Functions of Anti-MAGE T-Cells Induced in Melanoma Patients under Different Vaccination Modalities

Thierry Connerotte, Aline Van Pel, Danièle Godelaine, Eric Tartour, Sophie Lucas, Kris Thielemans, Gerold Schuler, and Pierre G. Coulie

Abstract
Tumor regressions have been observed in a small proportion of melanoma patients vaccinated with a MAGE-A3 peptide presented by HLA-A1, administered as peptide, ALVAC canarypox virus containing a MAGE-A3 minigene, or peptide-pulsed dendritic cells (DC). There was a correlation between tumor regression and the detection of anti–MAGE-3.A1 CTL responses. These responses were monoclonal and often of a very low magnitude after vaccination with peptide or ALVAC, and usually polyclonal and of a higher magnitude after DC vaccination. These results suggested that, at least in some patients, surprisingly few anti–MAGE-3.A1 T-cells could initiate a tumor regression process. To understand the role of these T cells, we carried out a functional analysis of anti–MAGE-3.A1 CTL clones derived from vaccinated patients who displayed tumor regression. The functional avidities of these CTL clones, evaluated in lysis assays, were surprisingly low, suggesting that high avidity was not part of the putative capability of these CTL to trigger tumor rejection. Most anti–MAGE-3.A1 CTL clones obtained after DC vaccination, but not after peptide or ALVAC vaccination, produced interleukin 10. Transcript profiling confirmed these results and indicated that approximately 20 genes, including CD40L, prostaglandin D2 synthase, granzyme K, and granzyme H, were highly differentially expressed between the anti–MAGE-3.A1 CTL clones derived from patients vaccinated with either peptide-ALVAC or peptide-pulsed DC. These results indicate that the modality of vaccination with a tumor-specific antigen influences the differentiation pathway of the antivaccine CD8 T-cells, which may have an effect on their capacity to trigger a tumor rejection response.

Introduction
Therapeutic vaccination of metastatic melanoma patients with measurable disease has thus far met objective but limited success. We focused on vaccination with tumor-specific antigens encoded by MAGE-A cancer germ line genes. These genes are expressed in many tumors of various histologic types, in male germ line cells, and at least 1,000-fold less in other normal tissues. Antigens encoded by these genes ought to be strictly tumor-specific because male germ line cells do not carry HLA molecules and therefore do not present antigenic peptides to T lymphocytes. Antigenic peptide MAGE-A3168–176, referred to below as MAGE-3.A1, is encoded by the gene MAGE-A3 and presented to CD8 T lymphocytes by HLA-A1 molecules. It has been used in vaccines consisting of either the antigenic peptide administered without adjuvant (1), or of ALVAC mini–MAGE-1/3, a recombinant canarypox virus containing a minigene encoding peptide MAGE-3.A1 (2), or of monocyte-derived dendritic cells (DC) pulsed with the peptide (3). Out of 99 vaccinated patients, 24 showed some evidence of tumor regression but only 6 had a partial or complete tumor response according to Response Evaluation Criteria in Solid Tumors guidelines (Table 1). Other therapeutic vaccination trials in patients with melanoma yielded similar proportions of tumor regressions and tumor responses (4–8).

To understand why vaccination is followed by tumor regression in some patients, and not in others, it is important to understand the immunologic consequences of vaccination. Antivaccine T cells can be monitored as to their quantity or quality. Thus far, for vaccination with antigen MAGE-3.A1, we focused on estimating the number of antivaccine T cells in peripheral blood mononuclear cells for all patients, and in tumors for selected patients (9–11). To detect these cells among peripheral blood mononuclear cells, we used a sensitive approach based on in vitro restimulation with the antigenic peptide under limiting dilution condition, followed by labeling with tetramers, and cloning (9). In patients vaccinated with peptide MAGE-3.A1, an antivaccine CTL response was found in 1 out of 5 patients who showed tumor regression and in 0 out of 10 who did not (Table 1; ref. 12). In patients vaccinated with ALVAC mini–MAGE-1/3, anti–MAGE-3.A1 CTL responses were found in 3 out of 4 patients who showed tumor regression and in 1 out of 11 who did not (Table 1; refs. 12, 13). All these CTL responses were monoclonal. Even among those vaccinated patients who showed a CTL response, many had a very low frequency of anti–MAGE-3.A1 CTL, ranging between 10–6 and 10–5 of blood CD8 T-cells (9, 13). In patients vaccinated with DC pulsed with peptide, CTL responses were found in all four patients who showed tumor regression and in none of the five who did not, with frequencies ranging between 10–5 and 10–3 of CD8 T-cells (Table 1; refs. 11, 14). Three of these four CTL responses were polyclonal (14). Altogether, these results indicated that these MAGE-3.A1–containing vaccines were often poorly immunogenic and that there was a correlation between the detection of antivaccine CTL responses and the occurrence of tumor regressions (Table 1; ref. 12).

Our working hypothesis is that the vaccinations caused the tumor regressions through the detected antivaccine CTLs. The results summarized above suggested that, at least in some...
vaccinated patients, a surprisingly low number of anti–MAGE-3.A1 T-cells sufficed to trigger a tumor rejection response. Among other possibilities, these rare antivaccine T cells could do so as a result of a particularly high affinity for the MAGE-3.A1 antigen, or a very high lytic activity against melanoma cells.

Here, we present the results of functional analyses carried out on these anti–MAGE-3.A1 T-cells. Our first objective was to find explanations for their putative antitumor activity in vivo, keeping in mind their low frequencies in the vaccinated patients. Our second objective was to examine whether different vaccination modalities with the same antigen resulted in different functions being exerted by the antivaccine T cells. The very low number of anti–MAGE-3.A1 T-cells in most of our vaccinated patients prevents robust ex vivo functional analyses. Therefore, we present here the results of experiments carried out on a representative collection of 16 anti–MAGE-3.A1 CTL clones, derived from nine melanoma patients vaccinated with either peptide, ALVAC mini–MAGE-1/3, or peptide-pulsed DC (Table 1). Eight of these nine patients showed tumor regression following vaccination. The CTL clones were screened for their lytic activity, functional avidity, cytokine secretion, and gene expression profiles.

### Materials and Methods

**Patients and CTL clones.** Patient CP64 was vaccinated with peptide MAGE-3.A1 injected without adjuvant (9). Patients EB81, LAU47, NAP33, and NAP36 were vaccinated with ALVAC mini–MAGE-1/3 (2). Patients 04, 06, and 09 received autologous DC pulsed with peptide and tetanus toxoid (3). DC were generated from adherent monocytes cultured with granulocyte-macrophage colony-stimulating factor and interleukin (IL)-4, and maturated with monocyte-conditioned medium and tumor necrosis factor-a (TNF-a).

<table>
<thead>
<tr>
<th>Vaccine</th>
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<th>CTL responses † in patients with</th>
<th>Ref.</th>
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*Tumor responses according to Response Evaluation Criteria in Solid Tumors guidelines are indicated between parentheses. †(M) indicates that the antivaccine CTL responses were monoclonal. 
†This CTL clone proliferated less than the others and could not be included in the experiments of Fig. 1 and in one experiment of Fig. 4A. 
‡Maturation with monocyte-conditioned medium + TNF-α, except for patient LB2586, for which the DC were matured with IL-1β, IL-6, TNF-α, and prostaglandin E₂. 
§Eighty-three antivaccine CTL clones were obtained from this patient, of which 70 (84%) corresponded to clonotypes 1, 2, 4, and 5 (14). 
¶Twenty-two antivaccine CTL clones were obtained from this patient, of which 13 (59%) corresponded to clonotypes 3, 4, and 6 (14). 
∫Twenty-eight antivaccine CTL clones were obtained from this patient, of which 23 (82%) corresponded to clonotypes 1, 2, and 3 (14). 
††Two-tailed Fisher's exact test.
followed by cloning (13). They were maintained by weekly restimulations with peptide-pulsed HLA-A*1 melanoma cells M22-MEL-3.0, allogenic LG2-EBV-B cells as feeder cells, IL-2, IL-4, and IL-7. Some clones were initially derived without IL-4 but with phytohemagglutinin A (PHA-IA 16 at 0.125 μg/mL; Murex Biotech), and then restimulated as above. We verified, by cloning bulk CTL populations with and without IL-4 and PHA, that these differences had no effect on our results, and in particular on IL-10 production.

Figure 2A includes published results obtained with 22 other anti-MAGE CTL clones, which were used to identify the corresponding MAGE antigenic peptides. References for these CTL clones and their peptide titration experiments can all be found at the Cancer Immunity Peptide Database.7


Binding of peptides to HLA molecules was tested in an in vitro HLA refolding assay. Briefly, biotinylated complexes of recombinant HLA molecules refolded around an antigenic peptide are immobilized on streptavidin-coated microwells, incubated for 90 s in 25 mmol/L of citrate, 150 mmol/L of NaCl at pH 3.1 to release the peptide and β2-microglobulin moieties, and incubated overnight with various concentrations of peptides in a Tris buffer (200 mmol/L) at pH 7, containing 4 μg/mL of human β2-microglobulin. The amount of reanimated HLA molecules was then measured by adding monoclonal antibody W6/32 (2.5 μg/mL), which recognizes a conformational epitope present on HLA class I heavy chains only if they are complexed with β2-microglobulin and an antigenic peptide, followed by rabbit anti-mouse immunoglobulin antibodies coupled to peroxidase.

**Functional assays.** All tests, including gene profiling, were performed on CTL clones collected 7 to 9 days after restimulation with antigen, feeder cells, and growth factors, when cell proliferation had stopped. Lytic activity was tested with classical 3HCr release assays and incubation of effector with targets over 4 h at 37°C. In Fig. 1B, E8B1-EBV-B target cells were incubated for 30 min at room temperature with various concentrations of peptide MAGE-3.A1 (EVDIPGHL) before adding the CTL clones. Cytokine production was tested on 20,000 CTLs incubated overnight in microwells precoated with OKT3, washed, and resuspended in PBS + 1% human serum. Anti-CD40L antibodies coupled to PE (clone 89-76; BD Biosciences) or control isotype IgG1 coupled to PE (BD Biosciences) were added for 15 min at 4°C. Cells were washed, fixed, and analyzed by flow cytometry.

**Gene expression analysis.** For gene profiling, 5 × 106 cells of each CTL clone were activated for 6 h on inosulphosphorylated monoclonal anti-CD3 antibody UCHT1 (R&D Systems). RNA was extracted using the TriPure Isolation Reagent (Roche Diagnostics Belgium) and its quality assessed on agarose RNase-free gels.

Biotin-labeled cRNA was prepared from 5 μg of RNA using the one-cycle target labeling procedure (Affymetrix, P/N 900493), and hybridized to HG-U133A gene chips (Affymetrix), which were then scanned on the Gene Array Scanner (Hewlett Packard). Data acquisition and processing was conducted with Affymetrix Microarray Suite 5.0 and EXCEL. For real-time quantitative PCR (qPCR), RNA was extracted from 5 × 106 cells activated for 6 h with UCHT1 as above, using the TriPure Isolation Reagent (Roche), reverse transcription with the Powerscript Reverse Transcriptase (Clontech Laboratories), and RNase H digestion (Invitrogen SA). PCR amplifications were conducted in a final volume of 25 μL with 0.6 units of HotGoldStar DNA polymerase (Eurorgenate SA), 300 mmol/L of each primer, 100 mmol/L of probe, 200 μmol/L of deoxynucleotide triphosphate, and 5 mmol/L of MgCl2 in an ABI Prism 7700 Sequence Detector (Applied Biosystems) under standard conditions: 94°C for 10 min, 45 cycles of 94°C for 15 s, and 60°C for 1 min. The following oligonucleotides were used (5′ to 3′ sense, antisense, and probe, respectively): β-actin, ATTCGGCAAGAGATGAGAAG, GTCATACCTCCTGGTCTGCTGA, and FAM-TCAGATCTCATTGGTCTCCT-GAGCT-TAMRA; granzyme K (Gzmk), GACATGTTCGCTGCCAGGATGT, CAGGGCTTTTGTTGGCAACAC, and FAM-ATACTTCCTCGGGGTATCAG-TAMRA; granzyme H (GzmH), TGTCCTCAATGAGCATTCTGAGCAAC, CTGGAGGTGTCCTTTTGTGT, and FAM-TAAGGAGCTGACCCAAGGT-TAMRA; prostaglandin D2 synthase (PGD2S), CCTCTTATTGACATGATGGTCA, CAYGATCGATCAGTGGTCTG, and FAM-AGGATTCCTCG-CGTGCTAACT-G-TAMRA.

**Results**

**Anti–MAGE-3.A1 CTL clones.** The 16 anti–MAGE-3.A1 CTL clones included in this study are listed in Table 1. All of them were derived from blood lymphocytes of vaccinated melanoma patients, and described in previous reports (9, 13, 14). All these CTL clones were derived from patients deemed to have mounted an antivaccine CTL response on the basis of a ≥10-fold increase in the blood frequency of anti–MAGE-3.A1 T-cells after vaccination, or of the presence of repeated clonotypes in a set of postvaccination anti–MAGE-3.A1 CTL clones (12). All clones, with the exception of CTL clone 1 from patient NAP36, were derived from patients who showed tumor regression following vaccination. All clones had different TCRB gene rearrangements (data not shown), were lytic, and recognized melanoma cells that naturally expressed the MAGE-3.A1 antigen.

The CTL clones of this study were derived from nine patients (Table 1). For six patients, the anti–MAGE-3.A1 CTL responses were

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monoclonal (Table 1), which means that the analyzed CTL clone represented the complete antivaccine T-cell response. For three patients, the antivaccine CTL responses were polyclonal (Table 1), and the three or four CTL clones that we analyzed for each patient represented 59% to 84% of their antivaccine T-cell responses (Table 1).

Comparing functional avidities of anti–MAGE-3.A1 CTL clones induced by peptide, ALVAC, or DCs. Our previous results indicated that antigen MAGE-3.A1 administered as peptide without adjuvant or as ALVAC mini–MAGE-1/3 was poorly immunogenic because (a) a small proportion of the patients mounted an antivaccine CTL response, (b) the frequencies of the antivaccine T cells were often low, in the range of $10^{-5}$ to $10^{-7}$ of blood CD8 T-cells, and (c) these responses were monoclonal (9, 12, 13). On the contrary, they were polyclonal in three of four patients vaccinated with DC pulsed with peptide (14). Therefore, we hypothesized that the peptide-ALVAC vaccines preferentially primed anti–MAGE-3.A1 CTL precursors with a high-affinity T-cell receptor (TCR), whereas the peptide-pulsed DC would have primed CTL displaying a wider range of affinities.

**Figure 1.** Functional avidity of anti–MAGE-3.A1 CTL clones. A, lytic activity against melanoma cell line MZ2-MEL 3.0, which expresses genes HLA-A*0101 and MAGE-A3. No exogenous peptide was added. B, lytic activity against HLA-A1+ EB81-EBV–transformed B cells incubated with the indicated concentrations of peptide MAGE-3.A1. The CTL to target ratio was 10:1. Shaded areas, the ranges of peptide concentrations required to obtain 50% maximal lysis of the target cells. Left, CTL clones derived from melanoma patients vaccinated with peptide alone or with ALVAC mini–MAGE-1/3. Right, CTL clones derived from patients vaccinated with DCs pulsed with peptide MAGE-3.A1.
To address this point, we estimated CTL functional avidities in lysis assays. Functional avidity, which is easy to measure and usually consists of titrating the antigenic peptide on antigen-presenting cells, cannot be considered as a reliable surrogate marker for the intrinsic affinity of the TCR. Because it integrates several variables such as TCR affinity itself, the influence of coreceptors such as CD8, the role of accessory adhesion molecules, or topographical distribution of the TCR at the cell surface, it is a good measure of the efficacy of antigen recognition by a CTL. In a first set of experiments, we used HLA-A1+ MAGE-A3+ melanoma target cells and compared four anti–MAGE-3.A1 CTL clones derived from patients vaccinated with peptide or ALVAC (peptide-ALVAC group) with nine clones derived from patients vaccinated with peptide-pulsed DC (DC group; Fig. 1A). All clones displayed similar lytic activities, with the exception of two clones from the DC group, for which the maximal levels of lysis were slightly lower (Fig. 1A). In a second set of assays, the target cells were HLA-A1+ EBV-transformed B cells incubated with increasing amounts of peptide MAGE-3.A1. The concentrations of peptide required to obtain 50% maximal lysis were similar for the two groups of CTL clones, ranging between 30 and 300 nmol/L for the peptide-ALVAC group, and between 80 and 300 nmol/L for the DC group (Fig. 1B). We concluded that the peptide-ALVAC or DC vaccines stimulated anti–MAGE-3.A1 CTL precursors of similar avidity. These functional avidities were not very high, as compared with those reported in the literature for anti-MAGE CTL clones tested under similar conditions and recognizing 22 other HLA/MAGE peptide combinations (Fig. 2A).

This difference did not result from a poor binding of peptide MAGE-3.A1 to HLA-A1 molecules because in an in vitro assay, the peptide induced the refolding of recombinant HLA-A1 molecules as efficiently as of HLA-B35 molecules and as efficiently as two other MAGE peptides did it for HLA-A2 molecules (Fig. 2B). As indicated in the legend of Fig. 2B, two of these other HLA-peptide combinations were recognized by CTL clones displaying a much higher functional avidity than that of the anti–MAGE-3.A1 CTLs. We concluded that high avidity was unlikely to be crucial to the capacity of the anti–MAGE-3.A1 CTL to trigger tumor regressions.

Anti–MAGE-3.A1 CTL clones of the peptide-ALVAC and DC groups differ in their pattern of cytokine secretion. Screening the panel of CTL clones for their production of cytokines, we observed an unexpected difference between the peptide-ALVAC and DC groups of clones. Stimulation of the clones for 20 hours with the HLA-A1+ MAGE-A3+ melanoma cells MZ2-MEL.0 led to the production of IL-10 by all of the 11 DC CTL clones, whereas only 2 of the 5 peptide-ALVAC clones produced a detectable amount of IL-10 (Fig. 3A). This difference in IL-10 production between the two groups of clones was statistically significant (P < 0.0002). No significant difference was observed for the production of IL-5, IL-4, IL-2, IFN-γ, and TNF-α (Fig. 3A).

Because a few reports describe the secretion of IL-10 by human CD8+ lymphocytes displaying suppressive activity (16–19), we tested whether our IL-10–producing anti–MAGE-3.A1 CTL clones inhibited the proliferation of cocultured allogenic bulk populations of CD4+CD25+ T-cells, with negative results (Fig. 3B). As positive controls, these experiments included suppressive CD4+CD25+Foxp3+ clones, which did not produce IL-10.9

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9 S. Lucas, P.G. Coulie, unpublished observations.

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Figure 2. Functional avidities of anti–MAGE-3.A1 vs. other anti-MAGE CTL clones. A, concentrations of peptide required to obtain 50% of maximal lysis of EBV-transformed B cells. The anti–MAGE-3.A1 CTL clones (●) correspond to the 13 clones shown in Fig. 1, plus clones MZ2-CTL-20/38 (42) and EB81-CTL-103 (C; ref. 13). For the 22 other anti-MAGE CTL clones, which recognize 21 different HLA/MAGE peptide combinations listed in Materials and Methods, values correspond with published results, obtained in experiments conducted with the same method as that used in Fig. 1B, i.e., lysis of EBV-transformed B cells expressing the appropriate HLA class I molecules and incubated with increasing concentrations of the relevant antigenic peptide. Bars, geometric means. A two-tailed Student’s t test was used to compare the two groups. B, concentrations of peptides required to refold HLA molecules in vitro, as an estimation of their affinity for these molecules. Immobilized HLA molecules were stripped of their antigenic peptides, washed, and incubated with β2-microglobulin and the indicated concentrations of antigenic peptides. Proper refolding was measured with the conformation-dependent anti-HLA class I monoclonal antibody W6/32. Peptide MAGE-3.A1 binding to HLA-A*0101 was compared with two other HLA/peptide combinations recognized by CTL clones displaying higher functional avidities than the anti–MAGE-3.A1 CTL clones, with EC50's of 0.03 nmol/L for MAGE-A2185–194 on B35 and 0.2 nmol/L for MAGE-C2191–200 on A2, and to peptide MAGE-A1054–262 recognized on A2 by a CTL clone with a functional avidity (EC50 of 100 nmol/L) similar to that of the anti–MAGE-3.A1 CTLs (mean EC50 of 133 nmol/L).
Gene expression profiling. We used gene arrays to examine whether other genes were differently expressed between the anti-MAGE-3.A1 CTL clones of the peptide-ALVAC and DC groups after activation for 6 hours by insolubilized anti-CD3 antibodies. This protocol of T-cell activation was chosen to give the same stimulus to all CTLs before examining gene expression, and to avoid the presence of stimulating or feeder cells which would have contributed to the gene profiling. Four clones of the peptide-ALVAC group were included, the fifth clone could not be tested because of poor proliferation. Among the 11 clones of the DC group, we selected 5 with a good proliferation out of the 9 that produced >0.15 ng/mL of IL-10 (Fig. 3A). Unsupervised hierarchical clustering analysis failed to separate the nine clones into the peptide-ALVAC and DC groups. To identify differentially expressed genes, we applied a supervised method focusing on genes with a large difference of expression between the two groups. Selection criteria were (a) mean expression value five times higher in one set of clones compared with the other one, (b) genes expressed in every clone from the high expression subset, and (c) no overlapping expression values between the two groups. Twenty-three genes were selected with this procedure (Table 2).

To verify that these 23 genes were not selected by chance, we used a balanced permutation analysis, applying our three selection criteria to random groups of four and five samples picked up from our total of nine. Out of the 126 possible balanced permutations, 12 representative ones were tested. The mean number of genes selected per permutation was 1.5 (0–4), indicating a 95% confidence interval of 1.5 ± 0.7 genes per test. Therefore, we estimated that the proportion of genes identified by chance in our reference analysis was 6.5 ± 4% (false discovery rate). We can conclude with 95% confidence that 90% of the genes sorted in our supervised selection were not identified by chance.

Approximately half of the 23 genes that were differentially expressed by the peptide-ALVAC versus DC clones code for proteins contributing to the differentiation or effector functions of T lymphocytes. Two genes coding for granzymes were more expressed by the peptide-ALVAC CTL group, whereas 21 genes including IL-10 were more expressed by the DC group (Table 2). These results suggested that the anti-MAGE-3.A1 CTL clones derived from patients vaccinated under different modalities have different functional properties.

To consolidate our conclusion, we applied the well-known significance analysis of microarrays (SAM) software (20). Here, each gene receives a score based on its differential expression between groups, relative to the standard deviation of measurements within each group. Genes with scores greater than a threshold are deemed potentially significant. To estimate the false discovery rate, the proportion of genes selected by chance is calculated with
permutation analysis. In a first analysis, 100 of the 126 possible permutations were tested with a false discovery rate set at 9%, and a minimal fold change at 5 to match the empirical criterion of our supervised analysis. This procedure kept only 12 genes more expressed by the DC clones, and all of them matched with our supervised analysis, as indicated in the last column of Table 2. The 11 other genes identified as differentially expressed in our supervised analysis were not selected by SAM because of a high standard deviation of the gene expression levels in one or the other of the two very small groups of clones. In a second SAM analysis, carried out with a minimal fold change of 2 but with the same other variables, 51 genes came out as more expressed by the DC CTL clones (data not shown). Altogether, the SAM analysis confirmed that the anti–MAGE-3.A1 CTL clones derived from the two groups of patients had slightly different gene expression profiles.

Quantitative expression analysis of selected genes. To confirm some of the gene expression results, quantitative real-time reverse transcription-PCR was applied to the complete panel of anti–MAGE-3.A1 CTL clones, after activation for 6 hours with insolubilized anti-CD3 antibodies (Fig. 4A). The microarray experiments indicated that the granzyme K and granzyme H genes were more expressed in the peptide-ALVAC CTL clones than in the DC group. Of note, all clones expressed the perforin and granzyme B genes equally well (data not shown). Granzyme K and granzyme H are serine proteases present together with granzymes A, B, and M in the lytic granules of human CTL and natural killer (NK) cells (21, 22). As shown in Fig. 4A, there are clear differences between the levels of expression of genes granzyme K and granzyme H by the peptide-ALVAC and DC groups of CTL clones. It is worth noting that the same CTL clones showed the highest levels of granzyme K or granzyme H expression in the microarray and qPCR experiments, and that the clones that did not express these genes according to the microarray analysis were found to contain approximately one granzyme K or granzyme H mRNA copy per cell in the qPCR. Altogether, the results confirmed a differential expression of granzyme K and granzyme H by the two groups of anti–MAGE-3.A1 CTL clones.

One of the genes found to be more expressed in the DC group of clones was PGD2S, reported to be expressed in human Th2 but not Th1 lines (23). As shown in Fig. 4A, qPCR confirmed the difference: clones of the DC group had a mean of 30 copies of PGD2S mRNA per cell.

### Table 2. Genes differentially expressed in two groups of anti–MAGE-3.A1 CTL clones

<table>
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<tr>
<th>Probe set</th>
<th>Description</th>
<th>Peptide-ALVAC group</th>
<th>Ratio</th>
<th>DC group</th>
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<td>3.6</td>
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**NOTE:** Gene expression analysis (Affymetrix array HG-U133A) was performed on four peptide-ALVAC and five DC CTL clones. The latter were selected on the basis of their production of IL-10 (>0.15 ng/mL on Fig. 2). Cells were stimulated for 6 h by insolubilized anti-CD3 antibodies. Affymetrix flags are reported in bold-type characters for significant expression, italic-type for absence of expression, and medium-type for marginal expression. Data were normalized to obtain a mean gene expression value of 10 for each CTL clone. A set of 23 genes were then selected using three criteria: (a) mean expression value at least five times higher in one group of clones compared with the other; (b) genes expressed in every clone from the high expression group, and (c) no overlapping of expression values between the two groups.

*Genes selected with the SAM software on 100 permutations, with a false discovery rate of 9%, and a minimal fold change of 5.
per cell, whereas the others had <0.5 copy per cell. However, three DC clones expressed very little PGD2S mRNA: clone 04 CTL4, which produced <0.15 ng/mL of IL-10 and was not included in the microarray analysis, and clones 09 CTL3 and LB2586 CTL1, which were also expressing low PGD2S levels in the microarray experiment (Table 2).

It is worth noting that the differential expression of the granzyme K, granzyme H, and PGD2S genes did not depend on the activation of the clones, as it was also observed without stimulation (data not shown).

Anti-CD40L labeling. To confirm the difference in CD40L gene expression, we performed anti-CD40L labeling on 15 of the 16 CTL clones, with and without stimulation for >6 hours with insolubilized anti-CD3 antibodies. Seven out of the 11 DC clones up-regulated cell surface CD40L, whereas none of the 4 peptide-ALVAC clones did so (Fig. 4B). Out of the four CD40L-negative DC clones, three were not included in the microarray (low IL-10 secretion) and the fourth was a 04 CTL 2 clone, with the lowest level of CD40L expression among the five profiled DC clones. Altogether, the cell surface labeling results are in line with those of the gene profiling, but the difference observed between the two groups of clones (up-regulation of CD40L in 0/4 versus 7/11) was not statistically significant ($P = 0.077$, two-tailed Fischer's exact test).

Discussion

Our main objective at the outset of this study on anti–MAGE-3.A1 CTL clones derived from vaccinated melanoma patients was to gain some insight into the functional properties of these T cells, keeping a close eye on what could trigger tumor rejection responses. We were particularly interested in the clones corresponding to the very low-level monoclonal responses observed in regressor patients, such as clones CP64 CTL 1, EB81 CTL 35, LAU147 CTL 1, NAP33 CTL 2, and LB2586 CTL 1 (9, 11, 13). We were mindful that our work would suffer from two serious limitations: the low number of clones that we could study, and the fact that we were analyzing established T-cell clones instead of $ex vivo$ unmanipulated lymphocytes. The very low frequencies of antivaccine T cells in most of our vaccinated patients have thus far precluded us from performing $ex vivo$ analyses of anti–MAGE-3.A1 CTL. Our secondary objective was to
compare the functions of human CD8+ T-cells induced against the same epitope through vaccinations under different modalities.

Comparing the CTL clones for their functional avidity, using either tumor cells or peptide-pulsed EBV-B cells as targets in lysis assays, did not reveal a clear difference between anti–MAGE-3.A1 T-cells induced in vivo with antigenic peptide alone, even though this only concerns a single clone, with ALVAC mini–MAGE-1/3, or with peptide-pulsed DC. Two clones from the DC group lysed fewer tumor cells compared with the other clones, but required the same amount of peptide for half-maximal lysis of EBV-B cells. An unexpected conclusion from our study is that none of the anti-MAGE-3.A1 CTL clones had superior avidity. On the contrary, as shown in Fig. 2B, it was lower than that of most of the other anti-MAGE CTL clones that we and others have analyzed with the same methodology, i.e., peptide titration on EBV-transformed B cells. Inasmuch as these assays reflect TCR affinity, the results would be compatible with some degree of central tolerance to MAGE-3.A1. This, in turn, could result from the expression of several MAGE genes in human medullary thymic epithelial cells (24). It is noteworthy that other MAGE genes, including MAGE-A1 and MAGE-A4, are also expressed in the human thymus even though we observed that CTL clones against MAGE-A1 or MAGE-A4 antigenic peptides had higher avidities (EC50 ranging between 0.1 and 50 nmol/L) than the anti–MAGE-3.A1 CTL. In addition, not all anti-MAGE-A3 CTL display low functional avidity as high-avidity clones, with EC50’s of 0.03 and 0.3 nmol/L on Fig. 2A, recognize MAGE-A3 peptides on HLA-B*3501 and B*4001, respectively (25, 26). Clearly, we are still little closer to understanding the basis of this avidity difference between anti–MAGE-3.A1 and other anti-MAGE CTL clones. We nevertheless conclude that for the 13 anti–MAGE-3.A1 CTL clones analyzed here, which were all vaccine-induced and present in patients showing postvaccination tumor regressions, high functional avidity is not an explanation to their putative capability to trigger a tumor rejection response.

Our current hypothesis is that the vaccine-induced anti–MAGE-3.A1 T-cells, despite their relatively low affinity, triggered the priming or restimulation of other antitumor CTL (27). Indeed, searching for other immune effectors of tumor regression in vaccinated melanoma patients, we observed that in all seven patients analyzed, the frequencies of their blood antitumor CTL, i.e., lytic effectors that recognize autologous melanoma cells but not autologous B cells or NK target K562, ranged from 10^{-4} to 3 \times 10^{-3} of the CD8 T-cells, that is, 10 to 10,000 times higher than the antivaccine CTL in the same patients (28). From patient EB81, that had shown nearly complete tumor regression following vaccination with ALVAC-mini–MAGE-1/3, we derived a set of antitumor CTL clones, which turned out to be directed mainly against MAGE-C2 and gp100 antigens (28). Using TCR-specific PCR, the frequency of antitumor CTL inside metastases was found to be ~10,000 times higher than that of the antivaccine T cells. Some of these antitumor CTL were already present in a metastasis resected before vaccination. Interestingly, new clonotypes appeared after vaccination (10). Similar observations have now been made in another vaccinated patient (11). These results suggest that the antivaccine CTL may not be the principal effectors that kill the bulk of the tumor cells. They may exert their effect mainly by creating local conditions enabling priming or restimulation of large numbers of other CTL which then proceed to destroy the tumor (27). In this scenario, antivaccine CTL must have capacities that are not shared by the antitumor T cells that are already present at the tumor sites prior to vaccination.

Several reports have indicated that high-avidity CTL display superior in vivo antitumor efficacy (for example, see refs. 29–31). Our results do not contradict these observations. In patient EB81, the antitumor CTL clones directed against MAGE-C2 or mutated antigens have a high functional avidity, with peptide EC50 in the nanomolar range (32). Therefore, in this patient, tumor regression was associated with the activation of antitumor CTL that were present at much higher frequencies than antivaccine T cells, and had a higher avidity. However, we do not believe that activation of high-avidity CTL clones suffices to trigger tumor rejection, because in patient EB81, high-avidity tumor-specific CTL were already present prior to vaccination.11

The other result of this study is that the vaccine-induced anti-MAGE-A3.A1 CTL clones had slightly different functions according to the vaccination modality, as approximately 20 genes were differentially expressed between the anti–MAGE-3.A1 CTL clones derived from patients vaccinated with either peptide-ALVAC or peptide-pulsed DC. The first difference was that clones derived from patients vaccinated with peptide or ALVAC mini–MAGE-1/3 expressed the genes encoding the orphan granzyme H and granzyme K, whereas the other clones did not or much less so. GzmH has no structural equivalent in the mouse and seems to be uniquely human. It was shown to be expressed at high levels in NK cells, at low levels in CD4+ but not in CD8+ T-cells, and was not induced by classical T-cell activators (33). Our results are therefore the first indication that GzmH could also be expressed in CD8+ CTL. Its function has only recently been described, with recombinant protein tested in reconstituted in vitro systems. It induces a caspase-independent cell death program in K562 cells, without cleavage of Bid or release of cytochrome c from mitochondria (34). In adenovirus-infected cells, it cleaves the adenovirus DNA-binding protein, which is required for viral DNA replication (35). In humans, Gzmk is expressed in NK cells, in a subset of CD4+ T-cells, and in a subpopulation of memory CD8+ T-cells (21, 36). When delivered into cells, Gzmk induces a caspase-independent apoptosis, with processing of Bid by Gzmk itself, and release of cytochrome c from mitochondria (37). It will be interesting to find out whether GzmkH and Gzmk proteins are present in some tumor-specific CTL in vivo and, should this be the case, whether it confers a superior antitumor activity to them.

Another difference between the anti–MAGE-3.A1 CTL clones of the peptide/ALVAC and DC groups was the selective expression by the latter of a set of genes including IL-10. In vivo, the effects of IL-10 seem to be mainly to limit exaggerated immune and inflammatory reactions. In doing so, it may however promote viral persistence (38) or inhibit antitumor immunity (39). It was shown to be produced by human CD8+ lymphocytes displaying suppressive activity (16–19). Our IL-10–producing CTL clones had no detectable suppressive activity, as shown in Fig. 3B. Moreover, they expressed very low Foxp3 levels when in resting conditions, contrary to our suppressor CD4+ clones (data not shown). We conclude that IL-10 production by the DC-induced anti–MAGE-3.A1 clones is not part of a suppressor T-cell phenotype. Instead, the link between the DC vaccines and

10 Unpublished observations.

11 Corbère et al., manuscript in preparation.
IL-10 production could be IL-12. The group of G. Trinchieri showed that, in vitro, human CD4+ or CD8+ T-cell clones producing high levels of both IL-10 and IFN-γ could be generated if IL-12 was added during the first days of stimulation (40). Priming in the presence of IL-12 was also shown to induce IL-10 expression by murine CD8 T-cells (41). It would be interesting to see whether, in the human setting, priming with IL-12 also leads to the expression of other genes coexpressed with IL-10 in our clones.

Our results indicate that it is meaningful to carry out functional analyses on T-cell clones derived from rare tumor-specific lymphocytes. Somewhat surprisingly, stable established clones keep distinct phenotypes, even after multiple rounds of restimulation with antigen, feeder cells, and cytokines such as IL-2, IL-4, and IL-7, indicating that the vaccination modality affected the differentiation of anti-MAGE-3-A1 T-cells. This is not surprising, and it may be important to continue monitoring the immunologic T-cell levels of vaccinated patients using methods that can pick up these differences.

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Functions of Anti-MAGE T-Cells Induced in Melanoma Patients under Different Vaccination Modalities

Thierry Connerotte, Aline Van Pel, Danièle Godelaine, et al.


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