

TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

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The central research theme of our group is the study of tumor antigens recognized by T lymphocytes. Besides our continued effort to identify additional antigens of interest, we mainly want to address a number of fundamental or mechanistic issues that have a direct impact on the utilization of such antigens as cancer vaccines in human patients. These antigens consist of peptides that are presented by MHC molecules at the cell surface and derive from intracellular proteins that are degraded by the proteasome. The intracellular pathway leading from the protein to the peptide/MHC complex is known as “antigen processing”. We are currently studying the processing of several human tumor antigens by the proteasome, and we are particularly interested by the processing differences we have observed between the standard proteasome, which is present in most cells, and the immunoproteasome which is found in some dendritic cells and in cells exposed to interferon-gamma.

We are also studying a mouse preclinical model of cancer immunotherapy, where we try to define the optimal conditions to induce effective anti-tumor responses by various vaccination approaches against defined antigens. This led us to uncover a powerful mechanism of tumor resistance which is based on tryptophan catabolism by indoleamine-2,3 dioxygenase, an enzyme that we found frequently expressed in tumors. The resulting local tryptophan shortage appears to prevent the proliferation of lymphocytes at the tumor site. Inhibitors of indoleamine-2,3 dioxygenase are currently being tested in vivo for their ability to counteract this tumor resistance mechanism.

To obtain the most relevant information from such preclinical models, we are trying to build a new mouse melanoma model where tumors expressing a given antigen could be induced, using a transgenic system based on Cre-lox recombination. This should recapitulate the long-term host/tumor relationship that occurs in humans when a tumor slowly develops within a normal tissue.

Building on our expertise in antigen processing and presentation, we have also developed a collaboration with the Unité de Rhumatologie of the Cliniques Universitaires St-Luc to study antigen presentation by dendritic cells in Systemic Lupus Erythematosus (SLE), both in mouse models of SLE and in human patients.

Differential processing of tumor antigens by the standard proteasome and the immunoproteasome

Jacques Chapiro, Benoit Guillaume, Sandra Morel, Fanny Piette

Antigens recognized by cytolytic T lymphocytes (CTL), such as viral or tumor antigens, usually

consist of peptides of 8-10 amino acids in length, which are presented by MHC class I molecules at the cell surface. Because such peptides derive from intracellular proteins, a processing step is required before they can be exposed to the cell surface in association with MHC molecules. Firstly, the peptide is produced, as a result of the degradation of the parental protein by the proteasome. Secondly, it is taken up by a dedicated transporter named TAP, and translocated inside the endoplasmic reticulum where it meets and associates with newly synthesized MHC molecules.

The first step of cleavage by the proteasome is crucial in that cleavage location determines the precise sequence of the final antigenic peptide. We have observed that this cleavage may occur differently in some cells, depending on their proteasome content. The proteasome comes in two forms : the standard proteasome, which is found in most cells, and the immunoproteasome, which is expressed by mature dendritic cells and by cells exposed to interferon-gamma (IFN γ).

We previously reported that a class-I restricted antigenic peptide derived from an ubiquitous human protein was processed efficiently by the standard proteasome but not by the immunoproteasome. As a result, the relevant epitope is not presented efficiently by mature dendritic cells, which contain immunoproteasomes (1). This could explain how certain potentially autoreactive CTL can escape tolerance induction in the thymus and fail to be activated in the periphery. We have now extended those observations to other epitopes derived from self proteins, and to several antigenic peptides of interest for cancer immunotherapy, including HLA-A2-restricted epitopes derived from tyrosinase, Melan-A^{MART1} and gp100. These results were obtained from in vitro experiments where synthetic peptides of about 20 amino acids, encompassing the epitope, are digested with highly purified preparations of either standard proteasomes or immunoproteasomes. The presence of the antigenic peptide in the digests is tested with the relevant CTL after pulsing on target cells (Fig. 1). It is then confirmed by HPLC and mass spectrometry. Consistent with their poor processing by the immunoproteasome, those epitopes are not presented efficiently to CTL by cells containing immunoproteasomes, such as tumor cells treated with IFN γ for 7 days, or cells transfected with cDNAs encoding the three immunoproteasome subunits, β 1i (LMP2), β 2i (MECL1), and β 5i (LMP7).

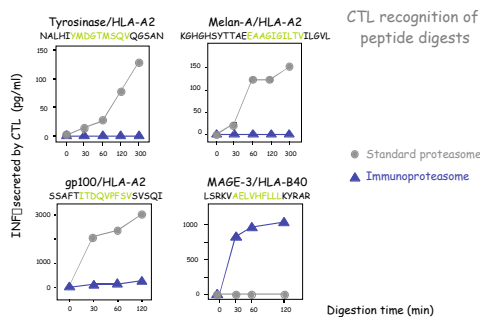


Fig.1. see text for explanations

On the contrary, we showed that another tumor epitope, which is derived from MAGE-3 and presented by HLA-B40, is processed by the immunoproteasome but not by the standard proteasome (Fig. 1). Accordingly, this epitope is presented to CTL only by tumor cells pre-treated with IFN γ (2).

Our observations indicate that the pool of antigenic peptides presented at the cell surface may differ substantially according to the proteasome type that is predominant in the cell. This may have major implications for immunotherapy, particularly for cancer immunotherapy, as it means that the peptide repertoire presented by tumor cells may differ from the repertoire presented by antigen-presenting cells (3). The peptide repertoire of tumor cells themselves may vary according to the localization of the tumor (e.g. primary tumor versus lymph node metastasis) and its level of exposure to IFN γ . Therefore, it appears essential for the success of cancer immunotherapy to study those processing differences in detail, so as to define the most effective vaccination strategy for each epitope and to use the appropriate combination of antigens in order to minimize the risk of tumor escape by proteasome switching.

Identification of new antigens recognized by autologous CTL on human melanoma

Wenbin Ma, Nathalie Vigneron (in collaboration with P. Coulie)

Melanoma line EB81 expresses several antigens recognized by autologous CTL. By using a cDNA expression cloning approach, we identified the antigens recognized by two of them. These antigens correspond to two distinct peptides derived from MAGE-C2, a gene with a cancer-germ-line expression pattern, which is expressed in about 40% of melanomas and 30% of bladder carcinomas. This gene had been isolated earlier by a genetic subtraction approach between tumor and normal material, and was not known yet to encode tumor antigens. Both peptides are presented by HLA-A2, which is the most frequent HLA allele in Caucasians. Because of their strict tumor-specificity and their wide expression in tumors, these new antigens represent promising targets for cancer immunotherapy.

Melanoma line LG2-MEL also expresses several antigens recognized by autologous CTLs. One of them consists of a peptide derived from tyrosinase and presented by HLA-B*3503. We have identified another antigen of LG2-MEL as a peptide presented by HLA-B*4403 and resulting from a point

mutation in gene OS-9 (4). This gene is expressed in various normal tissues. It is located on chromosome 12 in the vicinity of the CDK4 locus and is frequently co-amplified with CDK4 in human sarcomas. The mutation, a C-to-T transition, changes a proline residue into a leucine at position 446 of the OS-9 protein. Mutated transcripts were found in all the melanoma sublines of LG2-MEL. None of the 184 tumor samples collected from other cancer patients expressed the mutated transcript, indicating that this is a rare mutational event. Interestingly, some of the melanoma sublines of LG2-MEL have lost the wild-type allele of gene OS-9. Those sublines appear to grow faster in vitro than the sublines that retained the wild-type allele, suggesting that this loss of heterozygosity may favor tumor progression. The mutation we have identified in gene OS-9 might therefore participate in the oncogenic process by affecting the function of this potential tumor-suppressor gene.

TNF-mediated toxicity after massive induction of specific CD8+ T cells following immunization of mice with a tumor-specific peptide

Catherine Uyttenhove, Dominique Donckers, Luc Pilotte

In order to optimize the vaccination modalities applied in cancer immunotherapy trials, we have continued our systematic assessment of the efficacy of various immunogens to induce CTL responses in mice against defined tumor antigens. In the course of those studies, we immunized mice with antigenic peptide P815E, which is presented by H-2Kd and recognized by tumor-specific CTL raised against P815 tumor cells. This peptide is encoded by the ubiquitously expressed gene MsrA and carries a mutated residue conferring tumor specificity. Unexpectedly, we observed a severe toxicity occurring in the early hours after the third injection, resulting in the death of most mice within 24 h (5). The toxic syndrome was reminiscent of TNF-induced shock, and the sera of ill mice contained high levels of TNF. Toxicity was prevented by injection of neutralizing anti-TNF Abs, confirming the involvement of TNF. Depletion of CD8+ T cells could also prevent toxicity, and ex vivo experiments confirmed that CD8+ lymphocytes were the major cellular source of TNF in immunized mice. Tetramer analysis of the lymphocytes of immunized mice indicated a massive expansion of P815E-specific T cells, up to >60% of circulating CD8+ lymphocytes. A similar toxicity was observed after massive expansion of specific CD8+ T cells following immunization with another P815 peptide, which is encoded by gene P1A and was injected in a form covalently linked to an immunostimulatory peptide derived from IL-1. We conclude that the toxicity is caused by specific

CD8+ lymphocytes, which are extensively amplified by peptide immunization in a QS21-based adjuvant and produce toxic levels of TNF upon further stimulation with the peptide. Our results suggest that immunotherapy trials involving new peptides should be pursued with caution and should include a careful monitoring of the T cell response.

A novel tumor immune escape mechanism based on tryptophan degradation by indoleamine 2,3 dioxygenase

Catherine Uyttenhove, Luc Pilotte, Ivan Théate, Dominique Donckers, Nicolas Parmentier, (in collaboration with Vincent Stroobants and Didier Colau)

It has been shown that T lymphocytes undergo proliferation arrest when exposed to tryptophan shortage, which can be provoked by indoleamine 2,3-dioxygenase (IDO), an enzyme that is expressed in placenta and catalyzes tryptophan degradation. Local tryptophan depletion by IDO expression has therefore been proposed as a natural immunosuppressive mechanism promoting tolerance of the fetus during pregnancy. Expression of IDO is also induced in many cells by interferon-gamma, and could thereby participate in the regulation of immune responses.

To determine whether tumors might use this mechanism to escape T-cell mediated immune responses, we measured the expression of IDO by RT-PCR in a series of murine and human tumor cell lines. We found that many lines were positive. Moreover, when we tested a large series of human tumor samples by immunohistochemistry with an IDO-specific antibody, we observed that a vast majority stained positive, including all prostatic, colorectal, pancreatic and cervical carcinomas.

Using the well-characterized model system of mouse tumor P815, where the antigen encoded by gene P1A is the major target of the tumor rejection response, we observed that expression of IDO by P815 tumor cells prevents their rejection by pre-immunized mice. This effect can be partly reverted by systemic treatment of mice with an inhibitor of IDO, in the absence of noticeable toxicity. These results suggest that the efficacy of therapeutic vaccination of cancer patients could be improved by concomitant administration of an IDO inhibitor.

Development of a mouse melanoma model for immunotherapy

Ivo Huijbers (in collaboration with Paul Krimperfort (NKI, Amsterdam) and Anne-Marie Schmitt-Verhulst (CIML, Marseille))

Immunotherapy represents an attractive approach for the treatment of cancer, in particular melanoma. Preclinical studies of various strategies of immunotherapy rely on model systems where tumor cells grown *in vitro* are inoculated into syngeneic mice. However, this does not recapitulate the long-term host-tumor relationship that occurs in patients during tumor development. In order to have a model system more relevant to the human situation, we are trying to develop a mouse strain in which we can induce melanomas expressing a tumor antigen of interest.

One of the most common site of genetic lesions in human melanoma is the *INK4A/ARF* locus, which encodes two distinct tumor suppressor proteins p16^{INK4A} and p14^{ARF}. Genetic disruption of this locus predisposes mice to the formation of various tumor types, but is not sufficient to induce melanoma unless the Ras-pathway is specifically activated in melanocytes. In order to have a fully controlled model system for melanoma, we planned to generate transgenic mice in which the deletion of the *Ink4a/Arf* genes and the melanocyte-specific expression of both activated Harvey-Ras^{G12V} and a well characterized antigen is spatially and temporally regulated by a fusion protein between the Cre-recombinase and the tamoxifen responsive hormone-binding domain of the estrogen receptor (CreER^{DP}). The antigen is encoded by *P1A*, a gene expressed in several tumors but silent in normal tissues except testis and placenta. The tumor induction in these mice will be performed by topical administration of tamoxifen, which should be sufficient to induce the essential genetic rearrangements in melanocytes necessary to establish neoplastic transformation.

Six transgenic lines were generated, harboring a construct of respectively the tyrosinase promoter with two enhancer elements, a CreER^{DP} fusion gene flanked by loxP sites, a V12 mutated H-Ras gene, an internal ribosomal entry site and the P815A-antigen encoding gene, P1A. The expression and regulation of the CreER^{DP} fusion gene was analyzed by crossing these mice to a Rosa26 Cre reporter strain. In three transgenic lines specific blue staining could be observed in melanocytes after topical treatment of the ear by 4-hydroxytamoxifen. This implied that the CreER^{DP} fusion gene was expressed specifically in melanocytes and activated upon treatment with its ligand. In order to determine whether the transgene was still intact and functional after Cre-recombination, these transgenic

lines were crossed with a CMV-Cre-deleter strain. In one transgenic line a break in the CreER^{DP} gene was observed after recombination. In the other two lines, H-ras and P1A were still detected after Cre-recombination implying that the transgene was arranged in such a way that the tyrosinase-promoter was now driving the expression of genes H-ras and P1A. These lines are now being crossed to a homozygous conditional *Ink4a/Arf* knock-out background in order to induce melanoma formation by applying 4-hydroxytamoxifen to the skin.

Antigen presentation by dendritic cells in Systemic Lupus Erythematosus

Bernard Lauwerys (in collaboration with Frédéric Houssiau, Unité de Rhumatologie)

Systemic Lupus Erythematosus (SLE) is an autoimmune disorder characterized by overt polyclonal B cell activation and autoantibody production against nuclear antigens. We are studying the involvement of dendritic cells in the impaired central and peripheral tolerance which is characteristic of the disease. Studying strains of mice that are congenic for different SLE susceptibility loci and were developed by E. K. Wakeland (University of Texas), we observed that dendritic cells from one of these strains (Sle3) are characterized by an increased gene expression of BAFF, a cytokine that promotes B cell survival and activation. The role of these dendritic cells in the induction of antibody production is currently under investigation. Another aspect will be a detailed analysis of the antigen presentation capacity of dendritic cells from BWF1 mice, which also develop SLE. Besides the expression of surface markers, we will investigate the capacity of those dendritic cells to induce central tolerance in the thymus by negative selection, using P1A-TCR transgenic mice that have been developed within the group.

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