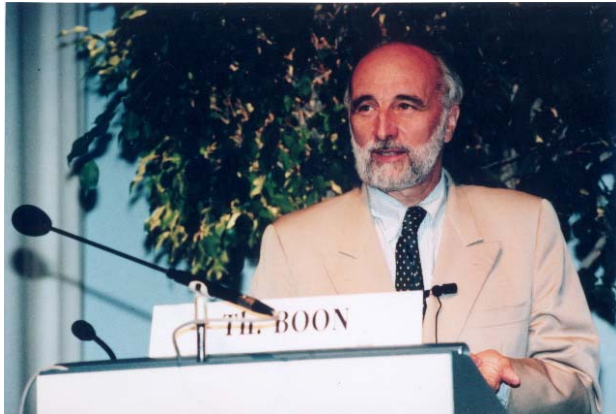


Ludwig Institute for Cancer Research (LICR),

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. Ludwig investigators are recognized leaders in many areas of science, involving genetics, bioinformatics, immunology, virology, cell biology and signal transduction.



Thierry Boon

TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

Benoît VAN DEN EYNDE, Member

Bernard LAUWERYS, Senior Investigator

Catherine UYTENHOVE, Senior Investigator

Guy WARNIER, Veterinarian, Associate Investigator

Fanny PIETTE, Research Assistant

Luc PILOTTE, Research Assistant

Jacques CHAPIRO, Postdoctoral Fellow

Wenbin MA, Postdoctoral Fellow, until April

Ivan THEATE, Postdoctoral Fellow

Edus WARREN, Visiting Scholar, until January

Benoît GUILLAUME, Student

Ivo HUIJBERS, Student

Tanja NASLUND, Student, until February

Nicolas PARMENTIER, Student

Nathalie VIGNERON, Student

Christine CORBIAU, Technician

Dominique DONCKERS, Technician



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

We have also recently described a new aspect of the proteasome function in antigen processing, which is the production of antigenic peptides made of two non-contiguous segments. The production of such peptides involves the excision of a peptidic segment and the splicing of the two fragments. We showed that this excision and splicing process is done by the proteasome, presumably by a mechanism of transpeptidation. The study of additional peptides produced by splicing is ongoing.

We are also studying a mouse preclinical model of cancer immunotherapy, where we try to define the optimal conditions to induce effective anti-tumor responses by various vaccination approaches against defined antigens. This led us to uncover a powerful mechanism of tumor resistance which is based on tryptophan catabolism by indoleamine-2,3 dioxygenase, an enzyme that we found frequently expressed in tumors. The resulting local tryptophan shortage appears to prevent the proliferation of lymphocytes at the tumor site. Treatment of mice with inhibitors of indoleamine-2,3 dioxygenase can counteract this tumor resistance mechanism. These results suggest that a treatment based on the inhibition of this enzyme could be combined with approaches of cancer immunotherapy in order to increase the tumor response rate.

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

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

Jacques Chapiro, Vincent Stroobant, Benoit Guillaume, Wenbin Ma, Fanny Piette

Antigens recognized by cytolytic T lymphocytes (CTL), such as viral or tumor antigens, usually consist of peptides of 8-10 amino acids in length, which are presented by MHC class I molecules at the cell surface. Because such peptides derive from intracellular proteins, a processing step is required before they can be exposed to the cell surface in association with MHC molecules. Firstly, the peptide is produced, as a result of the degradation of the parental protein by the proteasome. Secondly, it is taken up by a dedicated transporter named TAP, and translocated inside the endoplasmic reticulum where it meets and associates with newly synthesized MHC molecules. The first step of cleavage by the proteasome is crucial in that cleavage location determines the precise sequence of the final antigenic peptide. We have observed that this cleavage may occur differently in some cells, depending on their proteasome content. The proteasome comes in two forms: the standard proteasome, which is found in most cells, and the immunoproteasome, which is expressed by mature dendritic cells and by cells exposed to interferon-gamma (IFN γ).

We previously reported that a class-I restricted antigenic peptide derived from an ubiquitous human protein was processed efficiently by the standard proteasome but not by the immunoproteasome. As a result, the relevant epitope is not presented efficiently by mature dendritic cells, which contain immunoproteasomes (1). This could explain how certain potentially autoreactive CTL can escape tolerance induction in the thymus and fail to be activated in the periphery. We have now extended those observations to 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 IFN γ (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. In other cases, the difference lies in the efficiency of cleavage at the C-terminal end of the antigenic peptide.

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

An antigenic peptide produced by peptide splicing in the proteasome

Nathalie Vigneron, Vincent Stroobant, Jacques Chapiro (in collaboration with Annie Ooms and Gérard Degiovanni, Université de Liège, Belgium)

By stimulating blood lymphocytes of a melanoma patient with autologous tumor cells, we isolated a clone of CD8 cytolytic T lymphocytes that recognizes an antigenic peptide presented by HLA-A32 and derived from glycoprotein gp100. This peptide, whose sequence is RTKQLYPEW, is present on several melanoma lines and was found to be composed of two non-contiguous segments of the gp100 protein. Its production requires the excision of four amino acids and splicing of the residual fragments (4). The antigenic peptide could be produced *in vivo* by electroporating the 13-amino acid precursor RTKAWNRQLYPEW into EBV-B cells. Proteasome inhibitors lactacystin and epoxomicin prevented the recognition of target cells electroporated with the 13-amino acid peptide, indicating that proteasome activity was required for the splicing of this precursor. Moreover, the digests produced after incubation of the 13-amino acid precursor with highly purified proteasomes strongly stimulated IFN γ production by the CTL. Analysis of these digests by tandem mass spectrometry clearly

demonstrated the presence of the spliced peptide in the reaction mixture. Thus, the proteasome appears to produce the antigenic peptide RTKQLYPEW by excision and splicing. By incubating proteasomes with sets of two distinct fragments each containing a different portion of the precursor peptide, we could show that the energy required for the creation of the new peptide bond was recycled from one of the bonds that are cleaved during the excision process. These data suggest that the splicing occurs via a transpeptidation mechanism involving an acyl-enzyme intermediate. Our results reveal an unanticipated aspect of the proteasome function, which increases the diversity of antigenic peptides presented to T cells.

We are currently studying in more details this novel activity of the proteasome. We are also extending our observation to additional peptides that are also produced by splicing. This is done in collaboration with two research groups from USA.

Identification of new antigens recognized by autologous CTL on human melanoma

Wenbin Ma, Nathalie 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 (5). 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.

Melanoma line LG2-MEL also expresses several antigens recognized by autologous CTLs. We previously reported the molecular definition of three distinct antigens recognized by some of these CTL clones. One of them consists of a peptide derived from tyrosinase and presented by HLA-B35.

Another is a peptide corresponding to a mutation in a gene expressed ubiquitously, named OS9 (6). A third is a peptide produced by splicing of two non-contiguous fragments of melanocytic protein gp100 (4) (see above). We have now identified a fourth antigenic peptide expressed by this melanoma line and recognized by a CTL clone restricted by HLA-B35. The antigenic peptide, which is 9-amino acid long, has the sequence LPHSSSHWL and is also derived from melanocyte differentiation protein gp100.

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

Catherine Uyttenbove, Luc Pilotte, Ivan Théate, Dominique Donckers, Nicolas Parmentier, Vincent Stroobant, Didier 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. These results suggest that the efficacy of therapeutic vaccination of cancer patients could be improved by concomitant administration of an IDO inhibitor.

Development of an inducible mouse melanoma model for immunotherapy

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

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

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

Eighteen transgenic lines were generated by three different strategies based on the use of a construct containing the tyrosinase promoter, a CreER^{AD} fusion gene flanked by loxP sites, a V12 mutated H-Ras gene, an internal ribosomal entry site and the P815A-antigen encoding gene, P1A. After crossing with a mouse strain containing a conditional *INK4A/ARF* locus flanked by loxP sites, one transgenic line was found to develop cutaneous melanomas after subcutaneous injection of tamoxifen, in 8 out of 33 treated animals. The induced tumors are deleted for

INK4A/ARF, express the activated *Ras* and express P1A.

This line is now being backcrossed to the H-2d background in order to be used as a model for immunotherapy. In parallel, we have developed a strain of mice transgenic for the P1A-specific T cell receptor, which will be useful for such studies.

Antigen presentation by dendritic cells in Systemic Lupus Erythematosus

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

Systemic lupus erythematosus (SLE) is an autoimmune disorder that is characterized by overt polyclonal B cell activation and T cell-driven autoantibody production against nuclear antigens. We are investigating the involvement of dendritic cells (DC) in the impaired peripheral tolerance leading to the activation of autoreactive CD4 T cells in SLE. Studying strains of mice that are congenic for different SLE susceptibility loci and were developed by E.K. Wakeland (University of Texas, Southwestern Medical Center at Dallas), we observed that DC from one of these strains (*Sle3*) significantly impact CD4 T cell activation and apoptosis as compared to control DC. Further work will focus on the identification of the genetic mechanisms underlying these observations, taking advantage of the availability of newly produced subcongenic strains, carrying smaller intervals of the *Sle3* region.

In parallel, the availability of large amounts of PBMC collected from untreated patients with active disease gives us the opportunity to carry on experimental procedures aiming at the identification of new physiopathological targets in SLE, using high-density cDNA microarrays (Affymetrix). In order to increase the sensibility of that procedure, PBMC from SLE patients are sorted by flow cytometry in lymphocyte subsets, prior to RNA extraction and hybridization of the slides. Interpretation of the results will be facilitated by the knowledge of numerous SLE susceptibility regions in the human genome that contain genes of particular interest.

Finally, we recently identified a new gene encoding a potential decoy receptor for MIF (Macrophage migration Inhibitory Factor), a cytokine that plays important roles in inflammatory and tumoral responses. We currently investigate the functions of that novel molecule, targeting its potential inhibitory

activity of MIF actions in inflammation and tumor growth.

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. Schultz ES, Chapiro J, Lurquin C, Claverol S, Burlet-Schiltz O, Warnier G, Russo V, Morel S, Levy F, Boon T, Van den Eynde BJ, van der Bruggen P. *The production of a new MAGE-3 peptide presented to cytolytic T lymphocytes by HLA-B40 requires the immunoproteasome.* **J Exp Med** 2002;195:391-9.
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. 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.
6. Nathalie Vigneron, Annie Ooms, Sandra Morel, Gérard Degiovanni and Benoît J. Van den Eynde. *Identification of a new peptide recognized by autologous cytolytic T lymphocytes on a human melanoma* **Cancer Immunity** 2002; 2 : 9.
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. Bilsborough J, Uyttenhove C, Colau D, Bousso P, Libert C, Weynand B, Boon T, van den Eynde BJ. *TNF-mediated toxicity after massive induction of specific CD8+ T cells following immunization of mice with a tumor-specific peptide.* **J Immunol** 2002;169:3053-60.
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.
11. Brandle D, Brasseur F, Weynants P, Boon T, Van den Eynde B. *A mutated HLA-A2 molecule recognized by autologous cytotoxic T lymphocytes on a human renal cell carcinoma.* **J Exp Med** 1996;183:2501-8.

GENES EXPRESSED IN CANCER AND GERMLINE CELLS

Etienne De PLAEN, Assistant member
Charles DE SMET, Assistant member

Axelle LORIOT, Student
Olga KHOLMANSKIKH, Student
Claudine BLONDIAUX, Technician



Human tumors express specific antigens arising from the activation of genes, such as MAGE genes, that are normally expressed only in germ cells. As germ cells are not subject to scrutiny by the immune system, antigens encoded by these genes are strictly tumor-specific. We are trying to identify new genes that present the same pattern of expression as MAGE genes. Additionally, efforts are devoted to determining both the function of "cancer-germline" genes, and the mechanisms leading to their activation in tumors.

Isolation and characterization of new cancer-germline genes

Screening procedures based on differential expression profiling allowed the isolation of several genes with cancer and germline specific expression (1-4). Most of these genes are located on the X chromosome, and have their normal site of expression in spermatogonia, the pre-meiotic stage of sperm development. This restricted expression is likely related to the chromosomal location of these genes, as the X chromosome is inactivated in post-meiotic sperm cells.

MAGE-A1 acts as a transcriptional repressor

MAGE-A1, the first characterized cancer-germline gene, belongs to a family of twelve genes located on the X chromosome in region q28 (5,6). To analyze the functions of MAGE-A1, we searched to identify protein partners of this protein. Using yeast two-hybrid screening, we found interaction between MAGE-A1 and transcriptional regulator SKIP. SKIP is an adaptor protein that connects DNA-binding proteins such as Smad3, the Vitamin D receptor, CBF1 or MyoD, to proteins that activate or repress transcription. A repression complex including histone deacetylases (HDAC) is known to bind to

SKIP and CBF1. In the presence of the intracellular part of Notch1 (Notch1-IC), the repression complex is detached from SKIP by Notch1-IC and recruitment of an activation complex including histone acetyltransferases (HAT) is facilitated by SKIP. To examine whether MAGE-A1 could interfere with a signalling pathway involving SKIP, we expressed MAGE-A1 in mammalian cells in which Notch1-IC binds to SKIP fused to the Gal4 DNA-binding domain and activates a *CAT* reporter gene containing Gal4-binding sites near its promoter (Fig. 1).

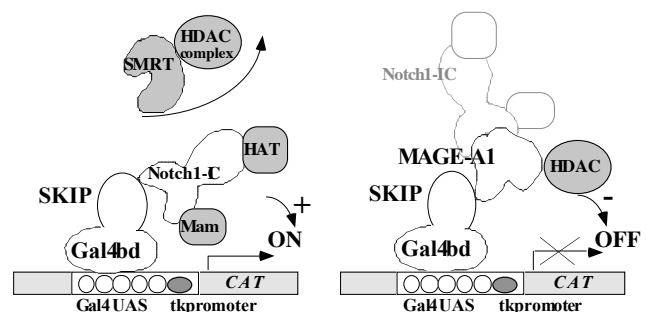


Fig. 1. MAGE-A1 counteracts Notch1-IC transactivation and recruits histone deacetylases

We found that MAGE-A1 inhibited the Notch-1/SKIP transcriptional activation. Deletion analysis

indicate that binding to SKIP is required to observe MAGE-A1-mediated repression of Notch1-IC transactivation. Moreover, MAGE-A1 was found to actively repress transcription by binding and recruiting histone deacetylase 1. Our results suggest that by binding to SKIP and by recruiting histone deacetylases, MAGE-A1 protein present in the nucleus could repress genes implicated in development and spermatogenesis. We are now trying to identify the genes that are regulated by MAGE-1 by using an inducible transfected *MAGE-A1* gene and the microarray technology.

Genome hypomethylation in the activation of cancer-germline genes in tumors

Studies on the transcriptional regulation of cancer-germline genes, such as *MAGE-A1*, showed that CpG methylation is an essential component of their repression in normal somatic tissues (7,8). Activation of these genes in tumors is associated with the genome-wide DNA demethylation process which often accompanies tumorigenesis (9). Recently, we found that hypomethylated CpGs in tumors are not randomly distributed but are clustered into specific regions such as the promoter region of *MAGE-A1* (10). This site-specific hypomethylation is not due to a persistent targeted demethylating activity, but appears to result from a transient overall demethylation process followed by a permanent local inhibition of remethylation due to the binding of transcriptional activators (Fig. 2).

Using the antisense oligonucleotide technology, we are currently trying to identify the enzymes that are normally involved in maintaining CpG methylation within cancer-germline genes, with a special emphasis on the DNA methyltransferases DNMT1, DNMT3a and DNMT3b.

These studies should bring insight into the processes that lead to DNA methylation changes in cancer. Moreover, understanding the mechanisms that activate cancer-germline genes may help designing procedures to induce the expression of specific antigens on tumors, thereby facilitating their elimination by the immune system.

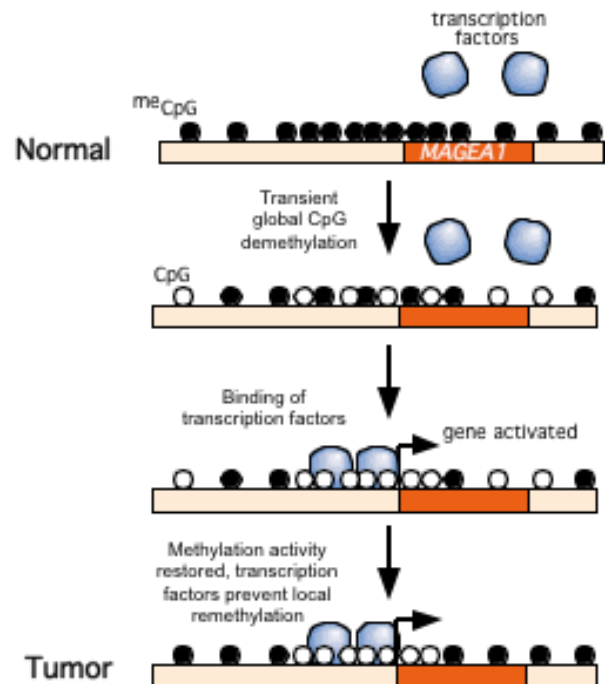


Fig. 2. Model for the stable activation of *MAGE-A1* in tumors.

Selected publications

1. Lucas S, De Smet C, Arden KC, Viars CS, Lethe B, Lurquin C, Boon T. *Identification of a new MAGE gene with tumor-specific expression by representational difference analysis.* **Cancer Res** 1998; 58:743-52.
2. Lethé B, Lucas S, Michaux L, De Smet C, Godelaine D, Serrano A, De Plaen E, Boon T. *LAGE-1, a new gene with tumor specificity.* **Int J Cancer** 1998; 76:903-8.
3. Martelange V, De Smet C, De Plaen E, Lurquin C, Boon T. *Identification on a human sarcoma of two new genes with tumor-specific expression.* **Cancer Res** 2000; 60:3848-55.
4. Lorient A, Boon T, De Smet C. *Five new human cancer-germline genes identified among 12 genes expressed in spermatogonia.* **Int J Cancer** 2003; 105:371-376.
5. De Plaen E, Arden K, Traversari C, Gaforio JJ, Szikora JP, De Smet C, Brasseur F, van der Bruggen P, Lethé B, Lurquin C, Brasseur R, Chomez P, De Backer O, Cavenee W, Boon T. *Structure, chromosomal localization, and expression of 12 genes of the MAGE family.* **Immunogenetics** 1994; 40:360-9.

6. Serrano A, Lethe B, Delroisse JM, Lurquin C, De Plaen E, Brasseur F, Rimoldi D, Boon T. *Quantitative evaluation of the expression of MAGE genes in tumors by limiting dilution of cDNA libraries.* **Int J Cancer.** 1999 Nov 26;83(5):664-9.
7. De Smet C, Courtois SJ, Faraoni I, Lurquin C, Szikora JP, De Backer O, Boon T. *Involvement of two Ets binding sites in the transcriptional activation of the MAGE1 gene.* **Immunogenetics** 1995; 42:282-90.
8. De Smet C, Lurquin C, Lethe B, Martelange V, Boon T. *DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter.* **Mol Cell Biol** 1999 ;19:7327-35.
9. De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T. *The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation.* **Proc Natl Acad Sci U S A** 1996; 93:7149-53.
10. De Smet C, Lorient 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;24:4781-4790.

IDENTIFICATION OF HUMAN TUMOR ANTIGENS

Pierre van der BRUGGEN, Member

Vincent STROOBANT, Postdoctoral Fellow

Nicolina RENKVIST, Postdoctoral Fellow

Zhaojun SUN, Postdoctoral Fellow

Yi ZHANG, Visiting Scientist

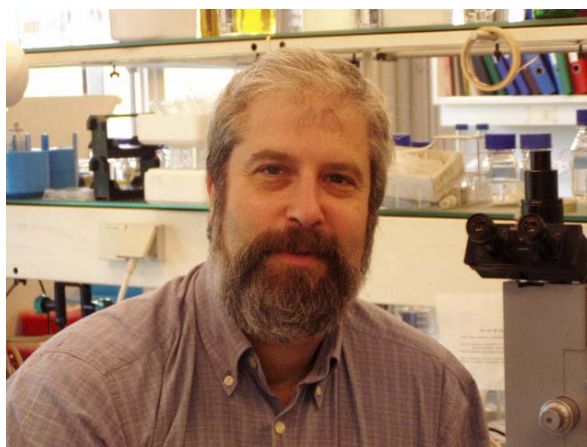
Claude WILDMANN, Research Associate

Nathalie DEMOTTE, Ph.D. Student

Hugues NICOLAY, Ph.D. Student (to February 2004)

Sabrina OTTAVIANI, Technician

Bénédicte TOLLET, Technician



The group led by Pierre van der Bruggen is defining antigenic peptides encoded by genes such as those of the MAGE family. Therapeutic vaccination of cancer patients with MAGE peptides is now in progress, and the identification of additional antigenic peptides is important to increase the range of patients eligible for therapy with peptides and to provide tools for a reliable monitoring of the immune response. The monitoring of the anti-vaccine T cell frequencies in patients vaccinated with protein is even more complex. The responses could be directed against a large number of HLA-peptide combinations, including many that are presently unknown. The group has undertaken a systematic effort to develop a reproducible monitoring approach with high specificity and sensitivity. The group is also involved in the study of functional defects of T cells.

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

Yi Zhang, Vincent Stroobant, Zhaojun Sun, Sabrina Ottaviani

“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 (2). As a result, a vast number of sequences are known that can code for tumor-specific shared antigens, but most of the encoded antigenic peptides have not been identified yet. The identification of a large number of antigenic peptides presented by HLA class I and class II molecules is likely to be important for the future of clinical trials with defined antigenic peptides. A large

set of peptide/HLA combinations will alleviate HLA restriction and widen the set of eligible patients. It will also facilitate the design of concurrent immunizations against several antigens. Such immunizations could increase the primary anti-tumor efficacy of the vaccine and also decrease the risk of tumor escape by loss of antigen expression.

We have used approaches that we have loosely named “reverse immunology” (3). They aim at identifying antigenic peptides recognized by T cells, using gene sequences as starting point. We have focused this search on the cancer-germline genes, which are expected to code for tumor-specific shared antigens on the basis of their pattern of expression.

Search for antigenic peptides recognized by CD8⁺ T cells

We stimulated CD8⁺ T lymphocytes with dendritic cells transduced with viral vectors containing complete MAGE-coding sequences. As this requires the processing of the antigen by the dendritic cells, we surmised that the peptides that would be identified would also be processed in the tumors expressing the MAGE genes. A difficulty of the use of recombinant viruses is the activation of

CTL precursors directed against viral antigens. We circumvented this problem by using different vectors for the stimulation of the microcultures, for the lytic assay with the responder T cells, and for the cloning step. This procedure is summarized in Figure 1. Dendritic cells were infected with either an adenovirus, a canarypoxvirus or a lentivirus, and they were used to stimulate microcultures of autologous CD8⁺ T lymphocytes (4, 5). After three weekly stimulations, the responder cells were tested for lysis on autologous EBV-B cells infected with vaccinia-MAGE. Positive microcultures were cloned. To identify the antigenic peptide, the resulting CTL clone

was tested for lysis of autologous EBV-B cells pulsed with a complete set of peptides of 16 amino acids that overlap by 12. When a peptide scored positive, shorter peptides were synthesized to identify the shortest optimal peptide. To identify the HLA presenting molecule, the CTL clones were tested for stimulation by cells transfected with the MAGE cDNA together with cDNAs coding for the possible HLA presenting molecules. Finally, relevant tumor targets were used in a lysis assay to ascertain that the antigen was also processed by tumor cells.

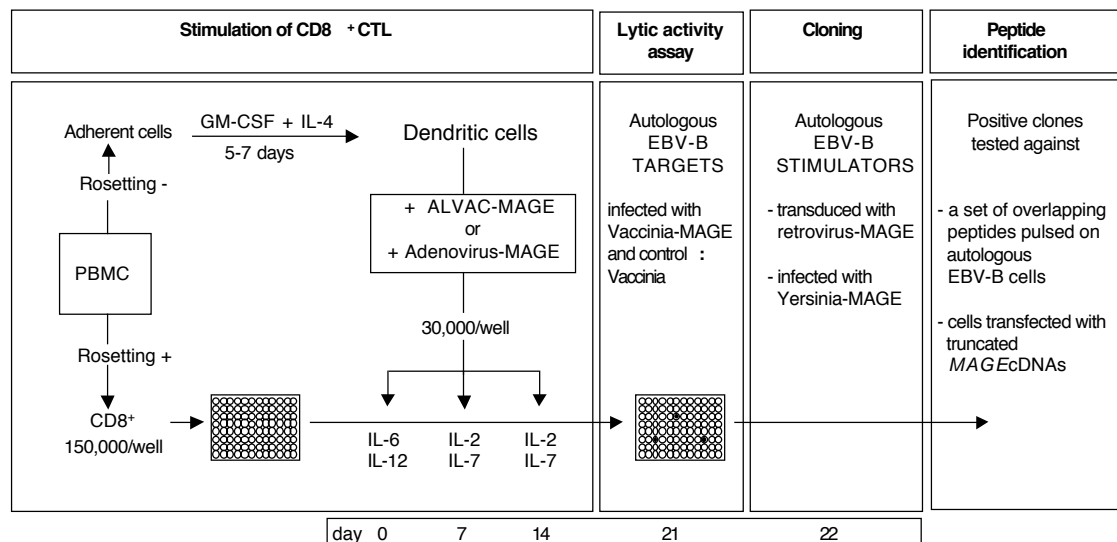


Fig. 1. Overview of the procedure to obtain anti-MAGE CD8⁺ CTL clones by stimulation with dendritic cells infected with viral vectors carrying a MAGE coding sequence

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

We have identified a MAGE-3.B40 antigen which is the first example of a tumor-specific antigen

exclusively presented by tumor cells expressing the immunoproteasome (6). This work was done in collaboration with the group of Benoît Van den Eynde.

We have found that MAGE-1 peptide SAYGEPKRL is recognized by CTL clones that are restricted either by HLA-Cw3, Cw6 or Cw16 (3). The presentation of the same peptide by different HLA molecules may be frequent for HLA-C molecules, because they are more closely related to each other in

the peptide-binding region than HLA-A and B molecules. But for HLA-A and B also this may occur more frequently than usually thought. MAGE-3 peptide MEVDPIGLY is presented to different CTL by HLA-B*4402, B*4403, and B*1801 (3). MAGE-1 peptide EADPTGHSY was found to be recognized by different CTL on HLA-A1 and B35 molecules, and to bind to HLA-A29 (3). The same was found for the MAGE-3 homologous peptide EVDPIGHLY.

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

Search for antigenic peptides recognized by CD4⁺ T cells

Studies in several animal models have demonstrated an important role for CD4⁺ T cells in inducing and maintaining anti-tumor immunity. It is therefore possible that the addition of antigenic peptides presented by class II to those presented by class I will improve the efficacy of therapeutic anti-tumor vaccination. To identify new HLA-peptide combinations, we used dendritic cells loaded with a recombinant MAGE protein to stimulate autologous CD4⁺ T lymphocytes (7). After four weekly stimulations, the responder cells were tested for their ability to secrete IFN- γ upon stimulation with the antigen, and the positive microcultures were cloned. The procedure is summarized in Figure 2.

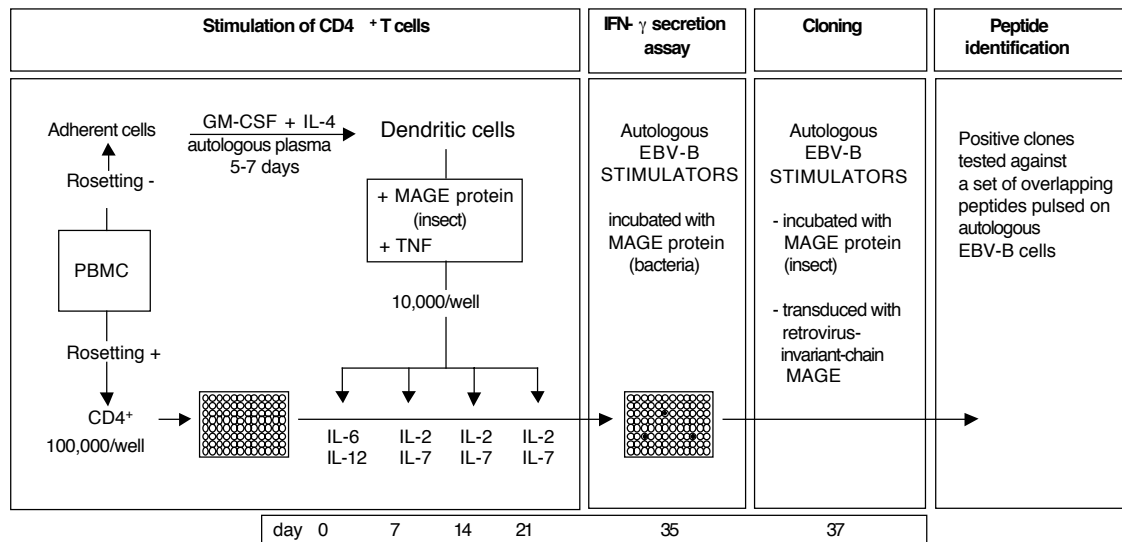


Fig. 2. Overview of the procedure to obtain anti-MAGE CD4⁺ T cell clones by stimulation with dendritic cells loaded with a whole protein

To identify the antigenic peptide, the positive clones were stimulated with a set of peptides of 16 amino acids that overlapped by 12 and covered the entire MAGE protein sequence. The positive peptide

was then tested for recognition on several Epstein-Barr virus immortalized B cell lines (EBV-B cell lines) to identify the HLA presenting molecule. Because a large number of the CD4⁺ T cells that were obtained

in our first experiments appeared to be directed against bacterial contaminants, we chose to alternate the sources of protein used at the various stages of the procedure. For example, to stimulate the lymphocytes, we used a MAGE protein produced in insect cells, and to test the specificity of the responder lymphocytes, we used a protein produced in bacteria. Microcultures that specifically produced IFN- γ after stimulation with the MAGE protein were cloned by limiting dilution using autologous EBV-B stimulator cells either loaded with the MAGE protein used during the stimulation step, or transduced with a retroviral construct encoding a truncated human invariant chain (Ii) fused with the MAGE protein.

MAGE-1 and MAGE-3 antigenic peptides identified by this procedure are listed in a database (<http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm>) (8). They include a MAGE-3 peptide presented by HLA-DP4, which is expressed by more than 70% of Caucasians. This peptide could not have been found by a peptide stimulation approach because no consensus anchor residue was known for HLA-DP4.

A novel approach to identify antigens recognized by CD4 T cells using complement-opsonized bacteria expressing a cDNA library

Pierre van der Bruggen (in collaboration with Lisette van de Corput from Leiden University, and Pascal Chaux)

We propose a new sensitive and rapid approach using the exogenous pathway to take up and process proteins encoded by a cDNA library expressed in bacteria. We hypothesized that, after opsonization with complement, recombinant bacteria can be endocytosed via receptor-mediated uptake by Epstein-Barr virus immortalized B cells to allow protein processing and presentation. To validate this approach, we made use of a minor histocompatibility antigen encoded by the human male-specific gene DBY. A recombinant bacteria library was constructed and screened with a DBY-specific CD4 T cell clone. We were able to identify bacteria expressing DBY diluted into a 300-fold excess of bacteria expressing a non-relevant gene. Screening of a bacterial library using a DBY-specific CD4 T cell clone resulted in the isolation of several DBY cDNAs (6).

The normal anti-MAGE-3.A1 repertoire

Sabrina Ottaviani, Claude Wildmann (a project started by Christophe Lonchay)

Cancer-germline gene *MAGE-3* codes for tumor-specific antigens recognized on many tumors by T lymphocytes. A MAGE-3 antigen presented by HLA-A1 has been used in several vaccination trials on metastatic melanoma patients. Only a small minority of patients have shown evidence of tumor regression. Attempts to correlate the tumor rejections with the CTL response against the vaccine have been hampered by the low level of these responses. To estimate not only the frequency but also the T cell receptor diversity of the naive precursors directed against MAGE-3.A1, we developed an approach aimed at obtaining a large number of CTL clones from very low frequency precursors. More than three billion peripheral blood mononuclear cells (PBMC) were obtained from several blood samples of a non cancerous individual. An HLA-A1/MAGE-3 tetramer was used to label large numbers of T cells and the positive cells were enriched by magnetic sorting. The selected fraction was then passed through a flow cytometer to further enrich the A1/MAGE-3 tetramer-positive cells among the CD8 cells. A number of positive cells were cloned. A fraction of the proliferating clones were tetramer-positive and showed lytic activity on cells pulsed with the peptide. These CTL clones invariably lysed the melanoma cells also, indicating that their avidity was high enough to recognize the level of antigen expressed by tumor cells. This led to an estimate of 4×10^{-7} of the CD8 T cells for the anti-MAGE-3.A1 CTLp in this individual (9). To evaluate the diversity of the naive anti-MAGE-3.A1 repertoire, we examined the TCR sequences of the 24 lytic anti-MAGE-3.A1 CTL clones obtained in this individual. Among the total of 23 clones, only two had the same TCR α and β sequences. The results indicate that it is very likely that the repertoire comprises more than 100 clonotypes. On this basis it is possible to use not only the frequency of CTL precursors in the blood but also the presence of dominant clonotypes to ascertain in patients the existence of anti-MAGE-3.A1 responses as low as 10^{-6} of CD8.

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

Nathalie Demotte, Sabrina Ottaviani, Claude Wildmann

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

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

Yi Zhang, Nicolina Renkvist, Zhaojun Sun, Hugues Nicolay, Sabrina Ottaviani, in collaboration with Didier Colau

Patients injected with class II-restricted peptides

For therapeutic vaccination trials, the vaccine can consist of defined antigenic peptides. This approach greatly facilitates the monitoring of the T cell response, because the presumed target of the T cells is completely defined. It allows the use of HLA-peptide tetramers to detect T cell responses in patients. HLA class II tetramers have been more difficult to obtain than HLA class I tetramers. Didier Colau has recently succeeded in obtaining a DP4.MAGE-3 tetramer, which was produced in insect cells. It stained specifically relevant CD4 clones. We have evaluated the anti-vaccine T cell frequencies in melanoma patients, who have been injected with dendritic cells pulsed with the MAGE-3 peptide presented by HLA-DP4. Blood cells from

vaccinated patients were labeled *ex vivo* with the multimers and the multimer-positive CD4 T cells were sorted by flow cytometry, multiplied in clonal conditions and tested for their specific recognition of the MAGE-3 antigen. In one patient, the frequency of anti-MAGE-3.DP4 T cells peaked at 1/1,400 of the CD4 T cells, representing at least an increase of 3,000-fold of the frequency found before immunization. TCR analysis of the anti-MAGE-3.DP4 clones from this patient established that the response was highly polyclonal.

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. The monitoring of the anti-vaccine T cell frequencies in patients vaccinated with protein is complex. The responses could be directed against a large number of HLA-peptide combinations, including many that are presently unknown. Therefore, the tetramer approach used for the detection of CD8 or CD4 is not appropriate. We have undertaken a systematic effort to develop a reproducible monitoring approach with high specificity and sensitivity. It combines sorting of living blood T cells producing IFN- γ after a short antigenic stimulation and detailed functional analyses of the cells amplified in clonal conditions. Using this approach, we have estimated the anti-vaccine CD4 T cell frequencies in the five patients who showed tumor regressions after injection of a MAGE-3 protein without adjuvant. Blood cells were stimulated overnight with autologous dendritic cells loaded with protein MAGE-3. The IFN- γ -secreting CD4 T cells were sorted by flow cytometry, multiplied in clonal conditions and tested for their specific recognition of MAGE-3. Anti-MAGE-3 CD4 T cells were detected in one patient after vaccination. Surprisingly the 13 anti-MAGE-3 clones, which corresponded to five different TCR clonotypes, recognized the same peptide presented by HLA-DR1. The frequency of the anti-MAGE-3 CD4 T cells was estimated at 1/60,000 of the CD4 T cells in post-vaccination blood samples, representing at least an 80-fold increase of the frequency found before immunization. The frequencies were confirmed in experiments where, instead of the protein, the peptide was used to stimulate the blood cells. The frequency of the dominant CD4 clonotype was also confirmed by a

direct PCR evaluation on cDNA extracted from blood cells. The blood cells of the four other clinical responders were screened using our specific and sensitive monitoring approach, but no anti-MAGE-3 T cell was detected. We are now defied to explain why several patients with evidence of tumor regression following vaccination have no detectable anti-vaccine T cells in their blood.

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;254:1643-7
2. Boël P, Wildmann C, Sensi ML, Brasseur R, Renauld JC, Coulie P, Boon T, van der Bruggen P. *BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes.* **Immunity** 1995;2:167-75.
3. 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.
4. Chaux P, Luiten R, Demotte N, Vantomme V, Stroobant V, Traversari C, Russo V, Schultz E, Cornelis GR, Boon T, van der Bruggen P. *Identification of five MAGE-A1 epitopes recognized by cytolytic T lymphocytes obtained by in vitro stimulation with dendritic cells transduced with MAGE-A1.* **J Immunol** 1999;163:2928-36.
5. Breckpot K, Heirman C, De Greef C, van der Bruggen P, Thielemans K. *Identification of new antigenic peptide presented by HLA-Cw7 and encoded by several MAGE genes using dendritic cells transduced with lentiviruses.* **J Immunol** 2004;172:2232-7.
6. van de Corput L, Chaux P, van der Meijden ED, De Plaen E, Falkenburg JHF, van der Bruggen P. *A novel approach to identify antigens recognized by CD4 T cells using complement-opsonized bacteria expressing a cDNA library.* **Leukemia** 2004;in press.
7. 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.
8. Zhang Y, Chaux P, Stroobant V, Eggermont AM, Corthals J, Maillère B, Thielemans K, Marchand M, Boon T, van der Bruggen P. *A MAGE-3 peptide presented by HLA-DR1 to CD4+ T cells that were isolated from a melanoma patient vaccinated with a MAGE-3 protein.* **J Immunol** 2003;171:219-225.
9. Lonchay C, van der Bruggen P, Connerotte T, Hanagiri T, Coulie PG, Colau D, Lucas S, Van Pel A, Thielemans K, van Baren N, Boon T. *Correlation between tumor regression and T cell responses in melanoma patients vaccinated with a MAGE antigen.* **Proc Natl Acad Sci U S A** 2004;in press.
10. 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.

THERAPEUTIC VACCINATION OF CANCER PATIENTS WITH TUMOR-SPECIFIC ANTIGENS

Marie MARCHAND, Senior Clinical Investigator

Nicolas van BAREN, Clinical Investigator
Jérôme DEGUELDRE, Clinical Research Associate
Christian VERFAILLE, Project Manager
Sylvie HOPPE, Secretary



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). While 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, T lymphocytes and tumor samples collected at different timepoints during vaccination can be analyzed in detail, which improves our understanding on what happens in patients who experience regression of metastatic lesions, and which may explain why this does not happen in the majority of patients with overall disease progression. This knowledge can then be used to design new vaccination modalities.

Therapeutic vaccination with MAGE tumor antigens

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 330 patients have been included in these multicentric 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, 1 partial response and 3 mixed responses (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 on a 4 week basis to 5 patients, without any evidence of melanoma 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.

In a future clinical trial, the MAGE-3.A1 peptide will be mixed with an immunostimulatory CpG-containing oligonucleotide to try to increase its immunogenicity. This new and promising adjuvant activates antigen presenting cells after binding to Toll-like receptor 9, and is thought to enhance CTL responses.

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 study LUD 99-003. 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 complete response lasting for more than 1 year. Thus this vaccine does not seem to induce more regressions than the MAGE-3.A1 peptide, but it does not require that the patient carries a specific HLA type. We will now mix this recombinant protein with adjuvant AS15 containing an immunostimulatory CpG nucleotide, and combine 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 (Study LUD 02-002).

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. We plan to investigate in a new trial whether increasing the dose of ALVAC would result in improved immunological and clinical responses.

Summary of relevant observations and perspectives

Immunization with MAGE peptides, the MAGE-3 recombinant protein or the ALVAC recombinant viral vector, is devoid of significant toxicity. A minority of melanoma patients (about 20 %) show regression of metastatic lesions following immunization, whatever the MAGE vaccine used. About 10% of the patients show complete or partial clinical responses. Some of these lasted for several years. 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. CTL

responses can be detected in a minority of patients vaccinated either with peptide or ALVAC virus. The responses appear to be weak and are mainly monoclonal. The relative frequency of CTL responders versus non-responders is higher in patients who had tumor regressions (4).

Selected publications

1. Boon T., Coulie P.G., 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., Beauvain M., Dietrich P-Y., Russo V., Kerger J., Masucci G., Jäger E., De Greve J., Atzpodien J., Brasseur F., Coulie P.G., van der

Bruggen P., and 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 : 219-230.

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

4. Coulie P.G., 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*. **Immunological Reviews** 2002; 188 : 33-42.

5. Marchand M., Punt C.J., Aamdal S., Escudier B., Kruit W.H., Keilholz U., Hakansson L., van Baren N., Humblet Y., Mulders P., Avril M.F., Eggermont A.M., 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.

ANALYSIS OF T CELL RESPONSES OF VACCINATED CANCER PATIENTS *

Aline VAN PEL, Associate Member

Danièle GODELAINE, Senior Investigator

Didier COLAU, Senior Investigator

Javier CARRASCO, Student

David BASTIN, Technician

Vinh HA THI, Technician

Maria PANAGIOTAKOPOULOS, Technician

Christophe LURQUIN, Associate Investigator

Bernard LETHE, Associate Investigator

Francis BRASSEUR, Associate Investigator

Marie-Claire LETELLIER, Technician

Madeleine SWINARSKA, Technician



* In association with the group of Pierre Coulie, ICP: see **Human tumor immunology** (research at ICP).

The identification of antigens recognized on human tumors by autologous T lymphocytes has opened the way for therapeutic vaccination strategies involving defined tumor antigens such as the MAGE antigens (1, 2). Tumor regressions have been observed in a minority of treated melanoma patients. Such clinical responder patients have been found following immunization with peptides and recombinant ALVAC viruses. In some patients, a monoclonal T cell response was observed and the level of the response appeared to be stable during the course of the vaccination protocol (3, 4). Since detectable T cell responses occurred more frequently in patients who show signs of tumor regression than in those who did not, we consider the possibility that the limiting factor for the anti-tumor effect of the vaccine is the intensity of the CTL response to this vaccine (5, 6). Improving the efficacy of such vaccines would thus critically depend on their capacity to trigger a robust immune response. A novel approach to vaccination is to exploit the potentiality of dendritic cells that are widely accepted to be particularly effective in presenting antigens to T cells and immunize cancer patients with a sample of their autologous dendritic cells charged with tumor antigens. We initiated a collaboration with G. Schuler and B. Schuler-Thurner at the University of Erlangen (Germany), who vaccinated advanced stage IV melanoma patients with mature, monocyte-derived dendritic cells pulsed with MAGE peptides and observed regression of some metastases(7). As shown in our previous report, an immune response was found in a few regressing patients(8). These results prompted us to initiate a new collaboration with K. Thielemans (VUB, Belgium), who vaccinated less advanced patients with the same protocol.

Methods for evaluation of T-cell responses in vaccinated cancer patients

To establish whether there is a correlation between tumoral regressions and T-cell responses against the vaccine antigen, we evaluated the responses of patients vaccinated with a MAGE-3 antigenic peptide, with a recombinant virus coding for this peptide or with MAGE peptide-pulsed dendritic cells.

The recent development of tetrameric peptide-MHC class I and class II complexes (tetramers) allows the direct identification of antigen-specific T-cells. The technology has been introduced and is still being developed by Didier Colau.

To detect low-level responses, blood lymphocyte microcultures were stimulated with the antigenic peptide in limiting dilution conditions (Mixed Lymphocyte Peptide Culture, MLPC), followed by tetramer analysis, cloning of the tetramer-positive cells, and T-cell receptor (TCR) sequence analysis of the cytolytic T lymphocyte (CTL) clones that showed

strict specificity for the vaccine antigen (for method, see P. Coulie, ICP report).

Christophe Lurquin and Bernard Lethé focused their efforts on detailed analysis of frequencies of characterized T-cell clones in blood, metastases and non tumoral tissue samples, using 'clonotypic' polymerase chain reaction (PCR) amplifications specific for the V α and V β rearrangements of relevant TCR. These PCR amplifications on cDNA were sensitive enough to detect one CTL expressing a given TCR mixed with 3x10⁷ PBMC of normal donor and they were highly specific for the given TCR insofar as no product was amplified with cDNA prepared from 3x10⁷ PBMC of 5 unrelated donors.

Previous results showed that a monoclonal CTL response against a MAGE-3 antigen presented by HLA-A1 was observed by the *in vitro* tetramer analysis in a melanoma patient who showed partial rejection of a large metastasis after treatment with a vaccine containing only the tumor-specific antigenic peptide. Our results proved that the vaccination induced at least a 100-fold amplification of an anti-MAGE-3.A1 CTL and that vaccines containing only a tumor-specific antigenic peptide can elicit a CTL response (3)

A melanoma patient vaccinated with a MAGE-3 recombinant canarypox virus

Patient EB81 had about 70 cutaneous metastases when she was vaccinated with a recombinant canarypox virus of the ALVAC type containing a minigene encoding the MAGE-3.A1 peptide. Repeated injections of ALVAC were followed by vaccinations with the MAGE-3 peptide. Ten months after the first vaccine, all the metastases had become undetectable except an enlarged lymph node which was resected. A similar vaccination protocol alternating virus and peptide injections was carried on until now.

Blood lymphocytes collected before vaccination were analyzed with the MLPC-tetramer method. No anti-MAGE-3 CTL could be detected among 10⁷ CD8⁺ cells, suggesting a frequency similar to that found in normal donors (4). Two different anti-MAGE-3.A1 CTL clones were identified in postimmune blood. One, CTL 35, was found in more than 95% of the analyzed independent microcultures, suggesting that this MAGE-3.A1 response was essentially monoclonal.

PCR amplifications specific for the TCR 35 V α and V β rearrangements were applied directly to cDNA obtained from groups of PBMC. In the first post-ALVAC sample, the frequency of CTL C35 rose to 3.6 x 10⁻⁶ of the CD8⁺, suggesting that the ALVAC vaccination induced at least a 30 times amplification of anti-MAGE-3.A1 T cells. This frequency, that remained stable during the peptide vaccinations, rose to 1.1x10⁻⁵ after a boost of ALVAC vaccinations given one year later and remained stable thereafter.

By stimulating blood lymphocytes from melanoma patient EB81 with autologous tumor cells (MLTC), a series of CTL clones that specifically lysed autologous melanoma cells were isolated. Some of these CTL clones recognized peptides presented by HLA-A2 and encoded by gene MAGE-C2, another cancer-germline gene which is expressed at high level by the melanoma cells of the patient. We analyzed the TCR V β gene expression of this set of anti-MAGE-C2 CTL clones. One of them, named CTL 16, seemed to be amplified after vaccination since it was retrieved many times as independent clones from the postimmune blood but was not found in preimmune blood samples. Clonotypic RT-PCR amplifications of TCR 16 V α and V β rearrangements indicated an average frequency of the corresponding CTL in blood throughout the whole time of vaccination of ~3.5 x 10⁻⁵ among the CD8⁺.

To investigate the involvement of the MAGE-C2-specific CTL in the tumor regression process observed in this patient after vaccination against a MAGE-3 antigen, we have analyzed the frequencies of the anti-MAGE-3.A1 CTL 35 and the anti-MAGE-C2.A2 CTL 16 in the resected metastatic lymph node which presented histological signs of regression. Sections of 7 μ m thick and ~80 mm², fragments of such sections and groups of cells excised from sections with laser microdissection were tested by RT-PCR for the presence of TCR 35 and TCR 16. We obtained a frequency of ~1/32,500 CD8 for anti-MAGE-3 CTL, which represents a 20-fold enrichment relative to blood frequency at the time of the metastasis resection. Moreover, the anti-MAGE-C2 CTL was at least 275-times enriched in the resected sample with a frequency higher than 1/100 CD8 in the lymph node tissue and a frequency >1/5 CD8 in tissue regions strongly invaded by tumoral cells.

During this year, we confirmed and extended these results by analyzing TCR β cDNA libraries performed on the RNA of various metastases of patient EB81. These assays allowed the estimation of the frequencies of various anti-tumor CTL within

metastases. They also enabled us to considerably enlarge the diversity of activated T lymphocyte clones repeatedly found in either the same metastasis, or in other metastases resected before and after vaccination. T lymphocyte clones above a frequency of 1% of all the T cells were not rare. Some of these clonotypes were not found before vaccination neither in blood nor in tumor. Among the frequent clones, one appeared to be directed against a mutated antigen, but most of them were directed against various MAGE-C2 epitopes, accounting for up to 20% of the T cells present in the metastasis. In this patient who is still disease-free four years after the onset of vaccinations, some of the intra-tumor clonotypes were enriched up to 1000-fold relative to their frequencies in the blood.

A melanoma patient vaccinated with dendritic cells pulsed with a MAGE-3 peptide presented by HLA-A1

Melanoma patient MMB02 had about 20 cutaneous metastases on her right leg when she was vaccinated by K. Thielemans and his coworkers at the VUB with mature, monocyte-derived dendritic cells pulsed with MAGE-3.A1 peptide according to a protocol set up by Gerold Schuler at Erlangen (Germany). At the end of the first cycle of 6 vaccinations, some metastases started to regress while new ones appeared. Vaccinations were continued and the patient kept showing evidence of a mixed response. To improve our understanding of the process leading to occasional tumor regressions that occur following vaccination, we investigated the presence of anti-vaccine and anti-tumor CTL in the blood and inside metastases collected at various time points.

In the blood, the frequency of anti-MAGE-3.A1 T cells among CD8 cells raised from less than 3.4×10^{-7} before vaccination to 9.3×10^{-7} after 3 vaccinations, to 2.6×10^{-6} after 6 vaccinations and 5.4×10^{-6} after another cycle of 6 vaccinations. At this time point, only one anti-MAGE-3.A1 clonotype was detected, a monoclonality that contrasts with the polyclonality observed in Schuler's patients (8). On the other hand, the frequency of anti-tumor CTL, i.e. lytic effectors that recognize the autologous melanoma cells but not autologous B cells nor NK target K562, was 2.2×10^{-4} before and 2×10^{-4} after 6 vaccinations. Thus, in the same patient, the frequency of blood anti-tumor CTL was more than two orders of magnitude above the frequency of anti-vaccine CTL and was already present before vaccination.

Cutaneous metastases removed after the second cycle of vaccinations allowed the isolation of a few TIL clones able to specifically recognize the autologous tumor cells. None of these clones was directed against the vaccine antigen. One of them was shown to be directed against a new tumor-specific antigen encoded by gene *MAGE-C2* and presented to CTL by HLA-B44: peptide SESIKKKVL. This anti-MAGE-C2.B44 CTL was also found in the blood taken after 6 vaccinations. RT-PCR specific for TCR sequences of that anti-MAGE-C2-B44 CTL clone is being set up and will allow estimation of the frequency of that clone in the blood samples taken before and after immunization, as well as in cutaneous metastases resected before and after vaccination. This should help to evaluate the potential contribution of that clone to the tumor regressions that are observed occasionally after vaccination.

If the frequency and the diversity of the anti-vaccinal and anti-tumoral CTL are important features to understand the mechanisms underlying tumor regressions, the functional activity of these CTL is another important aspect to be analyzed. In this prospect, we have undertaken the analysis of anti-MAGE-3.A1 CTL from vaccinated patients for expression of some phenotypic markers (CD25, CD69, CD28, CD27, CCR7, CD45...) that have been associated to the CTL activation status or particular levels of maturation, in order to search for a correlation with CTL expansion capacities, lysis and secretion profile of cytokines.

Selected publications

1. Boon T, Cerottini JC, Van den Eynde B, van der Bruggen P, Van Pel A. *Tumor antigens recognized by T lymphocytes*. **Annu Rev Immunol** 1994 ; 12 : 337-65.
2. 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.
3. Coulie PG, Karanikas V, Colau D, Lurquin C, Landry C, Marchand M, Dorval T, Brichard V, 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.
4. 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.

5. 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.

6. 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.

7. Thurner B, Haendle I, Roder C, Dieckmann D, Keikavoussi P, Jonuleit H, Bender A, Maczek C, Schreiner D, von den Driesch P, Brocker EB, Steinman RM, Enk A, Kampgen E, Schuler G. *Vaccination with mage-3.A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma.* **J Exp Med** 1999 ; 190 :1669-78.

8. Godelaine ., 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.

CYTOKINES IN IMMUNITY AND INFLAMMATION

Jean-Christophe RENAULD, Member

Jacques VAN SNICK, Member, Guest Investigator

Laure DUMOUTIER, Postdoctoral Fellow

Jamila LOUAHED, Postdoctoral Fellow

Laurent KNOOPS, Student

Valérie STEENWINCKEL, Student

Amel TOUNSI, Student

Brigitte de LESTRE, Technician

Monique STEVENS, Technician

Emiel VAN ROOST, Technician

Francine WELLEKENS, Technician



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

Jean-Christophe Renauld, Jamila Louahed, Laurent Knoops, Valérie Steenwinckel, Brigitte de Lestré, Monique Stevens, Emiel Van Roost

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 (1). 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 and in the lungs of IL-9

transgenic mice. This observation is reminiscent of mice that are prone to the development of diseases that are characterized by the production of autoantibodies, such as Systemic Lupus Erythematosus, and suggests that IL-9 might play a role in some autoimmune processes (2).

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 (3).

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. Current studies indicate that IL-9 promotes asthma through both IL-13-dependent and IL-13-independent pathways. 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. Phase I clinical trials using anti-IL-9 antibodies produced in our laboratory have been initiated in collaboration with Medimmune.

IL-9 receptor and signal transduction

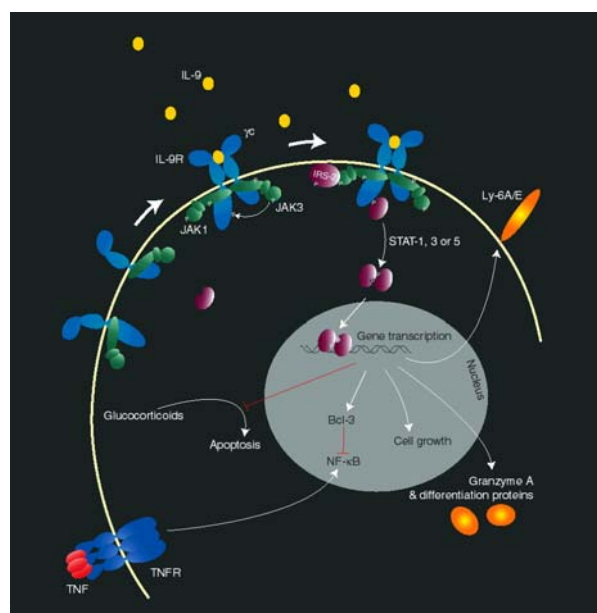
Jean-Christophe Renauld, Laurent Knoops, Monique Stevens, Emiel Van Roost

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 (4). 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 γ_c . This induces the phosphorylation of the JAK1 and JAK3 tyrosine kinases, which are associated with IL-9R and γ_c , 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 (5).

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.



Anti-apoptotic activity of I-309receptor and signal transduction

Jean-Christophe Renauld, Amel Tounsi, Jacques Van Snick, Jamila Louabed

Incidentally, our studies of this particular model of the regulation of cell death by cytokines, lead them to purify another protein called I-309, originally described as a human chemotactic factor, and that turned out to exert a significant anti-apoptotic activity

for thymic lymphomas (6). 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

Jean-Christophe Renault, Jacques Van Snick, Laure Dumoutier, Jamila Louabed, Laurent Knoops, Amel Tounsi, Monique Stevens, Emiel Van Roost

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- κ B

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- κ B transcription factors and its induction by IL-9 represents a novel mechanism of NF- κ B regulation by cytokines, and a new crosstalk between the JAK/STAT and NF- κ B signal transduction pathway (7). 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 (8).

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 (9) and protection against experimental hepatitis (L. Dumoutier, unpublished results). Recombinant human IL-22 was produced (with D. Colau, LICR) and its crystallographic structure solved. Despite its structural homology with IL-10, IL-22 fails to recapitulate any of IL-10 biological activities.

Analysis of the genome databases leads to the identification of a new receptor belonging to the IL-10 receptor family (10). 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-10R β . Anti-IL-10R β antibodies indeed block the IL-22-induced acute phase response in HepG2 cells (9). 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-10R β 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 β , 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.

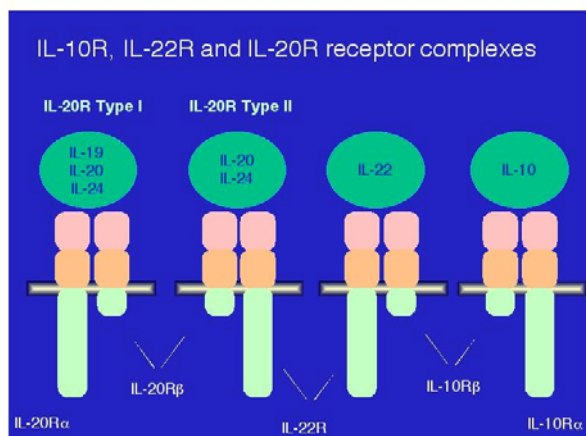


Fig. 2

In addition to its role in IL-22 binding and signalling, the IL-22R chain also forms a functional heterodimeric receptor complex by associating with IL-20R β , 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 (11). In addition, IL-20 and IL-24 can also bind to other complexes consisting of IL-20R α and IL-20R β . This promiscuity in cytokine receptor usage is illustrated in Fig 2 (see also ref. 12 for a review of this new cytokine family).

LICR2: a new cytokine receptor mediating antiviral activities

Jean-Christophe Renault, Laure 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 receptor 2). This receptor

binds new cytokines designated IFN- λ 1-3, and mediates the same activities as those mediated by the receptors for IFN- α and β , including antiviral and antiproliferative activities (13), raising the possibility of therapeutic applications in viral infections and cancer.

Selected publications

1. Renault JC, van der Lugt N, Vink A, van Roon M, Godfraind C, Warnier G, Merz H, Feller A, Berns A, Van Snick J. *Thymic lymphomas in interleukin 9 transgenic mice*. **Oncogene** 1994; 9:1327-32.
2. Vink A, Warnier G, Brombacher F, Renault JC. *Interleukin 9-induced in vivo expansion of the B-1 lymphocyte population*. **J Exp Med**. 1999; 189:1413-23.
3. Richard M, Grecis RK, Humphreys NE, Renault 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.
4. Renault JC, Druetz C, Kermouni A, Houssiau F, Uyttenhove C, Van Roost E, Van Snick J. *Expression cloning of the murine and human interleukin 9 receptor cDNAs*. **Proc Natl Acad Sci U S A** 1992; 89:5690-4.
5. Demoulin JB, Van Roost E, Stevens M, Groner B, Renault 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.
6. Van Snick J, Houssiau F, Proost P, Van Damme J, Renault JC. *I-309/T cell activation gene-3 chemokine protects murine T cell lymphomas against dexamethasone-induced apoptosis*. **J Immunol** 1996; 157:2570-6.
7. Richard M, Louahed J, Demoulin JB, Renault JC. *Interleukin-9 regulates NF-kappaB activity through BCL3 gene induction*. **Blood** 1999; 93:4318-27.
8. Louahed J, Grasso L, De Smet C, Van Roost E, Wildmann C, Nicolaides NC, Levitt RC, Renault JC. *Interleukin-9-induced expression of M-Ras/R-Ras3 oncogene in T-helper clones*. **Blood** 1999; 94:1701-10.
9. Dumoutier L, Van Roost E, Colau D, Renault 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.

10. 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.

11. 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.

12. Renauld JC. *Class II cytokine receptors and their ligands: key antiviral and inflammatory modulators*. **Nature Rev. Immunol.** 2003; 3:667-676.

13. Dumoutier L, Tounsi A, Michiels T, Sommereyns C, Kotenko SV, and 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; 279:32269-32274.

SIGNAL TRANSDUCTION GROUP: STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS

Stefan CONSTANTINESCU, Associate Member

Katharina KUBATZKY, Postdoctoral Fellow (Delori Fellow)

Virginie MOUCADEL, Postdoctoral Fellow (Marie Curie Fellow)

Yohan ROYER, PhD Student

Judith STAERK, PhD Student

Alexandra DUSA, PhD Student

Nadine SEUBERT, Student, (Univ. Würzburg, Germany)

Mingli LI, Research Assistant

Yan YIN, Research Assistant

Loic GARCON, Marie Curie Visiting Student, Institut Gustave Roussy, Villejuif, France

Yaniv MALKA, Marie Curie Visiting Student, Tel-Aviv University, Israel

Sylvie HOPPE, Administrative Assistant



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). These kinases phosphorylate downstream proteins, like receptors themselves, signal transducers and activators of transcription (STAT) proteins and a variety of other signaling proteins. We study the signal transduction mechanisms and biologic functions of cytokine receptors such as the receptors for erythropoietin (Epo), thrombopoietin (Tpo), interleukin 2 (IL2) and interleukin 9 (IL9) and their involvement in diseases such as Polycythemia Vera or leukemias. 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. We also study the mechanisms by which STAT proteins become constitutively activated and how they function in transformed hematopoietic or patient-derived leukemia cells.

Determination of the interface and orientation of the activated erythropoietin receptor dimer

Nadine Seubert, Yohan Royer, Judith Staerk, Katharina Kubatzky, Virginie Mouchadel

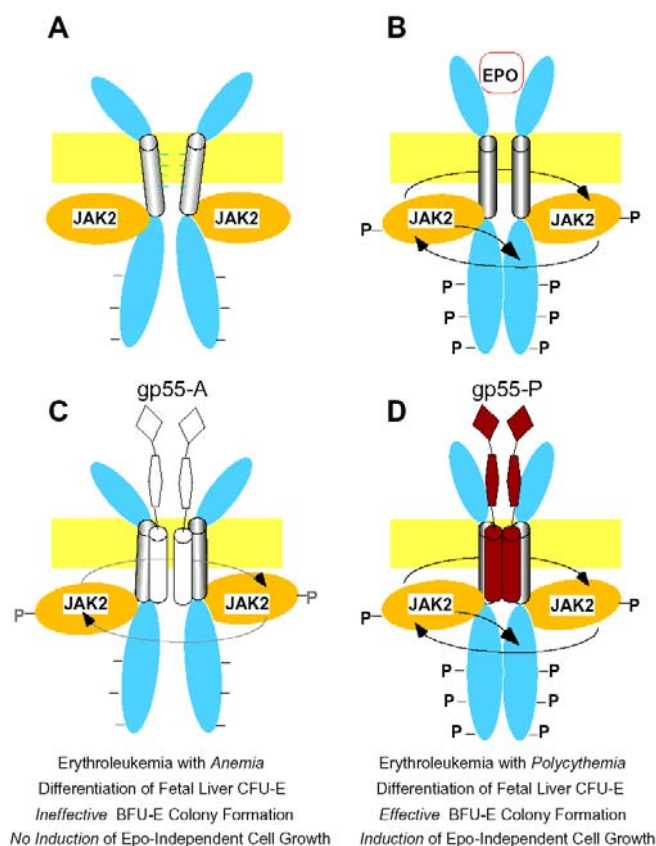
The determination of the interface of the active erythropoietin receptor (EpoR) dimer has been a priority during the last year (1). Epo binding to the erythropoietin receptor (EpoR) results in survival, proliferation and differentiation of erythroid progenitors into mature red blood cells. We have shown that, in the absence of Epo, the cell-surface EpoR is dimerized in an inactive conformation,

which is stabilized by interactions between the TM sequences (2). Epo binding to the extracellular EpoR domain induces a conformational change of the receptor, which results in the activation of cytosolic JAK2 proteins (Figure 1A and B). The α -helical orientation of the (TM) and cytosolic JM domains is crucial for receptor activation (3). In collaboration with Lily Huang and Harvey Lodish, Whitehead Institute, Cambridge, MA, USA, we have identified a number of key residues in the EpoR cytosolic JM domain, which are required for switching on the activity of JAK2 and initiate signaling (4).

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 (1).

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. This allows the prediction of the positions (on an α -helix) of the residues that will be in the interface of the activated EpoR dimer. We have generated seven different constructs where all seven possible orientations were imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR. All seven fusion proteins are expressed when transduced in cytokine-dependent cell lines and reach the cell-surface. However, only two of the

seven fusion proteins showed activity represented by stimulation of proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells (1). The predicted dimeric interfaces of the two active fusion proteins are very close, emphasizing the notion that a unique dimeric EpoR conformation is 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. This approach of exploring orientation-dependent signaling is now applied in our group for the determination of the active interfaces of the thrombopoietin and G-CSF receptors.



(A) The erythropoietin receptor (EpoR) is an inactive dimer on the cell-surface in the absence of ligand due to interactions between the transmembrane (TM) domains (interrupted line). Cytosolic Janus kinase 2 (JAK2) is bound to the receptor juxtamembrane domain and stimulates receptor folding and traffic to the cell-surface.

(B) Erythropoietin (Epo) binding induces a conformational change in the extracellular domain which is transmitted via the α -helical TM domain to receptor residues in the juxtamembrane domain that contact JAK2 and switch its activity on. Activated JAK2 phosphorylates (-P) itself, the receptor cytosolic domain and many other signaling proteins leading to survival, proliferation and differentiation of erythroid progenitors into mature red blood cells.

(C) Co-expression of the EpoR with the gp55-A envelope protein of the Spleen Focus Forming Virus results in cell-surface complex formation due to specific interactions between the TM domains. The EpoR transmembrane dimer, which maintains the EpoR inactive, is disrupted by the interaction with gp55-A. The receptor acquires a conformation, which allows weak constitutive activation resulting in erythroleukemia with low numbers of red blood cells (*anemia*).

(D) Co-expression of the EpoR with the viral gp55-P envelope protein results in cell-surface complex formation due to specific interactions between the TM domains. In this complex the EpoR acquires a dimeric conformation very similar to that induced by Epo, which results in strong constitutive activation of EpoR signaling leading to erythroleukemia and massive production of mature red blood cells (*polycythemia*).

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

Katharina Kubatzky, Judith Staerk, Mingli Li, Alexandra Dusa

To define the interfaces of the active and inactive EpoR dimers we performed cysteine scanning mutagenesis of the extracellular juxtamembrane and TM domains (5). 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 (5). The same residues were found to be in the interface of the active coiled-coil-EpoR fusion proteins (1). These data show that in the native structure the TM domains of the EpoR are closely interacting with each other.

The structure of the cytosolic domains of cytokine receptors remains a mystery. Due to our previous work showing that the junction between the EpoR TM and intracellular domain is rigid (3) we hypothesized that the important structured segments of the cytosolic domain must contain the cytosolic JM segment. We have cloned the cDNAs coding for the seven coiled-coil-EpoR fusion proteins in the pET31b vector in order to produce recombinant fusion proteins in quantities amenable for biophysical and structural studies. In collaboration with the group of Steven Smith, SUNY, Stony Brook, NY, USA, we will determine the NMR structure of the EpoR TM and cytosolic JM domains in the active and inactive coiled-coil-EpoR fusion proteins.

Signaling by the thrombopoietin receptor

Judith Staerk

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. In contrast, only EpoR can support efficient formation of mature red cells. Since both EpoR and TpoR are members of the homodimeric class of cytokine receptors we have started to compare signaling and gene expression induced by these two receptors as well as the orientation of their intracellular domains in the activated state. We have already identified a major difference between TpoR and the EpoR, namely that several orientations of the TpoR are compatible with inducing cell proliferation but only a restricted dimeric conformation is compatible with the induction of intercellular adhesion. By constructing chimeric receptors we attempt to identify the relevant sequences which confer to the TpoR and EpoR their specific biologic activities in immature hematopoietic progenitors and committed erythroid progenitors, respectively

Signaling by the IL9R via the common γ_c chain (γ_c) and traffic of cytokine receptors to the cell-surface

Yohan Royer

γ_c is a cytokine receptor that is shared by the receptor complexes of several cytokines, such as IL2, IL4, IL7, IL9, IL15 and IL21. γ_c binds and activates JAK3. Humans that lack the γ_c or have mutations in JAK3 develop severe combined immunodeficiency (SCID). We study the regions important in the IL9R and the γ_c for JAK1 and JAK3 interactions. For both of them, the region comprising Box1 and Box2 is necessary for the JAKs binding. In contrast, the region between the juxtamembrane domain and Box1 has quite different sequence requirements. While IL9R is rather similar to the EpoR (3), γ_c does not require hydrophobic residues before Box1. Finally, by confocal microscopy, the distribution of the γ_c on the cell surface is totally different than the one of the IL9R or the EpoR. The γ_c forms big patches whereas the IL9R and the EpoR exhibit a normal diffuse distribution, as we have shown in collaboration with Pierre Courtoy. Paraformaldehyde fixation was required for the appearance of the big patches in the case of the γ_c , suggesting that the receptor is localized in membrane regions where formaldehyde can not block diffusion and aggregation of membrane proteins. This observation could reveal different compartmentalization of receptors on the cell-surface.

Traffic and cell-surface expression of the EpoR critically depend on the ability of EpoR to bind JAK2 intracellularly (4). In the absence of JAK2, the receptor does not get transported from the endoplasmic reticulum (ER) to the Golgi apparatus and does not acquire EndoH resistance. We have observed that, in hematopoietic cells, overexpressing cognate JAK proteins, leads to enhanced cell-surface localization of several cytokine receptors (i.e. TpoR, IL9R, IL2R, γ_c). The IL9R α which requires JAK1 for signaling is expressed at higher levels on the cell-surface when JAK1 but not JAK2 or JAK3 is overexpressed. 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.

Sequence-specific interactions between transmembrane domains

Alexandra 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 (6). In collaboration with Yoav Henis, Tel-Aviv University, Israel, we have shown that both the gp55-A and gp55-P TM domains specifically interact with the TM domain of the EpoR (Figure 1C and D)(7). 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 (8). 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 represent a powerful selection.

Constitutive activation of JAK-STAT signaling pathways and genes targeted by STAT5 in transformed hematopoietic and patient-derived leukemia cells

Virginie Moucadel, Judith Staerk

Cytokine stimulation of cytokine receptors, induces transient activation of the JAK-STAT pathway. In contrast several mutant forms of cytokine receptors have been isolated that signal constitutively (i.e. EpoR R129C or TpoR S498N) [reviewed in 9]. Such receptors are permanently dimerized in an activated state and induce the biologic effects of the wild type receptors as well as leukemic cell transformation. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously, in the absence of any cytokine. In the transformed cells many of the transient signaling events induced by cytokines are detectable permanently, i.e. ligand-independent phosphorylation of JAK and STAT proteins or high levels of nuclear activated STATs especially STAT5 and STAT3. A similar picture has been noted in patient derived leukemia cells. The critical questions we would like to answer concern the mechanisms by which the JAK-STAT remain permanently activated in transformed cells and which genes are regulated by constitutively active STAT proteins in leukemic cells. Using chromatin 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 not only to N3 sites, which is characteristic of ligand-activated STAT5 (12). Furthermore, while cytokines such as Epo activate both STAT5A and STAT5B, we have recently observed that in transformed hematopoietic cells it is mainly STAT5B that is constitutively active (12).

We are attempting to identify the promoters actually bound by STAT proteins in living cells in physiologic and pathologic situations. We use a modified version of the chromatin immunoprecipitation assay pioneered by Alex Varshavsky in conjunction with DNA microarray gene 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 expressed in bicistronic retroviral vectors that allow wide expression of cDNAs at physiologic levels.

Selected publications

1. Seubert N, Royer R, Staerk J, Kubatzky, KF, Moucadel V, Krishnakumar S, Smith SO, Constantinescu SN. *Active and inactive orientations of the transmembrane and cytosine domains of the erythropoietin receptor dimer.* **Mol Cell** 2003;12:1239-50.
2. Constantinescu SN, Keren T, Socolovsky M, Nam H, Henis YI, Lodish HF. *Ligand-independent oligomerization of cell-surface erythropoietin receptor is mediated by the transmembrane domain.* **Proc Natl Acad Sci U S A** 2001;98:4379-84.
3. 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.
4. Huang LJ, Constantinescu SN, Lodish HF. *The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor.* **Mol Cell** 2001;8:1327-38.
5. Kubatzky, KF, Liu W, Li M, Royer Y, Simmerling C, Smith SO, Constantinescu SN. *Structure of the extracellular juxtamembrane region of the erythropoietin receptor : novel constitutive active receptor mutants. Submitted EMBO J, 2004.*
6. Constantinescu SN, Wu H, Liu X, Beyer W, Fallon A, Lodish HF. *The anemic Friend virus gp55 envelope protein induces erythroid differentiation in fetal liver colony-forming units-erythroid.* **Blood** 1998;91:1163-72.
7. Constantinescu SN, Keren T, Russ, W, Ubarretxena-Belandia I, Malka Y, Kubatzky, K, Engelman DM, Lodish, HF, Henis YI. *The Epo receptor transmembrane protein modulates complex formation with viral anemic and polycythemic gp55 proteins.* **J Biol Chem** 2003; 278:43755-63.
8. Constantinescu SN, Liu X, Beyer W, Fallon A, Shekar S, Henis YI, Smith SO, Lodish HF. *Activation of the erythropoietin receptor by the gp55-P viral envelope protein is determined by a single amino acid in its transmembrane domain.* **EMBO J** 1999;18:3334-47.
9. Constantinescu SN, Moucadel V. *STAT signaling by erythropoietin.* In **Signal Transducers and Activators of Transcription (STATs) : Activation and Biology** (Editors PB Sehgal, T Hirano, DE Levy) 2003;12:575-593, Kluwer Academic Publishers, New York.
10. Royer Y, Menu C, Liu X, Constantinescu SN. *High throughput Gateway bicistronic retroviral vectors for stable expression in mammalian cells : exploring the biologic effects of STAT5 overexpression.* **DNA and Cell Biol** 2004;23:355-65.

Administration and general services

Administration and secretariat

Dario FLOREAN, Administrator

Aline BIEN, Accounting and Administrative Assistant

Sylvie HOPPE, Scientific Secretary

Nathalie KRACK, Librarian

Carine LEMOINE, Scientific and Administrative Assistant

Arnaud ROKS, Accounting Assistant

Geneviève SCHOONHEYDT, Administrative Assistant

Valérie WINAND, Scientific Secretary

Contact :

UCL 7459, Avenue Hippocrate 74, 1200 Bruxelles, Belgium

Tel : +32 (2) 764 7459

Fax : +32 (2) 764 9405

Pre-clinical Research Resources Group:

Guy WARNIER, Veterinarian and Group Head

Jean -Pierre. MATTHEWS, Technical Assistant

Phone SIRICHANTO, Technical Assistant

Kéota SOUVANNARAJ, Technical Assistant

Keoprasith SOUVANNARAJ, Technical Assistant

Technical support

Jo LEJEUNE, Head of Computer Systems

Paul WAUTERS, Laboratory Manager

André TONON, FACS Operator

Jacques VAN CLEVE, Research Assistant in Bioinformatics

Jean-Luc GERMIS, Technician

Manuel ORVIZ, Senior Technical Assistant

Antoinette VAN GOETSENHOVEN, Technical Assistant

Jean-Claude VERHELST, Administrative and Technical Assistant

M. AHLOUMOU, Laboratory Helper

Mustafa HAMED, Laboratory Helper

Samira JEBARI, Laboratory Helper

Pascale MAHIEU-GAUDY, Laboratory Helper

Encarnacion MARIANINI, Laboratory Helper

Rafaëlla REYES PRIETO, Laboratory Helper