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IFN-γ Responses in Peptide-treated Melanoma Patients Measured by an ELISPOT Assay Using Allogeneic Dendritic Cells

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Abstract. *Background: Several melanoma-specific peptides are currently used in clinical trials. However, the monitoring of the T cell response remains non-standardised and is often limited by shortage of cells. Materials and Methods: We established an IFN-γ ELISPOT assay to detect the CD8$^+$ T cell response in HLA-A2-positive melanoma patients using pre-frozen, peptide-loaded HLA-A2-positive but otherwise allogeneic, monocyte-derived dendritic cells (DC) as antigen-presenting cells. We tested HLA-A2-positive stage III or IV melanoma patients before and after peptide immunotherapy. Results: The number of EBV and influenza-specific IFN-γ-spots were comparable irrespective of the use of autologous or allogeneic HLA-A2 immature DCs when using purified CD8$^+$ cells as responder cells, but a high allogeneic background was seen when using PBMC. We observed modifications of the in vitro response to the melanoma peptides in three out of four responding patients, while virus responses remained constant; however, similar results were seen in the group with progressive disease. Conclusion: This demonstrates the possibility of monitoring an immune response by using allogeneic DCs, reducing the consumption of patient cells. The in vitro IFN-γ responses increased in response to the peptide therapy, however this could not be correlated to clinical outcome.*

Abbreviations: ELISPOT, enzyme-linked immunospot assay; PBMC, peripheral blood mononuclear cells; IFN, interferon; DC, dendritic cell.

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Several melanoma-specific HLA-A2 peptides have been identified and these are currently used in different clinical trials, either as direct peptide immunotherapy or in combination with dendritic cells (DC). Anti-tumor CD8$^+$ cells have long been thought to be the major effector cells in tumor rejection (1, 2), and adoptively transferred CD8$^+$ T cells have been shown to promote long-term immunity *in vivo* (3). However, the monitoring of the T cell response remains non-standardised and is often limited by the shortage of patient cells. Several methods are available to measure CD8$^+$ T cell responses, including limiting dilution assays followed by cytotoxicity assays or cytokine measurements (4), or direct assays like ELISPOT (5) or tetramers (reviewed in 6). These methods either use peripheral blood mononuclear cell (PBMC) populations which may vary in cell constitution or, when autologous DC are used (7, 8), consume large numbers of cells from the patient. The need for effective antigen-presenting cells is of essence. To avoid the use of many patient cells the use of HLA-transfected insect cells as APC have been tried (9, 10). However, expression of co-stimulatory molecules needs to be tightly regulated not to reduce the sensitivity of the assay. Other APC that have been used are T2 cells, hybrids between a B-lymphoblastoid cell line and a T cell leukemia cell line, which do express both MHC class II and costimulatory molecules, but give a moderate to high background (9).

Cryopreserved PBMC have been used in several studies and have been shown to be similar to fresh cells in their capacity to produce IFN-γ (11, 12) and allow the testing of blood samples from different time-points, *e.g.* during a treatment, in the same experiment. The aim of this study was to develop a sensitive assay to detect antigen-specific CD8$^+$ T cells in cells from peripheral blood from patients included in a peptide-based vaccine trial. DC have been

shown to be the most potent antigen-presenting cells (13) and can be produced from monocytes by culturing them in IL-4 and GM-CSF (14). They express high levels of MHC class I and costimulatory molecules even at the immature state and can be frozen and still maintain their functionality (15, 16). We established an ELISPOT assay to detect the $CD8^+$ response in melanoma patients using pre-frozen allogeneic HLA-$A2^+$ monocyte-derived dendritic cells (DC) as antigen-presenting cells. By using immature cells we minimized the allogeneic response. Our results show that IFN-γ-producing $CD8^+$ T cells could be detected after stimulation with peptide-loaded allogeneic DC, and the responses were higher after peptide immunotherapy. However, the responses could not be correlated to clinical outcome of treatment in the eight patients tested.

Table I. *Stimulation of LT12 clone with monocyte-derived DC*.*

Number of LT12 cells added to well	Number of IFN-γ spots/well		
	Exp. 1	Exp.2	Exp. 3
0	1	6	0.3
5	21	12	7
50	111	57	61.3
500	NC**	372	340

*5000 DC pre-incubated with influenza peptide (2x1 μg/ml) for 24+4h were added to each well and different densities of LT12 T cell clone (CD8+, influenza-specific, HLA A2-restricted) were added, and incubated in ELISPOT plate 18h. Results represent the mean of triplicate wells. **Not countable, >400 spots.

Materials and Methods

Reagents. Reagents for ELISPOT were anti-human IFN-γ mAb 1-DIK, mAb 7B6-1-Biotin and Streptavidin-ALP from MABTECH (Stockholm, Sweden). Antibodies used in FACS against CD14, CD83, CD86 and CD1a were from Becton Dickinson (Bedford, MA, USA); anti-CD83 was from Dako (Trappes, France).

Peptides. All peptides were HLA-A2-specific. All patients were injected with the peptides Tyrosinase 368-376 (YMDGTMSCV), the modified Melan-A/MART-1 26-35 (ELAGIGILTV) Na-17a 9-mer (VLPDVFIRC) and Na-17a 10-mer (VLPDVFIRCV), except for patient 6 who was only given injections of Tyrosinase and Melan-A. The modified Melan-A/MART-1, carrying a substitution of Ala for Leu at position 2 from the Nh2 terminus, has been shown to give a higher $CD8^+$ response than the natural peptide (17). Responses to all peptides as well as the control peptides Influenza matrix 58-66 (GILGFVFTL) and EBV BMLF1 280-288 (GLCTLVAML) were tested *in vitro*. All peptides were synthesized by Synt:em (Synt:em, Nîmes, France).

Subjects. All patients were HLA-$A2^+$ with stage III/ IV malignant ocular melanoma, except one (patient 6) who had cutaneous melanoma. Patients were injected according to the following schedule: 300 μg of each peptide, 80% *s.c.* at one site, 20% *i.d.* at one site, distant from tumour. Injections were made once a week during the first five weeks, then every two weeks. Leucapheresis was made after informed consent, before onset of treatment and after three months of treatment. Cells from the two time-points were tested in the same experiment. Patients 2-4 had stable disease within the three months of testing while patients 1 and 5 showed a sustained improvement of disease. The two well-responding patients were also tested 9 months after onset of treatment. Patients 6-10 had progressive disease. Cells from healthy HLA-$A2^+$ blood donors were used for experimental set-up tests as well as for the preparations of DC used in the experiments with the patient samples.

Preparation of $CD8^+$ cells. For experimental set-up tests, mononuclear cells from heparinized peripheral blood were isolated by Ficoll-Paque (Pharmacia-Upjohn, Stockholm, Sweden) gradient centrifugation. For freezing, the cells were put at 10^7 cells/ml in tissue-culture media (TCM=RPMI supplemented with Gentamicin (25 mg/ml), 2mM L-glutamine and 10% heat-inactivated foetal calf serum) containing 10% Dimethyl sulphoxide (DMSO) and frozen gradually 1˚C/minute to -80˚C in a freezing container (Nalgene Cryo 1˚C, Nalge Company, USA). Cells from leucapheresis were used from the patients. After thawing, $CD8^+$ cells were separated with MACS-negative separation according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). We obtained between 85 and 95% purity as detected by FACS. The cells from the two time-points were analysed in the same ELISPOT plate.

Preparation and cultivation of DC. Monocytes were separated from leucopack by adhesion to plastic for 2 hours, and non-adherent cells were washed away with PBS. Cells were plated at 1 million CD14+/CD45+ cells /ml after washing in PBS-HSA. Monocytes were cultured in IL-4 and GM-CSF (14) for 7 days with cytokines replaced every second day and then frozen in 90% human serum and 10% DMSO. The cells were HLA-A2-positive as detected in FACS (not shown). By using frozen DC we could use the same batch for several experiments. After thawing, DC were loaded with peptide (1 μg/ml), added at 24 hours and again at 4 hours before the experiment, and then washed before transfer to ELISPOT-plates.

ELISPOT. The reverse ELISPOT assay was performed as described before (18). Briefly, the coating mAbs were diluted to a concentration of 10 μg/ml in sterile, filtered (0.45 mm) phosphate-buffered saline (PBS, pH 7.2), and 100 μl per well were added onto nitro-cellulose plates (Multiscreen, Millipore Corp., Bedford, MA, USA). Plates were incubated overnight at 4˚C and thereafter unbound antibodies were washed away with filtered PBS. Fifty μl of the pre-stimulated DC suspension (5,000 DC/well) and 50 μl of the separated $CD8^+$ cells or the PBMC (50,000 cells/well) were added to each well and the plates were incubated for 18 hours at 37˚C. The cells were washed away and 100 μl of the biotinylated mAbs (1 μg/ml) were added and incubated for two hours at room temperature (RT). Thereafter, the plates were washed and incubated for 90 minutes at RT with 100 μl of streptavidin-alkaline-phosphatase (MABTECH, Stockholm, Sweden) diluted 1/1000 in PBS. Unbound conjugate was removed by another series of washings and finally 100 μl of BCIP/NBT substrate solution (Bio-Rad, Richmond, USA) were added and incubated at RT until dark

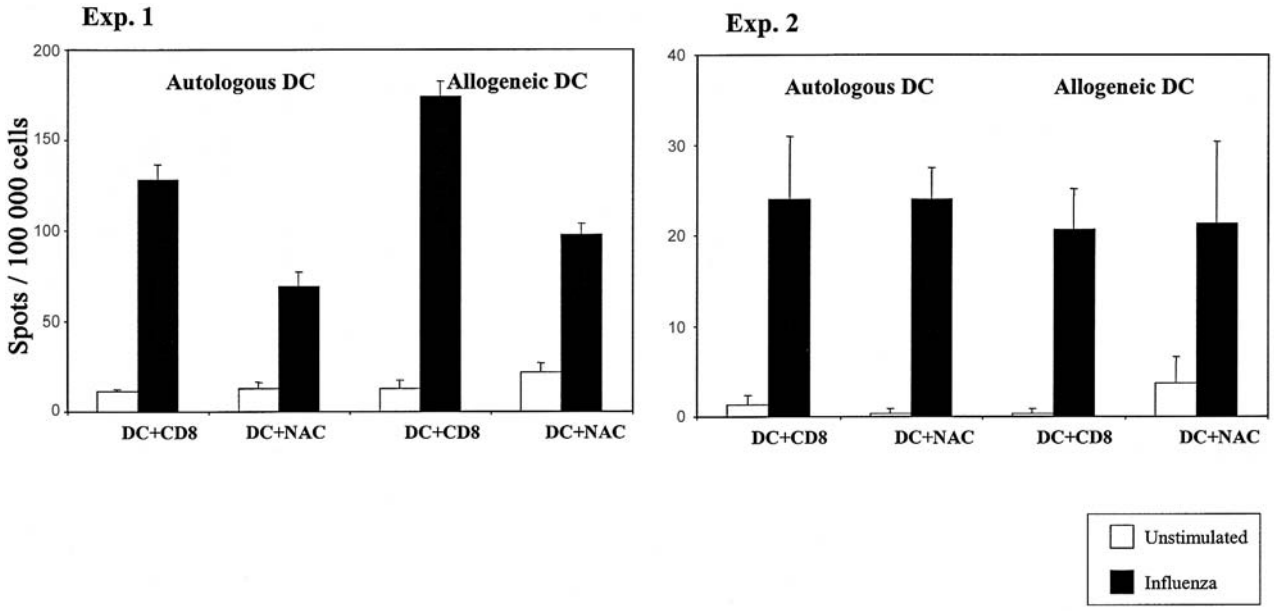

Figure 1. *Comparison of allogeneic or autologous DCs as APC. The IFN-γ response was compared in response to the individual's own or allogeneic HLA-A2$^+$ peptide-pulsed DC. No major differences were seen. NAC = non-adherent PBMC.*

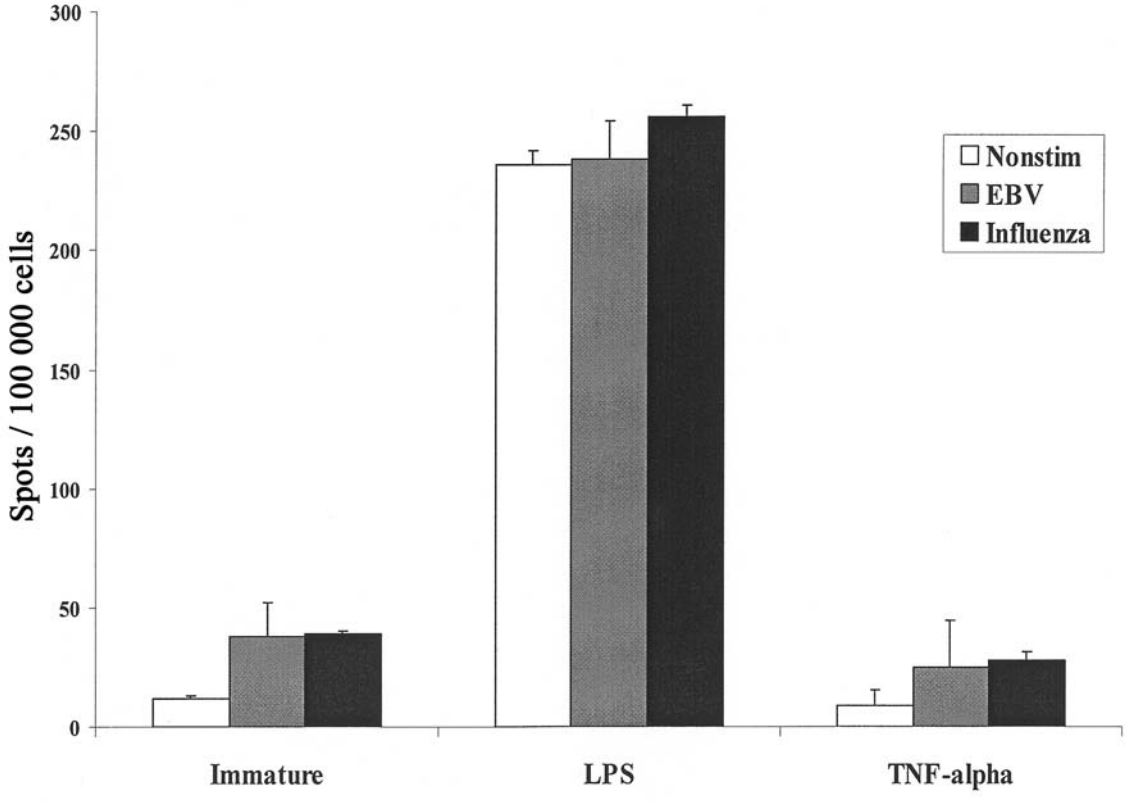

Figure 2. *Matured or immature DCs as APC. LPS or TNF-α was added at 24h and peptide was added at 24 and 4 h before harvesting of DC. CD8+ cells and pre-incubated DC were incubated for 18h in ELISPOT plates. Results represent mean IFN-γ spots of triplicates +SD.*

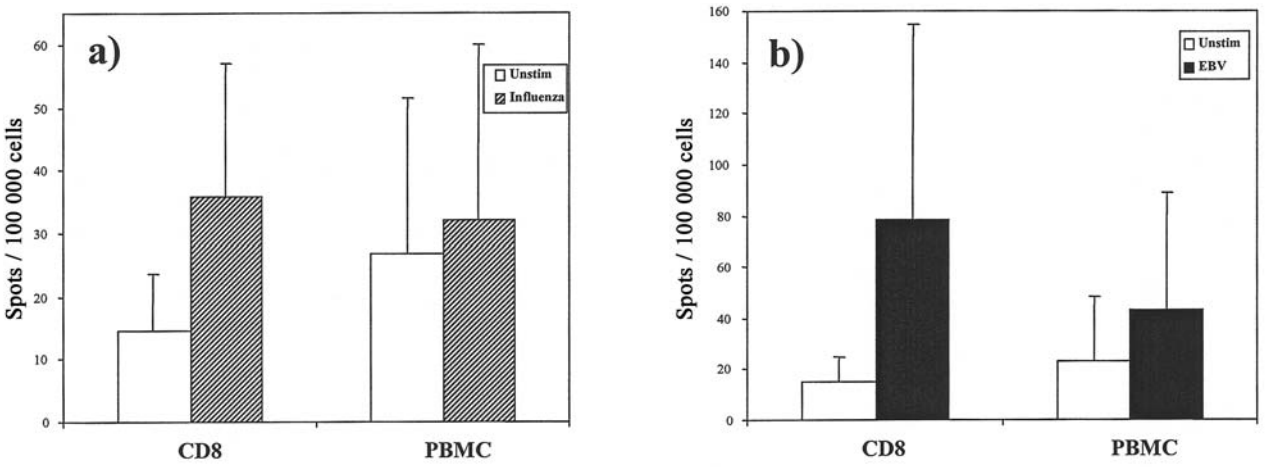

Figure 3. *Purification of CD8+ cells increases sensitivity of the allogeneic ELISPOT. Response to peptide-stimulated DCs was compared for purified CD8+ T cells (MACS negative selection) or PBMC. Only responding patients are included, n=5 for EBV, n=6 for influenza. Cells were incubated for 18h in ELISPOT plates in triplicates. Filled bars represent stimulated cells and unfilled bars represent unstimulated cells. Results represent mean values +SD.*

spots emerged (1-2 hours). The colour development was stopped by repeated washings with tap-water. After drying, the spots were counted in a dissection microscope (x40).

Results

Dendritic cells. DC made from two HLA-A2$^+$ donors were used. DC at day 7 were CD1a$^+$, CD14$^-$, MHC class I$^+$, MHC class II$^+$, CD83$^-$, CD86$^+$ and CD40$^+$ as measured by FACS (data not shown). The DCs were tested for antigenic presentation capacity together with the human CD8$^+$ T cell clone LT12 (Table I). The number of spots in response to influenza peptide-pulsed DC were in the same magnitude as the theoretical number of LT12 cells added to the wells supporting a strong antigen-presenting capacity of the DC.

Allogeneic or atologous DC. The MHC class I restriction was tested and verified since peptide responses were inhibited when adding anti-MHC class I antibodies (mAb W6.32, data not shown). To compare efficiency of antigen presentation between autologous and allogeneic DC, the IFN-γ response from both CD8$^+$ cells and non-adherent cells (NAC, PBMC after removal of adherent cells used for DC preparations) to influenza peptide was compared between autologous DC and allogeneic, HLA-A2-positive DC pulsed with peptides (Figure 1). The responses were shown to be very similar whether autologous or allogeneic DC were used, both for CD8+ cells and NAC. However, the background was higher with NAC and the allogeneic DC. We also tested the addition of maturation stimuli to the DC at the same time as the addition of peptide (Figure 2) to see if it would increase the specific CD8+ T cell response to the peptide. However, TNF-γ did not increase the specific response and LPS only increased the number of background spots. The responses to EBV and influenza peptide-pulsed DC were compared between separated CD8+ cells and PBMC (Figure 3). The background values (allogeneic response, without peptide added) for PBMC were generally higher than for CD8+ cells; mean for CD8+ cells 12.7, range 2.7-34, while the mean for PBMC was 48.8, range 3-204 per well. As expected, the number of virus-specific IFN-γ spots were higher in wells with purified CD8$^+$ cells than PBMC, however, it was not as high as would be expected if all responding cells in the PBMC cultures were CD8+ cells, since 10% of PBMC would be expected to be CD8+ cells.

Melanoma patients. The response to melanoma peptides before and after 3 months of peptide vaccination was tested in ten patients. The allogeneic response was low in all except one patient (patient 2, 140 spots per 100,000 cells) and this individual was excluded from the study. In one patient (patient 8) we obtained too few CD8$^+$ cells to perform the ELISPOT assay.

Both increases and decreases of melanoma peptide responses could be detected, in both the clinically responding and the non-responding group (Figure 4). In several individuals, increases of the melanoma response could be detected while the response to EBV or influenza remained constant (patients 1, 3, 4, 6, 7, 9).

In the two highly responding patients, patients 1 and 5, we also tested the CD8+ response after 9 months. No response could be detected either at 3 or at 9 months in patient 5 (not shown). In patient 1 the response to

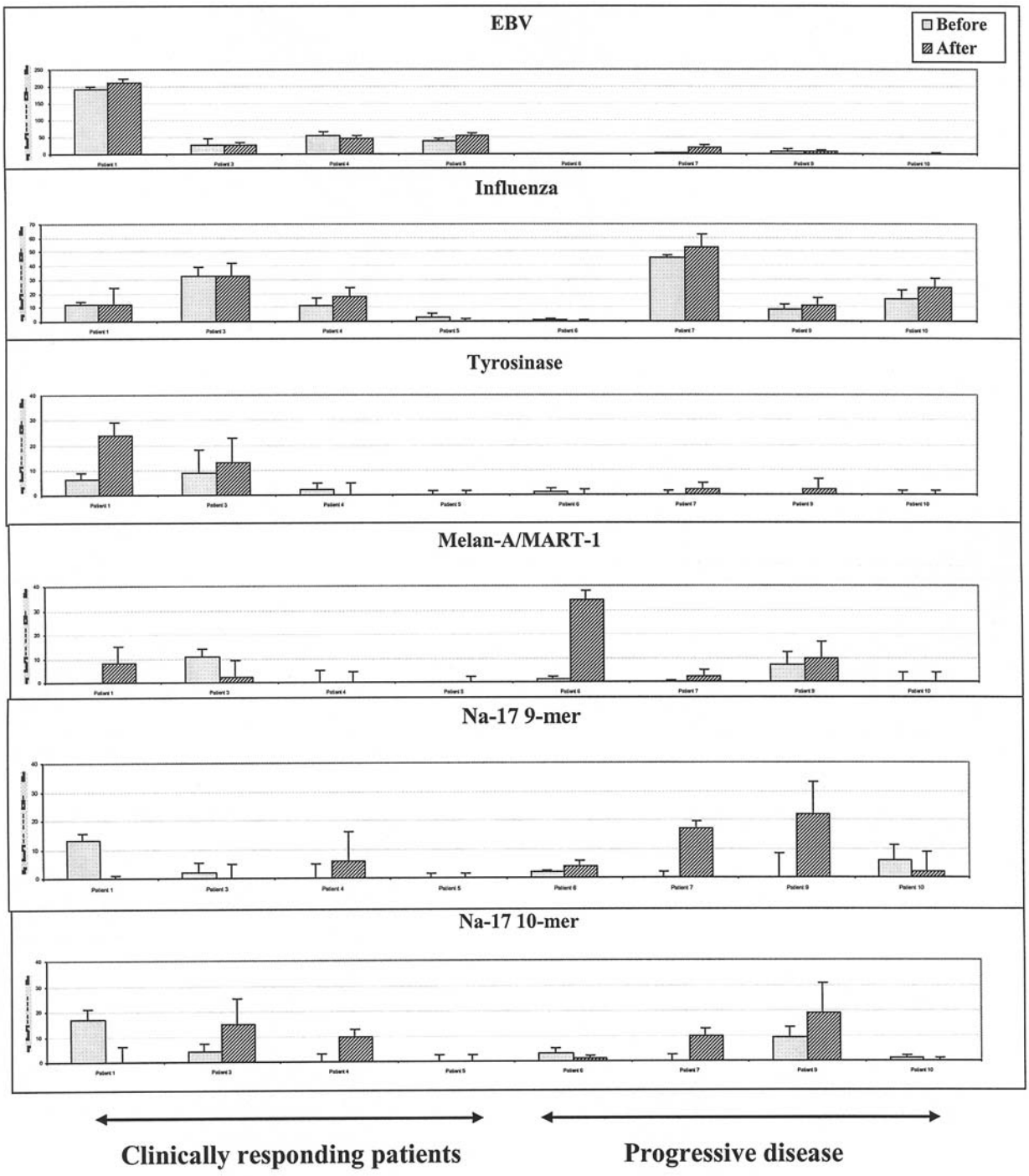

Figure 4. *Peptide responses in melanoma patients before and after peptide therapy. Results represent IFN-γ spots in ELISPOT / 100,000 CD8+ cells as measured by FACS. CD8+ cells were enriched by MACS-negative selection and the purity was between 85 and 95%.*

Tyrosinase increased from 3 to 24 spots after 3 months, and after 9 months this response was 15 spots. No increase of IFN-γ-producing cells was detected in response to the other peptides.

Discussion

Our results show that allogeneic DCs can be used as APC in an ELISPOT assay. The use of allogeneic instead of

autologous DCs could be argued not to be optimal for the assay, since the allogeneic response might disturb the interpretation of the results. However, this might in many cases be the only way to obtain sufficient number of cells for an *in vitro* production of DC, which can be used for T cell stimulation. The use of DCs from healthy individuals instead of the patient's own DCs may even improve the sensitivity of the assay, as it has been reported that DCs from cancer patients may have a decreased capability of antigen presentation (19, 20) which may be manifested as a decreased detection of influenza-reactive T cells (21). Furthermore, our results indicate that there was very little difference between autologous and allogeneic DCs, when a pure CD8+ population was used. Additionaly, the use of pure DCs and CD8+ T cell populations gives less variation of cell composition than the use of PBMC as APC, as in the classical ELISPOT. The resulting population after a single Ficoll Paque separation is variable in terms of number of monocytes and other cell populations that can function as APC or give help in the form of co-stimulatory molecules or cytokines.

Our DCs, which were produced in serum-free medium, were CD86+ and CD83-. However, these DCs were highly capable of inducing a specific CD8+ response, as indicated by EBV and/or influenza-specific IFN-γ spots. We used no further maturation stimuli, since TNF-α or LPS-treated DCs did not increase the number of antigen-specific spots. After LPS treatment the cells were fully mature, but this resulted in a higher background, probably due to an increased allogeneic reaction. However, different combinations of IL-1, IL-6, TNF-α and PGE2 (22) could be tried to further optimise the T-stimulatory capacity, though this is likely to induce more unspecific spots as well. The response to DCs without or with peptide showed that a purified CD8+ cell population gives a more specific response compared to adding PBMC to the DCs, since the background is lower and the absolute numbers in the stimulated wells are higher in the wells with purified CD8+ cells.

The responses to the peptides in the peptide-vaccinated patients showed a great variability, but an increase after vaccination to some peptides were seen in most patients. However, the stable responses to EBV and influenza shows the reproducibility of the method. In some patients, a pre-vaccine response was detected, which also has been shown in previous studies (23). The responses were similar when we divided the patients into a responding and a non-responding group. This suggests that measurement of IFN-γ-producing CD8+ cells might not be the only parameter decisive for the clinical outcome. We saw great variations in $CD8^{low}$ cells (not shown), probably indicating large differences in NK cell numbers. NK cells can kill tumor cells directly as well as generate tumor-specific cytotoxic T lymphocytes (24). Other factors that are essential for the clinical outcome include the level of costimulatory molecules on the DCs, the level of MHC class I expression on the tumor cells, mutations leading to loss of expression of the vaccination peptides and the existence of tumor-specific CD4+ cells (25).

Comparisons of tetramer techniques and ELISPOT have shown that responses in ELISPOT are correlated to the number of CD45RO+, or memory cells, and should be a good indicator of active effector cells (26). Before vaccination $CD8^+$ cells from patients often display a naïve phenotype, which shifts to a memory phenotype after vaccination (27). However, since memory cells can change CD45 isoform expression patterns *in vitro* (28, 29), the expression of IFN-γ could be a better way to measure the occurrence of effector cells.

We used manual enumeration of spots, which can be laborious and is subject to human error, especially if many spots are present in one well. This is usually not the case with peptide-specific responses as in this study. However, computer-assisted image analyzers are now available (30, 31), possibly giving a more objective enumeration, but this needs further evaluation (32).

In conclusion, we showed that allogeneic DCs can be used as antigen-presenting cells in a system with purified $CD8^+$ cells as long as they are not mature. The use of allogeneic DCs as APC could also be employed in other cytokine-detection assays such as cytokine secretion assay. Increases in peptide responses could be seen in three responding patients after treatment, while the response to EBV and influenza remained constant. However, we also saw increases in the non-responding group. This suggests that the monitoring of the $CD8^+$ response might not give the full answer to whether there are cells with tumor-killing capacity in the patient, and the ELISPOT probably needs to be combined with testing of other parameters such as NK cell numbers or expression of MHC class I or peptide in tumoral tissue.

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