Research

at the

de Duve Institute

and

Brussels Branch of the Ludwig Institute for Cancer Research

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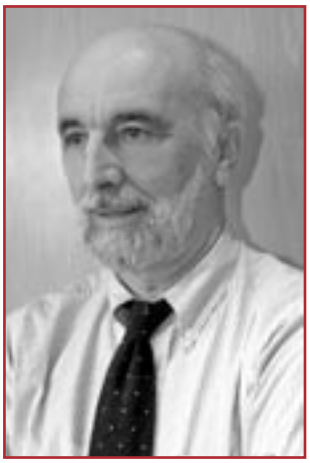
LUDWIG INSTITUTE FOR CANCER RESEARCH

BRUSSELS BRANCH

Cancer is a major concern in human health. The prospects for bringing cancer under control require linked innovative basic and clinical research. In this view, Daniel K. Ludwig created in 1971 the Ludwig Institute for Cancer Research, an international organization bringing together scientists and clinicians from around the world. Many Ludwig investigators are leaders in many areas of science, involving genetics, bioinformatics, immunology, virology, cell biology and signal transduction.



Faithful to the organizing principles laid down by Mr Ludwig, the Institute conducts its research through nine Branches, located in seven countries. The Branch structure allows the Institute to interact with a number of different research and clinical environments. Each Branch is focused on a research program defined by the Branch Director in relation with the overall objectives of the Institute. The Branches are established in association with University Hospitals, to stimulate close collaborations between research laboratories and the clinic. By organizing and controlling its own clinical trials programs, the Institute has indeed created a continuum that integrates laboratory and clinical research.

The biological properties of any given cancer cell constantly change, allowing tumors to spread and become more aggressive. To overcome these obstacles, the Ludwig Institute has developed a broad-based discovery program that seeks to understand the full complexity of cancer. Research is organized according to the four major programmatic themes that define the Institute: genetics, cell biology, cell signalling and immunology.

Branch staffs vary in size from 30 to over 90, and internationally the Institute employs some 600 scientists, clinicians and support personnel. The quality of the research is monitored on an ongoing basis by the Institute's Scientific Committee and by an external peer review process.

The Brussels Branch of the Institute was created in 1978. It is composed of 90 members and is headed by Thierry Boon, Branch Director.

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TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

Building up on the molecular definition of tumor antigens recognized by T cells, our group mainly focuses on two aspects of tumor immunology, namely the processing of tumor antigens and the study of animal models to optimize cancer immunotherapy and evaluate tumor resistance mechanisms.

Tumor antigens recognized by Cytolytic T Lymphocytes (CTL) 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". Our group focuses on the proteasome and recently described a new mode of production of antigenic peptides by the proteasome, based on cutting and pasting peptide fragments to form a new spliced peptide.
The first example was a peptide derived from human melanocyte protein gp100. This antigenic peptide is nine-amino acid long and is produced by the splicing of two fragments that were initially non-contiguous in the parental protein. The splicing is made by the proteasome, is tightly coupled to the proteolytic reaction, and appears to occur by transpeptidation involving an acyl-enzyme intermediate. We also described a second example of spliced peptide, which is a minor histocompatibility antigen, and where the two fragments are rearranged before splicing. We are currently working on additional spliced peptides. We are also studying the processing differences between the standard proteasome, which is present in most cells, and the immunoproteasome which is found in dendritic cells and in cells exposed to interferon-gamma. Several tumor antigens - including spliced peptides - were found to be processed differently by the two proteasome types, usually because of a preferential

cleavage made by one or the other proteasome within the antigenic peptide itself. We also study the splicing capacity of the two proteasome types.

Translation of knowledge on tumor antigens into efficient cancer immunotherapy requires additional studies on the various strategies that can be used. Some of these studies can be done in preclinical animal models. The study of such a model allowed 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 to be 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 can be used in vivo to counteract this tumor resistance mechanism. We are searching for new IDO inhibitors that could be developed clinically. We also study additional tumor resistance mechanisms.

The currently available murine models are limited by the fact that they are based on transplantation of tumor cells grown in vitro into a healthy animal. This does not recapitulate the long-term host/tumor relationship that occurs in humans when a tumor slowly develops within a normal tissue. To circumvent this limitation and obtain more relevant information from such preclinical models, we have build a new mouse melanoma model where tumors expressing a given antigen can be induced, using a transgenic system based on Cre-lox recombination.

DIFFERENTIAL PROCESSING OF TUMOR ANTIGENS BY STANDARD PROTEASOMES, IMMUNOPROTEASOMES AND INTERMEDIATE PROTEASOMES

B. Guillaume, V. Stroobant, W. Ma

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 through 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 (IFNg).

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). We have now extended those observations to several antigenic peptides of interest for cancer immunotherapy, including HLA-A2-restricted epitopes derived from tyrosinase, Melan-AMART1 and gp100.

On the contrary, we showed that other tumor epitopes, which are derived from MAGE-3 and MAGE-C2, are processed by the immunoproteasome but not by the standard proteasome and therefore are presented to CTL only by tumor cells pre-treated with IFNg (2). By analyzing the peptidic fragments produced after in vitro digestion with the two proteasome types, we found that the differential processing can result from two mechanisms. In some cases, one of the proteasome types predominantly cleaves within the sequence of the epitope, resulting in its destruction (2). In other cases, the difference lies in the efficiency of cleavage at the C-terminal end of the antigenic peptide. These observations may have major implications for cancer immunotherapy, as they imply 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 IFNg. It is therefore essential 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. We have also observed the presence in many tumor lines of proteasome types that are intermediate between the standard proteasome and the immunoproteasome. These intermediate proteasomes contain only some of the three catalytic subunits of the immunoproteasome, i.e. only ß5i or ß1i and ß5i. In terms of production of antigenic peptides, these intermediate proteasomes produce more or less the same peptides as the immunoproteasome, and therefore explain the recognition of tumor cells by CTL directed against these peptides in the absence of IFNg. However, we have also identified two antigenic peptides that are produced exclusively by intermediate proteasomes.

ANTIGENIC PEPTIDES PRODUCED BY PEPTIDE SPLICING IN THE PROTEASOME

A. Dalet, V. Stroobant (in collaboration with E. Warren, Fred Hutchinson Cancer Research Center, Seattle, USA)

By studying the antigen recognized by a CTL clone isolated from a melanoma patient, we identified an antigenic peptide composed of two non-contiguous fragments of the same protein, namely the melanocytic protein gp100. The production of this peptide requires the excision of an intervening fragment of 4 amino acids and the splicing of a fragment of 3 residues with a fragment of 6 residues. We have shown that this splicing is exerted by the proteasome and can be reproduced in vitro by incubating a precursor peptide with purified proteasomes. Splicing is coupled directly to peptide bond cleavage by the proteasome and appears to occur by transpeptidation involving an acyl-enzyme intermediate (Fig. 1) (4). The splicing reaction appears not to involve a particular motif, but rather to result from a low-efficiency reversal of the proteolysis reaction. Its occurrence is depending only on the occurrence of peptide cleavage.

We have also identified a second antigenic peptide produced by peptide splicing in the proteasome (5). This peptide is recognized by CTL directed against a minor histocompatibility antigen. The CTL was isolated from a multiple myeloma patient treated with HLA-identical bone marrow transplantation. The peptide is encoded by the polymorphic region of a gene ubiquitously expressed. Again it is made by the joining of two fragments that are initially non-contiguous in the parental protein. In addition, the two fragments are inverted in the spliced peptide, i.e. the fragment that was more N-terminal in the parental protein ends up at the C-terminal side of the spliced peptide, and vice-versa. We showed that splicing

and transposition could be reproduced in vitro with purified proteasomes. The splicing mechanism based on transpeptidation immediately after peptide bond cleavage is compatible with a transposition of the fragments prior to splicing. Together with the previous description of a peptide produced by protein splicing of FGF-5, this is the third example of antigenic peptide produced by splicing. These results indicate that spliced peptides are not uncommon and may represent a significant part of the peptide repertoire presented by MHC class I molecules.

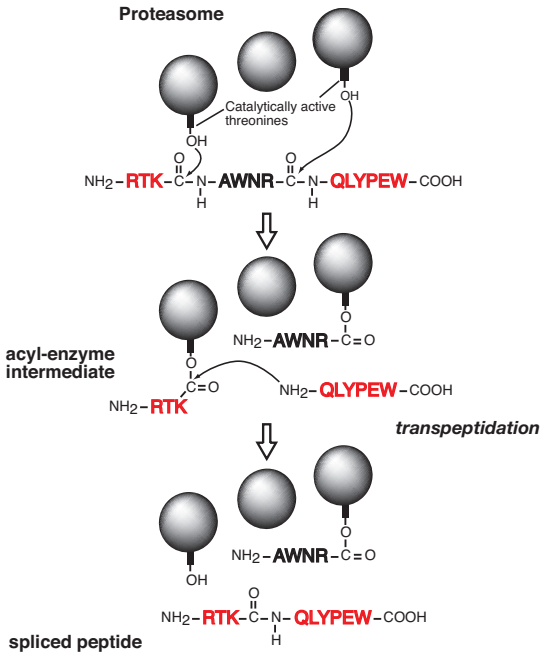

Figure 1. Model of the peptide-splicing reaction in the proteasome. The active site of the catalytic subunits of the proteasome is made up of the side-chain of a threonine residue, which initiates proteolysis by performing a nucleophilic attack on the carbonyl group of the peptide bond. An acyl- enzyme intermediate is formed, which is then liberated by hydrolysis. In the peptide-splicing reaction, a second peptide fragment appears to compete with water molecules for performing a nucleophilic attack on the acyl-enzyme intermediate, resulting in a transpeptidation reaction producing the spliced peptide. Experimental support for this model of reverse proteolysis includes evidence that the energy required to create the new peptide bond is recovered from the peptide bond that is cleaved at the amino-terminus of the excised fragment, and that the amino-terminus of the other fragment needs to be free for transpeptidation to occur.

IDENTIFICATION OF NEW ANTIGENS RECOGNIZED BY AUTOLOGOUS CTL ON HUMAN MELANOMA

W. Ma, N. Vigneron (in collaboration with P. Coulie)

Melanoma patient EB81 was vaccinated with a MAGE-type antigen and showed regression of all cutaneous metastases. Blood lymphocytes collected after the regression were stimulated with autologous tumor cells, and CTL clones were obtained. Surprisingly, none of these clones was directed against the antigen used for vaccination. Using a cDNA expression cloning approach, we identified the antigens recognized by three of them. These antigens correspond to three distinct peptides all derived from MAGE-C2, a gene with a cancer-germline expression pattern, which is expressed in about 40% of melanomas and 30% of bladder carcinomas. Two peptides are presented by HLA-A2, and one by HLA-B57 (6). Because of their strict tumor-specificity and their wide expression in tumors, these new antigens represent promising targets for cancer immunotherapy. The processing of two of these peptides is dependent on the immunoproteasome (2).

A NOVEL TUMOR IMMUNE ESCAPE MECHANISM BASED ON TRYPTOPHAN DEGRADATION BY INDOLEAMINE 2,3 DIOXYGENASE

C. Uyttenhove, L. Pilotte, I. Théate, D. Donckers, N. Parmentier, V. Stroobant, D. Colau

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme that catalyses rapid tryptophan degradation. Because tryptophan can freely cross the plasma membrane, IDO expression results in a local depletion of tryptophan in the extracellular medium surrounding the expressing cell. Tryptophan depletion was shown to impair T lymphocyte proliferation, and therefore IDO expression represents a powerful immunosuppressive mechanism that accounts, for example, for maternal tolerance to allogeneic fetuses, where IDO expression by placenta was found to play an essential role. Expression of IDO can be induced by interferon-gamma in many cellular types, including macrophages and dendritic cells, and appears to play a prominent role in immune regulation.

We have observed that many human tumors express IDO in a constitutive manner (7). To determine whether IDO expression provides tumor cells with a survival advantage by allowing their escape from immune rejection in vivo, we used the well-characterized model system of mouse tumor P815, where the antigen encoded by gene P1A is the major target of the 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 (7). These results suggest that the efficacy of therapeutic vaccination of cancer patients could be improved by concomitant administration of an IDO inhibitor. We are currently searching for new IDO inhibitors that could be developed at the clinical level. We also study the expression of IDO in normal mature dendritic cells, making use of a novel monoclonal antibody we have raised against human IDO (in collaboration with the group of Jean-Christophe Renauld).

AN INDUCIBLE MOUSE MODEL OF MELANOMA EXPRESSING A DEFINED TUMOR ANTIGEN

C. Powis de Tenbossche, C. Hervé, S. Goriely (in collaboration with A.-M. Schmitt-Verhulst, CIML, Marseille)

Cancer immunotherapy based on vaccination with defined tumor antigens has not yet shown strong clinical efficacy, despite promising results in preclinical models. This discrepancy might result from the fact that available preclinical models rely on transplantable tumors, which do not recapitulate the long-term host-tumor interplay that occurs in patients during progressive tumor development and results in tumor tolerance. To create a faithful preclinical model for cancer immunotherapy, we generated a transgenic mouse strain developing autologous melanomas expressing a defined tumor antigen recognized by T cells (8). We chose the antigen encoded by P1A, a well-characterized murine cancer germline gene. To transform melanocytes, we aimed at simultaneously activating the Ras pathway and inactivating tumorsuppressor Ink4a/Arf, thereby reproducing two genetic events frequently observed in human melanoma. The melanomas are induced by s.c. injection of 4-OH-tamoxifen (OHT). By activating a CreER recombinase expressed from a melanocyte-specific promoter, this treatment induces the loss of the conditional Ink4a/Arf gene in melanocytes. Because the CreER gene itself is also flanked by loxP sites, the activation of CreER also induces the deletion of its own coding sequence and thereby allows melanocyte-specific expression of genes H-ras and P1A, which are located downstream on the same transgene. All melanomas induced in those mice with OHT show activation of the Ras pathway and deletion of gene Ink4a/

Arf. In addition, these melanomas express P1A and are recognized by P1A-specific T lymphocytes. This model will allow to characterize the interactions between the immune system and naturally occurring tumors, and thereby to optimize immunotherapy approaches targeting a defined tumor antigen. We have now optimized the induction of melanoma in this model, and we reach an incidence of 70-80% tumors. In many tumor-bearing mice, we observed an accumulation of immature myeloid cells bearing both the CD11b and Gr1 markers. We are characterizing those cells, which might correspond to the myeloid-derived suppressor cells described in other models. In parallel, we have developed a strain of mice transgenic for the P1A-specific T cell receptor, which will be useful for such studies.

PHYSIOPATHOLOGY OF SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

B. Lauwerys, I. Gutierrez-Roelens, V. Badot, A.-L. Maudoux (in collaboration with F. Houssiau, Unité de Rhumatologie)

SLE is a systemic autoimmune disorder of unknown etiology. From a biological point of view, the disease is characterized by overt polyclonal B cell activation and CD4 T cell-driven production of specific autoantibodies directed against constituents of the chromatin. These antibodies (in particular the double-stranded DNA antibodies) are pathogenic and associated with the most severe manifestations of the disease. In order to better understand the underlying molecular pathways, we performed analyses of global gene expression on sorted CD4 T and B cells from SLE patients as compared to controls and patients with rheumatoid arthritis (RA), using Genechip U133 Plus 2.0 arrays. We also performed similar experiments on synovial tissue from SLE patients with arthritis. We found the presence of a strong type 1 interferon signature in SLE samples, i.e. the presence of numerous interferon-induced genes, as previously observed in SLE PBMC by other groups. We are currently investigating the physiopathological pathways that are dysregulated by the over-expression of these genes, using PBMC from patients and animal models of the disease.

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TUMOR GENETICS GROUP

Human tumors express specific antigens arising from the activation of genes, such as MAGE, BAGE, GAGE and LAGE/NY-ESO1, that are normally expressed only in germ cells. As germ cells are not subject to scrutiny by the immune system, antigens encoded by these genes are strictly tumor-specific. Our group has developed methods to identify genes that are specifically expressed in tumors and germ cells (1, 2). Most of these genes have their normal site of expression in spermatogonia, the pre-meiotic stage of sperm development, and are located on the X chromosome (3). Efforts are now devoted to determining the function of «cancer-germline» genes and deciphering the mechanism leading to their activation in tumor cells.

To analyze the functions of a MAGE protein, MAGE-A1, we searched to identify binding partners of this protein. Using yeast two-hybrid screening, we found an interaction between MAGE-A1 and transcriptional regulator SKIP (4). SKIP is an adaptor protein that connects DNA-binding proteins to proteins that activate or repress transcription. Results obtained by transient transfection in HeLa cells indicate that by binding to SKIP and by recruiting histone deacetylase 1, protein MAGE-A1 present in the nucleus can act as a potent transcriptional repressor. In addition, we have observed an interaction between MAGE-A1 and DNA methyltransferases (DNMT). Since recruitment of DNMT3A by the Myc transcription factor has been shown to repress the p21Cip1 promoter (5), we are now trying to evaluate whether promoters could be repressed by MAGE-A1 in the presence of DNMT.

It has been recently published that the germline-specific gene BORIS (Brother Of the Regulator of Imprinted Sites) might be responsible for the activation of most cancer-germline genes, including MAGE-A1 (6). In collaboration with Charles De Smet and his group, we have evaluated the frequency of BORIS activation in melanoma by quantitative RT-PCR (7). BORIS activation was detected in 27% (n = 63) of melanoma tissue samples. Surprisingly, many melanoma samples expressed MAGE-A1 and other cancer-germline genes in the absence of BORIS activation, suggesting that BORIS is not an obligate factor for activation of these genes in melanoma. Moreover, we could not induce expression of MAGE-A1 by forced expression of BORIS in two melanoma cell lines, one sarcoma cell line, immortalized human keratinocytes and normal human fibroblasts. It appears therefore that BORIS is neither necessary nor sufficient for the activation of cancer-germline genes.

Finally, in collaboration with Nicolas Van Baren and Francis Brasseur, we are analyzing the molecular mechanisms by which IFN-γ, TGF-β, IL1-β and TNF-α reduces the expression of melanocyte differentiation genes. We are also evaluating, in collaboration with Pierre van der Bruggen, the consequences of cytokine exposure for recognition of melanoma cells by cytolytic T lymphocytes.

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REGULATION OF T LYMPHOCYTE FUNCTION IN TUMORS

We identified in the early 1990s the first gene coding for a human tumor antigen recognized by cytolytic T lymphocytes (CTL). It was named MAGE-A1. We spent several years to define antigenic peptides encoded by genes such as those of the MAGE gene family. These peptides have been used in therapeutic vaccination trials of cancer patients. Efforts have been devoted to set up assays that accurately monitor T cell responses to cancer vaccines, including regulatory T cells.

We further analyzed the properties of anti-tumor CTL clones. The differentiation of naive T cells into memory and effector cells is marked by changes in the expression of surface molecules such as CCR7 and CD45. We found that the expression of CD45RA on CCR7$^-$ CD8$^+$ T cells is indicative of the time elapsed since the last antigenic stimulation rather than the signature of a terminally differentiated status with an incapacity to proliferate.

Anti-tumor T cells are present in tumor metastases that are progressing. This spontaneous anti-tumor T cell response must become ineffective at one point, possibly because the effector cells have become unable to exert their function, a state known as anergy. This anergy could result from inhibitory processes elicited by tumor cells. The group is currently involved in the study of T cell anergy. We have identified a novel mechanism causing anergy of human tumor-infiltrating lymphocytes, and established a new approach to correct this anergy in vitro. We observed that exhausted CTL clones and anergic tumor-infiltrating lymphocytes had lost the colocalization of T cell receptor (TCR) and CD8. Effector function and TCR-CD8 colocalization were restored with competitive galectin binders, such as sugars, suggesting that the binding of TCR to galectin plays a role in the distancing of TCR from CD8. Administration of competitive galectin binders may be a therapeutic option to induce a more efficient and long-lasting anti-tumor immune response.

In the 1970s it became clear that T lymphocytes, a subset of the white blood cells, were the major effectors of tumor rejection in mice. In the 1980s, human anti-tumor cytolytic T lymphocytes (CTL) were isolated in vitro from the blood lymphocytes of cancer patients, mainly those who had melanoma. Most of these CTL were specific, i.e. they did not kill non-tumor cells. This suggested that they target a marker, or antigen, which is expressed exclusively on tumor cells. We started to study the anti-tumor CTL response of a metastatic melanoma patient and contributed to the definition of several distinct tumor antigens recognized by autologous CTL. In the early 1990s, we identified the gene coding for one of these antigens, and defined the antigenic peptide (1). This was the first description of a gene, MAGE-A1, coding for a human tumor antigen recognized by T lymphocytes.

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 are therefore referred to as "cancer-germline genes". They encode tumor specific antigens, which have been used in therapeutic vaccination trials of cancer patients (2). A large set of additional cancer-germline genes have now been identified by different approaches, including purely genetic approaches. As a result, a vast number of sequences are known that can code for tumor-specific shared antigens. The identification of a larger set of antigenic peptides, which are presented by HLA class I and class II molecules and recognized on tumors by T lymphocytes, could be important for therapeutic vaccination trials of cancer patients and serve as tools for a reliable monitoring of the immune response of vaccinated patients. To that purpose, we have used various approaches that we have loosely named "reverse immunology" , because they use gene sequences as starting point (3).

The group of Stefan Constantinescu found that a mutation in the kinase JAK2 is present in a majority of *Polycythemia vera* patients. An abnormal peptide, resulting from the JAK2 mutation, could be expressed on the cell surface of the malignant clone present in the bone marrow of *Polycythemia vera* patients. This would correspond to a new tumor antigen, able to be targeted by cytolytic T lymphocytes. The research project of Vijay Singh in our group, in collaboration with N. Van Baren and S. Constantinescu, is to evaluate whether such an abnormal peptide exists on *Polycythemia vera* cells, to characterize it, and to isolate cytolytic T lymphocytes that recognize this peptide and kill abnormal cells.

Human tumor antigens recognized by $CD4^+$ or $CD8^+$ T cells are being defined at a regular pace worldwide. Together with colleagues at the de Duve Institute, we read the new publications and incorporate the newly defined antigens in a database accessible at <http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm>

ANALYSIS OF THE T CELL RESPONSES OF CANCER PATIENTS

$CD8^+$ T cells

J. Carrasco (till end 2005), D. Godelaine, V. Ha Thi, in collaboration with A. Van Pel, K. Thielemans (Vrije Universiteit Brussel) and B. Neyns (Vrije Universiteit Brussel)

We analyzed the T cell response in one patient who regressed upon vaccination with autologous dendritic cells pulsed with MAGE-3.A1 and MAGE-3.DP4 peptides. This patient developed a mixed tumor response, with disappearance/reduction of metastases and appearance of new metastases (Figure 1). In many MAGE-vaccinated patients who show tumor regression, the frequency of anti-vaccine T cells is very low. We wondered how such a low number of T cells could provide the main component of the specific effectors destroying the tumor cells. This led us to evaluate the frequency of all the T cells that recognized the

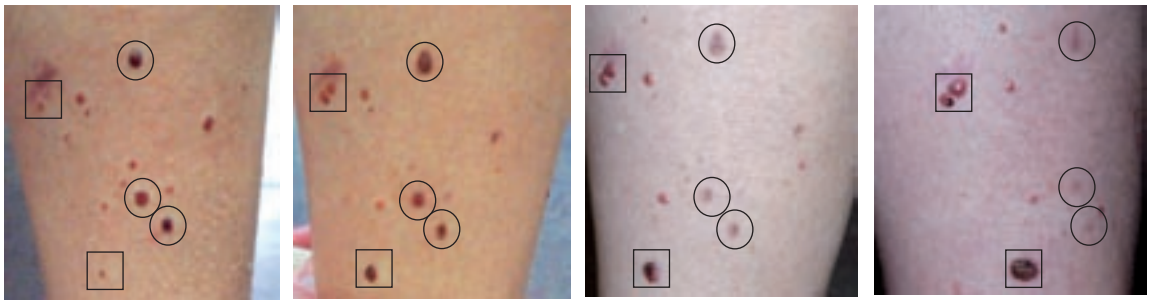

Figure 1. Development of a mixed tumor response in patient LB2586. Regressing lesions on the posterior face of the right leg are encircled. The squares frame lesions that progressed over the same period of time.

autologous tumor cell line. We performed an in-depth longitudinal analysis of the anti-vaccine and anti-tumor responses, in the blood and in tumor sites of this patient (4). Before vaccination, the patient already had high frequencies of anti-tumor T cells in the blood. Skin metastases contained T cells but they were apparently inactive in destroying tumor cells. Upon vaccination, a modest anti-vaccine response was observed and this response lasted over the observation period of three years, with no evident concentration of anti-vaccine T cells at tumor sites. Interestingly, a new anti-tumor CTL clone appeared in the blood after vaccination and was found to be enriched by more than 1,000-fold in the metastases. This anti-tumor CTL was directed against a previously unknown antigen, a MAGE-C2-derived peptide presented by HLA-B44 (Figure 2) (5).

Three other MAGE-C2 antigenic peptide were identified with CTL isolated from melanoma patients who displayed impressive CTL responses against these antigens, reaching frequencies in a tumor metastasis of several percent of CD8 T cells. Whether this makes MAGE-C2 derived antigens particularly useful for anti-tumoral vaccines will have to be determined by clinical experimentation.

The CD8 T cell response observed in this patient reinforce an hypothesis proposed by T. Boon and P. Coulie: anti-vaccine CTL are not the effectors that kill the tumor cells but their arrival at the tumor site containing exhausted anti-tumor CTL, generates conditions allowing

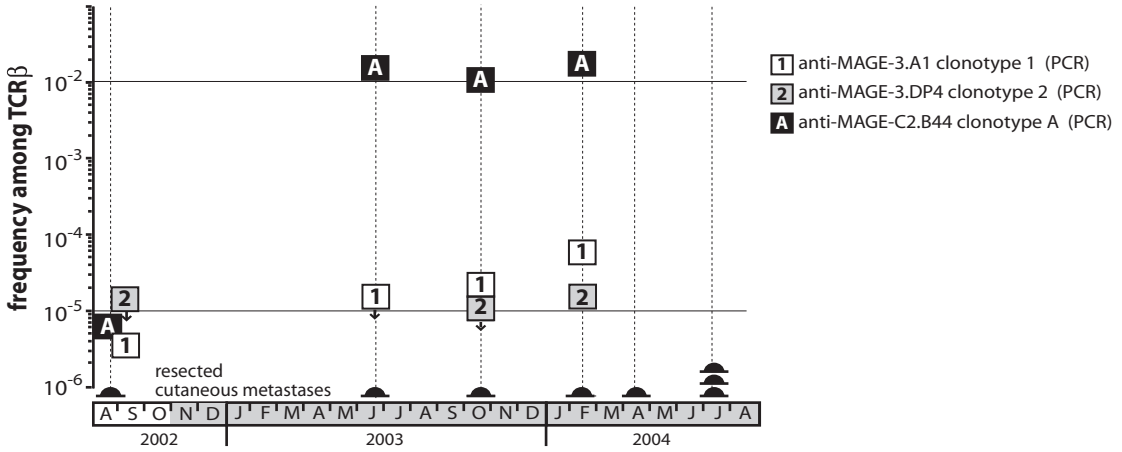

Figure 2. Frequency and diversity of anti-vaccine and anti-tumor T cells in various skin metastases of patient LB2586. The frequencies were estimated by PCR using primers specific for the CD3/TCRβ region of each clonotype.

the reawakening of the exhausted CTL and/or activation of new anti-tumor CTL clones, some of them contributing directly to tumor destruction.

CD4⁺ T cells

V. François, S. Ottaviani, N. Renkvist (till end 2005), D. Colau, P. van der Bruggen, in collaboration with S. Lucas, J. Stockis, G. Schuler (University of Erlangen, Germany), K. Thielemans (Vrije Universiteit Brussel) and B. Neyns (Vrije Universiteit Brussel)

We have produced HLA-DP4 fluorescent multimers loaded with the MAGE-A3243-258 peptide and designed a method to analyze low-frequency specific CD4 T cell responses in vaccinated cancer patients, by ex vivo staining of the blood cells with the fluorescent multimers and amplification of the sorted multimer⁺ CD4 clones. The specificity of the clones was assessed by their ability to secrete cytokines or to upregulate activation markers upon contact with the MAGE-3 antigen (6). Using this approach, low frequencies of about 1 out of 1 million CD4 T cells could be detected (Figure 3). One advantage of the multimer approach is that antigen-specific CD4 T cells are detected independently of their effector functions. It allows therefore the detection of anti-vaccine

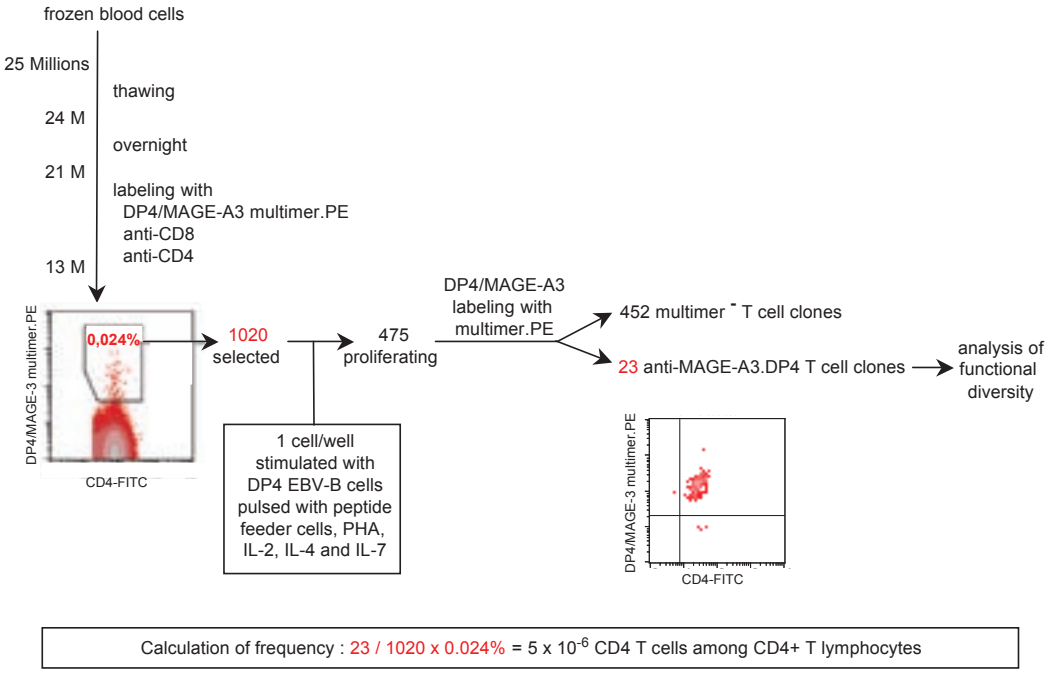

Figure 3. Overview of the procedure used to isolate anti-MAGE-A3.DP4 T cell clones. The numbers indicated correspond to an experiment performed with blood cells of a vaccinated patient. Frozen blood cells were thawed and kept overnight in culture medium. Cells were collected and labeled with DP4 fluorescent multimers folded with a MAGE-A3 peptide (DP4/MAGE-A3), anti-CD8 and anti-CD4 antibodies. Multimer⁺ CD8⁻ CD4⁺ cells were selected by flow cytometry and distributed at one cell per microwell. After three weekly stimulations, cloned T cells that had proliferated were screened with the multimer. Functional diversity of the clones was assessed by their ability to release cytokines IFN-γ, IL-10 and IL-2 upon contact with the antigen. We calculated the frequency by multiplying the fraction of cloned cells that yielded anti-MAGE-A3.DP4 clones with the fraction of the CD4 T cells that were considered multimer⁺ by flow cytometry analysis.

CD4 T cells secreting no cytokines, including for instance regulatory T cells. We have analyzed the anti-vaccine CD4 T cell response of 14 patients enrolled in four different clinical trials. The patients were injected either with peptides, with or without adjuvant, or dendritic cells pulsed with peptides. All the vaccines contained at least the synthetic MAGE-A3243-258 peptide presented to CD4 T cells by HLA-DP4 molecules. We wondered if the type of vaccine had any influence on the frequency and functional phenotype of anti-MAGE-3.DP4 CD4 T cells.

The anti-MAGE-A3.DP4 T cell responses were polyclonal with frequencies ranging from 1/500,000 to 1/500 CD4 blood T lymphocytes. Their functional diversity was high, but we found no correlation between the type of vaccine and the functional phenotype of the anti-vaccine T cells. Twelve out of 196 clones expressed CD25 in resting state, upregulated CD25 upon stimulation but released no cytokine, suggesting that they are MAGE-A3-specific regulatory T (T_{reg}) cell clones. We searched for functions or markers that would identify T_{reg} clones. A suppression assay based on peptide stimulation was designed and used to screen a set of clones, which were also analyzed for cytokine secretion, *FOXP3* protein expression and for the demethylation of the first intron of *FOXP3*. All but one clone that had an in vitro suppressive activity expressed CD25 in resting state, stained positive for *FOXP3*, released no cytokine upon stimulation, and had a demethylated *FOXP3*. This is the first demonstration that vaccination can induce vaccine-specific T cells with in vitro suppressive activity.

SIGNIFICANCE OF THE CD45RA EXPRESSION ON MEMORY AND EFFECTOR CD8$^+$ T CELLS

J. Carrasco (till end 2005), D. Godelaine, P. van der Bruggen, in collaboration with A. Van Pel

It was reported that the differentiation of naive T cells into memory and effector cells is marked by changes in the expression of surface molecules such as CCR7 and CD45. The quality of the immune response to viruses and tumors was often evaluated with the concept that the CD45RA$^+$CCR7$^-$ cells are the terminally differentiated, most effective CD8$^+$ T cells for the destruction of tumor cells and virus-infected cells (Figure 4). We re-examined this issue by studying, after T-cell receptor stimulation, the

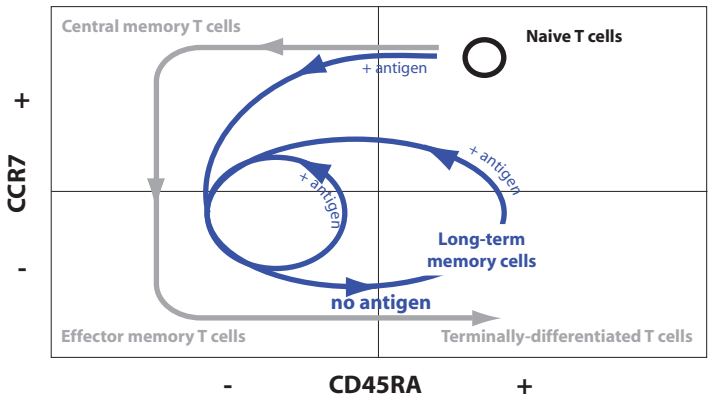

Figure 4. Model for the significance of CCR7 and CD45RA expression on CD8 T cells. The linear differentiation model proposed by Lanzavecchia & Sallusto (Nature, 1999), and Champagne & Pantaleao (Nature, 2001), is indicated in grey. The pathways proposed on the basis of our observations are indicated in blue.

time course of CD45RA and CCR7 expression both on blood CD8 T cells and on CTL clones directed against the MAGE-3 antigen (7). Our results indicate that $CD45RA^+CCR7^-$ $CD8^+$ T cells are resting memory cells which, upon antigenic stimulation and during the next 10 days, can proliferate, lose CD45RA and transiently acquire CCR7. In the absence of further antigenic stimulation, they progressively re-express CD45RA during the 10 following weeks and become $CD45RA^+CCR7^-$. We conluded that the expression of CD45RA on $CCR7^-CD8^+$ T cells is indicative of the time elapsed since the last antigenic stimulation rather than a terminally differentiated status with incapacity to proliferate. This observation leads to a re-interpretation of the significance of the presence of $CD45RA^+CD8^+$ T cells in patients with viral infections or cancer.

A MECHANISM CAUSING ANERGY OF $CD8^+$ T LYMPHOCYTES

N. Demotte, D. Colau, S. Ottaviani, D. Godelaine, C. Wildmann, I. Jacquemart, G. Wieërs, V. Singh, P. van der Bruggen, in collaboration with V. Stroobant, P. Courtoy (ICP, Cell Unit), P. Van Der Smissen (ICP, Cell Unit), I.F. Luescher (Ludwig Institute for Cancer Research, Lausanne Branch), C. Hivroz (Institut Curie, Paris), J.-L. Squifflet (Cliniques Universitaires Saint-Luc) and M. Mourad (Cliniques Universitaires Saint-Luc)

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. The labeling of these inactive CTL by an HLA-peptide tetramer was strongly reduced, even though the amount of T cell receptor (TCR) at their surface is similar (8).

We have identified a novel mechanism causing anergy of T lymphocytes, including human tumor-infiltrating lymphocytes, and established new approaches to correct this anergy in vitro. This type of anergy appears to be a transient status during the normal stimulation cycle of T lymphocytes. We observed that from day 1 after antigen stimulation, cytolytic T lymphocytes clones (CTL) lose the capacity to secrete cytokines and in some case their cytolytic activity. These functions are recovered gradually and are usually completely restored by day 14. We noticed that the TCR and the CD8 co-receptor were co-localized at the cell surface of functional CTL but, on the contrary, distant at the cell surface of non-functional CTL.

Human CD8 tumor-infiltrating T lymphocytes (TIL) were isolated from tumor ascites or solid tumors and compared with T lymphocytes from blood donors. TCR were distant from CD8 on the cell surface of TIL, whereas TCR and CD8 were co-localized on blood T lymphocytes. We observed that these TIL were anergic, being unable to secrete INF-γ or other cytokines after non-specific stimulation with anti-CD3 and anti-CD28 antibodies (Figure 5).

On the basis of several publications, we hypothesized that the absence of TCR-CD8 co-localization at the cell surface of non-functional CTL and anergic TIL is due to the loss of TCR mobility trapped into a lattice of glycoproteins (including TCR) clustered by extracellular galectin-3. To test this hypothesis, we incubated anergic CTL and TIL with a disaccharide ligand of galectin-3, N-acetyllactosamine. Treated CTL and TIL recovered the TCR-CD8 co-localization and the capacity to secrete IFN-γ and other cytokines after stimulation. These results were recently published (Figure 6) (9).

The overexpression of galectin-3 by tumor cell lines and the presence of galectin-3 in ascites and tumors have been shown in many studies and confirmed in our laboratory. Our observations indicate that TIL can recover ex vivo their effector functions with galectin li-

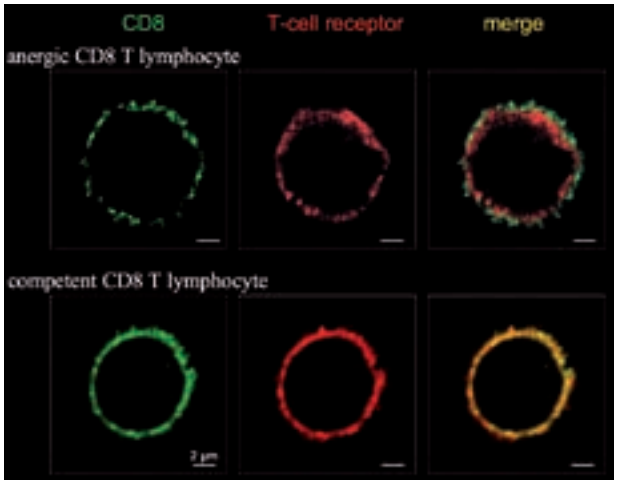

Figure 5. Absence of colocalization of the T cell receptor and CD8 co-receptors at the surface of anergic CD8 T lymphocytes.

gands and suggest that treatment of cancer patients with galectin ligands could correct TIL anergy. It is possible that peptide vaccination combined with galectin ligands induce a more efficient and long-lasting anti-tumoral immune response. It is also possible that treatment of cancer patients with galectin ligands, without peptide vaccination, will also trigger a broad activation of existing anti-tumor T cells, which could destroy the tumor. Examining whether correcting anergy of tumor infiltrating lymphocytes with drugs as simple as oligosaccharides can be translated into a clinical application will be our next challenging goal.

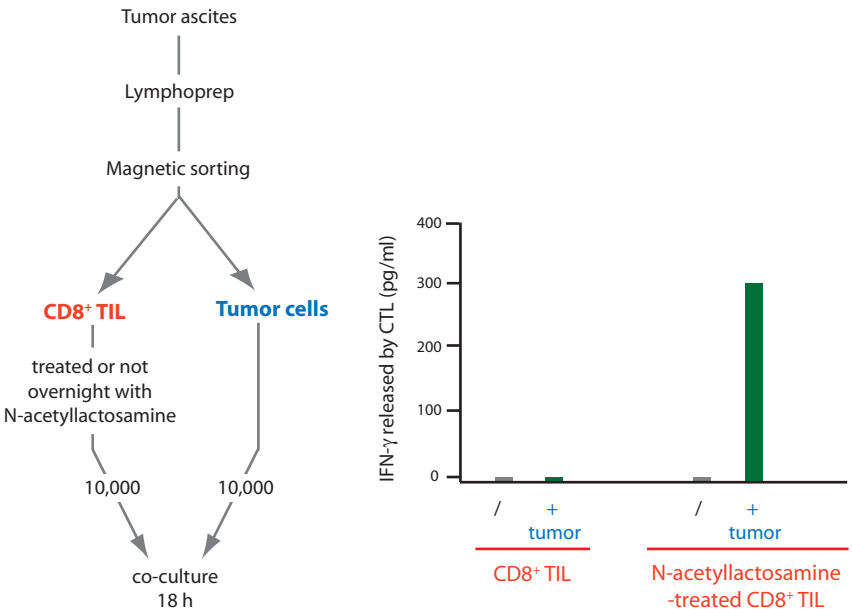

Figure 6. Recovery of effector function by «sugar-treated» tumor infiltrating T lymphocytes.

THEILERIA PARVA CANDIDATE VACCINE ANTIGENS RECOGNIZED BY IMMUNE BOVINE CYTOTOXIC T LYMPHOCYTES

A Collaboration between The International Livestock Research Institute (ILRI), Nairobi, Kenya, The Institute for Genomic Research (TIGR), Rockville, USA, Merial, Lyon, France, The Wellcome Trust Centre for Human Genetics, Oxford, United Kingdom, The Centre for Tropical Veterinary Medicine, Edinburgh, United Kingdom, The Ludwig institute for Cancer Research, Brussels, Belgium, D. Colau, C. Wildmann, and P. van der Bruggen

East Coast fever, caused by the tick-borne intracellular apicomplexan parasite *Theileria parva*, is a highly fatal lymphoproliferative disease of cattle. The pathogenic schizont-induced lymphocyte transformation is a unique cancer-like condition that is reversible with parasite removal. Schizont-infected cell-directed $CD8^+$ CTL constitute the dominant protective bovine immune response after a single exposure to infection. However, the schizont antigens targeted by *T. parva*-specific CTL are undefined (Figure 7).

To identify antigens, cells transiently transfected with schizont cDNA were screened with CTL from live vaccine-immunized cattle of diverse bovine leukocyte antigen (BoLA) MHC class I genotypes (10). In a first approach, a cDNA library was constructed in Brussels with RNA extracted from schizont. Pools of cDNA were transfected either in immortalized bovine skin fibroblasts or in monkey COS cells. The first screening of this library was performed in Brussels with CTL imported from Kenya and the other screening were performed at ILRI, Kenya. In a second approach, genes that were predicted to contain a secretion signal, by using preliminary sequence data of the *T. parva* chromosome, were cloned, transiently transfected in antigen-presenting cells and tested for recognition by CTL. The approach was based on the observation that the schizont lies free in the host cell cytoplasm whereby secreted parasite proteins would directly access the host cell MHC class I antigen processing and presentation pathway. Five candidate vaccine antigens were identified. $CD8^+$ T cell responses to these antigens were boosted in *T. parva*-immune cattle resolving a challenge infection and, when used to immunize naive cattle, induced CTL responses that correlated with survival from a lethal parasite challenge. These data provide a

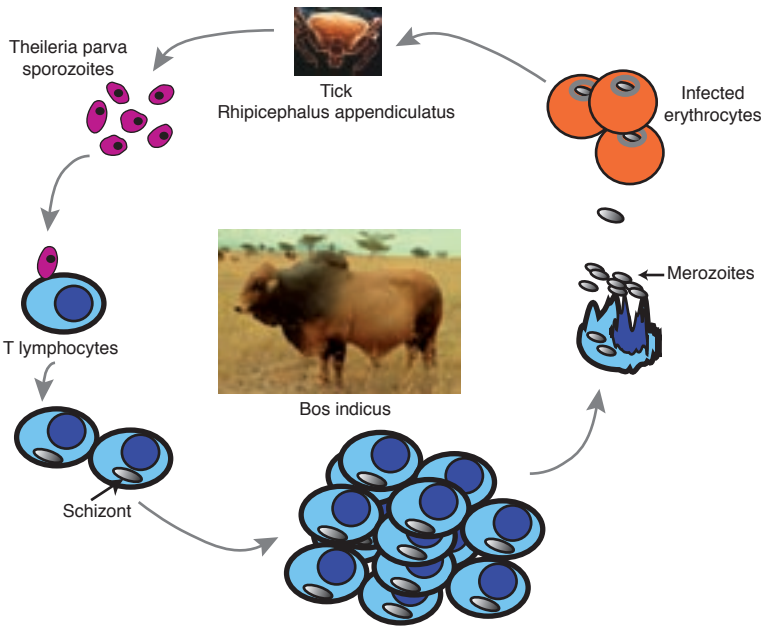

Figure 7. Life cycle of *Theileria parva*, a parasite responsible of the East Coast fever.

basis for developing an anti-East Coast fever subunit vaccine. To monitor anti-vaccine T cell responses in immunized animals, bovine MHC-peptide multimers have been constructed and produced in Brussels. The specificity of these multimers was validated by staining of relevant and non-relevant CTL clones. Experiments are in progress to define the optimal conditions to detect CTL in PBL from immunized animals.

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THERAPEUTIC VACCINATION AND TUMOR EXPRESSION PROFILING GROUP

Tumor cells carry antigens such as MAGE antigens that are absent from normal tissues, and that can be targeted by cytolytic T lymphocytes (CTL). Whilst it is possible to make such CTL recognize and kill autologous tumor cells in vitro, the precise way to induce an effective CTL response against a MAGE antigen in cancer patients is not known yet. In clinical vaccination trials, patients with a MAGE-expressing cancer, often melanoma, are treated repeatedly with a MAGE vaccine. These trials have two main objectives. First, the effectiveness of various vaccination modalities can be assessed by following the clinical evolution of the tumor, by analyzing whether a specific CTL response to the vaccine antigen occurred, and by determining whether immunological and clinical responses are correlated. Secondly, these trials allow crucial biological material to be collected from vaccinated patients. Blood samples provide anti-tumoral CTL clones, which can be functionally characterized. Tumor samples can be analyzed by expression microarrays and immunohistology, which allows to study the interaction between the tumor environment and the immune cells at the transcriptional level. New vaccination modalities can then be defined based on the knowledge acquired from these analyses.

THERAPEUTIC VACCINATION WITH MAGE TUMOR ANTIGENS

In collaboration with J.F. Baurain (Centre du Cancer, Cliniques Universitaires St Luc) and the group of P. Coulie (Cellular Genetics Unit, de Duve Institute). The clinical trial program was set up and a large part of it was carried out by M. Marchand.

We have set up small-scale clinical trials aimed at evaluating the toxicity, the antitumoral effectiveness and the immunological response in cancer patients immunized with MAGE vaccines involving either peptides, a recombinant protein or a recombinant viral vector. A total of about 380 patients have been included in these multicenter trials.

Clinical trials with the MAGE-3.A1 peptide

In a pilot study, the synthetic Mage-3.A1 peptide was administered to 45 HLA-A1 patients with MAGE-3 expressing melanoma, by subcutaneous (s.c.) and intradermal (i.d.) injections of 100 or 300 µg of peptide on three occasions at monthly intervals. No significant toxicity was reported. Of the 25 melanoma pa-

tients with measurable disease who received all 3 immunizations, seven displayed tumor regressions. We observed 3 complete responses (CR), 1 partial response (PR) and 3 mixed responses i.e., a regression of some metastases while others appear, progress, or stabilize (MxR).

Other vaccination modalities involving the same peptide were investigated in melanoma patients with measurable disease. This peptide was mixed with the immunological adjuvant MPL + QS21 and injected intramuscularly at 4-week intervals to 5 patients, without any evidence of tumor regression. A combination of the MAGE-3.A1 and MAGE-1.A1 peptides was administered s.c. and i.d. every 3 weeks to 11 patients. Two of them experienced tumor regression (1 CR, 1 MxR). MAGE-3.A1 was injected s.c. and i.d. every 10-11 days instead of every 3-4 weeks to analyze whether vaccination at higher frequency could improve the clinical response rate. Among 21 patients treated, three had regressions of tumor lesions (3 MxR). The same peptide was associated with the HLA class II-restricted MAGE-3.DP4 peptide, in order to induce both CD8 and CD4 T cell responses, hoping for an improved immunological and antitumoral effectiveness. None of the 7 patients evaluable after 9 i.d. and s.c. vaccinations given every 10-11 days had tumor regression.

Initial assessment of the CTL responses induced by vaccination with the MAGE-3.A1 peptide was hampered by the lack of sensitivity of available CTL monitoring techniques.

More recently, a new approach with improved sensitivity, involving lymphocyte-peptide culture and the use of HLA/peptide tetramers, was used to document a significant increase in CTLp frequency in a patient who showed tumor regression following vaccination with this peptide at high frequency. This method also showed that the CTL response was monoclonal. It was extended to 19 other patients who received this peptide without adjuvant. None had a detectable CTL response, indicating that this vaccine is weakly immunogenic.

In another study, patients with completely resected primary or regional metastatic melanoma with a high risk of relapse have been vaccinated with the MAGE-3.A1 peptide injected i.d. and s.c. every 2 weeks on 6 occasions. The purpose was to analyze whether vaccination of melanoma patients with less advanced disease in the adjuvant setting would improve the immunological response to a peptide vaccine. No CTL response was detected by our tetramer assay in the 6 patients who have received the complete treatment, including 3 patients with a resected tumor that did not express the appropriate antigen and who are assumed to be immunologically naive.

An ongoing study tests a cocktail of 8 different HLA-A2 restricted peptides mixed with an immunological adjuvant, either CpG7909 or Montanide ISA51. CpG7909 is an immunostimulatory CpG-containing oligonucleotide, which activates antigen presenting cells after binding to Toll-like receptor 9, and is thought to enhance CTL responses. Montanide is similar to incomplete Freund's adjuvant. It forms a water-in-oil emulsion with the peptides in solution, which allows to administer the vaccine mix as a long-lasting depot into the skin. The vaccine is injected on 6 occasions by i.d. and s.c. routes, at 2-week intervals. Fourteen patients are planned in each treatment arm. The purpose is to determine whether the adjuvanted multipeptide vaccine increases the CTL responses, and whether improved tumor response rates will be achieved. Fourteen patients have already received the peptides + CpG7909 vaccine, which was well tolerated. Three of them have shown evidence of tumor regression (all MxR). CTL responses against at least one of the 8 tumor antigens were detected in 6 patients, none of whom had a tumor response. A majority of these CTL responses were directed at the NY-ESO-1.A2 antigen. The second treatment arm in which Montanide is combined with the peptides is ongoing. Seven patients have already been included.

Clinical trials with the MAGE-3 protein

In a phase I/II trial, the recombinant Mage-3 protein was tested as a vaccine in patients with MAGE-3 expressing cancer, mainly melanoma. The patients received either 30, 100 or 300 µg of the protein, with or without the immunological adjuvants MPL and QS21, repeatedly by intramuscular injection. No severe toxicity was reported. Among 33 evaluable melanoma patients, four experienced regressions of metastatic lesions, 2 partial and 2 mixed responses. A partial response was also observed in a patient with metastatic bladder cancer.

The clinical efficacy of the MAGE-3 protein injected i.d. and s.c. without adjuvant in non-visceral melanoma patients was tested in another study. Patients received 300 µg of MAGE-3 protein on 6 occasions at 3-week intervals. Five out of 26 evaluable patients have shown regressions, including 1 partial response and 4 mixed responses. Thus this vaccine does not seem to induce more regressions than the MAGE-3.A1 peptide, but it does not require that the patient carries a specific HLA type. We then mixed this recombinant protein with adjuvant AS15 containing CpG 7909 in addition to MPL and QS21, and combined these i.m. injections with the administration of selected class I or class II peptides by i.d. and s.c. routes, which may result in the simultaneous activation of both CD8+ and CD4+ specific T lymphocytes. 11 patients were included in that study before its early closure. Three of them had a mixed response.

Clinical trial with an ALVAC-MAGE virus.

40 patients with advanced cancer, including 37 with melanoma, were vaccinated with a recombinant canarypox (ALVAC) virus containing a minigene that encodes the MAGE-1. A1 and MAGE-3.A1 antigens, followed by booster vaccinations with the 2 corresponding peptides. The treatment comprised 4 ALVAC injections followed by 3 peptides injections, all i.d. and s.c., separated by 3 weeks each. Local inflammatory reactions at the sites of ALVAC injection were common, but were moderate in intensity and transient in duration. Among the 30 melanoma patients who received at least 4 ALVAC vaccinations, six experienced regression of one or more melanoma metastases. Significant CTL responses were detected in 3 of 4 patients with regressions, and in only one of 11 patients with disease progression, which indicates a significant correlation between immune and antitumor responses.

Summary of relevant observations and perspectives

Immunization with peptides, or with the MAGE-3 recombinant protein, both with or without adjuvant, or with the ALVAC recombinant viral vector, is devoid of significant toxicity. A minority of vaccinated melanoma patients (about 10 to 20%) show regression of metastatic lesions. This frequency is far beyond the reported incidence of spontaneous regressions of melanoma metastases, estimated at 0.2-0.3%, indicating that these regressions are linked to the vaccinations. However, only 5% of the patients experience a true clinical benefit. Some of the remissions have lasted for several years. There is no evidence that one of the vaccines tested is more effective against the tumors than the others. CTL responses were detected in a minority of patients vaccinated either with peptides or with the ALVAC virus. These responses were often weak, and, in the case of the MAGE-3.A1 antigen, were observed mostly in patients who had tumor regressions.

The most likely explanation for the poor effectiveness of cancer vaccines shown until now is the fact that tumors have acquired the ability to resist destruction by antitumoral T cells, through unknown mechanisms (see « Analysis of T cell responses of vaccinated cancer patients

» below). Future strategies aimed at improving cancer immunotherapy will undoubtedly rely on the characterization of these resistance mechanisms, which should define new important therapeutic targets. Vaccination at earlier stages, when the patient has no more detectable tumor after surgery but has a high risk of relapse, is another strategy that is being developed.

EXPRESSION PROFILING OF TUMOR SAMPLES FROM VACCINATED PATIENTS

In collaboration with the group of P. Coulie (Cellular Genetics Unit, de Duve Institute)

Using the microarray technology, we have established the gene expression profile of a series of tumor samples, mainly cutaneous metastases, obtained from melanoma patients, usually before the vaccine treatment was started. It is expected that a comparative analysis between samples from patients who experienced either tumor regressions or no regression at all will help us to identify genes whose expression is predictive of tumor response to cancer vaccines. Such genes might provide clues about the mechanisms by which tumors can resist destruction by immune cells.

We also use the microarray data to characterize the inflammatory events that take place inside those metastases, and to understand the interaction between the tumor cells and the inflammatory cells at the tumor site. This approach is combined with systematic immunohistological analysis of adjacent cryosections, using antibodies directed against tumor cells, T and B cells, macrophages, blood vessels, and various molecules involved in inflammatory reactions. In addition, selected tumors can be further analyzed by laser capture microdissection of small groups of cells with defined immunohistological characteristics, followed by quantitative RT-PCR analysis.

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CYTOKINES IN IMMUNITY AND INFLAMMATION

The cytokine group studies the biology of Interleukin-9 (IL-9) and IL-22, two cytokines discovered at the Branch. IL-9 is a TH2 cytokine that plays a role in immune responses against intestinal parasites and asthma. IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, upregulates the production of acute phase reagents in the liver. Its activity in inflammatory responses is modulated by a specific antagonist, the IL-22 binding protein (IL-22BP). The role of IL-9 and IL-22 in inflammation is currently being investigated using transgenic and gene-targeted mice for these cytokines and their receptors.

INTERLEUKIN 9

J.-C. Renauld, J. Van Snick, L. Knoops, V. Steenwinckel, M. Stevens

Interleukin-9 (IL-9) was discovered in our group in 1989, through its ability to sustain antigen-independent growth of certain murine T helper clones. We further identified human IL-9 by cross-hybridization with the mouse gene. Although IL-9 did not turn out to be a T cell growth factor for freshly isolated T cells, it was found particularly potent on T cell lymphomas, as an anti-apoptotic agent. To determine the biological activities of this factor, we generated transgenic mice overexpressing this cytokine. Analysis of these animals disclosed three essential properties of IL-9: its tumorigenic potential in T lymphocytes, its stimulatory activity on a particular subset of B lymphocytes, and its activity on mast cells and eosinophils with consecutive implications in asthma.

IL-9-transgenic mice : T cell lymphomas

IL-9 transgenic animals showed normal T cell development and T cell numbers but spontaneously developed thymic lymphomas at low frequency (5%) when maintained in a conventional environment. Two lines of evidence indicate that IL-9 is not a conventional oncogene but rather favors tumor development in response to exogenous stimuli. First, the tumor incidence was significantly lower when mice were maintained under pathogen-free conditions. Secondly, all IL-9 transgenic mice developed T cell lymphomas when exposed to subliminal doses of a chemical carcinogen or to irradiation, that were innocuous in wild type mice. The above mentioned anti-apoptotic activity of IL-9 provides an attractive explanation for these observations, namely that IL-9 could lead to increased survival of abnormal cells ge-

nerated by exposure to minimal doses of oncogenic stimuli. The potential implication of IL-9 in oncology was also confirmed in human systems by its constitutive expression in Hodgkin lymphomas.

IL-9-transgenic mice : B1 cell expansion

Further analysis of these IL-9-transgenic mice showed that a particular B lymphocyte population, called B-1 lymphocytes and usually restricted to the peritoneal and pleuropericardial cavities, were dramatically expanded in response to IL-9 overproduction. In addition, such cells were also found in the blood and in the lungs of IL-9 transgenic mice. This observation is reminiscent of mice that are prone to the development of diseases that are characterized by the production of autoantibodies, such as Systemic Lupus Erythematosus, and suggests that IL-9 might play a role in some autoimmune processes.

IL-9-transgenic mice : parasite infections and asthma

In addition, IL-9 transgenic mice were found to harbor increased numbers of mast cells in the intestinal and respiratory epithelia, and were also characterized by a general hypereosinophilia. This phenotypic characteristic was found to increase the capacity of these animals to expel nematodes like Trichinella spiralis or Trichuris muris., suggesting that IL-9 administration could protect susceptible hosts against these parasites. This was confirmed by taking advantage of a new strategy of anti-cytokine vaccination: mice vaccinated against their own IL-9 failed to expel T.muris parasites and had a decreased eosinophilic response against the parasite (1).

The other side of the coin was the discovery that IL-9 overexpression such as that characterizing the IL-9 transgenic animals resulted in bronchial hyperresponsiveness upon exposure to various allergens. Recent studies indicated that IL-9 promotes asthma through both IL-13 -dependent and IL-13-independent pathways (2). The potential aggravating role of IL-9 in asthma was confirmed by genetic analyses performed by others and pointing to both IL-9 and the IL-9 receptor genes as major candidate genes for human asthma. In addition, we found that asthma patients produce increased amounts of IL-9. Phase I clinical trials using anti-IL-9 antibodies produced in our laboratory have been initiated in collaboration with Medimmune.

IL-9 RECEPTOR AND SIGNAL TRANSDUCTION

Jean-Christophe Renauld, Laurent Knoops, Tekla Hornakova, Monique Stevens

Analysis of the mode of action of IL-9 at the molecular level was initiated in 1992 by the cloning of the murine and human IL-9 receptor (IL-9R) cDNAs. By further dissecting the signal transduction cascade triggered by IL-9, we showed that, upon IL-9 binding, the IL-9R associates with a co-receptor protein called γc. This induces the phosphorylation of the JAK1 and JAK3 tyrosine kinases, which are associated with IL-9R and γc, respectively. A single tyrosine residue of the IL-9R is then phosphorylated and acts as a docking site for 3 transcription factors of the STAT family, STAT-1, -3 and -5, which become phosphorylated and migrate to the nucleus, where they activate the transcription of a number of genes. This pathway is common to many cytokines but is often dispensable for their biological activities. For IL-9, our group demonstrated that activation of the STAT transcription factors is crucial for all the effects of IL-9 studied on various cell lines, including positive and negative regulation of cell proliferation, as well as inhibition of corticoid-induced apoptosis in T cell lymphomas. Further analysis demonstrated that STAT-1, -3 and -5 play specific, redundant and synergistic roles in the different activities of IL-9 in vitro (3).

The pathways responsible for IL-9-induced proliferation were studied in details, and this process was found to depend mainly on the activation of STAT-5, on the recruitment of the IRS-1 adaptor, and on the activation of the Erk MAP-Kinase pathway.

The signal transduction pathway downstream the IL-9 receptor is illustrated in Fig. 1.

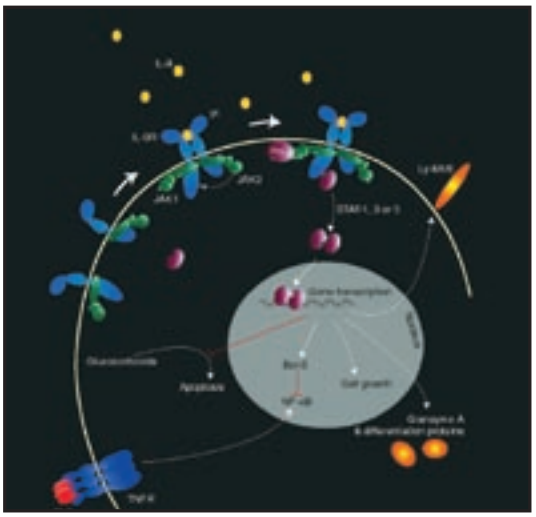

Figure 1. IL-9 receptor signalling.

ANTI-APOPTOTIC ACTIVITY OF I-309RECEPTOR AND SIGNAL TRANSDUCTION

J.-C. Renauld, A. Tounsi, J. Van Snick

Incidentally, our studies of this particular model of the regulation of cell death by cytokines, lead them to purify another protein called I-309, originally described as a human chemotactic factor, and that turned out to exert a significant anti-apoptotic activity for thymic lymphomas (4). However, I-309 and IL-9 trigger completely different pathways and it was shown that the I-309 anti-apoptotic activity was dependent on the activation of G-proteins and the Ras/MAPKinase pathway, whereas the IL-9-mediated effect was not. More recently, we showed that a viral protein related to human chemotactic factors (vMIP-I), and isolated from Herpes viruses that induce T cell tumors, has the same anti-apoptotic activity by binding to the I-309 receptor.

ROLE OF JAK OVEREXPRESSION IN TUMOR CELL TRANSFORMATION

J.-C. Renauld, L. Knoops, T. Hornakova, M. Stevens

Constitutive activation of the JAK-STAT pathway is frequent in cancer and contributes to oncogenesis. Some of our recent data indicate that JAK overexpression plays a role in such processes. Using a murine proB cell line that strictly depends on IL-3 for growth in vitro, cytokine-independent and tumorigenic clones were derived from a two-step selection process. Cells transfected with a defective IL-9 receptor acquired IL-9 responsiveness during a first step of selection, and progressed after a second selection step to autonomously growing tumorigenic cells. Microarray analysis pointed to JAK1 overexpression as a key genetic event in this transformation. Overexpression of JAK1 not only increased the sensitivity to IL-9 but most importantly allowed a second selection step towards cytokine-independent growth with constitutive STAT activation. This progression was dependent on a functional FERM and kinase JAK1 domain. Similar results were observed after JAK2, JAK3 and TYK2 overexpression. All autonomous cell lines showed an activation of STAT5, ERK1-2 and AKT but only TYK2-overexpressing cell lines showed a constitutive activation of STAT3. Thus, JAK overexpression can be considered as one of the oncogenic events leading to the constitutive activation of the JAK-STAT pathway. (5).

IL-TIF/IL-22 : A NEW CYTOKINE STRUCTURALLY RELATED TO IL-10

L. Dumoutier, M. de Heusch, J.-C. Renauld

Searches for genes specifically regulated by IL-9 in lymphomas lead to the cloning of a gene that turned out to encode a 179 amino acid long protein, including a potential signal peptide, and showing a weak but significant sequence homology with IL-10. This protein, originally designated IL-TIF for IL-10-related T-cell derived Inducible Factor, was later renamed IL-22. Recombinant human IL-22 was produced (with D. Colau, LICR) and its crystallograhic structure solved. Despite its structural homology with IL-10, IL-22 fails to recapitulate any of IL-10 biological activities. Biological activities of IL- 22 include the induction of acute phase proteins in liver (6) and protection against experimental hepatitis and colitis (L. Dumoutier, unpublished results). Among the different T cell subset, IL-22 was found to be preferentialy produced by TH17 cells raising some speculations about its potential role in autoimmune processes.

Analysis of genomic databases lead to the identification of a new receptor belonging to the IL-10 receptor family (7). This gene is located in the chromosome 6q24, at 24 kb from the IFNGR1 gene and at 152 kb from the IL-20R. It encodes a protein of 231 amino acid, showing 33 % and 34 % amino acid identity with the extracellular domains of the IL-22R and the IL-20R, respectively, but no cytoplasmic nor transmembrane domains were found. IL-22BP is highly expressed in the placenta, in the breast, in the mammary gland and in the skin. A specific interaction was demonstrated between insolubilized IL-22 and an IL-22-BP-Ig fusion protein. Moreover, recombinant IL-22BP could block IL-22 biological activity demonstrating that this protein can act as an IL-22 antagonist.

Although IL-22 does not share any biological activity with IL-10, these 2 cytokines share a common component of their respective receptor complex, IL-10Rß. Anti-IL-10Rß antibodies indeed block the IL-22-induced acute phase response in HepG2 cells (6). All receptor complexes for IL-10-related cytokines include a long chain and a short chain, based on the length of the cytoplasmic domain of these transmembrane proteins. IL-10Rß is a typical short chain component, with only 76 amino acids in the cytoplasmic domain, whose main function seems to consist in recruiting the Tyk2 tyrosine kinase. In addition to IL-10R ß, IL-22 signalling requires the expression of a long chain protein, called IL-22R and comprising a 319 amino acid long cytoplasmic domain. This chain associates with Jak1, and is responsible for the activation of cytoplasmic signalling cascades such as the JAK/STAT, ERK, JNK and p38 MAP kinase pathways. An unexpected feature of the IL-22R chain is the fact that the C-terminal domain of this receptor is constitutively associated with STAT3, and that STAT3 activation by this receptor does not require the phosphorylation of the receptor, in contrast to the mechanism of STAT activation by most other cytokine receptors (Dumoutier et al., submitted for publication).

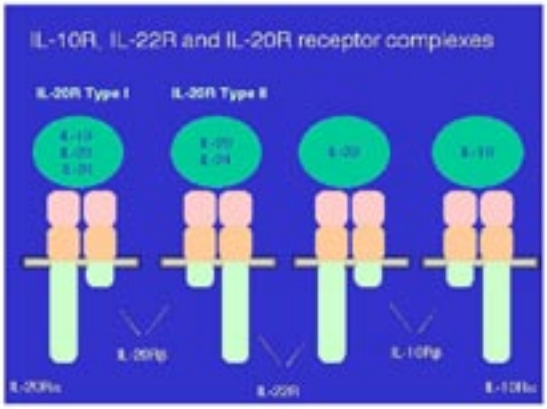

Figure 2. Receptor complexes for IL-10, IL-20 and IL-22

In addition to its role in IL-22 binding and signalling, the IL-22R chain also forms a functional heterodimeric receptor complex by associating with IL-20R ß, the second short chain member of the IL-10R-related receptor family. This complex mediates STAT-1 and –3 activation by IL-20 and IL-24, but not by IL-22 (8). In addition, IL-20 and IL-24 can also bind to other complexes consisting of IL-20Rα and IL-20Rß. This promiscuity in cytokine receptor usage is illustrated in Fig 2 (see also ref. 9 for a review of this cytokine family).

LICR2: A NEW CYTOKINE RECEPTOR MEDIATING ANTIVIRAL ACTIVITIES

J.-C. Renauld, L. Dumoutier

Type II cytokine receptors include receptors for type I and Ii interferons (IFNs) and for IL-10-related cytokines. These transmembrane proteins are almost exclusively related by their extracellular part, which consists of tandem fibronectin type II domains, whereas the cytoplasmic domain is associated with a tyrosine kinase of the Janus Kinase (JAK family). By screening genomic databases for similarity with the extracellular domain of these receptors, we identified a new receptor that we called LICR2 (Likely Interleukin or Cytokine rceptor 2). This receptor binds new cytokines designated IFN-λ1-3, and mediates the same activities as those mediated by the receptors for IFN-α and ß, including antiviral and antiproliferative activities (10), raising the possibility of therapeutic applications in viral infections and cancer.

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SIGNAL TRANSDUCTION AND MOLECULAR HEMATOLOGY GROUP

STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS

Cytokines and their receptors are critical for the formation of mature blood cells and for the function of the immune system. We study the structure and function of receptors for erythropoietin (Epo), thrombopoietin (Tpo), interleukins 2 and 9 (IL-2, IL-9) and Granulocyte Colony Stimulating Factor (G-CSF). Activation of these receptors is triggered by cytokine-induced changes in receptor dimerization/oligomerization, which lead to the activation of cytosolic Janus tyrosine kinases (JAKs). Regulation by JAK kinases of receptor traffic, the assembly of cell-surface receptor complexes, the mechanisms of dimerization of receptor transmembrane (TM) and cytosolic juxtamembrane (JM) domains, and mechanisms of JAK catalytic activation are major points of interest. The laboratory identified constitutively active mutants of JAK2 and of thrombopoietin receptor and is actively investigating the mechanisms by which JAK2 V617F and thrombopoietin receptor W515 mutants induce, in humans, Myeloproliferative Neoplasms, such as Polycythemia Vera or Primary Myelofibrosis.

THE MECHANISMS BY WHICH A MUTANT JAK2 INDUCES POLYCYTHEMIA VERA AND MYELOPROLIFERATIVE DISEASES IN HUMANS

C. Pecquet, J.-M. Heine

The JAK-STAT pathway is emerging as a key player in cancer, with several mutations in genes coding for JAKs being identified in the past three years (1). Janus kinases possess two kinase domains, one active and the other, denoted as the pseudokinase domain, inactive. JAK2, one of the four known JAKs (JAK1, JAK2, JAK3 and Tyk2) is crucial for signaling by several cytokine receptors, such as the erythropoietin receptor (EpoR), the thrombopoietin receptor (TpoR), the interleukin 3 receptor and the growth hormone receptor. JAKs are appended to the cytoplasmic juxtamembrane domains of receptors and are switched-on upon ligand binding to the receptors' extracellular domains.

Polycythemia Vera (PV), or the Vaquez disease, is characterized by excessive production of mature red cells and sometimes of platelets and granulocytes. Erythroid progenitors in PV are hypersensitive to or independent of erythropoietin (Epo) for proliferation and differentiation. Strikingly, the traffic of TpoR is defective in myeloid progenitors from PV. A hint that JAK2 or JAK2-binding proteins may be involved in PV came when we showed that the wild type JAK2 strongly promotes the maturation and cell-surface localization of TpoR, the very process that is defective in PV (2).

In collaboration with Prof. William Vainchenker and his INSERM unit at the Institut Gustave Roussy in Paris, we have been involved in the discovery of the *JAK2 V617F* mutation in a majority of *Polycythemia Vera* patients (3, 4). The mutation in the pseudokinase domain alters a physiologic inhibition exerted by the pseudokinase domain on the kinase domain. This mutant is found in >95% of PV patients and in 50% of Essential Thrombocythemia and Primary Myelofibrosis (PMF), two other diseases that belong to the myeloproliferative neoplasms (4). Strikingly, the homologous mutations in JAK1 and Tyk2 also enable these kinases to be activated without ligand-binding to cytokine receptors (5). These results suggested that point mutations in JAK proteins might be involved in different forms of cancers (1).

INVOLVEMENT OF TPOR IN MYELOPROLIFERATIVE DISEASES

C. Pecquet, M. Girardot

When the protein sequences of TpoR and the closely related EpoR were aligned, we realized that the TpoR contains a unique amphipathic motif (RWQFP) at the junction between the transmembrane and cytosolic domains. Deletion of this motif (delta5TpoR) results in constitutive activation of the receptor (6), suggesting that these residues maintain the receptor inactive in the absence of TpoR. Further stimulation with Tpo of the delta5TpoR leads to an excess of immature erythroid progenitors at the expense of megakaryocyte differentiation (6). In vivo, in reconstituted mice, the delta5TpoR induces massive expansion of platelets, neutrophils and immature erythroid progenitors and eventually myelofibrosis (Staerk et al., in preparation). Within the KWQFP motif (RWQFP in the human), the key residues that maintain the receptor inactive are the K/R and W residues; mutation of either of the two residues to alanine activates the receptor. We predicted that such mutations may exist in patients with myelofibrosis (6). Indeed, residue W515 has been found to be mutated to either leucine or lysine by the groups of D. G. Gilliland and A. Tefferi. Why the phenotype induced by TpoR W515 mutants is much more severe than that of JAK2 V617F is under investigation in our group.

At present, our laboratory is performing under the auspices of an ARC grant (Action de Recherche Concertée of the Université catholique de Louvain) with the St Luc Hospital departments of Hematology (Prof. Augustin Ferrant) and Clinical Biology (Prof. Dominique Latinne) a large study on the presence of JAK2 and TpoR mutations in patients with myeloproliferative neoplasms. Close collaborations with Drs. Laurent Knoops and Jean-Baptiste Demoulin are supported by the ARC project.

DETERMINATION OF THE INTERFACE AND ORIENTATION OF THE ACTIVATED EPOR, TPOR AND G-CSFR DIMERS

A. Dusa, J.-M. Heine, N. Caceres

Epo binding to the erythropoietin receptor (EpoR) results in survival, proliferation and differentiation of erythroid progenitors into mature red blood cells. In the absence of Epo, the cell-surface EpoR is dimerized in an inactive conformation, which is stabilized by inte-

ractions between the TM sequences. Epo binding to the extracellular EpoR domain induces a conformational change of the receptor, which results in the activation of cytosolic JAK2 proteins.

To identify the residues that form the interface between the receptor monomers in the activated EpoR dimer we have replaced the EpoR extracellular domain with a coiled-coil dimer of α-helices (7). Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (one), d (four), the register of the coiled-coil a-helices is imposed on the downstream TM α-helix and intracellular domain.

When each of the seven possible dimeric orientations were imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR, only two fusion proteins stimulated the proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells (7). Since the predicted dimeric interfaces of the two active fusion proteins are very close, a unique dimeric EpoR conformation appears to be required for activation of signaling. In this active conformation TM residues L241 and L244 and JM residue W258 are predicted to be in the interface.

Similar studies are undertaken for the related TpoR and G-CSFR. Like the EpoR, the TpoR is thought to signal by activation of JAK2, of several STATs (STAT1, 3 and 5) as well as of MAP-kinase, PI-3-kinase and AktB. However, TpoR and EpoR signal quite differently since only TpoR can induce hematopoietic differentiation of embryonic stem cells or stimulate the earliest stages of hematopoiesis in immature hematopoietic cells.

STRUCTURE AND FUNCTION OF JUXTA-MEMBRANE SEQUENCES IN TRANS-MEMBRANE PROTEINS

A. Dusa, J. Van Hees, C. Mouton, R.-I. Albu

To define the interfaces of the active and inactive EpoR dimers we performed cysteine scanning mutagenesis of the extracellular juxtamembrane and TM domains (10). We isolated three constitutively active novel mutants of the EpoR where residues L223, L226 or I227 were mutated to cysteine (10). These three mutants as well as cysteine mutants of residues 220-230 formed disulfide-bonded dimers. Cysteine-mediated maleimidyl crosslinking indicated that the first five TM residues are not helical and that the interface of the active EpoR dimer contains residues L241 and L244. Replacement of the first 4-5 predicted transmembrane residues of the EpoR, which form a helix-cap, with a stretch of leucine residues-which form an α-helix- leads to constitutive receptor activation (8).

These studies led to the notion that sequences flanking the transmembrane domain might play important roles in receptor function as «switch» regions and also may regulate transmembrane protein oligomerization. In a collaborative study with Jean-Noel Octave and Pascal Kienlen-Campard, we noted that the juxtamembrane and transmembrane domains of the Alzheimer's Precursor Protein (APP) contains three adjacent Gly-x-x-x-Gly motifs, that are predicted to promote tight dimerization of APP transmembrane domains. Replacement of the middle Gly-x-x-x-Gly motif by a Leu-x-x-x-Leu motif changed the dimerization interface of the APP transmembrane domain and abolished production of amyloidogenic peptides Abeta40 and Abeta42 (9). These results may be relevant for efforts to identify small molecules able to block the APP dimer into a dimer conformation that is unfavorable for the production of amyloidogenic peptides Aβ40 and Aβ42.

TRAFFIC OF CYTOKINE RECEPTORS TO THE CELL-SURFACE

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We have observed that, in hematopoietic cells, over-expression of JAK proteins leads to enhanced cell-surface localization of cytokine receptors (i.e. EpoR TpoR, IL9R, IL2R, γc). For some receptors, the effect of the cognate JAK is to promote traffic from the endoplasmic reticulum (ER) to the Golgi apparatus (12). For others, such as the TpoR, JAK2 and Tyk2 also protect the mature form of the receptor from degradation by the proteasome, and thus JAKs enhance the total amount of cellular receptor (2). In collaboration with Pierre Courtoy, we are employing confocal microscopy of epitope tagged receptors in order to define the precise intracellular compartments where receptors and JAKs interact. Our working hypothesis is that the N-terminus FERM domain of JAK proteins exerts a generic pro-folding effect on cytosolic domains of cytokine receptors. We are testing this hypothesis on several different cytokine receptors and are investigating the link between proper folding in the ER and transport to the cell-surface. Using a fetal liver retroviral cDNA library cloned in pMX-IRES-CD2, we are attempting to clone novel proteins that can regulate traffic and stability of TpoR.

RANDOM MUTAGENESIS APPROACHES TO STUDY INTERACTIONS BETWEEN TRANSMEMBRANE DOMAINS AND STRUCTURE OF JAK2 V617F

A. Dusa

Two transmembrane viral envelope proteins (gp55-P and gp55-A) belonging to the polycythemic (P) and anemic (A) Spleen Focus Forming Virus (SFFV) strains, can activate the EpoR when co-expressed in the same cell. In collaboration with Yoav Henis, Tel-Aviv University, Israel, we have shown that both the gp55-A and gp55-P TM domains specifically interact with the TM domain of the EpoR (Figure 1C and D). gp55-A weakly activates the receptor leading to erythroleukemia with low number of red blood cells (anemia). gp55-P fully activates the EpoR inducing erythroleukemia with elevated levels of red cells (polycythemia). The basis for this difference between gp55-P and gp55-A is represented by differences in specific binding of the TM domains to the TM domain of the EpoR. Taking advantage of this specific interaction we are constructing a genetic system where the TM sequence of gp55-P is randomized and tested for the ability to bind and activate the EpoR. In this system activation of EpoR signaling will result in cell survival and proliferation, which represents a powerful selection.

Second, we employ a random mutagenesis approach coupled to retroviral gene transduction in order to examine the sequence requirements at position V617 of JAK2 for constitutive activation. Mutants of JAK2 V617 to each of the other 18 amino acid residues were tested for constitutive kinase activity, for induction of cell proliferation and activation of STAT5. We identifed several other mutations (V617FW, V617L, V617I and V617I) which also lead to activation of JAK2 (10). Among those, only V617FW induces strong activation comparable to V617F and is resistant to the down-modulation effect of the negative regulator SOCS3. Since the V617F mutation also activated JAK1 and Tyk2 (5), we will test whether any of the potentially activating mutations may also activate JAK1 and Tyk2. We expect these results to shed light on the uniqueness of the JAK2 V617F in patients and on the structural requirements at position V617 of the JH2 region for activation.

CONSTITUTIVE ACTIVATION OF JAK-STAT SIGNALING PATHWAYS AND GENES TARGETED BY STAT5 IN TRANSFORMED HEMATOPOIETIC AND PATIENT-DERIVED LEUKEMIA CELLS

M. Girardot

Cytokine stimulation of cytokine receptors, induces transient activation of the JAK-STAT pathway. In contrast, oncogenic forms of receptors or of JAKs (JAK2 V617F) transmit a continuous signal which results in constitutive activation of STAT proteins. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors or JAKs in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously. In these transformed cells many of the transient signaling events induced by cytokines are detectable permanently, i.e. ligand-independent phosphorylation of JAK and STAT proteins or high levels of nuclear activated STATs especially STAT5 and STAT3. A similar picture has been noted in patient-derived leukemia cells. The critical questions we would like to answer concern the mechanisms by which the JAK-STAT remain permanently activated in transformed cells and which genes are regulated by constitutively active STAT proteins in leukemic cells. Using chromatin immunoprecipitation and sequencing of native promoters bound by STAT5 we noted that in transformed cells STAT5 can also bind to low affinity N4 sites (TTC-NNNN-GAA) not only to N3 sites, which are characteristic of ligand-activated STAT5. We are attempting to identify the promoters actually bound by STAT proteins in living cells in physiologic and pathologic situations. We use a modified version of the chromatin immunoprecipitation assay pioneered by Alex Varshavsky in conjunction with DNA microarray genomic profiling. The isolated genomic fragments are screened for the presence of STAT-binding sites and tested for the ability to regulate transcription of reporter genes. Newly identified genes regulated by such genomic sequences will be tested for function by cloning their cDNA expressed in bicistronic retroviral vectors that allow wide expression of candidate proteins at physiologic levels.

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