Research at

the de Duve Institute

and at

the Brussels Branch of

the Ludwig Institute for Cancer Research (LICR)

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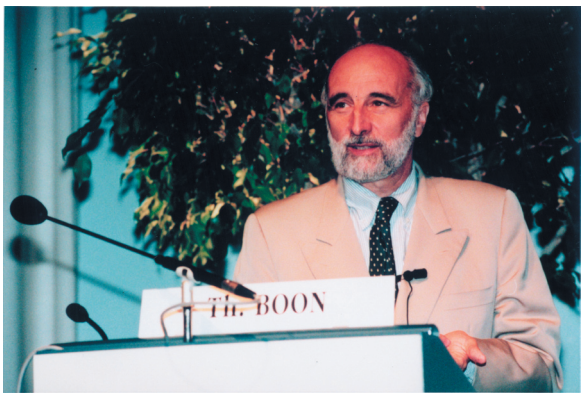
Ludwig Institute for Cancer Research (LICR)

Brussels branch

The Ludwig Institute for Cancer Research

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

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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. In the second example of spliced peptide, which is a minor histocompatibility antigen, the two fragments are rearranged before splicing.

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.

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.*

*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 and the 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 (IFNγ).

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 several antigenic peptides of interest for cancer immunotherapy, including HLA-A2-restricted epitopes derived from tyrosinase, Melan-A^{MART1} 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 IFNγ (2). By analysing 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 IFNγ. 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. We are currently characterizing these intermediate proteasomes, in terms of function and expression.

Antigenic peptides produced by peptide splicing in the proteasome

A. Dalet, V. Stroobant, (in collaboration with Edus 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 antige-

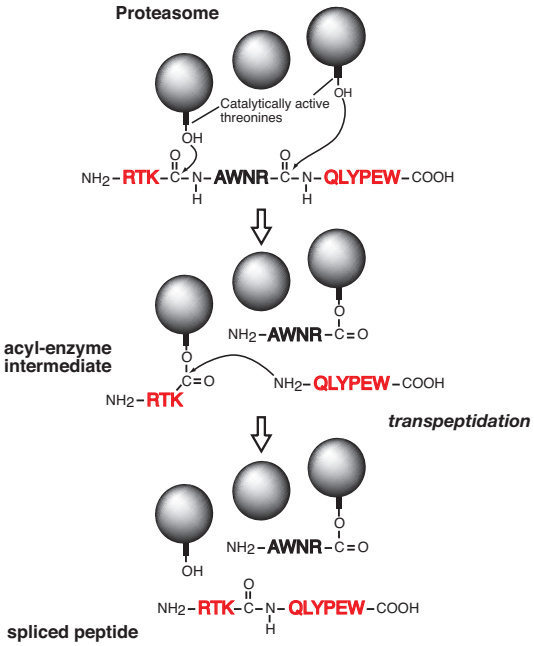

Figure 1. Model of the peptide-splicing reaction in the proteasome. The active site of the catalytic subunits of the proteasome is made up of the side-chain of a threonine residue, which initiates proteolysis by performing a nucleophilic attack on the carbonyl group of the peptide bond. An acyl-enzyme 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.

nic 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.

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-germ-line 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 of 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.

An inducible mouse model of melanoma expressing a defined tumor antigen

C. Hervé (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 germ line gene. To transform melanocytes, we aimed at simultaneously activating the Ras pathway and inactivating tumor suppressor 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. 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.

Selected publications

1. Morel S, Levy F, Burlet-Schiltz O, Brasseur F, Probst-Kepper M, Peitrequin AL, Monsarrat B, Van Velthoven R, Cerottini JC, Boon T, Gairin JE, Van den Eynde BJ. *Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells*. **Immunity** 2000;12:107-17.

2. Chapiro J, Claverol S, Piette F, Ma W, Stroobant V, Guillaume B, Gairin JE, Morel S, Burlet-Schiltz O, Monsarrat B, Boon T, Van den Eynde BJ. *Destructive cleavage of antigenic peptides either by the immunoproteasome or by the standard proteasome results in differential antigen presentation*. **J Immunol** 2006;176:1053-61.

3. Van den Eynde BJ, Morel S. *Differential processing of class-I-restricted epitopes by the standard proteasome and the immunoproteasome*. **Curr Opin Immunol** 2001;13:147-53.

4. Vigneron N, Stroobant V, Chapiro J, Ooms A, Degiovanni G, Morel S, van der Bruggen P, Boon T, Van den Eynde B. *An antigenic peptide produced by peptide splicing in the proteasome.* **Science** 2004;304:587-90.

5. Warren EH, Vigneron N, Gavin M, Coulie P, Stroobant V, Dalet A, Tybodi S, Xuereb S, Mito J, Riddel S, Van den Eynde BJ. *An antigen produced by splicing of noncontiguous peptides on the reverse order.* **Science** 2006:313,1444-48.

6. Ma W, Germeau C, Vigneron N, Maernoudt A-S, Morel S, Boon T, Coulie PG, Van den Eynde BJ. *Two new tumor-specific antigenic peptides encoded by gene MAGE-C2 and presented to cytolytic T lymphocytes by HLA-A2.* **Int J Cancer** 2004;109:698-702.

7. Uyttenhove C, Pilotte L, Theate I, Stroobant V, Colau D, Parmentier N, Boon T, Van den Eynde BJ. *Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase.* **Nat Med** 2003;9:1269-74.

8. Huijbers IJ, Krimpenfort P, Chomez P, van der Valk MA, Song JY, Inderberg-Suso EM, Schmitt-Verhulst AM, Berns A, Van den Eynde BJ. *An inducible mouse model of melanoma expressing a defined tumor antigen.* **Cancer Res** 2006;66:3278-86.

9. Probst-Kepper M, Stroobant V, Kridel R, Gaugler B, Landry C, Brasseur F, Cosyns JP, Weynand B, Boon T, Van Den Eynde BJ. *An alternative open reading frame of the human macrophage colony-stimulating factor gene is independently translated and codes for an antigenic peptide of 14 amino acids recognized by tumor-infiltrating CD8 T lymphocytes.* **J Exp Med** 2001;193:1189-98.

10. Van Den Eynde BJ, Gaugler B, Probst-Kepper M, Michaux L, Devuyst O, Lorge F, Weynants P, Boon T. *A new antigen recognized by cytolytic T lymphocytes on a human kidney tumor results from reverse strand transcription.* **J Exp Med** 1999;190:1793-800.

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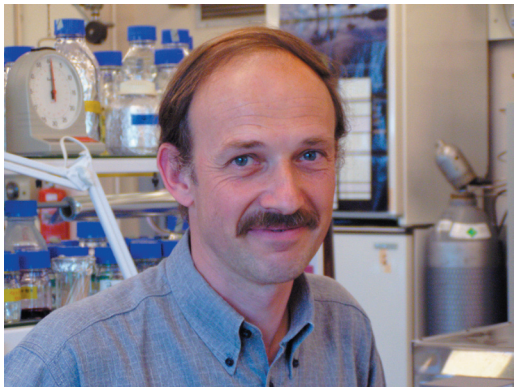

Human tumors express specific antigens arising from the activation of genes, such as MAGE, BAGE, GAGE and LAGE/NY-ESO1, which are normally expressed only in germ cells. As germ cells are not subject to scrutiny by the immune system, antigens encoded by these genes are strictly tumor-specific. The group of Etienne De Plaen and Charles De Smet has identified new genes that are specifically expressed in tumors and in germ cells. 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. Efforts are now devoted to deciphering the mechanism leading to the activation of "cancer-germline" genes in tumor cells and determining their function.

Charles De Smet and his group are studying the mechanisms leading to the activation of "cancer-germline" genes in tumors. It was previously shown by the group that these genes rely primarily on DNA methylation for their repression in normal somatic tissues, and that their activation in tumors is a consequence of the overall genome demethylation process that often accompanies tumorigenesis. Stable activation of cancer-germline genes in tumors does not require a permanent demethylating activity, but depends on the presence of specific transcription factors that maintain the promoter region unmethylated (1). Antisense-mediated knock-down experiments indicated that DNMT1 is the primary DNA methyltransferase to maintain methylation of cancer-germline genes (2), and that transient down-regulation of this enzyme suffices to induce stable activation of cancer-germline genes. This is supporting the view that hypomethylation of these genes in tumors results from a historical event of demethylation (2). The group is now trying to identify factors that induce DNA demethylation. Embryonic stem cells, which appear to have a demethylating activity, are currently tested as a potential source for the identification of such factors.

To analyze the functions of a MAGE protein, MAGE-A1, Etienne De Plaen and his group searched to identify binding partners of this protein. Using yeast two-hybrid screening, they found interaction between MAGE-A1 and transcriptional regulator SKIP (3). SKIP is an adaptor protein that connects DNA-binding proteins to proteins that activate or repress transcription. Their results suggest that by binding to SKIP and by recruiting histone deacetylase 1, protein MAGE-A1 present in the nucleus represses transcription. In addition, the group has 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 (4), the group is now trying to evaluate whether promoters could be repressed by MAGE-A1 in the presence of Dnmt.

Finally, in collaboration with Nicolas Van Baren and Francis Brasseur, the group is presently analyzing the molecular mechanisms by

which IFN-γ, TGF−β and TNF−α inhibits the expression of melanocyte differentiation genes. Data indicate that a soluble autocrine factor is released by the IFN-γ-treated melanoma cells. The group is in the process of isolating and characterizing this factor.

Selected publications

1. De Smet C, Loriot A, Boon T. *Promoter-dependent mechanism leading to selective hypomethylation within the 5' region of gene MAGE-A1 in tumor cells*. **Mol Cell Biol** 2004;24: 4781-90.

2. Loriot A, De Plaen E, Boon T, De Smet C. *Transient down-regulation of DNMT1 methyltransferase leads to activation and stable hypomethylation of MAGE-A1 in melanoma cells*. **J Biol Chem** 2006;281:10118-126.

3. Laduron S, Deplus R, Zhou S, Kholmanskikh O, Godelaine D, De Smet C, Hayward SD, Fuks F, Boon T, De Plaen, E. *MAGE-A1 interacts with adaptor SKIP and the deacetylase HDAC1 to repress transcription*. **Nucleic Acids Res** 2004;32:4340-50.

4. Brenner C, Deplus R, Didelot C, Loriot A, Viré E, De Smet C, Gutierrez A, Danovi D, Bernard D, Boon T, Pelicci PG, Amati B, Kouzarides T, de Launoit Y, Di Croce L, Fuks, F. *Myc represses transcription through recruitment of DNA methyltransferase corepressor*. **EMBO J** 2005;24:336-46.

IDENTIFICATION OF HUMAN TUMOR ANTIGENS

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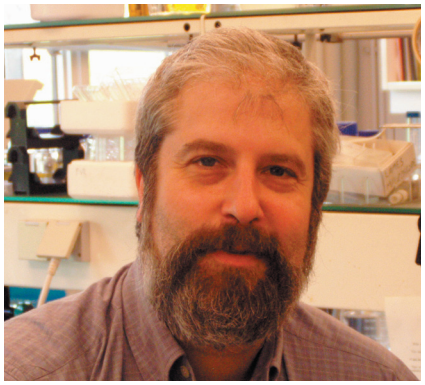


The group led by Pierre van der Bruggen has defined antigenic peptides encoded by genes such as those of the MAGE family. These peptides have been used in therapeutic vaccination trials of cancer patients and have served as tools for a reliable monitoring of the immune response of vaccinated patients. Efforts have been devoted to set up assays that accurately monitor CD4+ T cell responses to cancer vaccines. For patients vaccinated with a protein, we have validated a quantitative approach to isolate anti-vaccine T cells directed at all possible HLA-peptide combinations that could be targeted by the response. For patients vaccinated with a peptide, we have validated the use of the first HLA-DP4 tetramer, which was folded with a MAGE-3 peptide. By screening blood cells of vaccinated patients with this tetramer, we found that injections of the MAGE-3.DP4 peptide resulted in the activation and proliferation of specific T cells with various cytokine profiles, including IL-10-producing T cells and regulatory T cells. The group is currently involved in the study of functional defects of T cells. We have observed that human CTL clones lose their specific cytolytic activity and cytokine production under certain stimulation conditions. These inactive CTL simultaneously lose their labeling by an HLA-peptide tetramer, even though the amount of TCR-CD3 at their surface is not reduced. These results suggest the existence of a new type of functional defect of CTL.

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

S. Ottaviani, Z. Sun, V. Stroobant

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

immunizations against several antigens. Such immunizations could increase the primary anti-tumor efficacy of the vaccine.

We have used approaches that we have loosely named «reverse immunology» (2). They aim at identifying antigenic peptides recognized by T cells, using gene sequences as starting point. We have focused this search on the cancer-germline genes, which are expected to code for tumor-specific shared antigens on the basis of their pattern of expression.

Search for antigenic peptides recognized by CD8+ T cells

In a first approach, we stimulated $CD8^+$ T lymphocytes with dendritic cells transduced with viral vectors containing complete MAGE-coding sequences. As this requires the processing of the antigen by the dendritic cells, we surmised that the peptides that would be identified would also be processed in the tumors expressing the *MAGE* genes. A difficulty of the use of recombinant viruses is the activation of CTL precursors directed against viral antigens. We circumvented this problem by using different vectors for the stimulation of the microcultures, for the lytic assay with the responder T cells, and for the cloning step. This procedure is summarized in Fig. 1. Dendritic cells were infected with either an adenovirus or a canarypoxvirus, and they were used to stimulate microcultures of autologous $CD8^+$ T lymphocytes (3). After three weekly stimulations, the responder cells were tested for lysis on autologous EBV-B cells infected with vaccinia-MAGE. Positive microcultures were cloned. To identify the antigenic peptide, the resulting CTL clone was tested for lysis of autologous EBV-B cells pulsed with a complete set of peptides of 16 amino acids that overlap by 12. When a peptide scored positive, shorter peptides were synthesized to identify the shortest optimal peptide. To identify the HLA presenting molecule, the CTL clones were tested for stimulation by cells transfected with the MAGE cDNA together with cDNAs coding for the possible HLA presenting molecules. Finally, relevant tumor targets were used in a lysis assay to ascertain that the antigen was also processed by tumor cells.

In a second approach, a large number of

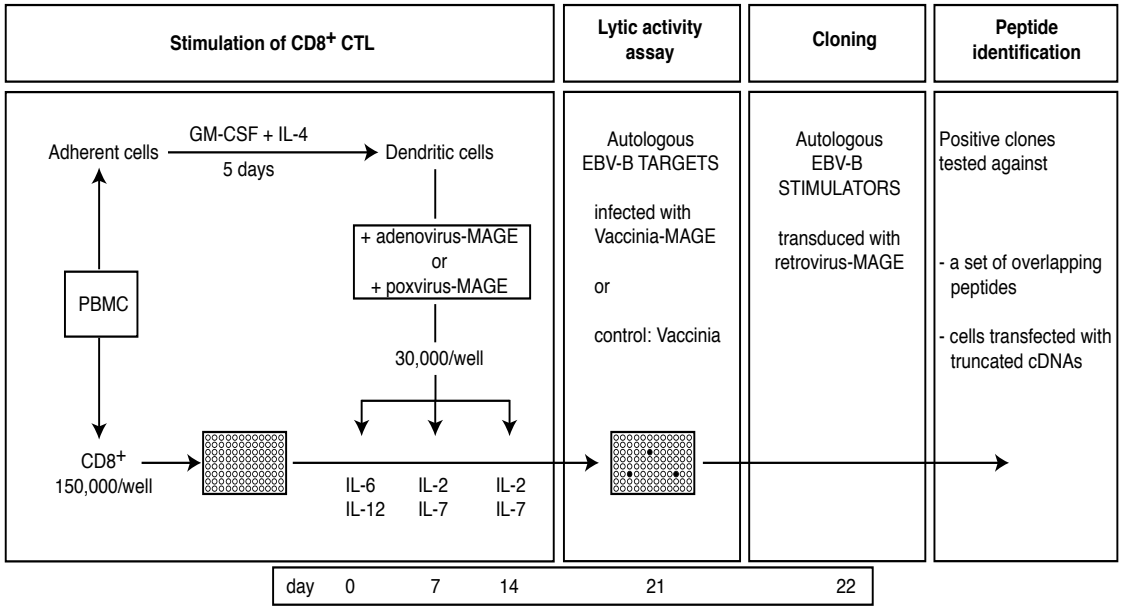


Figure 1. Overview of the procedure to obtain anti-MAGE $CD8^+$ CTL clones by stimulation with dendritic cells infected with viral vectors carrying a MAGE coding sequence.

T cells are incubated with HLA/peptide multimers conjugated to phycoerythrin (PE), and with anti-PE antibodies coupled to magnetic beads. The multimer-positive cells are first enriched by magnetic sorting. The selected cells are distributed in microwells and stimulated with peptide-pulsed autologous mature dendritic cells on days 0 and 7. The microcultures are screened on day 19 for the presence of cells specifically labeled with multimers (Fig. 2). Relevant tumor targets were used in a lysis assay to ascertain that the antigen was also processed by tumor cells (4).

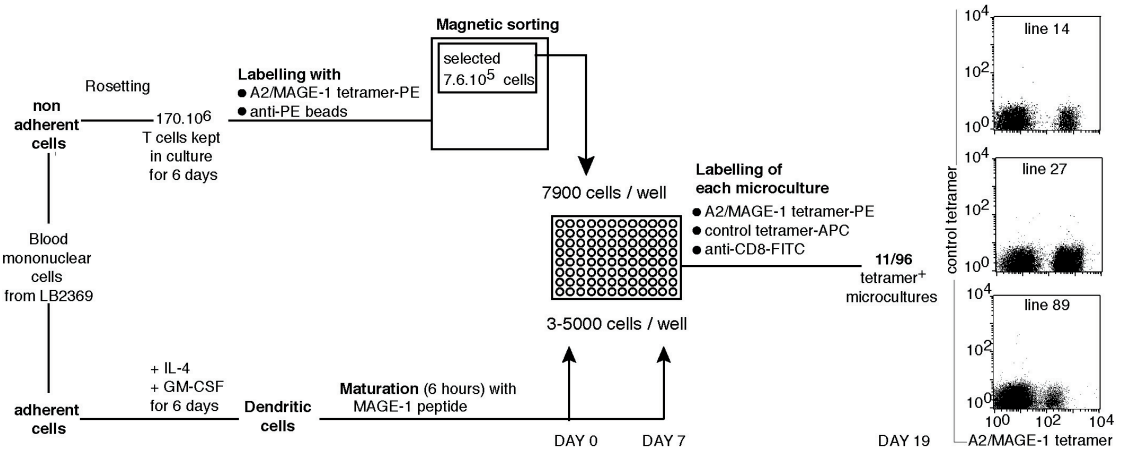


Figure 2. Overview of the procedure using HLA-peptide fluorescent multimers to isolate rare anti-MAGE-1 CD8⁺ CLTL clones.

Search for antigenic peptides recognized by CD4+ T cells

Studies in several animal models have demonstrated an important role for CD4⁺ T cells in inducing and maintaining anti-tumor immunity. It is therefore possible that the addition of antigenic peptides presented by class II to those presented by class I will improve the efficacy of therapeutic anti-tumor vaccination. To identify new HLA-peptide combinations, we used dendritic cells loaded with a recombinant MAGE protein to stimulate autologous CD4⁺ T lymphocytes (5). After four weekly stimulations, the responder cells were tested for their ability to secrete IFN-γ upon stimulation with the antigen, and the positive microcultures were cloned.

To identify the antigenic peptide, the positive clones were stimulated with a set of peptides of 16 amino acids that overlapped by 12 and covered the entire MAGE protein sequence. The positive peptide was then tested for recognition on several Epstein-Barr virus immortalized B cell lines (EBV-B cell lines) to identify the HLA presenting molecule.

MAGE-1 and MAGE-3 antigenic peptides identified by these procedures are listed in a database (http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm).

Study of the significance of the CD45RA expression on memory and effector CD8+ T cells.

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

The expression of CD45RA on CCR7⁻ human CD8⁺ memory T cells is widely consi-

dered to be a marker of terminal differentiation. We studied the time course of CD45RA and CCR7 expression on human antitumoral CTL clones and blood $CD8^+$ T cells after antigenic stimulation. Our results indicate that $CD45RA^+CCR7^-$ $CD8^+$ T cells are resting memory cells which, upon antigenic stimulation and during the next 10 days, proliferate, lose CD45RA and transiently acquire CCR7. In the absence of further antigenic stimulation, they progressively re-express CD45RA and become $CD45RA^+CCR7^-$ (6). We conclude that the expression of CD45RA on these cells is indicative of the time elapsed since the last antigenic stimulation rather than the incapacity to proliferate or a particularly high lytic potential. This concept leads to a reinterpretation of the significance of the presence of $CD45RA^+$ $CD8^+$ memory cells in patients affected by viral infections or by cancer.

Detection of CD4 T cell response in vaccinated cancer patients

D. Colau, V. François, S. Ottaviani

Patients injected with a protein

Immunizing patients with a MAGE-3 recombinant protein ought to induce T cell responses against several MAGE-3 peptides, including peptides recognized by CD4 T cells, and this might result in a more effective anti-tumor response. Moreover, protein vaccination alleviates the need to select patients according to their HLA, as many peptides presented by various HLA alleles are expected to be presented.

Quantitative evaluation of T cell responses of patients receiving anti-tumoral vaccination with a protein is difficult because of the large number of possible HLA-peptide combinations that could be targeted by the response. To evaluate the response of patients vaccinated with protein MAGE-3, we have developed an approach which involves overnight stimulation of blood T cells with autologous dendritic cells loaded with the protein, sorting by flow cytometry of the T cells that produce IFN-γ, cloning of these cells, and evaluation of the number of T cell clones that secrete IFN-γ upon stimulation with the antigen (8).

Patients injected with class II-restricted peptides

For therapeutic vaccination trials, the vaccine can consist of defined antigenic peptides. This approach greatly facilitates the monitoring of the T cell response, because the presumed target of the T cells is completely defined. It allows the use of HLA-peptide tetramers to detect T cell responses in patients. Although the production of soluble MHC class I multimers has rapidly expanded since their first use in 1996, the development of MHC class II complexes has proved to be more difficult probably due to the intrinsic structural instability of soluble class II molecules. We have developed a very sensitive approach using fluorescent HLA class II/peptide multimers to detect antigen specific CD4 T cells in cancer patients injected with vaccines containing the MAGE-$3_{243\text{-}258}$.DP4 peptide.

Production of HLA-DP4 multimers loaded with the MAGE-$3_{243\text{-}258}$ peptide

Dimers of soluble DP4.MAGE-3 complexes were produced in Drosophila S2 cells (Fig. 3A). The DPA1*0103 α and DPB1*0401 β chains were truncated to remove their transmembrane and cytosolic regions. Acidic and basic leucine zipper peptides were added at the C-terminus of the α and β chain respectively, to allow pairing of the chains. The MAGE-3 antigenic peptide was covalently linked to the N-terminal end of the β chain. The α chain was further modified by the addition of the murine IgG2a Fc domain to allow the dimerizaton of the DP4.MAGE-3 complex and further purification of the dimer by affinity chromatography. Fluorescent multimeric complexes (Fc Ig multimers) were produ-

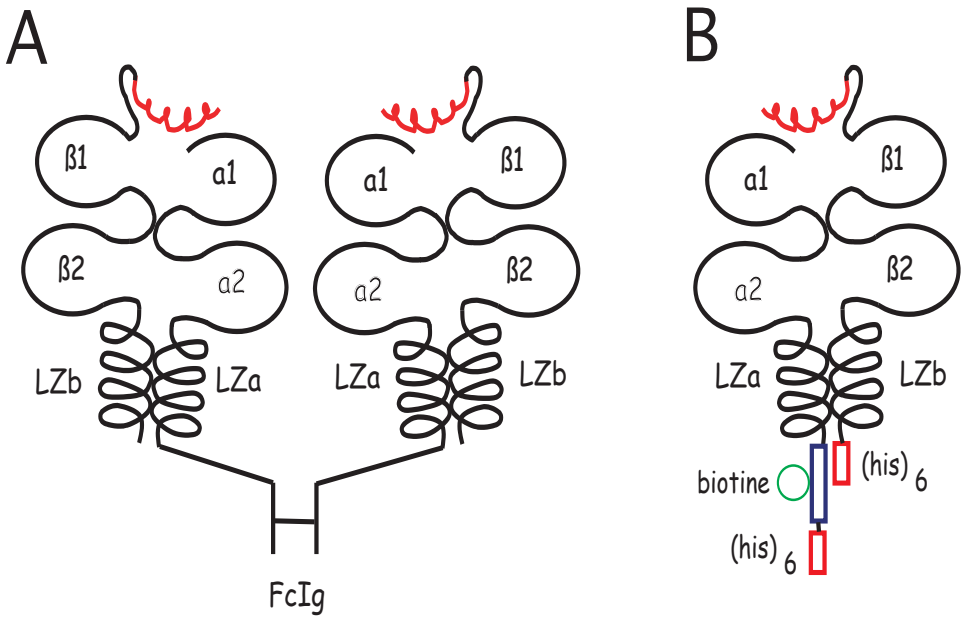


Figure 3. Schematic structures of DP4.MAGE-3 complexes.

ced by the addition of a biotinylated anti-Fc IgG2a antibody and PE labeled streptavidin. We also set up the construction of multimers from biotinylated DP4.MAGE-3 monomers (Fig. 3B). The structure of the DP4.MAGE-3 monomers was similar to the DP4.MAGE-3-FcIg construct with the following differences: the murine FcIg portion of the recombinant α chain, was exchanged for a sequence of six histidines and a tag for biotinylation followed by six histidines was fused to the C-terminus of the recombinant β chain. Monomers were produced in Drosophila S2 cells and purified by metal affinity chromatography. Fluorescent complexes (birA multimers) were obtained by the addition of PE labeled streptavidin. The specificity of the two multimers was demonstrated by staining of anti-MAGE-3.DP4 $CD4^+$ clones. Non-specific binding of the multimers to PBLs was evaluated on lymphocytes from a non cancerous $DP4^+$ patient. Staining with the DP4.MAGE-3 birA multimers showed a 5-fold lower level of non-specific binding (0.01% of the CD4 cells) in comparison to the DP4.MAGE-3-FcIg multimers (0.05%).

Analysis of the anti-MAGE-3.DP4 CD4 T cell responses in vaccinated cancer patients.

DP4.MAGE-3 birA multimers were used to stain *ex vivo* peripheral blood lymphocytes from melanoma patients injected with dendritic cells pulsed with several HLA class I and class II tumor antigenic peptides, including the $MAGE\text{-}3_{243\text{-}258}$ peptide (7). The multimer positive CD4 T cells were sorted and amplified in clonal conditions. Specificity of the clones was assessed by their ability to secrete cytokines or to upregulate activation markers upon contact with the MAGE-3 antigen. Using this approach, low frequencies of about $1x10^{-6}$ anti-MAGE-3.DP4 CD4 cells among CD4 cells could be detected.

Several patients injected with the MAGE-3.DP4 peptide, with or without adjuvant, or pulsed on dendritic cells were analyzed. The immune responses were polyclonal with frequencies ranging from $3x10^{-6}$ to $6x10^{-3}$. We found no correlation between the type of vaccine and the functional phenotype of the anti-vaccine T cells. A total of 200 $multimer^+$ clones were obtained that either upregulate activation marker CD25 upon contact with the MAGE-3.DP4 antigen, or release IFN-γ, IL-

10 or IL-2. Their functional diversity was high. Among these clones, 12 $CD4^+$ $multimer^+$ clones could be considered as regulatory T cell clones (Treg), as the rested cells expressed CD25 and foxp3, released no cytokine upon antigenic stimulation, and were able to suppress the proliferation of $CD4^+CD25^-$ T cells upon stimulation with anti-CD3/CD28 antibodies. This is the first report of human Treg clones with a known specificity that were induced by vaccination. Considering that expression of foxp3, in humans, was reported not to be sufficient to identify Treg cells, we took advantage of our large collection of anti-MAGE-3.DP4 clones to re-examine this question. We have designed an assay to measure the suppressive activity of the different clones after stimulation with antigenic peptides and in the presence of IL-2. In this assay, the proliferation of an indicator $CD4^+$ T cell clone upon stimulation with a MAGE-3.DR1 peptide was estimated by flow cytometry. This assay was performed together with an analysis of (a) foxp3 expression both at the protein and at the mRNA level, (b) the cytokines released upon antigenic stimulation, and (c) the diversity of the TCR repertoire.

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

N. Demotte, C. Wildmann, D. Colau

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 (9).

These first observations are now confirmed with 13 CD8 T cell clones and 20 CD4 T cell clones. Rested T cells have a $tetramer^{high}$ phenotype whereas all the clones become $tetramer^{low}$ after antigenic stimulation. A complete recovery of tetramer staining is usually observed within 2-3 weeks. The reduced tetramer staining is not the result of a downmodulation of the TCR. $Tetramer^{high}$ and $tetramer^{low}$ T cells are compared for their CD8 and TCR distribution on the membrane by confocal microscopy and electronic microscopy. In addition, microarray analyses are performed to compare the gene expression profiles of $tetramer^{high}$ and $tetramer^{low}$ cells.

Our results indicate that tetramers may fail to reveal some CD8 and CD4 specific T cells bearing the relevant TCR, even when such functionally arrested T cells retain the potential to participate in immune responses. They also suggest the existence of a new type of functional defect of T cells.

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, 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.

Two approaches were pursued to antigen identification, both dependent on screening of transiently transfected antigen-presenting cells with CTL from live vaccine-immunized cattle of diverse bovine leukocyte antigen (BoLA) MHC class I genotypes (10). For the 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 by using preliminary sequence data from one of the four *T. parva* chromosomes to contain a secretion signal, 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 signal peptide-containing parasite proteins would directly access the host cell MHC class I antigen processing and presentation pathway. Five candidate vaccine antigens that are the targets of MHC class I-restricted CTL from immune cattle 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 naïve cattle, induced CTL responses that significantly correlated with survival from a lethal parasite challenge. These data provide a basis for developing a CTL-targeted anti-East Coast fever subunit vaccine. In order to have tools 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.

Selected publications

1. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. *A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma.* **Science** 1991;254:1643-47.

2. van der Bruggen P, Zhang Y, Chaux P, Stroobant V, Panichelli C, Schultz ES, Chapiro J, Van den Eynde BJ, Brasseur F, Boon T. *Tumor-specific shared antigenic peptides recognized by human T cells.* **Immunol Rev** 2002;188:51-64.

3. Sun Z, Lethé B, Zhang Y, Russo V, Colau D, Stroobant V, Boon T, van der Bruggen P. *A new LAGE-1 peptide recognized by cytolytic T lymphocytes on HLA-A68 tumors.* **Cancer Immunol Immunother** 2005;55:644-52.

4. Ottaviani S, Colau D, van der Bruggen P. *A new MAGE-4 antigenic peptide recognized by cytolytic T lymphocytes on HLA–A24 carcinoma cells.* **Cancer Immunol Immunother** 2006;55:867-72.

5. Chaux P, Vantomme V, Stroobant V, Thielemans K, Corthals J, Luiten R, Eggermont AM, Boon T, van der Bruggen P. *Identification of MAGE-3 epitopes presented by HLA-DR molecules to CD4(+) T lymphocytes.* **J Exp Med** 1999;189:767-78.

6. Carrasco J, Godelaine D, Van Pel A, Boon T, van der Bruggen P. *CD45RA on human memory CD8 T cells is a marker for the time elapsed since the last antigenic stimulation.* **Blood** 2006;108:2897-905.

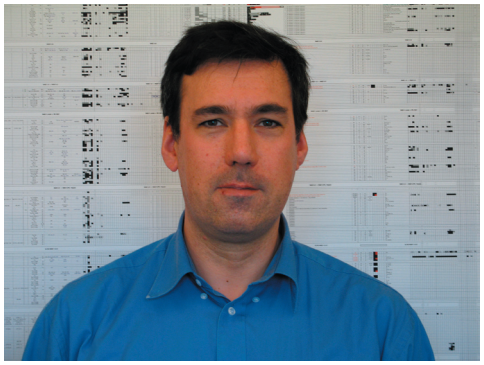
7. Zhang Y, Renkvist N, Sun Z, Schuler-Thurner B, Glaichenhaus N, Schuler G, Boon T, van der Bruggen P, Colau D. *A polyclonal anti-vaccine CD4 T cell response detected with HLA-DP4 multimers in a melanoma patient vaccinated with MAGE-3.DP4-peptide-pulsed dendritic cells.* **Eur J Immunol** 2005;35:1066-75.

8. Zhang Y, Sun Z, Nicolay H, Meyer RG, Renkvist N, Stroobant V, Corthals J, Carrasco J, Eggermont AM, Marchand M, Thielemans K, Wolfel T, Boon T, van der Bruggen P. *Monitoring of anti-vaccine CD4 T cell frequencies in melanoma patients vaccinated with a MAGE-3 protein.* **J Immunol** 2005;174:2404-11.

9. Demotte N, Colau D, Ottaviani S, Godelaine D, Van Pel A, Boon T, van der Bruggen P. *A reversible functional defect of $CD8^+$ T lymphocytes involving loss of tetramer labeling.* **Eur J Immunol** 2002;32:1688-97.

10. Graham SP, Pelle R, Honda Y, Mwangi DM, Tonukari NJ, Yamage M, Glew EJ, de Villiers EP, Shah T, Bishop R, Abuya E, Awino E, Gachanja J, Luyai AE, Mbwika F, Muthiani AM, Ndegwa DM, Njahira M, Nyanjui JK, Onono FO, Osaso J, Saya RM, Wildmann C, Fraser CM, Maudlin I, Gardner MJ, Morzaria SP, Loosmore S, Gilbert SC, Audonnet JC, van der Bruggen P, Nene V, Taracha EL. *Theileria parva candidate vaccine antigens recognized by immune bovine cytotoxic T lymphocytes.* **Proc Natl Acad Sci USA** 2006;103:3286-91.

THERAPEUTIC VACCINATION AND TUMOR GENE EXPRESSION PROFILING GROUP



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Tumor cells carry antigens such as MAGE antigens that are absent from normal tissues, and that can be targeted by cytolytic T lymphocytes (CTL) (1). 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, 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)

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 patients 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) (2).

Other vaccination modalities involving the same peptide were investigated in melanoma patients with measurable disease (3). 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 (4).

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. Four 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 (5).

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 (6). 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 (4).

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.

Gene expression profiling of tumor samples from vaccinated patients

Using the microarray technology, we have established the gene expression profile of a series of tumor samples, mainly cutaneous metastases, obtained from melanoma patients before the vaccine treatment was started. Comparative analysis between samples from patients who experienced either tumor regressions or no regression at all is ongoing. We hope to identify

genes whose expression is predictive of tumor response to cancer vaccines. The identity of such genes might help us to understand what happens in patients with tumor regression, and why this doesn't occur in patients without regression. 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.

Selected publications

1. Boon T, Coulie PG, van der Bruggen P, van Baren N. *Immunology of the cancer cell: T lymphocyte responses.* **In: Clinical Oncology, 2nd Edition. Eds: M. D. Abeloff, J.O. Armitage, A.S. Lichter, J.E. Niederhuber. Churchill Livingstone,** New York, 2000.

2. Marchand M, van Baren N, Weynants P, Brichard V, Dréno B, Tessier M-H, Rankin E, Parmiani G, Arienti F, Humblet Y, Bourlond A, Vanwijck R, Liénard D, Beauduin M, Dietrich P-Y, Russo V, Kerger J, Masucci G, Jäger E, De Greve J, Atzpodien J, Brasseur F, Coulie PG, van der Bruggen P, Boon T. *Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1.* **Int J Cancer** 1999;80:219-30.

3. Machiels JP, van Baren N, Marchand M. *Peptide-based Cancer Vaccines.* **Semin Oncol** 2002;29:494-502.

4. Coulie PG, Karanikas V, Lurquin C, Colau D, Connerotte T, Hanagiri T, Van Pel A, Lucas S, Godelaine D, Lonchay C, Marchand M, Van Baren N, Boon T. *Cytolytic T-cell responses of cancer patients vaccinated with a MAGE antigen.* **Immunol Rev** 2002;188:33-42.

5. Marchand M, Punt CJ, Aamdal S, Escudier B, Kruit WH, Keilholz U, Hakansson L, van Baren N, Humblet Y, Mulders P, Avril MF, Eggermont AM, Scheibenbogen C, Uiters J, Wanders J, Delire M, Boon T, Stoter G. *Immunisation of metastatic cancer patients with MAGE-3 protein combined with adjuvant SBAS-2: a clinical report.* **Eur J Cancer** 2003;39:70-7.

6. Kruit W, van Ojik H, Brichard V, Escudier B, Dorval T, Dréno B, Patel P, van Baren N, Avril M-F, Piperno S, Khammari A, Stas M, Ritter G, Lethé B, Godelaine D, Brasseur F, Zhang Y, van der Bruggen P, Boon T, Eggermont A, Marchand M. *Phase I/II study of subcutaneous and intradermal immunization with a recombinant MAGE-3 protein in patients with detectable non-visceral metastatic melanoma.* **Int J Cancer** 2005;117:596-604.

7. van Baren N, Bonnet MC, Dréno B, Khammari A, Dorval T, Piperno-Neumann S, Liénard D, Speiser D, Marchand M, Brichard VG, Escudier B, Négrier S, Dietrich PY, Maraninchi D, Osanto S, Meyer RG, Ritter G, Moingeon P, Tartaglia J, van der Bruggen P, Coulie PG, Boon T. *Tumoral and immunological response following baccination of melanoma patients with an ALVAC virus encoding MAGE antigens recognized by T cells.* **J Clin Oncol** 2005;23:9008-21.

ANALYSIS OF T CELL RESPONSES OF VACCINATED CANCER PATIENTS

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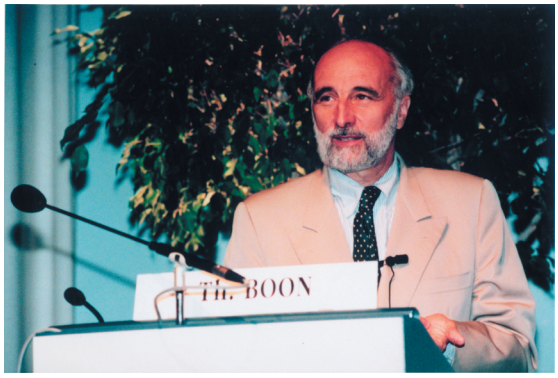

The work of our group is aimed at understanding why some metastatic melanoma patients show tumor regression following vaccination whereas most patients do not. Recent results suggest that a local immunosuppressive environment at the tumor sites may be the main barrier to the efficacy of immunotherapy. Our work is performed in close collaboration with the group of Pierre Coulie.

Study of the anti-vaccine and anti-tumoral T cell responses in melanoma patients vaccinated with an antigen encoded by gene MAGE-3

When we initiated our attempts to vaccinate metastatic melanoma patients with tumor-specific antigens, our belief was that very strong cytolytic T lymphocyte (CTL) responses would be required for tumor regressions to occur. Ten years later, our immunotherapy trials can be summarized as follows. Only a small proportion of patients shows tumor regression : about 20% of the patients show some evidence of regression and about 6% of the patients show a level of tumor regression that can be considered to be clinically beneficial.

Most of the patients who regress do so despite a frequency of anti-vaccine T cells in the blood which is lower than 1/100,000 of CD8 T cells. This frequency is remarkably stable over several months. The anti-vaccine CTL are usually a single T cell clone, except when the patients are vaccinated with tumor antigens presented by dendritic cells. There the response is usually polyclonal, but it is noteworthy that the rate of tumor regression is not higher.

In patients vaccinated with a recombinant ALVAC virus coding for an antigen of gene MAGE-3, we observed that detectable anti-MAGE-3 CTL responses showed correlation with tumor regression. But the paradox remained that tumor regressions were observed in patients who made very low CTL responses against the vaccine.

The analysis of patient EB-81 vaccinated with ALVAC-MAGE-3 indicated that this patient had, in addition to a blood frequency of anti-MAGE-3 CTL of about 1/300,000, a hundredfold higher frequency of CTL directed against other tumor antigens. Moreover, these "anti-tumor" CTL were already present before vaccination. The antigens recognized by the anti-tumor CTL of patient EB-81 were identified. Most of these CTL recognized antigens encoded by gene MAGE-C.2, another gene belonging to the same family as MAGE-3.

To understand better what happened in the

tumor, we used genetic approaches, namely PCR amplification of T cell receptor sequences, to evaluate the presence inside the metastases of patient EB-81 of the anti-vaccine CTL and of the main anti-MAGE-C2 CTL. The results were that the anti-vaccine CTL were barely enriched at the tumor sites relative to the blood whereas the anti-tumor CTL showed more than a hundredfold enrichment.

The results obtained in patient EB-81 have been completely confirmed in another patient treated with dendritic cells pulsed with a MAGE antigen.

These results led to a complete reversal of our views about the processes that lead to tumor regression. We now believe that as a melanoma evolves, there arises a spontaneous T cell response against specific tumor antigens. Thus, an immunosurveillance process occurs and probably results in the complete elimination of some tumors at an early stage. However, many tumors appear to escape this response. They manage to produce an immunosuppressive environment that renders ineffective the large number of T cells present in the tumor. A recent analysis of another patient indicates that the spontaneous T cell response against melanoma can occur at the stage of the primary tumor.

It appears that, in some vaccinated patients, a few anti-vaccine T cells manage to get a foothold in the tumor : they resist the local immunosuppressive conditions long enough to attack some tumor cells, and this results in a focal reversal of the immunosuppressive conditions. This in turn causes the restimulation and the proliferation of other anti-tumor T cells and it is these T cells that carry out the elimination of the bulk of the tumor cells (Fig. 1). To summarize, the anti-vaccine T cells serve only as a "spark" that activates the regression of the tumor.

A result of this process is that the anti-vaccine T cells are also restimulated. Hence the correlation between regression and anti-vaccine T cell responses.

Our results also indicate that a new wave of naïve anti-tumoral T cells may be stimulated and amplified in the course of the tumor regression process.

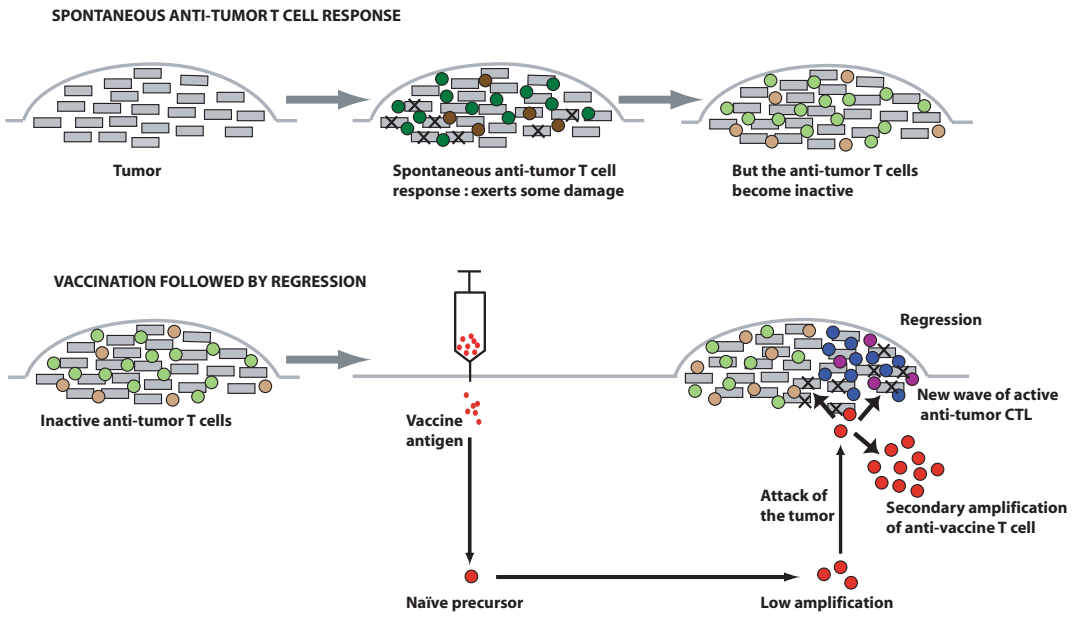

Figure 1

In conclusion, we suspect that the difference between the patients who show tumor regression following vaccination and those who do not, is the "tightness" of the immunosuppressive conditions at the tumor sites. This tightness may vary from one metastatic site to another, explaining why in many vaccinated patients some metastases regress whereas others don't.

In 2006, GlaxoSmithKline Biologicals reported the result of a randomized phase II adjuvant immunotherapy trial performed on non-small cell lung cancer patients. The 244 patients received several vaccinations with Mage-3 protein after complete surgical removal of their tumor. A statistically significant reduction of relapse within 2 years was observed in the vaccinated patients. Interestingly, the benefit was observed only for those patients where lymph node involvement had been observed at the time of surgery, and for the other patients from whom only minimal lymph node removal had been performed. We interpret this as evidence supporting the notion that the effectiveness of the vaccine depends on the presence in the tumor of anti-tumor T-cells resulting from an anterior spontaneous response. In those patients where the local lymph nodes are completely removed before the development of this spontaneous T cell response, the vaccine is ineffective.

Selected publications

1. Van Pel A, van der Bruggen P, Coulie PG, Brichard VG, Lethé B, van den Eynde B, Uyttenhove C, Renauld JC, Boon T. *Genes coding for tumor antigens recognized by cytolytic T lymphocytes.* **Immunol Rev** 1995;145:229-50.

2. Coulie PG, Karanikas V, Colau D, Lurquin C, Landry C, Marchand M, Dorval T, Brichard VG, Boon T. *A monoclonal cytolytic T-lymphocyte response observed in a melanoma patient vaccinated with a tumor-specific antigenic peptide encoded by gene MAGE-3.* **Proc Natl Acad Sci USA** 2001;98:10290-10295.

3. Karanikas V, Lurquin C, Colau D, van Baren N, De Smet C, Lethé B, Connerotte T, Corbière V, Demoitié MA, Liénard D, Dréno B, Velu T, Boon T, Coulie PG. *Monoclonal anti-MAGE-3 CTL responses in melanoma patients displaying tumor regression after vaccination with a recombinant canarypox virus.* **J Immunol** 2003;171:4898-4904.

4. Coulie PG, Karanikas V, Lurquin C, Colau D, Connerotte T, Hanagiri T, Van Pel A, Lucas S, Godelaine D, Lonchay C, Marchand M, van Baren N, Boon T. *Cytolytic T-cell responses of cancer patients vaccinated with a MAGE antigen.* **Immunol Rev** 2002; 188:33-42.

5. Godelaine D, Carrasco J, Lucas S, Karanikas V, Schuler-Thurner B, Coulie PG, Schuler G, Boon T, Van Pel A. *Polyclonal cytolytic T lymphocyte responses observed in melanoma patients vaccinated with dendritic cells pulsed with a MAGE-3.A1 peptide.* **J Immunol** 2003;171:4893-97.

6. Germeau C, Ma W, Schiavetti F, Lurquin C, Henry E, Vigneron N, Brasseur F, Lethé B, De Plaen E, Velu T, Boon T, Coulie PG. *High frequency of antitumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens.* **J Exp Med** 2005;201:241-248.

7. Lurquin C, Lethe B, De Plaen E, Corbière V, Théate I, van baren N, Coulie PG, Boon T. *Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen.* **J Exp Med** 2005;201(2):249-257.

8. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P. *Human T cell responses against melanoma.* **Ann Rev Immunol** 2006;24:175-208.

CYTOKINES IN IMMUNITY AND INFLAMMATION

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Valérie STEENWINCKEL, Student
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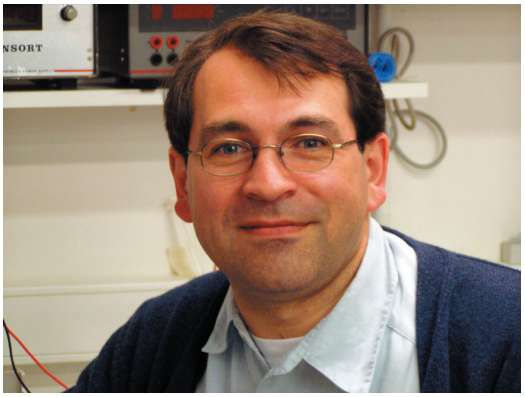

The cytokine group studies the biology of Interleukin-9 (IL-9) and IL-22, two cytokines discovered at the Branch. IL-9 is a TH2 cytokine that plays a role in immune responses against intestinal parasites and asthma. IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, upregulates 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 generated 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 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 (1).

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 (2).

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. 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

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

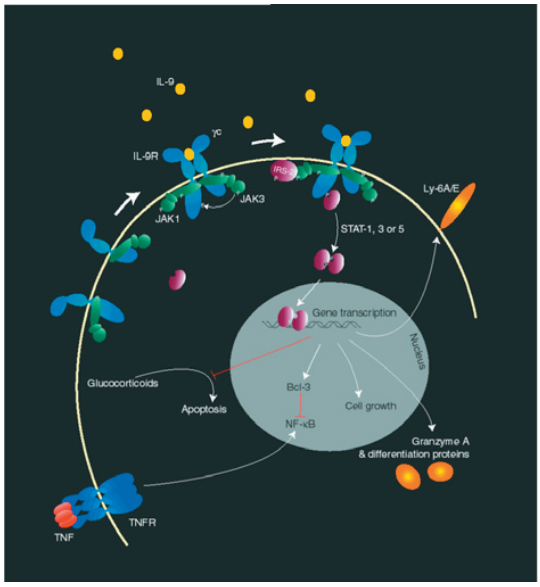

Figure 1.

Anti-apoptotic activity of I-309 receptor 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, led us 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. 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.

IL-9-induced genes

J.-C. Renauld, J. Van Snick, L. Dumoutier, L. Knoops, A. Tounsi, M. Stevens

To further characterize the mechanisms involved in the anti-apoptotic activity of IL-9 in this experimental model, we sought to identify genes induced by IL-9 in T cell lymphomas. Among the genes we identified, three open unexpected perspectives: BCL3, M-Ras and IL-TIF/IL-22.

BCL3 : indirect modulation of NF-κB

BCL3 is a gene originally identified at the breakpoint of translocations found in B cell leukemia, resulting in its transcriptional activation. The BCL3 protein interacts with NF-κB transcription factors and its induction by IL-9 represents a novel mechanism of NF-κB regulation by cytokines, and a new crosstalk between the JAK/STAT and NF-κB signal transduction pathway (4). BCL3 induction might play a role in the antiapoptotic activity of cytokines such as IL-4 and IL-9.

M-Ras : transcriptional regulation of the Ras-MAPKinase pathway

M-Ras is a new member of the Ras oncogene superfamily. The Ras proteins are known to regulate various cellular processes such as proliferation and apoptosis, when they are in their activated form, in association with a GTP molecule. Contrasting with the potent upregulation of M-Ras expression, M-Ras was not activated by IL-9 at the level of GTP binding. However, other cytokines such as IL-3 increased GTP binding to M-Ras, suggesting that M-Ras induction might represent a new mechanism of cooperativity between cytokines. Constitutively activated M-Ras mutants trigger the MAP Kinase pathway and induce proliferation of cytokine-dependent cells (5).

IL-TIF/IL-22 : a new cytokine structurally related to IL-10

IL-TIF is a new 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. Its expression is induced by IL-9 in thymic lymphomas, T cells and mast cells and by lectins in freshly isolated spleen cells. In addition, constitutive expression of IL-22 was detected by RT-PCR in thymus and brain, suggesting that the role of this new factor is not restricted to the immune system. Preliminary experiments showed that IL-22 induces STAT activation in various cell lines, suggesting that this factor might mediate some of the activities of IL-9. Biological activities of IL- 22 include the induction of acute phase proteins in liver (6) and protection against experimental heatitis (L. Dumoutier, unpublished results). 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.

Analysis of genome databases led 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-22BP-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.

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-20Rß, 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

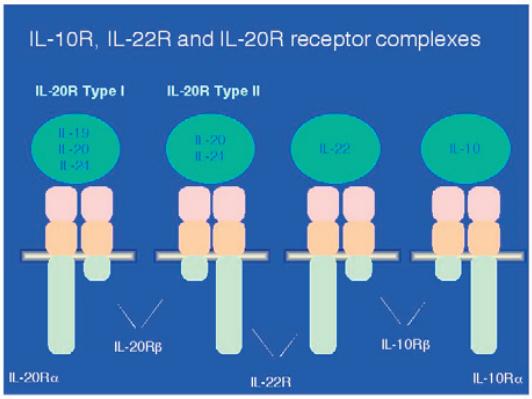

Figure 2.

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 new 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.

Selected publications

1. Vink A, Warnier G, Brombacher F, Renauld JC. *Interleukin 9-induced in vivo expansion of the B-1 lymphocyte population.* **J Exp Med** 1999;189:1413-23.

2. Richard M, Grencis RK, Humphreys NE, Renauld JC, Van Snick J. *Anti-IL-9 vaccination prevents worm expulsion and blood eosinophilia in Trichuris muris-infected mice.* **Proc Natl Acad Sci U S A** 2000;97:767-72.

3. Demoulin JB, Van Roost E, Stevens M, Groner B, Renauld JC. *Distinct roles for STAT1, STAT3, and STAT5 in differentiation gene induction and apoptosis inhibition by interleukin-9.* **J Biol Chem** 1999;274:25855-61.

4. Richard M, Louahed J, Demoulin JB, Renauld JC. *Interleukin-9 regulates NF-kappaB activity through BCL3 gene induction.* **Blood** 1999;93:4318-27.

5. Louahed J, Grasso L, De Smet C, Van Roost E, Wildmann C, Nicolaides NC, Levitt RC, Renauld JC. *Interleukin-9-induced expression of M-Ras/R-Ras3 oncogene in T-helper clones.* **Blood** 1999;94:1701-10.

6. Dumoutier L, Van Roost E, Colau D, Renauld JC. *Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor.* **Proc Natl Acad Sci U S A** 2000;97:10144-9.

7. Dumoutier L, Lejeune D, Colau D, Renauld JC. *Cloning and characterization of IL-22 binding protein, a natural antagonist of IL-10-related T cell-derived inducible factor/IL-22.* **J Immunol** 2001;166:7090-5.

8. Dumoutier L, Leemans C, Lejeune D, Kotenko SV, Renauld JC. *Cutting edge: STAT activation by IL-19, IL-20 and mda-7 through IL-20 receptor complexes of two types.* **J Immunol** 2001;167:3545-9.

9. Renauld JC. *Class II cytokine receptors and their ligands: key antiviral and inflammatory modulators.* **Nature Rev Immunol** 2003;3:667-76.

10. Dumoutier L, Tounsi A, Michiels T, Sommereyns C, Kotenko SV, Renauld JC. *Role of the Interleukin-28 Receptor tyrosine residues for antiviral and antiproliferative activity of IL-29/IFN-lambda 1: Similarities with type I Interferon signalling.* **J Biol Chem** 2004;279:32269-74.

SIGNAL TRANSDUCTION AND MOLECULAR HEMATOLOGY GROUP: STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS

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Cytokines and their receptors are critical for the formation of mature blood cells and for the function of the immune system. Signaling by cytokine receptors is triggered by ligand-induced changes in receptor dimerization/oligomerization, which induces the activation of cytosolic Janus tyrosine kinases (JAK). We study the signal transduction mechanisms and biologic functions of cytokine receptors such as the receptors for erythropoietin (Epo), thrombopoietin (Tpo), and Granulocyte-Colony-Stimulating Factor (G-CSF). The assembly of cell-surface receptor complexes, the structure and orientation of the transmembrane (TM) and cytosolic juxtamembrane (JM) domains, and the regulation by JAK kinases of receptor traffic are major focuses. The laboratory is actively investigating the mechanisms by which a JAK2 point mutant (V617F) induces Polycythemia Vera and myeloproliferative diseases in humans, as well as the role of TpoR mutants in myelofibrosis.

The mechanisms by which a mutant JAK2 induces Polycythemia Vera and myeloproliferative diseases in humans

J. Staerk, C. Pecquet

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. In addition to their role in signaling, JAKs appear to play chaperone roles for promoting traffic of cytokine receptors to the cell surface.

Polycythemia Vera (PV)(known also as 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 Epo 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 (1).

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 (2, 3). The mutation in the pseudokinase domain alters a physiologic inhibition exerted by the pseudokinase domain on the kinase domain (Figure 1B and C). This mutant is found in >80% of PV patients and in 50% of Essential Thrombocythemia and Idiopathic Myelofibrosis (IMF), two other diseases that belong to the myeloproliferative syndromes (Figure 1). Current projects include the determination of downstream signaling proteins activated by the mutant JAK2, and the characterization of

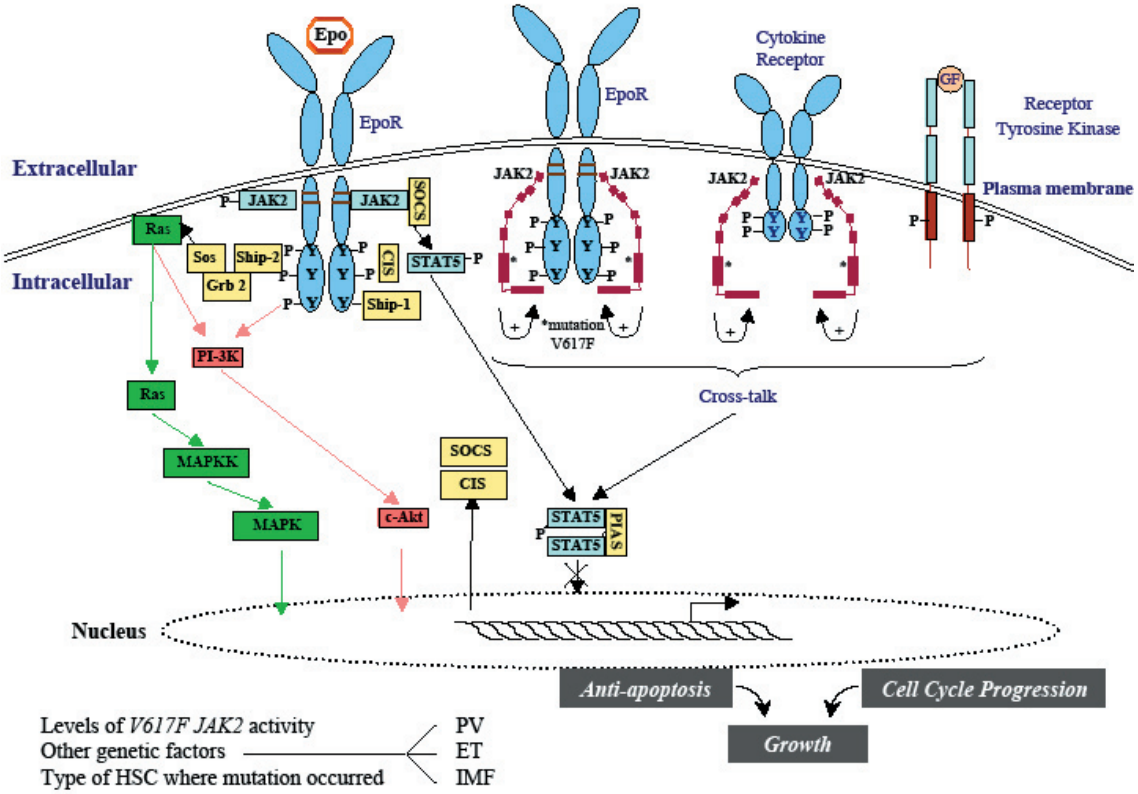

Figure 1. Cytokine receptors such as the erythropoetin receptor (EpoR) arrive at the cell surface after JAK2 had bound to their cytosolic domains early on in the secretory pathway. (A). When ligand (i.e. Epo for the EpoR) binds to the extracellular domain of the EpoR, the prebound JAK2 molecules trans-phosphorylate each other, become active and phosphorylate the receptors themselves and various signaling molecules, which become recruited to phosphorylated tyrosine residues of the receptor and of JAK2. Pathways that become activated are Ras-MAP-kinase, PI-3-kinase-AktB and STAT (STAT5 and STAT3), which upon dimerization are translocated to the nucleus and regulate gene expression. The end result is Epo-dependent activation of cell survival, proliferation and differentiation of erythroid progenitors. Normally JAK2 is inactive in the absence of the conformational change imposed by ligand activation of the receptor. (B,C). When V617 in the JH2 domain of JAK2 is mutated to phenylalanine (V617F) the inhibition exerted by the JH2 (pseudokinase domain) on JH1 is abolished and JAK2 can become active in the absence of ligand-activated receptors. Receptors such as EpoR (B) or others (C) such as thrombopoietin or G-CSF receptors can become phosphorylated and activated in the absence of ligand activation. (D). In cells that express the JAK2 V617F mutant, binding of growth factors such as Insulin-like growth factor 1 (IGF1) to its receptor IGF1R, a tyrosine kinase receptor, leads to the activation of the JAK-STAT pathway, which normally is only activated by cytokines and cell proliferation. This cross-talk between tyrosine kinase and cytokine receptors amplifies the proliferation of myeloid progenitors in patients with myeloproliferative diseases.

cytokine receptor signaling in the presence of the mutant and wild type JAK2 (Figure 1A and D). While the JAK2 wild type is down-modulated by SOCS3 (suppressor of Cytokine Signalling-3), we recently showed that signaling by JAK2V617F is potentiated by SOCS3 (5). Strikingly, the homologous mutations in JAK1 and Tyk2 also enable these kinases to be activated without ligand-binding to cytokine receptors (4). These results suggest that point mutations in JAK proteins may be involved in different forms of cancers and autoimmune diseases.

Involvement of TpoR in myeloproliferative diseases

J. Staerk, M. Girardot, N. Caceres

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, 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 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 and, indeed, the first reports from other groups and ours on mutations in this motif will soon be published. Thus, it is likely that among the JAK2 V617F-negative myelofibrosis patients some will harbor mutations in the TpoR RWQFP motif.

At present our laborarory 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 Clincal Biology (Prof. Dominique Latinne) a large study on the presence of JAK2 and TpoR mutations in patients with myeloproliferative diseases.

Determination of the interface and orientation of the activated EpoR and TpoR dimers

J. Staerk, A. Dusa, 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 interactions 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 (7, 8).

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 (8). Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (one), d (four), the register of the coiled-coil α-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 (8). 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.

The thrombopoietin receptor (TpoR) is essential for formation of platelets, for renewing hematopoietic stem cells and for expanding myeloid progenitors. 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.

We applied the coiled coil approach to determine the active interface of the TpoR. Unlike the EpoR, where essentially one conformation is productive for signaling, for the TpoR six of seven orientations were inducing cell proliferation. Closer examination showed that different biologic effects were induced by different orientations, i.e. megakaryocyte differentiation, renewal of early hematopoietic progenitors or cell-to-cell adhesion. These differently oriented dimers appear to activate separate pathways besides the common JAK2 molecule. Current experiments aim at using these dimers as baits to purify novel signaling proteins and at determining the in vivo effects, in mice of expressing the different TpoR dimers in hematopoietic stem cells.

Structural studies on the transmembrane and juxtamembrane cytosolic sequences of the EpoR

K. Kubatzky, A. Dusa

To define the interfaces of the active and inactive EpoR dimers we performed cysteine scanning mutagenesis of the extracellular juxtamembrane and TM domains (9). We isolated three constitutively active novel mutants of the EpoR where residues L223, L226 or I227 were mutated to cysteine. 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.

Traffic of cytokine receptors to the cell-surface: the common γ chain (γc) of the IL2/IL9 receptor complexes and TpoR

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We have observed that, in hematopoietic cells, over-expression of JAK proteins leads to enhanced cell-surface localization of cognate cytokine receptors (i.e. TpoR, IL9R, IL2R, γc). The IL9Rα which requires JAK1 for signaling is expressed at higher levels on the cell-surface when JAK1 but not JAK2 or JAK3 is overexpressed. For the common γc it is uniquely JAK3 that promotes traffic to the cell surface. In collaboration with Pierre Courtoy we are examining the intracellular location of receptors as a function of expression levels of JAKs. In the absence of JAK3 it appears that γc accumulates in an endosome-like vesicle. For the TpoR, both JAK2 and Tyk2 strongly promote traffic, stability and recycling of the mature TpoR, which co-localizes with recycling transferrin (1). JAK2 and Tyk2 exert similar effects on TpoR traffic, while JAK2 is 10 fold more potent in transmitting a signal from the TpoR (1). We are examining the possibility that at different stages of megakaryocyte differentiation, the ratio of JAK2 to Tyk2 may vary in order to modulate TpoR signaling. Furthermore, we are examining the mechanisms by which patients with myeloproliferative diseases have defective TpoR expression on their megakaryocytes and platelets.

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 attemtping to clone novel proteins that can regulate traffic and stability of TpoR.

Random mutagenesis approaches to study interactions between receptor transmembrane domains and the structure and function 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. 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 mutegensis 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 19 amino acid residues will be tested for constitutive kinase activity, for induction of cell proliferation and activation of STAT5. Since the V617F mutation also activated JAK1 and Tyk2 (4), 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 pathway remains permanently activated in transformed cells and which genes are regulated by constitutively active STAT proteins in leukemic cells. Using chromatin immuno-precipitation 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), and not only to N3 sites, which are characteristic of ligand-activated STAT5 (10). We are attempting to identify the promoters actually bound by STAT pro-

teins in living cells in physiologic and pathologic situations. We are using 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.

Selected publications

1. Royer Y, Staerk J, Costuleanu M, Courtoy PJ, Constantinescu SN. *Janus kinases affect thrombopoietin receptor cell surface localization and stability.* **J Biol Chem** 2005;280:27251-61.

2. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, Garcon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villeval JL, Constantinescu SN, Casadevall N, Vainchenker W. *A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera.* **Nature** 2005;434:1144-8.

3. Vainchenker W, Constantinescu SN. *A unique activating mutation in JAK2 is at the origin of Polycythemia Vera and allows a new classification of myeloproliferative diseases.* **Hematology** (Am. Soc. Hematol. Educ. Program) 2005;195-200.

4. Staerk J, Kallin A, Demoulin J-B, Vainchenker W, Constantinescu SN. *JAK1 and Tyk2 activation by the homologous Polycythemia Vera JAK2 V617F mutation: cross-talk with IGF1 receptor.* **J Biol Chem** 2005;280:41893-9.

5. Hookham MB, Elliott J, Suessmuth Y, Staerk J, Ward AC, Vainchenker W, Percy MJ, McMullin MF, Constantinescu SN, Johnston JA. *The myeloproliferative disorder-associated JAK2 V617F mutant escapes negative regulation by suppressor of cytokine signaling 3.* **Blood**. 2007;109:4924-9.

6. Staerk J, Lacout C, Smith SO, Vainchenker W, Constantinescu SN. *An amphipathic motif at the transmembrane-cytoplasmic junction prevents autonomous activation of the thrombopoietin receptor.* **Blood** 2006;107:1864-71.

7. Constantinescu SN, Huang LJ, Nam H, Lodish HF. *The erythropoietin receptor cytosolic juxtamembrane domain contains an essential, precisely oriented, hydrophobic motif.* **Mol Cell** 2001;7:377-85.

8. Seubert N, Royer Y, Staerk J, Kubatzky, KF, Moucadel V, Krishnakumar S, Smith SO, Constantinescu SN. *Active and inactive orientations of the transmembrane and cytosolic domains of the erythropoietin receptor dimer.* **Mol Cell** 2003;12:1239-50.

9. Kubatzky KF, Liu W, Goldgraben K, Simmerling C, Smith SO, Constantinescu SN. *Structural requirements of the extracellular to transmembrane domain junction for erythropoietin receptor function.* **J Biol Chem** 2005;280:14844-54.

10. Moucadel V, Constantinescu SN. *Differential STAT5 signaling by ligand-dependent and constitutively active cytokine receptors.* **J Biol Chem** 2005;280:13364-73.