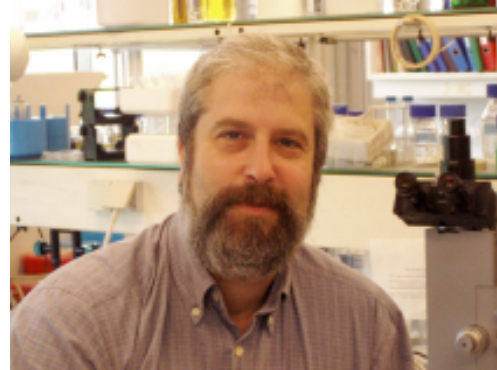


IDENTIFICATION OF HUMAN TUMOR ANTIGENS

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The group led by Pierre van der Bruggen is defining antigenic peptides encoded by genes such as those of the MAGE family. Therapeutic vaccination of cancer patients with MAGE peptides is now in progress, and the identification of additional antigenic peptides is important to increase the range of patients eligible for therapy and to provide tools for a reliable monitoring of the immune response. The group is also involved in designing reliable methods for the monitoring of the CD4⁺ T cell response to cancer vaccines, and in the study of functional defects of T cells.

New MAGE antigens recognized by CD8⁺ and CD4⁺ T cells

Yi Zhang, Vincent Stroobant, Christophe Panicelli, Sabrina Ottaviani, Tetsuto Kobayashi

“Cancer germline” genes such as those of the MAGE family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They encode shared tumor specific antigens, which have been used in therapeutic vaccination trials of cancer patients. The first antigens and genes that code for these antigens were identified with anti-tumor cytolytic T lymphocytes obtained from cancer patients (1). A few HLA class I-restricted antigenic peptides were identified by this “direct approach” (2). A large set of additional cancer-germline genes have now been identified by purely genetic approaches (3, 4). As a result, a vast number of sequences are known that can code for tumor-specific shared antigens, but most of the encoded antigenic peptides have not been identified yet. The identification of a large number of antigenic peptides presented by HLA class I and class II molecules is likely to be important for the future of clinical trials with defined antigenic peptides. A large set of peptide/HLA combinations will alleviate HLA restriction and widen the set of eligible patients. It will also facilitate the design of concurrent immunizations against several antigens.

Such immunizations could increase the primary anti-tumor efficacy of the vaccine and also decrease the risk of tumor escape by loss of antigen expression.

We have used approaches that we have loosely named “reverse immunology” (5). They aim at identifying antigenic peptides recognized by T cells, using gene sequences as starting point. We have focused this search on the cancer-germline genes, which are expected to code for tumor-specific shared antigens on the basis of their pattern of expression.

Search for antigenic peptides recognized by CD8⁺ T cells

We stimulated CD8⁺ T lymphocytes with dendritic cells transduced with viral vectors containing complete MAGE-coding sequences. As this requires the processing of the antigen by the dendritic cells, we surmised that the peptides that would be identified would also be processed in the tumors expressing the MAGE genes. A difficulty of the use of recombinant viruses is the activation of CTL precursors directed against viral antigens. We circumvented this problem by using different vectors for the stimulation of the microcultures, for the lytic assay with the responder T cells, and for the cloning step. This procedure is summarized in Figure 1. Dendritic cells were infected with either an adenovirus or a canarypoxvirus and they were

used to stimulate microcultures of autologous CD8⁺ T lymphocytes (6). After three weekly stimulations, the responder cells were tested for lysis on autologous EBV-B cells infected with vaccinia-MAGE. Positive microcultures were cloned. To identify the antigenic peptide, the resulting CTL clone was tested for lysis of autologous EBV-B cells pulsed with a complete set of peptides of 16 amino acids that overlap by 12. When a peptide scored positive, shorter peptides were synthesized

to identify the shortest optimal peptide. To identify the HLA presenting molecule, the CTL clones were tested for stimulation by cells transfected with the MAGE cDNA together with cDNAs coding for the possible HLA presenting molecules. Finally, relevant tumor targets were used in a lysis assay to ascertain that the antigen was also processed by tumor cells.

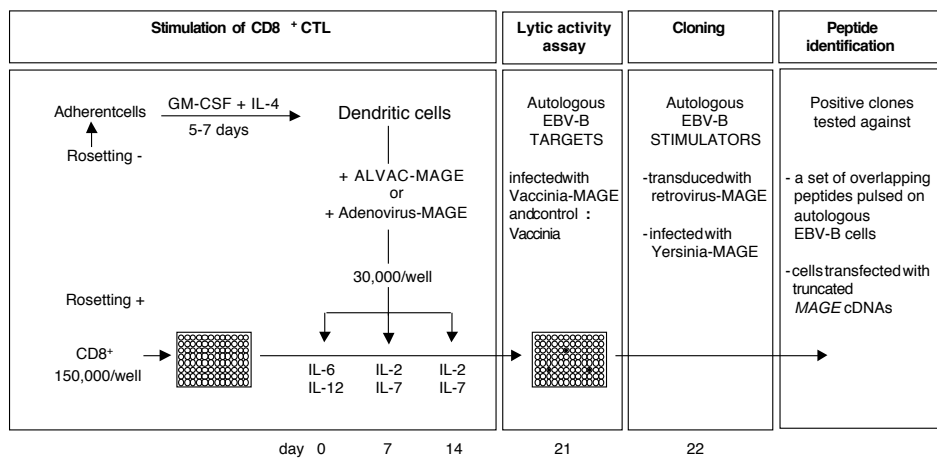


Fig. 1. Overview of the procedure to obtain anti-MAGE CD8⁺ CTL clones by stimulation with dendritic cells infected with viral vectors carrying a MAGE coding sequence

We have listed in a database class I-restricted antigenic peptides that are encoded by cancer-germline genes (<http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm>). MAGE-1 and MAGE-3 antigenic peptides are available for more than 90% of Caucasians.

We have identified a MAGE-3.B40 antigen which is the first example of a tumor-specific antigen exclusively presented by tumor cells expressing the immunoproteasome (7). This work was done in collaboration with the group of Benoît Van den Eynde.

We have found that MAGE-1 peptide SAYGEPKRL is recognized by CTL clones that are restricted either by HLA-Cw3, Cw6 or Cw16 (5). The presentation of the same peptide by different HLA molecules may be frequent for HLA-C molecules, because they are more closely related to each other in the peptide-binding region than HLA-A and B molecules. But for HLA-A and B also this may occur more frequently than usually thought. MAGE-3 peptide MEVDPIGLY is presented to different CTL by HLA-B*4402, B*4403, and B*1801 (5). MAGE-1 peptide EADPTGHSY was found to be recognized by different CTL on HLA-A1 and B35 molecules, and to bind to HLA-A29 (5). The same was found for the MAGE-3 homologous peptide EVDPIGHLY.

These results have consequences for the monitoring of the immune response of patients vaccinated with such tumor-specific shared peptides. A number of HLA-A1 patients were injected with MAGE-3.A1 peptide EVDPIGHLY, at a time when we did not know that it could be presented by B35 and A29 (5). The immune response was evaluated with HLA-A1 tetramers folded with the MAGE-3 peptide. Thus, A29 or B35-restricted responses against the peptide may have been missed.

Search for antigenic peptides recognized by CD4⁺ T cells

Studies in several animal models have demonstrated an important role for CD4⁺ T cells in

inducing and maintaining anti-tumor immunity. It is therefore possible that the addition of antigenic peptides presented by class II to those presented by class I will improve the efficacy of therapeutic anti-tumor vaccination. To identify new HLA-peptide combinations, we used dendritic cells loaded with a recombinant MAGE protein to stimulate autologous CD4⁺ T lymphocytes (8). After four weekly stimulations, the responder cells were tested for their ability to secrete IFN- γ upon stimulation with the antigen, and the positive microcultures were cloned. The procedure is summarized in Figure 2.

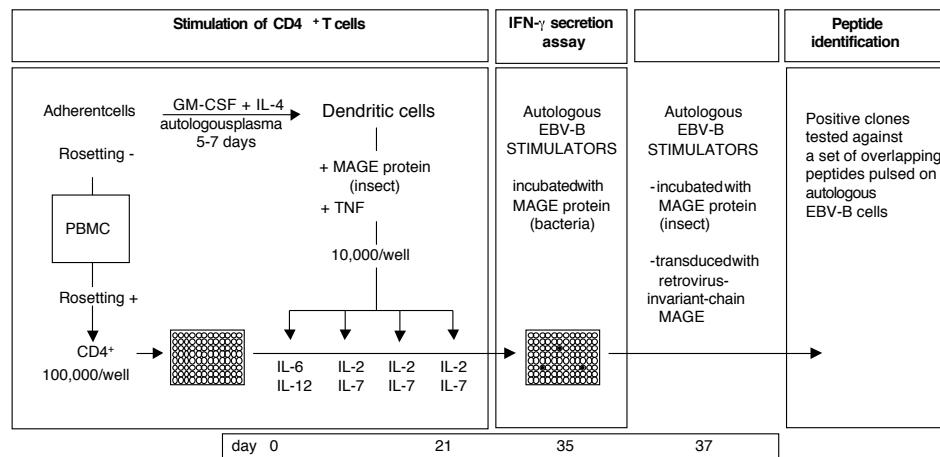


Fig. 2. Overview of the procedure to obtain anti-MAGE CD4⁺ T cell clones by stimulation with dendritic cells loaded with a whole protein

To identify the antigenic peptide, the positive clones were stimulated with a set of peptides of 16 amino acids that overlapped by 12 and covered the entire MAGE protein sequence. The positive peptide was then tested for recognition on several Epstein-Barr virus immortalized B cell lines (EBV-B cell lines) to identify the HLA presenting molecule. Because a large number of the CD4⁺ T cells that were obtained in our first experiments appeared to be directed against bacterial

contaminants, we chose to alternate the sources of protein used at the various stages of the procedure. For example, to stimulate the lymphocytes, we used a MAGE protein produced in insect cells, and to test the specificity of the responder lymphocytes, we used a protein produced in bacteria. Microcultures that specifically produced IFN- γ after stimulation with the MAGE protein were cloned by limiting dilution using autologous EBV-B stimulator cells either loaded with the MAGE

protein used during the stimulation step, or transduced with a retroviral construct encoding a truncated human invariant chain (Ii) fused with the MAGE protein.

MAGE-1 and MAGE-3 antigenic peptides identified by this procedure are listed in a database (<http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm>). They include a MAGE-3 peptide presented by HLA-DP4, which is expressed by more than 70% of Caucasians (9). This peptide could not have been found by a peptide stimulation approach because no consensus anchor residue was known for HLA-DP4.

The normal anti-MAGE-3.A1 repertoire

Christophe Lonchay

To estimate the frequency of anti-MAGE-3.A1 CTLp directed against a MAGE-3 peptide (EVDPIGHLY) presented by HLA-A1 in blood from normal donors, groups of about 200,000 purified CD8 cells were stimulated with autologous PHA-activated T cells incubated with peptide MAGE-3.A1, in the presence of IL-6 and IL-12 during the first week followed by two weekly restimulations in the presence of IL-2 and IL-7. After the third week the cultures were tested in a lysis assay against HLA-A1 peptide-pulsed EBV-B cells. On the basis of the fraction of positive groups, the frequency of CTLp directed against the MAGE-3.A1 antigen was estimated to be about 2×10^{-7} in the CD8 lymphocyte population.

Another estimate was obtained recently for an hemochromatosis patient, from whom a large number of blood lymphocytes was collected. Purified blood T cells were incubated with an A1/MAGE-3 tetramer. The very rare cells that were stained by the tetramer and also by anti-CD8 antibodies were sorted at one cell per well and restimulated with irradiated HLA-A1 cells pulsed with the MAGE-3.A1 peptide. On the basis of the number of T cell clones that multiplied and were stained by the tetramer, we estimated the frequency of the naive anti-MAGE-3.A1 CTLp at 6×10^{-7} of the CD8 T cells for. With about five liters of blood containing about 10^6 PBMC/ml with about 15% CD8 T cells, and about 2% of total lymphocytes being located in the blood, the body total number of CD8 cells is approximately 4×10^{10} . Therefore a blood frequency of 3×10^{-7} of the CD8 corresponds to 32,000 anti-MAGE-3.A1 precursors. In the course of this analysis, 15 anti-MAGE-3.A1 T cell clones were obtained, corresponding to 14 different T cell receptors (TCR). Using these numbers to estimate the diversity of the naive T cell repertoire against MAGE-3.A1, one can state with 90% certainty that the naive repertoire directed against MAGE-3.A1 consists of 40 to 400 different TCR.

A reversible functional defect of CD8⁺ T lymphocytes involving loss of tetramer labeling

Nathalie Demotte

We have observed that human CTL clones lose their specific cytolytic activity and cytokine production under certain stimulation conditions, while retaining an antigen-dependent growth pattern. These inactive CTL simultaneously lose their labeling by an HLA-peptide tetramer, even though the amount of TCR-CD3 at their surface is not reduced (10). The tetramer-negative cells recover tetramer staining and cytolytic activity after stimulation with tumor cells in the presence of a supernatant of activated lymphocytes. Our results suggest the existence of a new type of functional defect of CTL. They also indicate that tetramers may fail to reveal some CTL bearing the relevant TCR, even when such functionally arrested CTL retain the potential to participate in immune responses because their defect is reversible. We will analyze the ability of CTL to be labelled by tetramer during the days following the antigenic stimulation. We also plan to analyze the composition of lipid rafts of tetramer-positive and tetramer-negative cells. In addition, we will analyze tetramer-positive and tetramer-negative cells for differential expression of genes by microarray analyzes.

Detection of anti-vaccine CD4 T cell response in vaccinated patients

Yi Zhang

Patients injected with class II-restricted peptides

For therapeutic vaccination trials, the vaccine can consist of defined antigenic peptides. This approach greatly facilitates the monitoring of the T cell response, because the presumed target of the T cells is completely defined. It allows the use of HLA-peptide tetramers to detect T cell responses in patients. HLA class II tetramers have been more difficult to obtain than HLA class I tetramers, but have already been used to detect low frequencies of CD4 T cells. Didier Colau has recently succeeded in obtaining a DP4.MAGE-3 tetramer, which was produced in insect cells. It stained specifically relevant CD4 clones and we are using this tetramer to estimate the frequency of anti-vaccine CD4 T cells in patients injected with either the MAGE-3.DP4 peptide or dendritic cells pulsed with this peptide.

Patients injected with a protein

For therapeutic vaccination trials, the vaccine can also consist of the entire protein. This has the advantage that antigenic peptides binding to a broad set of HLA molecules can be processed from the vaccine, so that the patients do not have to be selected according to their HLA. However, the detection of the anti-vaccine T cells is more laborious and can not rely on the use of HLA-peptide tetramers. An interesting alternative assay uses bispecific antibodies that bind to the cell surface and capture cytokines immediately after their production. The cells are kept alive, can be cloned, and analyzed further for specificity and TCR expression. We have succeeded to detect anti-MAGE specific T cells using antigen-presenting cells pulsed with a peptide as stimulators. We are now trying to use dendritic cells loaded with a MAGE-3 protein so as to be able to monitor the immune response of patients vaccinated with an entire protein.

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