Mage-3 and Influenza-Matrix Peptide-Specific Cytotoxic T Cells Are Inducible in Terminal Stage HLA-A2.1+ Melanoma Patients by Mature Monocyte-Derived Dendritic Cells


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Mage-3 and Influenza-Matrix Peptide-Specific Cytotoxic T Cells Are Inducible in Terminal Stage HLA-A2.1+ Melanoma Patients by Mature Monocyte-Derived Dendritic Cells

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Dendritic cell (DC) vaccination, albeit still in an early stage, is a promising strategy to induce immunity to cancer. We explored whether DC can expand Ag-specific CD8+ T cells even in far-advanced stage IV melanoma patients. We found that three to five biweekly vaccinations of mature, monocyte-derived DC (three vaccinations of $6 \times 10^6$ s.c. followed by two i.v. ones of 6 and $12 \times 10^6$, respectively) pulsed with Mage-3A2.1 tumor and influenza matrix A2.1-positive control peptides as well as the recall Ag tetanus toxoid (in three of eight patients) generated in all eight patients Ag-specific effector CD8+ T cells that were detectable in blood directly ex vivo. This is the first time that active, melanoma peptide-specific, IFN-γ-producing, effector CD8+ T cells have been reliably observed in patients vaccinated with melanoma Ags. Therefore, our DC vaccination strategy performs an adjuvant role and encourages further optimization of this new immunization approach. *The Journal of Immunology, 2000, 165: 3492–3496.

Materials and Methods

Eligibility criteria

Inclusion criteria were stage IV cutaneous melanoma (i.e., distant metastases) progressive despite chemotherapeutic or immunotherapeutic treatment, HLA-A2.1+, Mage-3 mRNA expression in one or more metastasis, expected survival 4 mo or more, Karnofsky index 60% or more, age 18 years or older, informed consent given, and effective contraception use. Important exclusion criteria were active CNS metastasis, any significant psychiatric abnormality, severely impaired organ function; Abs to HIV-1/2, HTLV-1/2, hepatitis B virus, or hepatitis C virus; active autoimmune disease (except vitiligo); pregnancy; systemic chemotherapeutic, radiotherapeutic, or immunotherapeutic 4 wk or less before the study.

Clinical protocol, DC generation, and immunization schedule

The study was performed at the Departments of Dermatology in Erlangen, Würzburg, and Mainz. It was approved by the local ethics committees and the protocol review committee of the Ludwig Institute for Cancer Research (New York, NY) and was performed under the supervision of its Office of Clinical Trials Management (LUD 97-011). Five DC vaccinations were administered at 14-day intervals. To explore the toxicity and efficacy of the vaccine, the following two were given i.v. (6 and $12 \times 10^6$ DC for vaccinations 4 and 5, respectively). DCs were pulsed with all DC with Mage-3A2.1 tumor peptide (10) (FLWGPRALV, GMP quality, Clinalfa, Laufelfingen, Switzerland) as well as the IM A2.1 peptide (GILGFVFTL, Clinalfa, Laufelfingen, Switzerland) as a positive control.
Recall Ag specific proliferation and cytokine production. PBMC (3 and 10 $\times$ 10^6/well) were cultured with or without TT (at 0.1, 1, and 10 $\mu$g/ml) and pulsed on day 5 with $[^{3}H]$thymidine exactly as previously described (6).

Measurement of CD8$^+$ T cell responses in uncultured PBMC. IFN-γ-releasing (i.e., effector) Mage-3 (6) and IM peptide-specific (11), CD8$^+$ T cells were quantified with an ELISPOT assay exactly as previously described. HLA-A2.1-restricted Mage-3, IM, or HIV-1 gag (SLYNTVATL; as negative control) peptides were used at 10 $\mu$M. The number of spot-forming cells was calculated per 1 $\times$ 10^5 DC$^+$ T cells to allow comparison of frequencies at the different time points and with the results of other assays (see below).

Recall CD8$^+$ T cell responses in tissue culture: binding to HLA class I tetramer complexes. Soluble IM and Mage-3 HLA A2.1 tetramers were prepared, and binding to T cells was analyzed by flow cytometry at 37°C as previously described (12). Frozen aliquots of PBMC were thawed and analyzed following a 7-day culture in the presence of 10 $\mu$M Mage-3A2.1 or IM A2.1 peptide (vs no peptide as control). As shown in Fig. 2, the frequency of spot-forming cells was elevated about 6-fold in CD8$^+$ T cells (IL-2 (100 IU/ml), and IL-7 (10 ng/ml).

Recall CD8$^+$ T cell responses in tissue culture: semiquantitative assessment of CTL precursors (CTLp). The multiple microculture method developed by Romero et al. (13) was used, as previously described (6), to determine CTLp.

Statistical analysis

A repeated measurement ANOVA model was chosen, because samples were repeatedly taken from the same patients. This model allows adjustment for covariates that potentially modify the connection between the response and the background. The covariates included in the model were time (i.e., time point of blood sampling), peptide (i.e., IM or Mage-3), and PBMC (i.e., number of PBMC used for the analyses).

**Results**

Patients and DC vaccine

Twelve far-advanced stage IV melanoma patients were enrolled. Four died after two or three vaccinations, while the remaining eight patients (five in Erlangen, two in Wurzburg, and one in Mainz) received all five DC vaccinations at 14-day intervals (three vaccinations at 6 million DC s.c. followed by two i.v. ones with 6 and 12 million DC, respectively), and were thus fully evaluable. The DC were pulsed with Mage-3A2.1 and IM A2.1 peptides, and in all but three patients (05, 06, and 07) also with TT. All DC preparations fulfilled the release criteria described in Materials and Methods.

**Toxicity**

No major (more than grade II) toxicity occurred. However, we observed mild inflammatory reactions at the s.c. vaccination sites (all patients except 05), fever (all except 03), and also slight lymph node enlargements (all except 09) developing 2 days or more after vaccinations 1–3. Due to transient grade II fever (39.9°C) in patient 01, TT pulsing of the DC was omitted after the s.c. vaccinations. Nevertheless, transient grade II fever (in the absence of infection) still occurred after the fourth (first i.v.) DC injection, but was not observed at the final fifth vaccination when IM peptide was also omitted.

Clinical responses

No significant tumor responses were observed at the final evaluation 2–3 wk after the fifth vaccination. All patients showed disease progression except patient 07, whose extensive lymph node metastases appeared stable.

**Immune responses**

**TT specific, CD4$^+$ T cell responses.** In all five patients treated with TT-pulsed DC a significant boost of recall Ag-specific immunity was evident in proliferation assays (Fig. 1), indicating that the DC employed were indeed stimulatory. In sharp contrast, immunity to TT did not increase in patients 05, 06, and 07, whose DC had not been pulsed with TT (Fig. 1), emphasizing the Ag specificity of the DC vaccine.

**IM A2.1-peptide specific CD8$^+$ T cell responses.** To monitor the kinetics of the CD8$^+$ responses, fresh blood samples were drawn every 14 days and were analyzed by IFN-γ ELISPOT. As it was unlikely that Mage-3-specific CTL would be induced in the far-advanced melanoma patients, all DC were also loaded with the IM A2.1 peptide as a positive control. As shown in Fig. 2, the frequency of IM peptide-specific CD8$^+$ T cells increased in all patients following DC vaccination, albeit with varying degree and rapidity.

Recall assays employing tetramer and CTLp analyses provided evidence for the expansion of IM peptide-specific memory CTLs (Fig. 3 and Table I). This increase was not caused by intercurrent influenza infection, as shown by repetitive serological influenza tests.

**Mage-3A2.1-peptide specific CD8$^+$ T cell responses.** ELISPOT analysis of freshly drawn blood samples clearly revealed that Mage-3A2.1 peptide-specific, IFN-γ-releasing effector CD8$^+$ T cells increased in all patients upon vaccination (except in patient 06, who had significant frequencies of Mage-3A2.1-specific T cells already at onset; Fig. 2). Patients 01, 03, and 07 produced high frequencies of spot-forming cells ($\geq$1/1000 CD8$^+$ T cells),...
while the numbers in all other patients were 1 log lower (Fig. 2). Peak responses were often reached after the s.c. vaccinations (patients 03, 04, 05, 07, and 10) and declined upon successive i.v. injections (Fig. 2). In two patients (01 and 09) an alternative pattern appeared, in that a significant response was not detectable until the first and second i.v. vaccination (i.e., vaccinations 4 and 5, respectively; Fig. 2). The number of IFN-γ-releasing CD8+ T cells on a few occasions (sporadic low frequency in patient 04 at the time of vaccination 5, and significantly decreased frequencies only at the time of vaccinations 2 and 3 in patient 06 despite an interassay variability of $\pm 20\%$) for unknown reasons did not fit into the two patterns that had become apparent (i.e., initial increase followed by a decline vs increase until the end of the study).

In the three patients (05, 06, and 07) whose DC were not loaded with TT, varying levels of Mage-3A2.1 peptide-specific T cells were already present at the beginning of the study. Therefore, we do not know whether TT, which was included as a fail-safe internal control for immunization, was also critical in providing tumor-nonspecific help for CTL induction, e.g., by conditioning DC (1, 2). Recall cultures provided evidence for the expansion of Mage-3 specific precursors. After one in vitro restimulation, lytic effector cells were demonstrable in all except patient 10, but due to insufficient numbers of CD8+ T cells, the CTLp frequency and its change over time could not be evaluated critically, except in patients 01 (0 vs 30/10^7 CD8+ T cells) and 09 (0 vs 50/10^7 CD8+ T cells). The observed postimmunization frequency is well above the frequency found in individuals without cancer ($\sim 6/10^7$ CD8+ T cells) (14). In these two patients sufficient numbers of T cells were available to allow replication of the recall assay and confirmation by tetramer analysis of the increase in Mage-3A2.1-specific memory CD8+ T cells in patient 01 (0.04 vs 1.70%; see Fig. 3) as well as patient 09 (0.04 vs 0.14%; not shown). Of note is that in both patients despite a comparable frequency of Mage-3A2.1- and IM peptide-specific, IFN-γ-producing effector T cells (0.21 vs 0.28% in patient 1, 0.05 vs 0.12% in patient 09), the recall assays revealed a much higher percentage of IM peptide-specific CTL precursor cells (21.05% HLA-A2.1/IM-peptide tetramer binding cells vs only 1.7% HLA-A2.1/Mage-3 peptide tetramer-positive cells in patient 01 and 20.13 vs 1.14% in patient 09). These data indicate that the Mage-3A2.1-specific precursors proliferated much less in the vitro assays than the IM peptide-specific CTL precursors.

**Discussion**

To the best of our knowledge this is the first study employing a defined tumor Ag for vaccination that demonstrates the consistent induction of effector, IFN-γ-producing CD8+ T cells in blood ex vivo without prolonged in vitro stimulation. Of note is that the responses to the Mage-3 peptide appear comparable to the responses to the IM peptide that served as a positive control even though the Mage-3 specific CTL precursor frequency is very low even in noncancer patients (14). This has not previously been reported for either peptide (8, 15) or DC (3–6) vaccination. The lack of a significant clinical response despite induction of Mage-3A2.1-specific effectors could be explained by the recent finding that this

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**FIGURE 2.** Generation of IFN-γ-releasing, effector Mage-3A2.1 (left) as well as IM A2.1 peptide-reactive (right) CD8+ T cells upon vaccination with Mage-3/IM peptide-pulsed DC as demonstrated by ex vivo ELISPOT analysis of freshly drawn blood. The numbers of IFN-γ spot-forming cells above background/10^5 CD8+ T cells are given on the y-axis, whereas the x-axis indicates the time points of blood sampling (performed before therapy (pre), then every 14 days before the next vaccination (e.g., #2 means immediately before vaccination 2, i.e., 14 days after vaccination 1), and finally 14 days after therapy (post)). The patient code is given in parentheses. The increase in spot-forming cells upon vaccination was statistically highly significant ($p = 0.0088$ for Mage-3, $p = 0.0001$ for IM), as was the difference from the background ($p = 0.0050$ for Mage-3, $p = 0.0004$ for IM). The highest spot numbers reached are also highlighted as frequencies within CD8+ T cells (percentage of CD8+ T cells).
FIGURE 3. Increased memory CD8\(^+\) T cell responses to IM and Mage-3 peptides upon vaccination with IM/Mage-3A2.1 peptide-loaded DC, as demonstrated by HLA-A2.1/peptide tetramer analysis. Cryopreserved PBMC samples (patient 01) taken before therapy and 14 days after the fifth DC vaccination were thawed and assayed together after in vitro restimulation for 7 days with the respective peptides, IL-2, and IL-7. Expansion of IM- as well as Mage-3A2.1 peptide-specific CD8\(^+\) T cells post-vaccination is apparent (the percentage of tetramer-binding CD8\(^+\) T cells is noted in the upper right quadrant). Melan-A/Mart-1- and tyrosinase-peptide-specific CD8\(^+\) T cells were not detectable with respective tetramers, indicating the Ag specificity of the DC vaccination (not shown).

epitope is not well presented by most melanoma cells (16). Evidence for expansion of specific memory CD8\(^+\) T cells following vaccination was also obtained by in vitro recall assays using standard cytotoxicity as well as tetramer binding as a readout. Furthermore, expansion of TT-specific recall responses was evident in all patients vaccinated with DC who were pulsed also with TT.

A possible explanation for the effective immunization we observed may be our use of mature DC and the (superficial) s.c. administration route, whereas others have employed immature DC and i.v. or intranodal injection (3, 5). In a recently reported trial we used mature, Mage-3A1 peptide-loaded DC, but we failed to reliably demonstrate preactivated Ag-specific CD8\(^+\) T cells, as IFN-\(\gamma\)-producing CD8\(^+\) T cells in this previous study were found only in 2 of 11 patients and merely at a single time point (6). There are several potential explanations for this apparent difference. It might, for example, simply be easier to induce immunity to the Mage-3A2.1 epitope, as the CTLp frequency for this epitope is at least twice that for the Mage-3A1 peptide (14). Another possibility is that active CD8\(^+\) effectors were sequestered in (regressing) metastases in the Mage-3A1 trial (6), but not in the current trial where no regressions occurred. An alternative explanation is that the kinetics of the Mage-3A2.1-specific immune response are slower than those of the Mage-3A1 response, so that immunomonitoring 14 days after a preceding vaccination would still pick up elevated effectors in the case of immunization to Mage-3A2.1 but not following vaccination with Mage-3A1-pulsed DC. It is currently also unclear whether the constant detection of IFN-\(\gamma\)-producing Mage-3A2.1-specific CD8\(^+\) T cells directly ex vivo was perhaps primarily due to the fact that in the current study all 6 \(\times\) 10\(^6\) DC were given s.c., while in the previous trial (6) only half the DC were given s.c. and the other half were given intradermally. It is indeed noteworthy that a recent study used DC equivalent to those employed in the current study and also showed immunogenicity in healthy individuals upon s.c. injection (17).

We have identified in this study a DC vaccination strategy that clearly works to some extent, so that it is now much easier to address in future studies potentially important variables such as maturational state of DC, cell dose, and route and frequency of injections. Our observation that responses to Mage-3 or IM peptides (see Fig. 2) can decline significantly following the two final i.v. administrations of pulsed DC vaccinations confirms the findings of our previous Mage-3A1 DC vaccination trial (6) and implies that the i.v. route might indeed be counterproductive. The current finding that in two patients (01 and 09) a significant response was not detectable until the first and second i.v. vaccinations (i.e., vaccinations 4 and 5, respectively; see Fig. 2) points, however, to the alternative possibility that the biweekly vaccination schedule might be problematic independently of the route chosen. This concern is underscored by the recent observation in a murine model that frequent vaccinations after achieving a peak response can lead to a decrease in immunity (18). Therefore, it might be particularly critical to address this variable to exclude that too frequent injections of Ag-loaded DC induce activation-induced cell death of Ag-specific CD8\(^+\) T cells. Future studies will also require the use of peptides other than Mage-3 to assess whether the melanoma reactive cells can recognize and kill autologous tumor cells. Now that we have initial evidence for potent immunologic efficacy, we are confident that it will be relatively straightforward to rationally optimize the DC vaccination approach.

Acknowledgments
We are grateful to all patients for their confidence and cooperation. We thank H. Hinter for referring patients; T. L. Diepgen for statistical analysis; A. Knuth and T. Wölfel/W. Herr for help in establishing the semiquantitative CTL and ELISPOT assays, respectively; T. Boon, P. van der Bruggen, and A. Steinkasserer for helpful discussions; and H. F. Oetgen and E. Hoffman for their suggestions to improve the protocol and for supervising the trial.

References

Table I. Recall IM A2.1 peptide-specific CD8\(^+\) T cell responses in tissue culture

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\(a\) Cryopreserved blood samples taken before and after the five DC vaccinations were thawed simultaneously, restimulated once in vitro, and then assayed by tetramer staining (10) as well as the multiple microwell cytotoxicity method (13) to assess the frequency of IM peptide-specific CTLp. Note that in patient 06 IM peptide-specific T cells were low after therapy also in the ELISPOT analysis (see Fig. 2).

\(b\) ND. Analysis not possible due to shortage of cells or background problems.

\(c\) NE. Not evaluable as too few microwells could be tested (see Materials and Methods).


