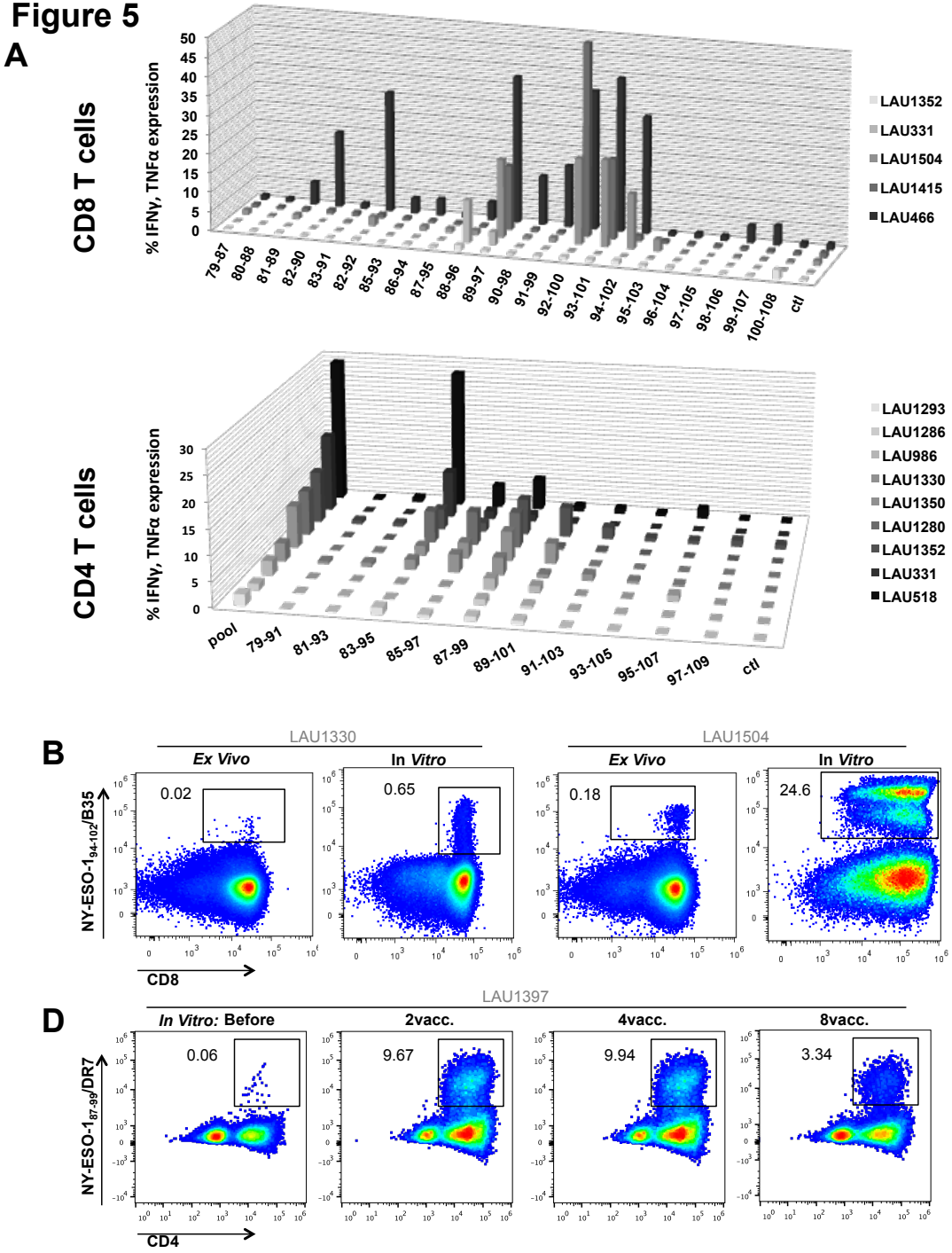

Figure 6

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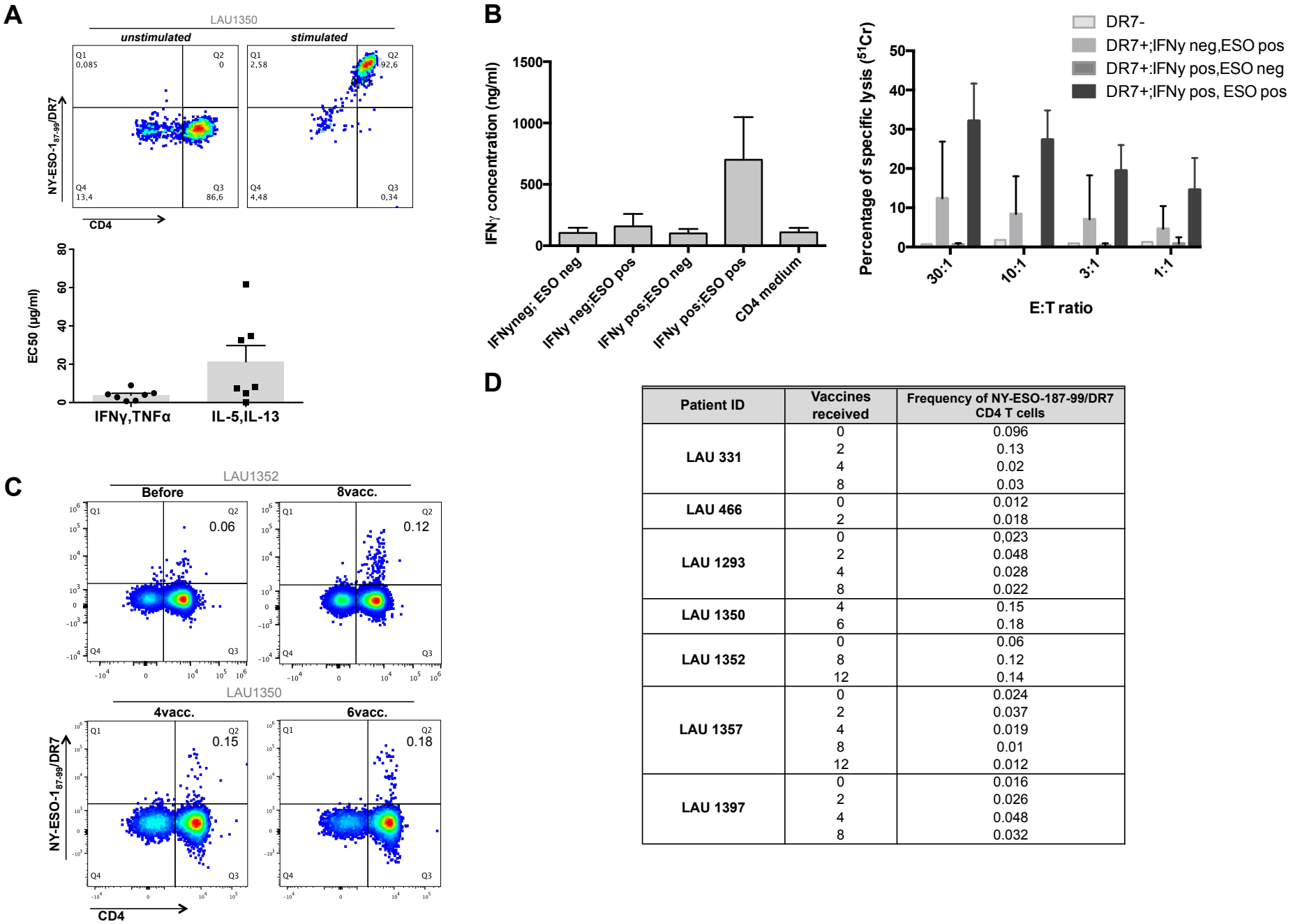

D

Patient ID	Vaccines received	Frequency of NY-ESO-187-99/DR7 CD4 T cells
LAU 331	0	0.096
	2	0.13
	4	0.02
	8	0.03
LAU 466	0	0.012
	2	0.018
LAU 1293	0	0,023
	2	0.048
	4	0.028
	8	0.022
LAU 1350	4	0.15
	6	0.18
LAU 1352	0	0.06
	8	0.12
	12	0.14
LAU 1357	0	0.024
	2	0.037
	4	0.019
	8	0.01
	12	0.012
LAU 1397	0	0.016
	2	0.026
	4	0.048
	8	0.032

Figure 7

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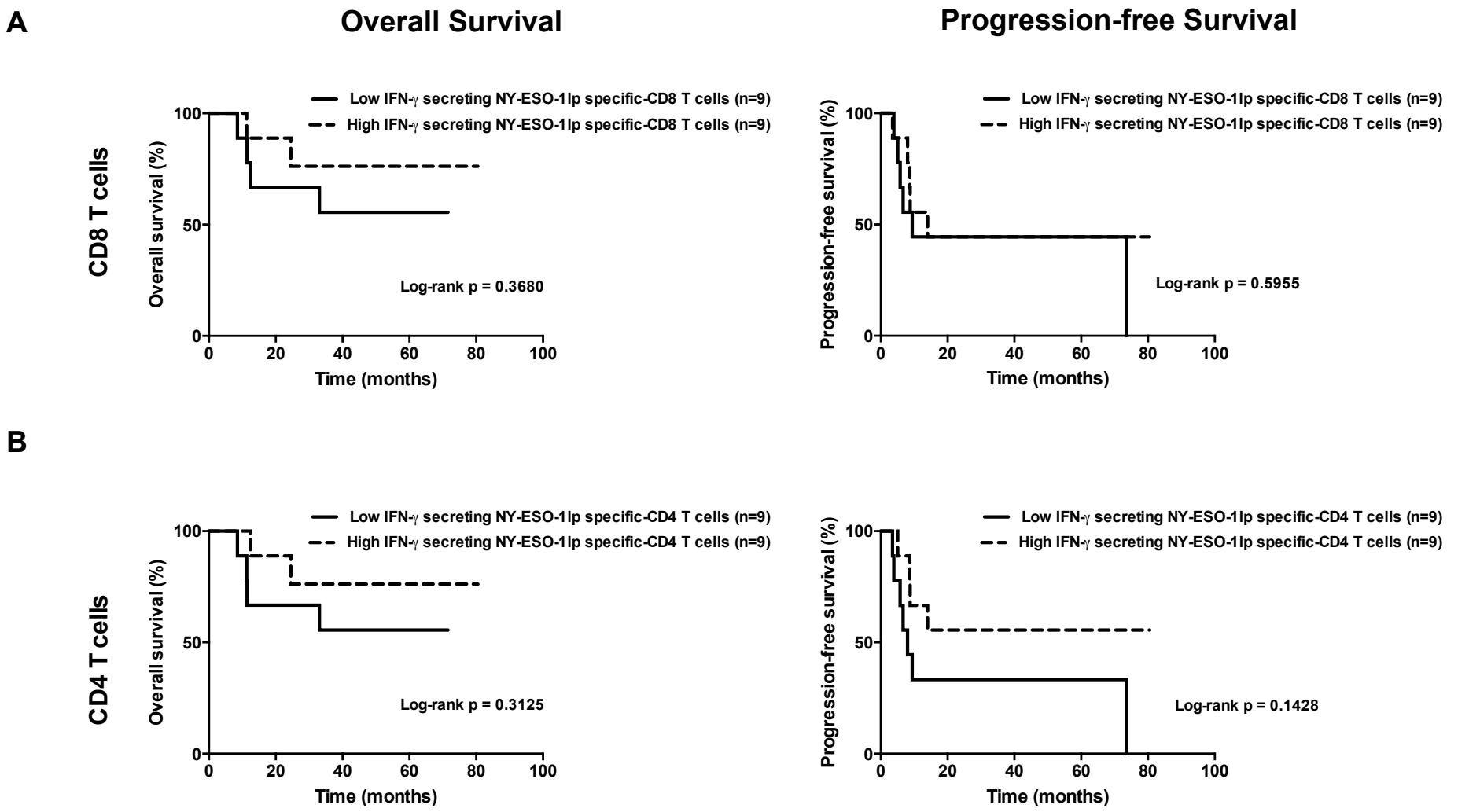

Supplemental Table I: HLA-typing of the patients. **Baumgaertner P., et al.**

Study vaccination groups	Patient ID	HLA typing
Group A (no IL-2)		
	LAU 986	A*0201 A30(19); B49(21) Bx; C*04 C*07; DRB1*0101, *1102; DPB1*0401, *1401, DRw52neg
	LAU 205	A*0201 A3; B*3501/27 B51; DRB1*0101, *13; DPB1*0401, *0402/0602; DRw52neg
	LAU 331	A*0201 A29(19); B8 B44(12); C*07 C*1601; DRB1*0301, *0701; DPB1*0101, *1101; DRw52pos
	LAU 518	A23(9) A31(19); B39(16) B44(12); C*05 C*07; DRB1*0401, *0404; DPB1*0401, *0601; DRw52neg
	LAU 1280	A*03 A*29; B*07 B*44; C*07 C*1601; DRB1*0801, *1501; DPB1*0401, *2001; DRw52neg
	LAU 1330	A1 A26(10); B44(12) B35; C*04 C*07; DRB1*0401, *12; DPB1*0201, *0401; DRw52neg
	LAU 1293	A3 A26(10); B13 B56(22); C*01 C*06; DRB1*0701, *0801; DPB1*0301, *0401; DRw52neg
	LAU 1286	A1 A*0201; B8 B63(15); C*07 C-; DRB1*1102, *1302; DPB1*0201, *1301; DRw52pos
	LAU 1352	A*0201 A3; B35 B51; C*04 C*16; DRB1*0701, *0801; DPB1*0401, *0402; DRw52neg
	LAU 1350	A*0205 A3; B14 B15; C*08 C*12; DRB1*0701, - ; DPB1*0401, -; DRw52neg
Group B (IL-2)		
	LAU 1357	A*01 A*01; B*07 B*44; C*05 C*07; DRB1*0701, *1501; DPB1*0401, *1601
	LAU 1397	A*0201 A1; B13 B57; DR *0701, *0701; DQ *0202, *03; DP *0401, *1701/ DRb neg; DR1 *07*01
	LAU 1408	A*01 A*24; B*14 B*44; DR *0102, *0701; DQ *0202, *05; DP *1101, *1701
	LAU 1415	A*01 A*33; B*08 B*58; DR *03, *13; DQ *0201, *0609; DP *0501, *2401; DR52 *0101, *0301; DRw52 pos
	LAU 1417	A*01 A*24; B*35 B*51; DR *0101, *1104; DQ *03, *05; DP *0402, *1001/ ; DRw52 neg
	LAU 466	A2 A26; B44(12) B35or75?; DR*0701, *1104; DQ*0202, *03; DP *0401, *1101/ DRw52 neg
	LAU 1394	A3 A68; B18 B51; DR*1104, *13; DQ *03, *06; DP *0402, *1001; DR52 *0101, *0202/ DRw52 neg
	LAU 1402	A*26 A*33; B*27 B*35; DR *04, *1303; DQ *03, *03; DP *0201, *0401; DRw52 neg
	LAU 1504	A*01 A*11; B*35 B*51; DR *04, *11; DQ *03, *03; DP *0301, *0402

Supplemental Table II: Number of vaccination cycles completed and vaccines administered.

	Patients completed Cycle 1	Patients completed Cycle 2	Patients completed Cycle 3	Patients received Boost	Total vaccines administered	Max number vaccines received per patient
All groups	16/19	12/14	6/8	1	153	18
Group A	9/10	8/9	4/6	1	97	18
Group B	7/9	4/5	2/2	0	56	12

Supplemental Table III: Toxicity profile, list of adverse events that were definitely, probably and possibly related to the study treatment, according to their CTCAE severity scale.

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SOC	CTCAE grade 1 adverse events			CTCAE grade 2 adverse events			CTCAE grade 3 adverse events			CTCAE grade 4 adverse events		
	n of events	n of patients	% of patients	n of events	n of patients	% of patients	nof events	n of patients	% of patients	n of events	n of patients	% of patients
CARDIAC DISORDERS	1	1	5	1	1	5	0	0	0	0	0	0
EAR AND LABYRINTH DISORDERS	1	1	5	0	0	0	0	0	0	0	0	0
EYE DISORDERS	5	2	11	0	0	0	0	0	0	0	0	0
GASTROINTESTINAL DISORDERS	35	12	63	5	2	11	1	1	5	0	0	0
GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS	577	19	100	30	10	53	1	1	5	0	0	0
INFECTIONS AND INFESTATIONS	1	1	5	0	0	0	0	0	0	0	0	0
INVESTIGATIONS	3	2	11	0	0	0	0	0	0	0	0	0
METABOLISM AND NUTRITION DISORDERS	2	1	5	0	0	0	0	0	0	0	0	0
MUSCULOSKELETAL AND CONNECTIVE TISSUE DISORDERS	79	11	58	15	7	37	0	0	0	0	0	0
NEOPLASMS BENIGN, MALIGNANT AND UNSPECIFIED (INCL CYSTS AND POLYPS)	1	1	5	0	0	0	0	0	0	0	0	0
NERVOUS SYSTEM DISORDERS	55	10	53	10	5	26	0	0	0	0	0	0
PSYCHIATRIC DISORDERS	3	3	16	0	0	0	0	0	0	0	0	0
RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS	4	1	5	1	1	5	0	0	0	0	0	0
SKIN AND SUBCUTANEOUS TISSUE DISORDERS	9	5	26	2	2	11	0	0	0	0	0	0
VASCULAR DISORDERS	5	4	21	0	0	0	0	0	0	0	0	0
Total AEs	781			64			2			0		

CTCAE: Common Terminology Criteria for Adverse Events (AEs), (Version 2.0, April 30, 1999)
SOC: System Organ Class.
The MedDRA terminology was developed as a medically validated medical terminology for utilization throughout the regulatory process. A SOC is the highest level of the hierarchy of MedDRA dictionary and distinguished by anatomical or physiological system, etiology, or purpose. CTCAE terms are grouped by MedDRA Primary SOCs. Within each SOC, AEs are listed and accompanied by descriptions of severity (Grade).

CTCAE severity (grade) scale: 1=mild, 2=moderate, 3=severe and 4=life threatening

Supplemental Table IV: Number of IL-2 injections administered and cumulative IL-2 dose received by patients in group B.

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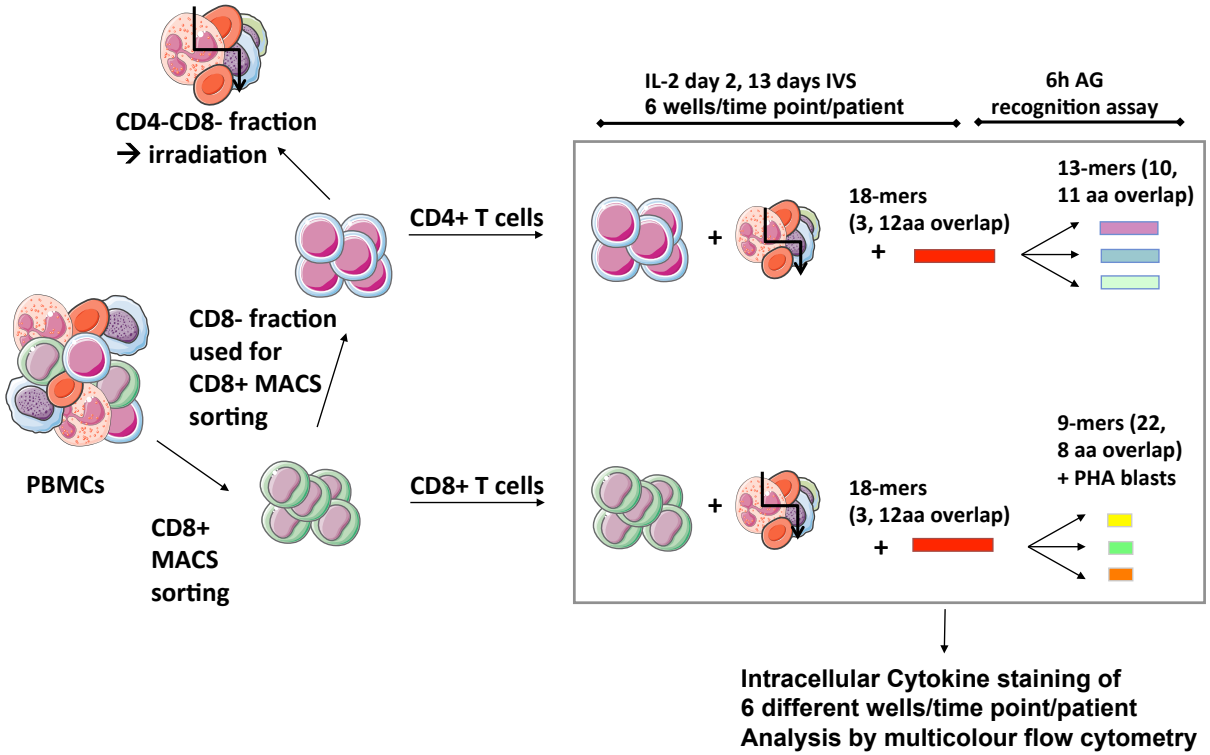
Study group	Patient ID	Vaccines received	IL-2 injections	Total cumulative IL-2 doses
Group B	**LAU 1357**	12	15 x 0.5 Mio UI/m^2 + 6 x 0.25 Mio UI/m^2	18 Mio UI
	LAU 1397	8	70 x 0.5 Mio UI/m^2	70 Mio UI
	LAU 1408	1	ND	0
	LAU 1415	5	36 x 0.5 Mio UI/m^2	36 Mio UI
	LAU 1417	8	70 x 1 Mio UI/m^2 + 27x 1.7 Mio UI/m^2	131.9 Mio UI
	LAU 466	2	10 x 1 Mio UI/m^2	16.7 Mio UI
	LAU 1394	4	30 x 1 Mio UI/m^2	63 Mio UI
	LAU 1402	4	25 x 1.5 Mio UI/m^2	75 Mio UI
	LAU 1504	12	ND	0

ND: not done

Supplemental Figure 1 **Baumgaertner P., et al.**



Supplemental Figure 1: IVS for the detection of antigen-specific CD4 and CD8 T-cells. CD8 T-cells were purified by positive selection using MACS isolation microbeads (Miltenyi) according to manufacturer's recommendations, followed by CD4 T-cell positive selection starting from the CD8 negative fraction, using the same method. Positive T-cells were cultured in IVS and CD4/ CD8 depleted PBMCs were irradiated (30 Gy) and used as feeders for stimulation of the cultures. T-cells (either CD4 or CD8) and autologous APCs were mixed at 1:1 ratio and co-cultured for 14 days with 3 18-mers (NY-ESO-$1_{79\text{-}96}$, NY-ESO-$1_{85\text{-}102}$, NY-ESO-$1_{91\text{-}108}$) spanning the entire vaccine NY-ESO-$1_{79\text{-}108}$ sequence, with 12 amino acids overlaps at 2 µM, in medium consisting of RPMI 1640 supplemented with 8% hear inactivated, pooled human serum. At day 2, 100 U/ml IL-2 was added and cultures were continued until day 14. Each individual initial culture was splitted separately when necessary. T-cell responses were evaluated after IVS in a 6-hour re-challenge experiment using overlapping peptide pools (2 µM final concentration) in the presence of Brefeldin A (10 µg/ml): for evaluation of CD4 T-cell responses 10 13-mer peptides overlapping by 11 amino acids were used, while for CD8 T-cell responses 22 9-mer peptides were pulsed on autologous PHA CD4 T-cell blasts for 1 hour, before addition to the CD8 T-cells. As negative control, each well was left unchallenged. As positive control, 2 wells for CD4 and CD8 T-cells were stimulated with PMA/Ionomycin in the presence of Brefeldin A and NY-ESO-1 restimulated T-cells were stained intracellularly for cytokines and analyzed by flow cytometry.

Supplemental Figure 2

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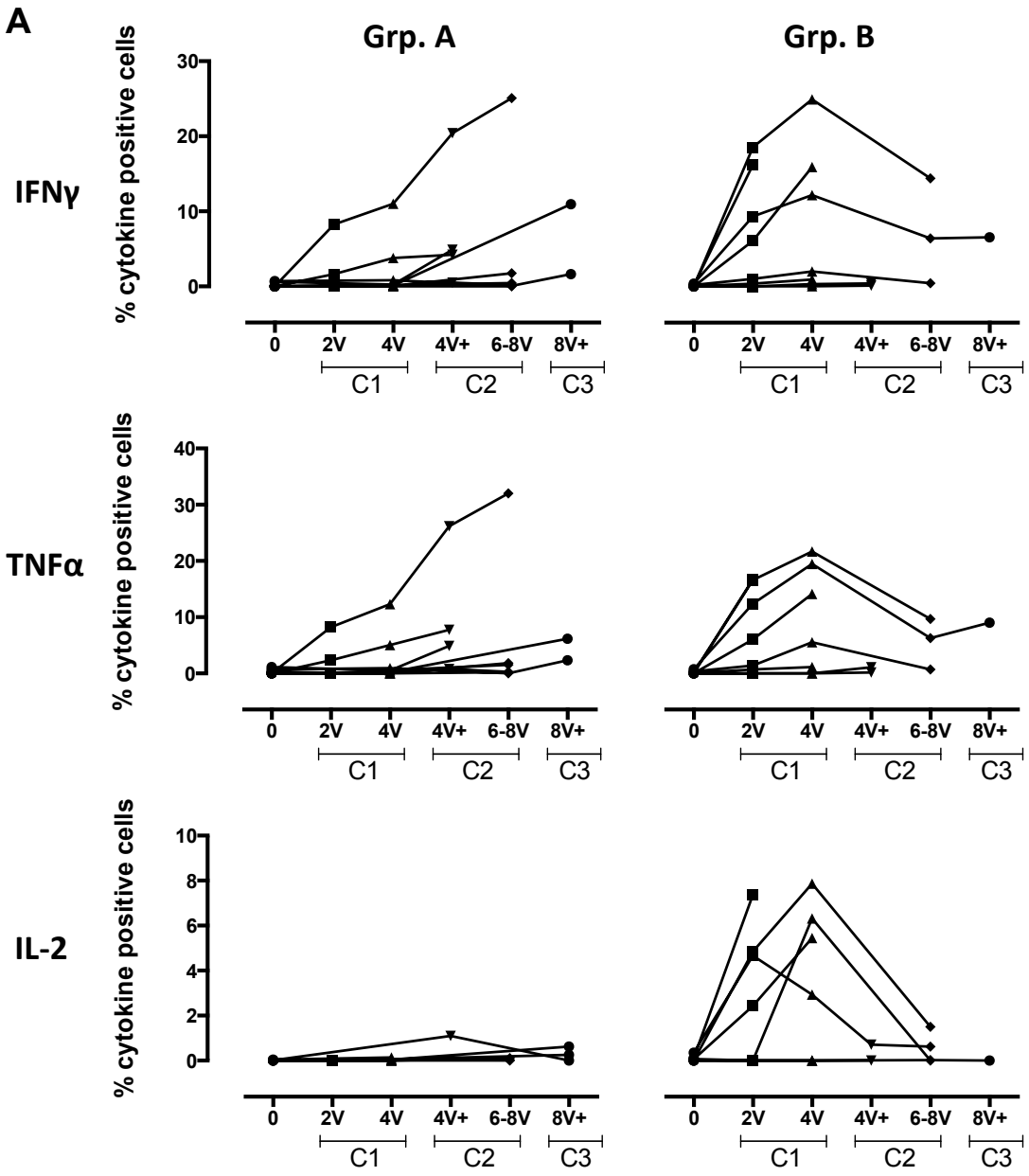

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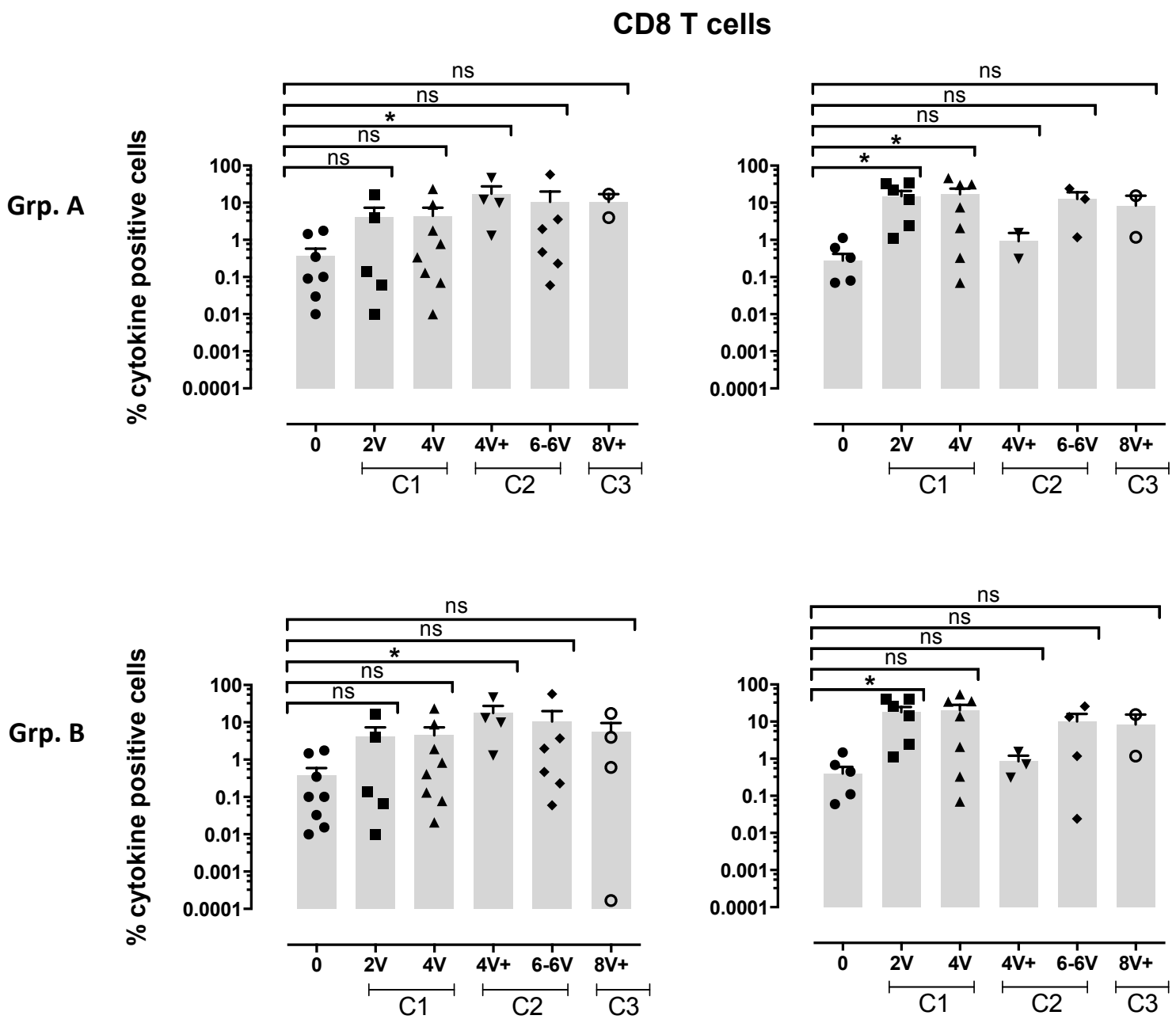
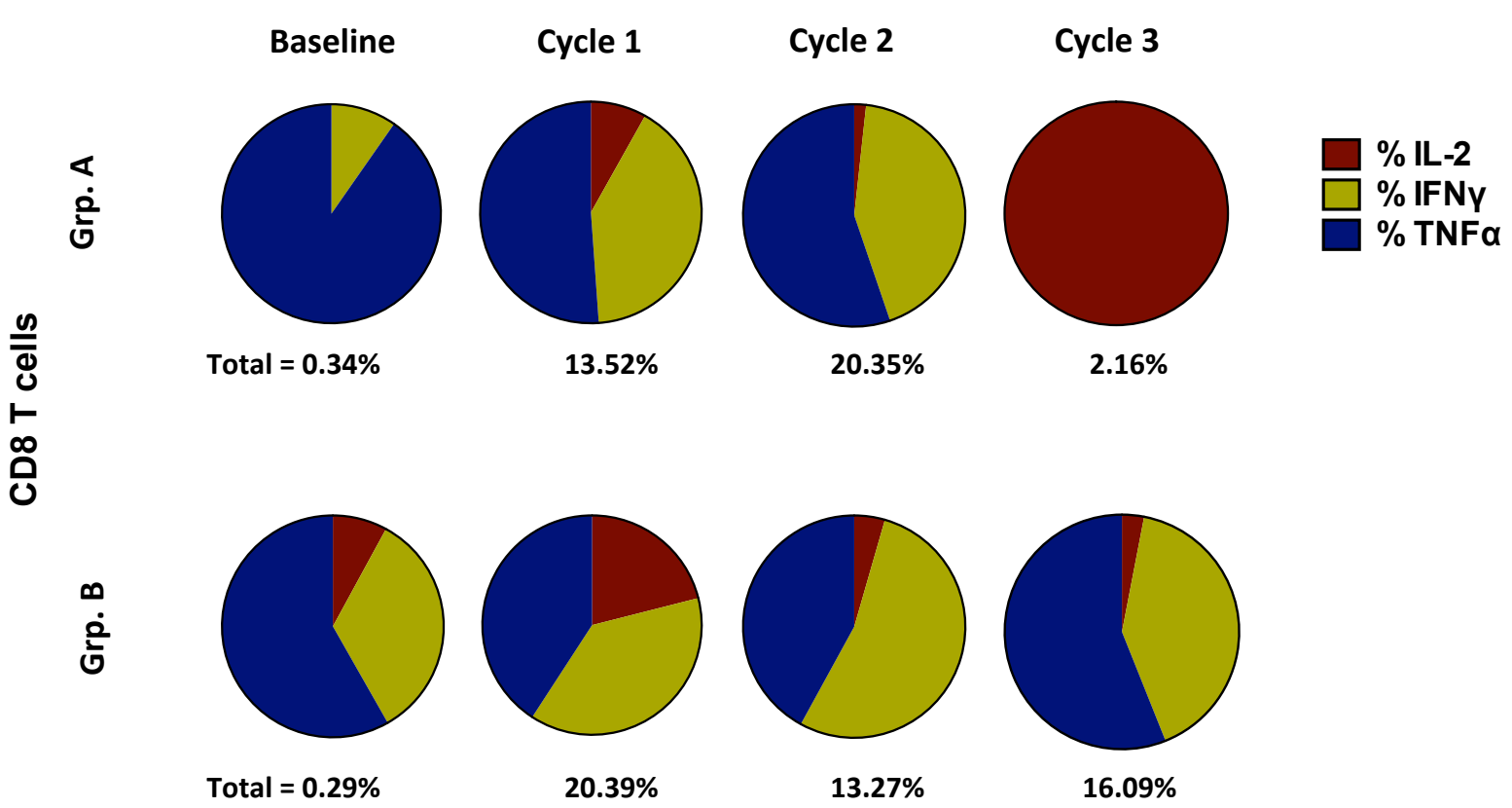

CD8 T cells
Grp. A
Grp. B
% cytokine positive cells
ns
*
100
10
1
0.1
0.01
0.001
0.0001
0
2V
4V
4V+
6-6V
8V+
C1
C2
C3



Supplemental Figure 2: Specific CD8 T-cell responses before and after vaccination with NY-ESO-1 LSP, in Groups A and B. **A.** Details of longitudinal NY-ESO-1-specific CD8 T-cell responses (IFNγ, TNFα, and IL-2) measured individually in each patient in Group A (left panels) and B (right panels), before and during vaccination. **B.** Polyfunctionality of NY-ESO-1-specific CD8 T-cell responses assessed as $IFN\gamma^{+}TNF\alpha^{+}$ or $IFN\gamma^{+}TNF\alpha^{+}IL\text{-}2^{+}$ cells, measured individually in each patient in Group A (left panels) and B (right panels), before and during vaccination. **C.** Quantification of the contribution of each individual cytokine (IFNγ, TNFα and IL-2) to the NY-ESO-1-specific CD8 T-cell response, before and during vaccination. The mean of the response for each cytokine is shown for patients in Group A (upper pies) and B (lower pies).

Supplemental Figure 3

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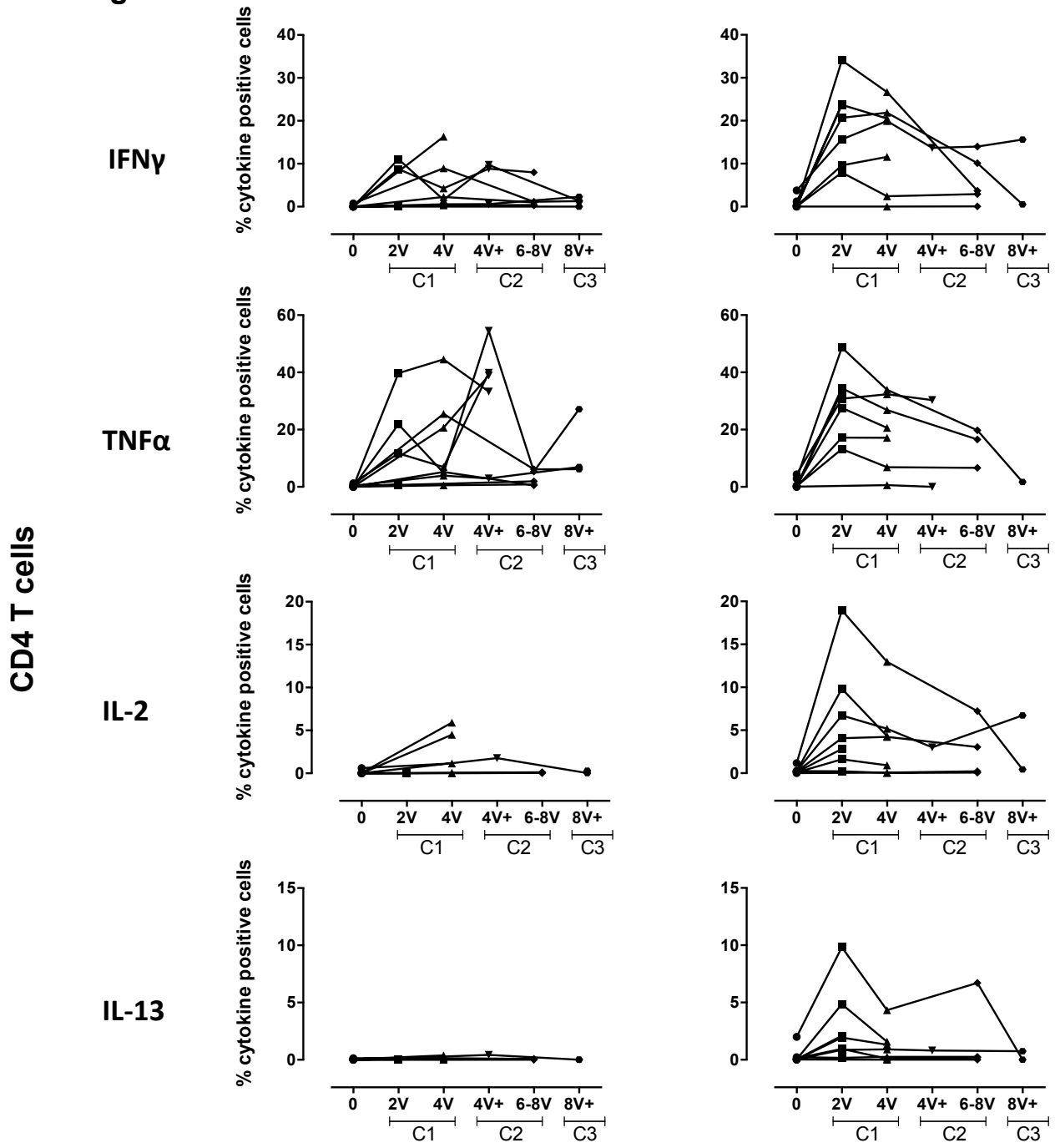
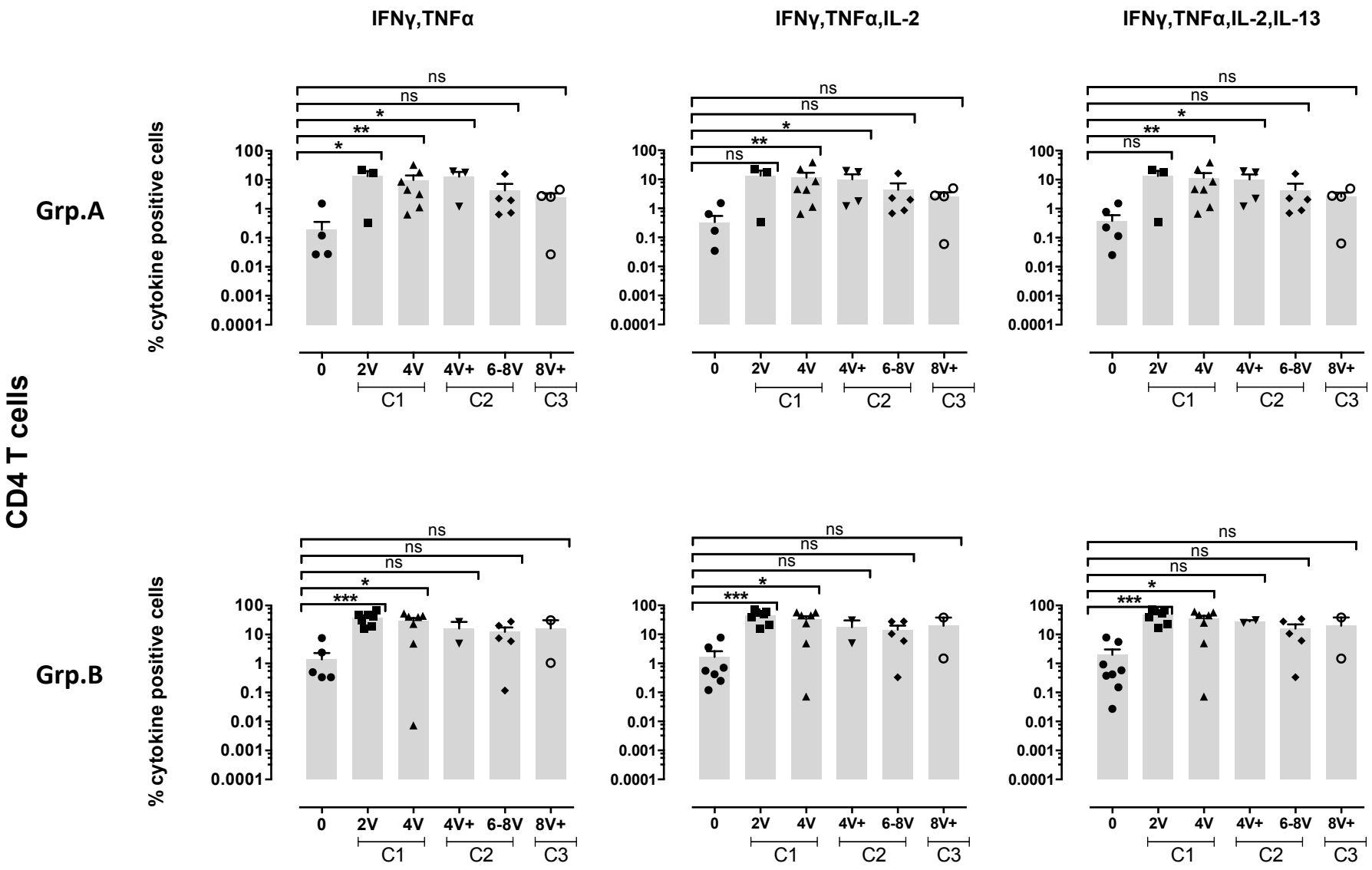
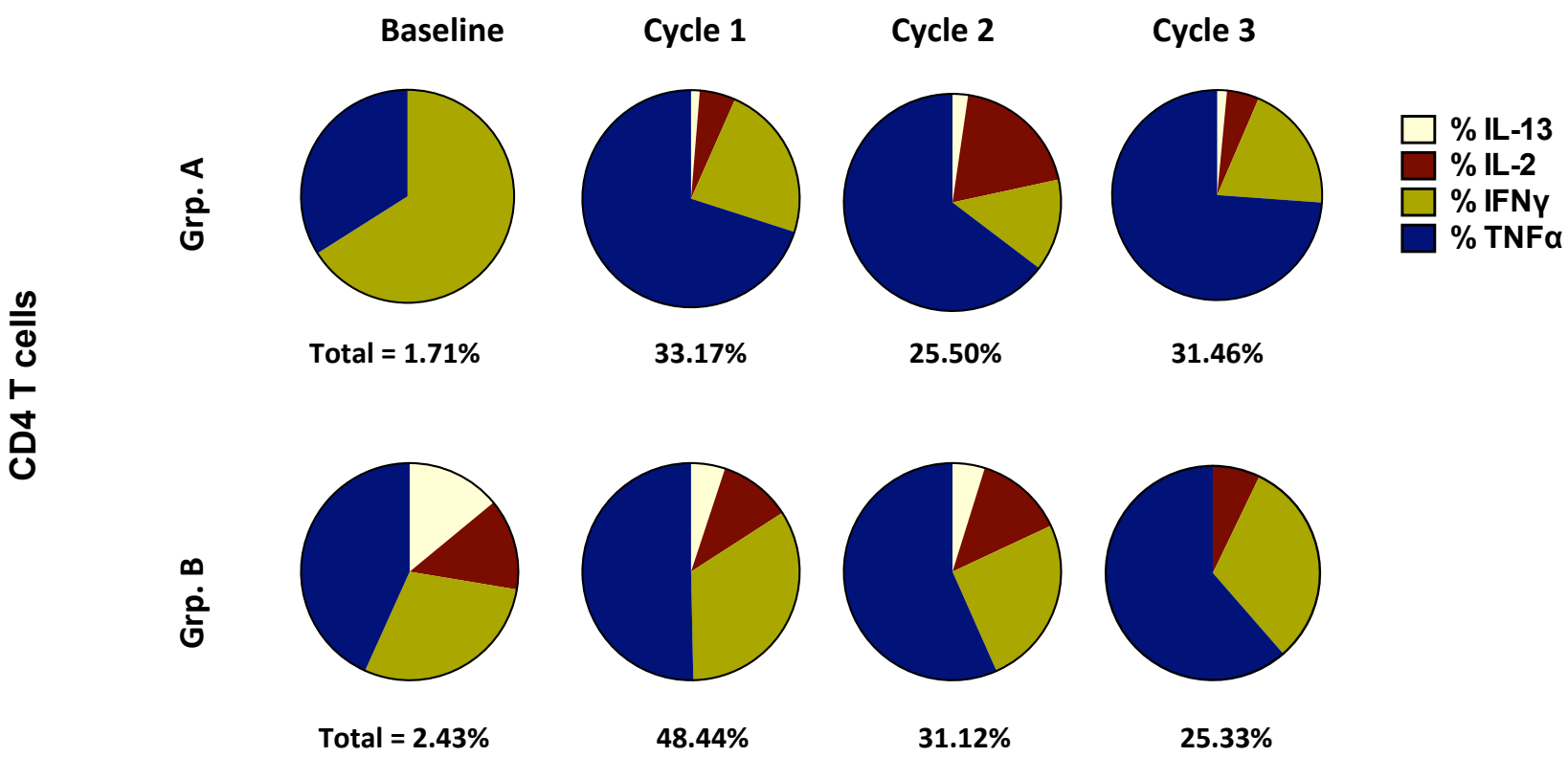
A

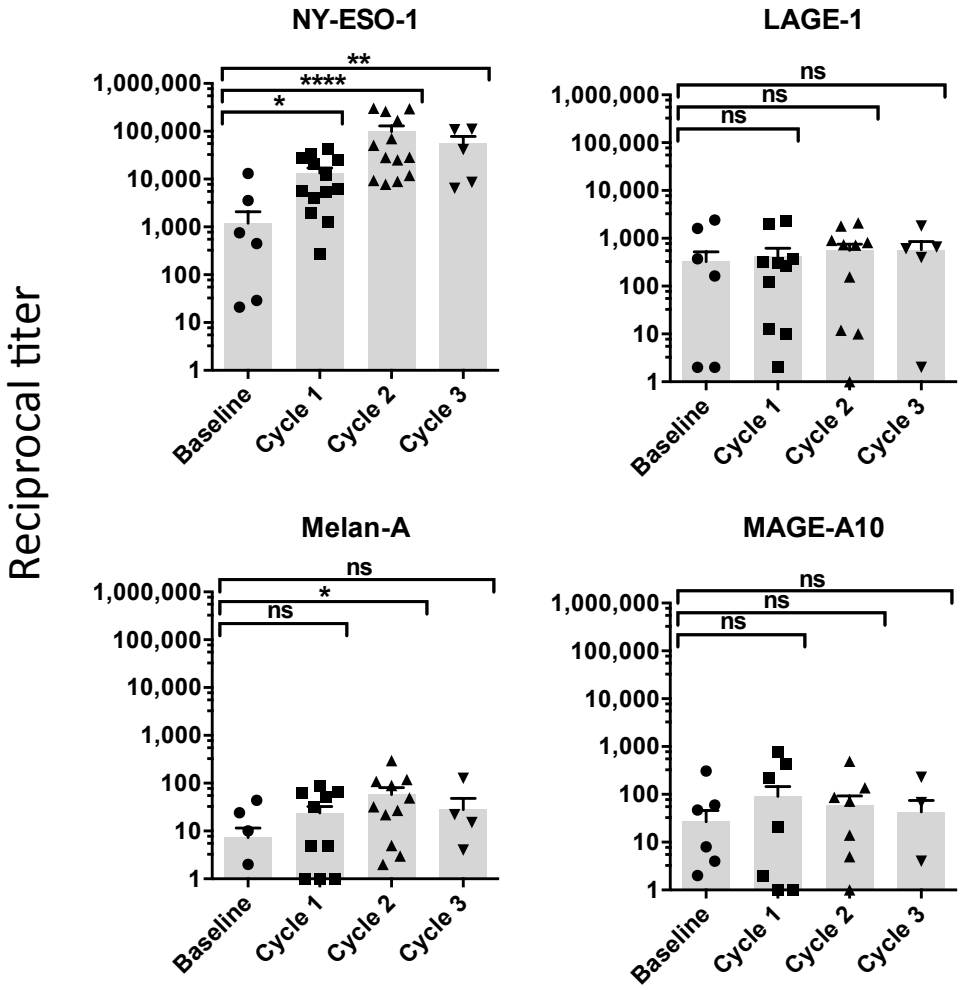


B

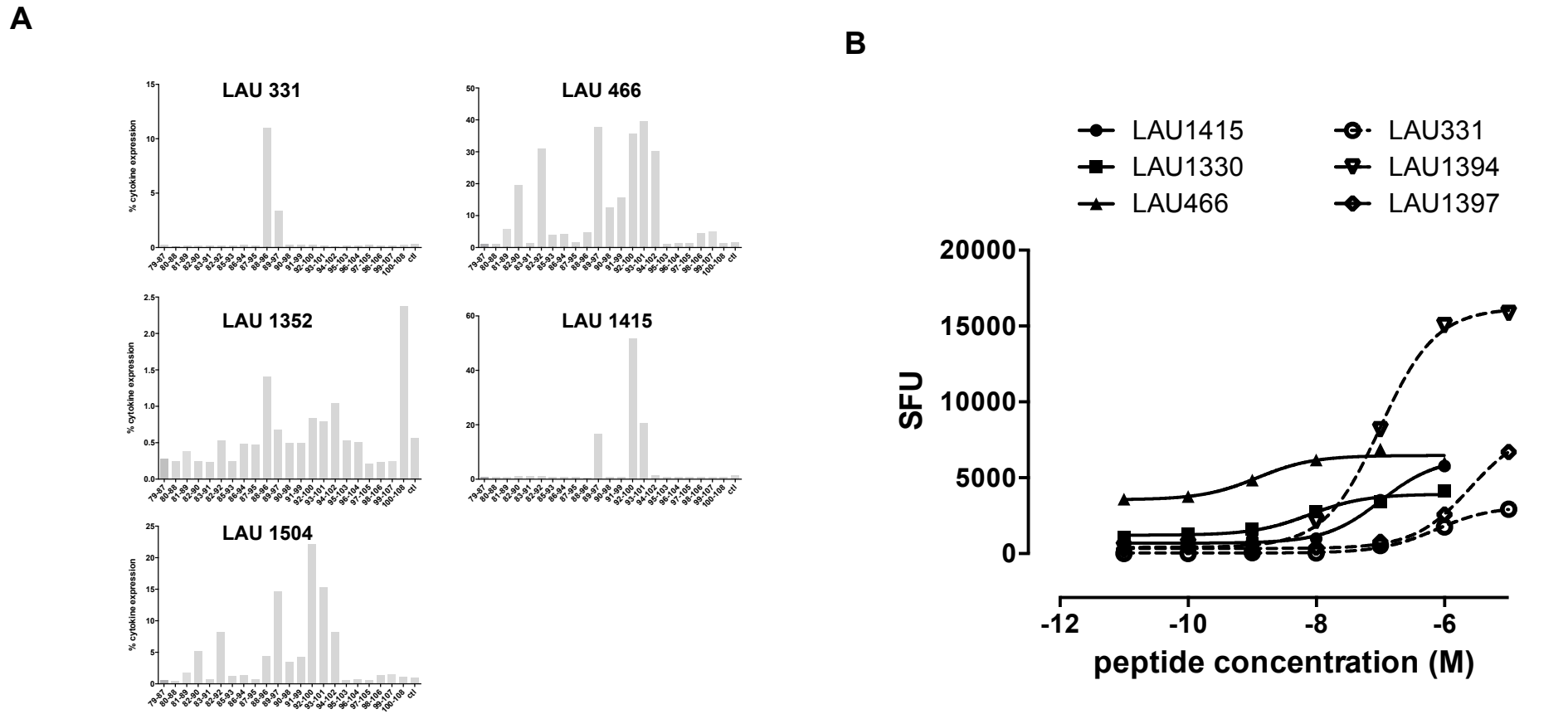

IFNγ,TNFα
IFNγ,TNFα,IL-2
IFNγ,TNFα,IL-2,IL-13
CD4 T cells
Grp.A
Grp.B
% cytokine positive cells
ns
*
**
***
100
10
1
0.1
0.01
0.001
0.0001
0
2V
4V
4V+
6-8V
8V+
C1
C2
C3



Supplemental Figure 3: Specific CD4 T-cell responses before and after vaccination with NY-ESO-1 LSP, in Groups A and B. **A.** Details of longitudinal NY-ESO-1-specific CD4 T-cell responses (IFNγ, TNFα, IL2 and IL-13) measured individually in each patient in Group A (left panels) and B (right panels), before and during vaccination. **B.** Polyfunctionality of NY-ESO-1 specific CD4 T-cell responses assessed as $IFN\gamma^{+}TNF\alpha^{+}$, or $IFN\gamma^{+}TNF\alpha^{+}IL\text{-}2^{+}$, or $IFN\gamma^{+}TNF\alpha^{+}IL\text{-}2^{+}IL\text{-}13^{+}$ cells, measured individually in each patient in Group A (left panel) and B (right panel), before and during vaccination. **C.** Quantification of the contribution of each individual cytokine (IFNγ, TNFα, IL-13 and IL-2) to the NY-ESO-1-specific CD4 T-cell responses, before and during vaccination. The mean of the response for each cytokine is shown for patients in Group A (upper pies) and B (lower pies).

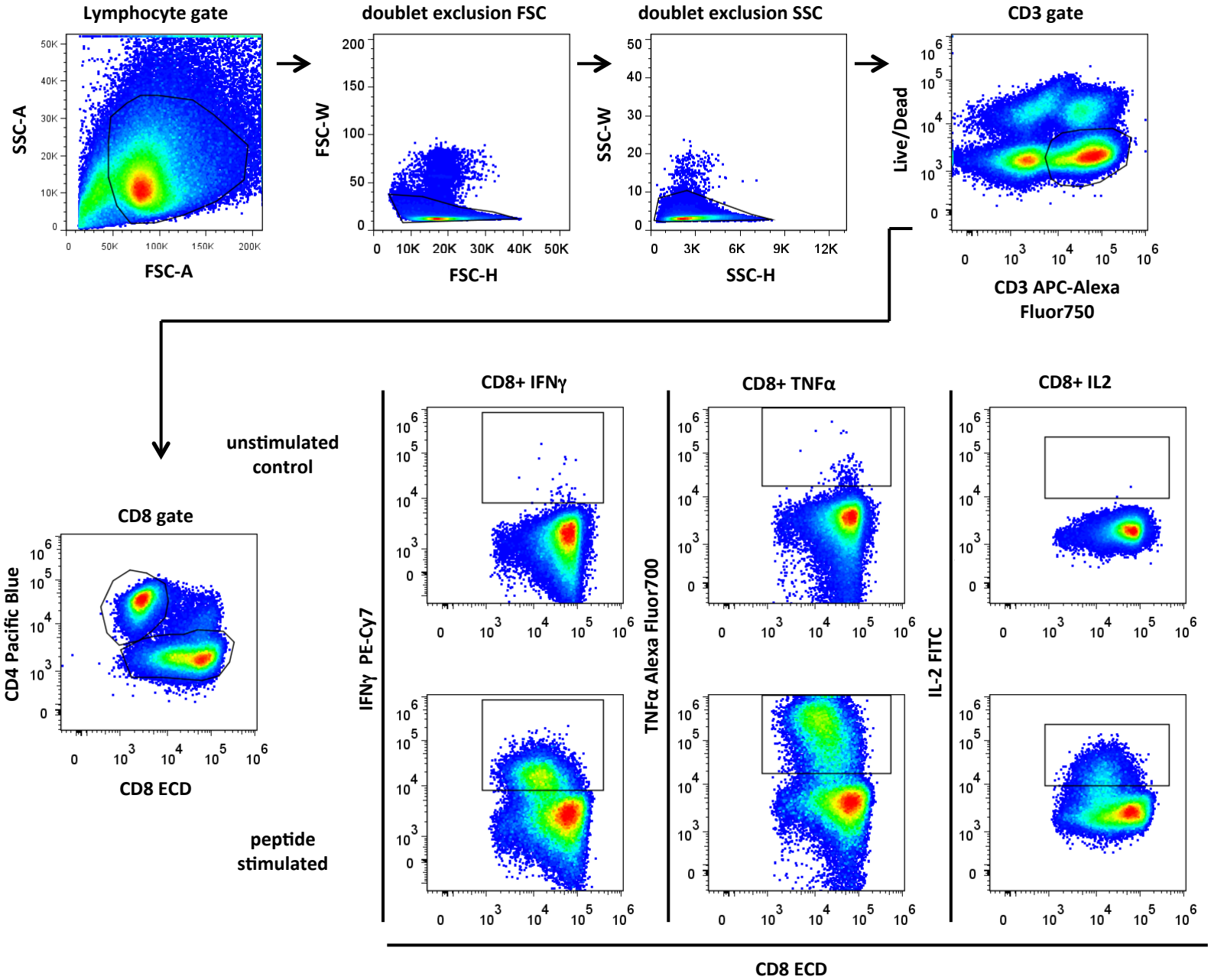


Supplemental Figure 4: Tumor antigen specific humoral responses. **A.** Plasma from patients before and during immunization was analyzed by ELISA for reactivity against recombinant NY-ESO-1, LAGE-1, Melan-A, and MAGE-A10 proteins. Reciprocal titers were determined as described in Materials and methods part.



Supplemental Figure 5: Mapping and avidity evaluation of NY-ESO-1-specific CD8 T-cell responses in individual patients. **A.** Using individual 9-mer overlapping peptides covering the entire NY-ESO-1 LSP sequence, NY-ESO-1-specific CD8 T-cell responses were mapped in 5 patients, by monitoring IFNγ$^+$ TNFα$^+$ CD8 T-cells after 6-hour peptide challenge. **B.** Using serial dilutions of the pool of 9-mer overlapping peptides covering the entire NY-ESO-1 LSP sequence, avidity of peptide recognition of NY-ESO-1-specific CD8 T-cell responses was assessed using ELISPOT assay for 6 patients.

Supplemental Figure 6 **Baumgaertner P., et al.**



Supplemental Figure 6: Gating strategy for identification of cytokine secreting T cells. The same strategy has been applied for both CD8 (shown heere) and CD4 T cells.