

Research at
the Brussels Branch of
the Ludwig Institute for Cancer Research (LICR)

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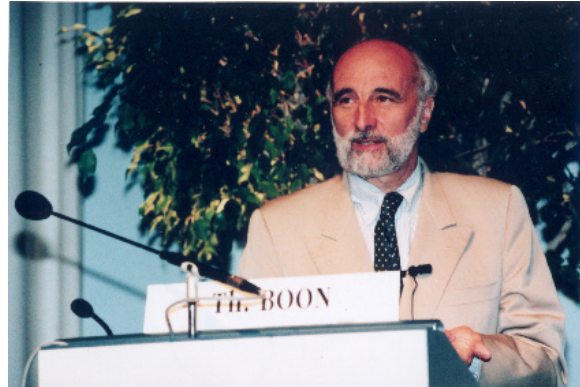
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The Ludwig Institute for Cancer Research

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.



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

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

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

The Brussels Branch of the Institute was created in 1978. It is composed of 84 members and is headed by Thierry Boon, Branch Director.

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TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

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

Tumor antigens recognized by Cytolytic T Lymphocytes (CTL) consist of peptides that are presented by MHC molecules at the cell surface and derive from intracellular proteins that are degraded by the proteasome. The intracellular pathway leading from the protein to the peptide/MHC complex is known as “antigen processing”. Our group focuses on the proteasome and recently described a new mode of production of antigenic peptides by the proteasome, based on cutting and pasting peptide fragments to form a new spliced peptide. The first example was a peptide derived from human melanocyte protein gp100. This antigenic peptide is nine-amino acid long and is produced by the splicing of two fragments that were initially non-contiguous in the parental protein. The splicing is made by the proteasome, is tightly coupled to the proteolytic reaction, and appears to occur by transpeptidation involving an acyl-enzyme intermediate. We are currently working on a second example of spliced peptide, where the two fragments are rearranged before splicing.

We are also studying the processing differences between the standard proteasome, which is present in most cells, and the immunoproteasome which is found in dendritic cells and in cells exposed to interferon-gamma. Several tumor antigens were found to be processed differently by the two proteasome types, usually because of a preferential cleavage made by one or the other proteasome within the antigenic peptide itself.

*Translation of knowledge on tumor antigens into efficient cancer immunotherapy requires additional studies on the various strategies that can be used. Some of these studies can be done in preclinical animal models. The study of such a model allowed us to uncover a powerful mechanism of tumor resistance, which is based on tryptophan catabolism by indoleamine-2,3 dioxygenase, an enzyme that we found to be frequently expressed in tumors. The resulting local tryptophan shortage appears to prevent the proliferation of lymphocytes at the tumor site. Inhibitors of indoleamine-2,3 dioxygenase can be used *in vivo* to counteract this tumor resistance mechanism.*

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

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

Jacques Chapiro, Vincent Stroobant, Benoît Guillaume, Benoît Vanholle, 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 immuno-

proteasomes (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 combina-

tion of antigens in order to minimize the risk of tumor escape by proteasome switching.

Antigenic peptides produced by peptide splicing in the proteasome

Nathalie Vigneron, Vincent Stroobant, Jacques Chapiro (in collaboration with Edus Warren, Fred Hutchinson Cancer Research Center, Seattle, USA)

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. In collaboration with Edus Warren (Seattle, USA) we recently found another peptide produced by splicing. This peptide, which is a human minor histocompatibility antigen corresponding to a polymorphism in gene SP110, is made of two peptide fragments that are non-contiguous in the parental protein. The two fragments are rearranged before splicing, so that they end up inverted in the spliced antigenic peptide. The proteasome appears responsible for this rearrangement/splicing, presumably by the same transpeptidation mechanism as above.

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-germline expression pattern, which is expressed in about 40% of melanomas and 30% of bladder carcinomas. Two peptides are presented by HLA-A2, and one by HLA-B57 (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. 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 (6).

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-Verhulst (CIML, Marseille))

Immunotherapy represents an attractive approach for the treatment of cancer, in particular melanoma. Preclinical studies of various strategies of immunotherapy rely on model systems where tumor cells grown *in vitro* are inoculated into syngeneic mice. However, this does not recapitulate the long-term host-tumor relationship that occurs in patients during tumor development. In order to have a model system more relevant to the human situation, we are 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 gene, which encodes two distinct tumor suppressor proteins p16^{INK4A} and p14^{ARF}. Genetic disruption of this gene 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 genera-

ted transgenic mice in which the deletion of the *Ink4a/Arf* gene 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* gene 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-2^d 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 Lauverys, Anne-Lise Maudoux (in collaboration with Frédéric Houssiau, 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

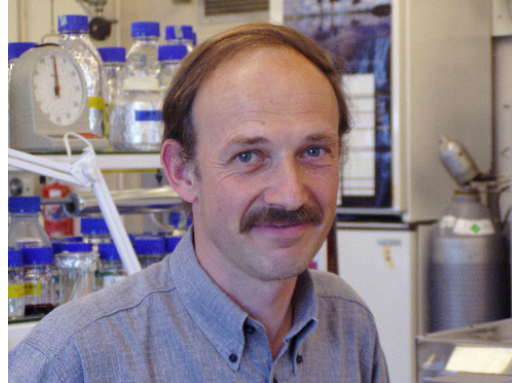
1. Morel S, Levy F, Burlet-Schiltz O, Brasseur F, Probst-Kepper M, Peitrequin AL, Monsarrat B, Van Velthoven R, Cerottini JC, Boon T, Gairin JE, Van den Eynde BJ. *Processing of some antigens by*

- 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. Vigneron N, Ooms A, Morel S, Ma W, Degiovanni G, Van den Eynde BJ. *A peptide derived from melanocytic protein gp100 and presented by HLA-B35 is recognized by autologous cytolytic T lymphocytes on melanoma cells.* **Tissue Antigens** 2005;65(2):156-62.
 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, Co-syns 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, Devuyt 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.

TUMOR GENETICS GROUP

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Human tumors express specific antigens arising from the activation of genes, such as MAGE, BAGE, GAGE and LAGE/NY-ESO1, that are normally expressed only in germ cells. As germ cells are not subject to scrutiny by the immune system, antigens encoded by these genes are strictly tumor-specific. The group of Etienne De Plaen and Charles De Smet is trying to identify new genes that are specifically expressed in tumors and germ cells. Screening procedures based on differential expression profiling allowed the isolation of several genes with cancer and germline specific expression (1). Most of these genes have their normal site of expression in spermatogonia, the pre-meiotic stage of sperm development, and are located on the X chromosome. Additionally, efforts are also devoted to determining the function of «cancer-germline» genes.

To analyze the functions of a MAGE protein, MAGE-A1, Etienne De Plaen and his group searched to identify binding partners of this protein. Using yeast two-hybrid screening, they found interaction between MAGE-A1 and transcriptional regulator SKIP (2). SKIP is an adaptor protein that connects DNA-binding proteins to proteins that activate or repress transcription. Their results suggest that by binding to SKIP and by recruiting histone deacetylase 1, protein MAGE-A1 present in the nucleus represses transcription. The group is now trying to identify the genes that are regulated by MAGE-A1 by using an inducible transfected MAGE-A1 gene and the microarray technology.

Charles De Smet and his group are studying the mechanisms leading to the activation of "cancer-germline" genes in tumors. It was previously shown by the group that these genes rely primarily on DNA methylation for their repression in normal somatic tissues, and that their activation in tumors is a consequence of the overall genome demethylation process

that often accompanies tumorigenesis. The group is now focusing on the mechanisms of demethylation of these genes in tumors. Stable activation of cancer-germline genes in tumors does not require a permanent demethylating activity, but depends on the presence of specific transcription factors that maintain the promoter region unmethylated (3). Antisense-mediated knock-down experiments indicated that DNMT1 is the primary DNA methyltransferase to maintain methylation of cancer-germline genes. Transient down-regulation of DNMT1 induced stable activation of cancer-germline genes, supporting the view that hypomethylation of these genes in tumors results from a historical event of demethylation.

Finally, the group investigates the gene expression profile of tumor samples and tumor cell lines obtained from melanoma patients who received experimental cancer vaccines. Using microarray and quantitative RT-PCR, the group is trying to identify genes involved in the resistance of tumors to their rejection by T lymphocytes.

Selected publications

1. Vialle-Castellano, A., Laduron, S., De Plaen, E., Jost, E., Dupont, S., Ameye, G., Michaux, L., Coulie, P., Olive, D., Boon, T. et al. *A gene expressed exclusively in acute B lymphoblastic leukemias*. **Genomics** 2004;83:85-94.
2. Laduron, S., Deplus, R., Zhou, S., Kholmanskikh, O., Godelaine, D., De Smet, C., Hayward, S.D., Fuks, F., Boon, T. and De Plaen, E. *MAGE-A1 interacts with adaptor SKIP and the deacetylase HDAC1 to repress transcription*. **Nucleic Acids Res**, 32, 4340-4350. **Print** 2004.
3. De Smet, C., Lorient, A. and 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

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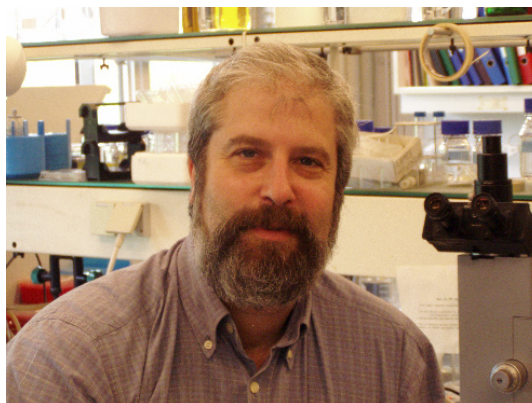
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Sabrina OTTAVIANI, Technician

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. Efforts are currently devoted to set up assays that accurately monitor CD4⁺ T cell responses to cancer vaccines. For patients vaccinated with a peptide, we recently validated the use of the first HLA-DP4 tetramer, which was folded with a MAGE-3 peptide. For patients vaccinated with a protein, we have validated a quantitative approach to isolate anti-vaccine T cells directed at all possible HLA-peptide combinations that could be targeted by the response. The group is also involved in the study of functional defects of T cells. We have observed that human CTL clones lose their specific cytolytic activity and cytokine production under certain stimulation conditions. These inactive CTL simultaneously lose their labeling by an HLA-peptide tetramer, even though the amount of TCR-CD3 at their surface is not reduced. These results suggest the existence of a new type of functional defect of CTL.

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

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 can-

cer patients (1-2). A few HLA class I-restricted antigenic peptides were identified by this “direct approach”. A large set of additional cancer-germline genes have now been identified by purely genetic approaches. As a result, a vast number of sequences are known that can code for tumor-specific shared antigens, but most of the encoded antigenic peptides have not been identified yet. The identification of a large number of antigenic peptides presented by HLA class I and class II molecules is likely to be important for the future of clinical trials with defined antigenic peptides. A large set of peptide/HLA combinations will alleviate HLA restriction and widen the set of eli-

gible patients. It will also facilitate the design of concurrent immunizations against several antigens. Such immunizations could increase the primary anti-tumor efficacy of the vaccine.

We have used approaches that we have loosely named «reverse immunology» (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

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

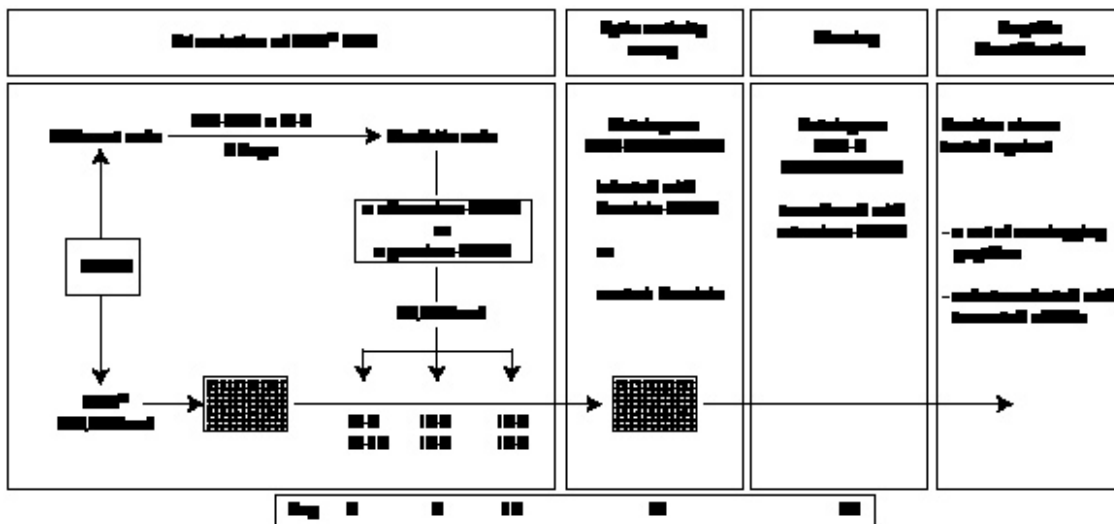


Figure 1. Overview of the procedure to obtain anti-MAGE CD8⁺ CTL clones by stimulation with dendritic cells infected with viral vectors carrying a MAGE coding sequence

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). After three weekly stimulations, the responder cells were tested for lysis on autologous EBV-B cells infected with vaccinia-MAGE. Positive microcultures were cloned. To identify the antigenic peptide, the resulting CTL clone

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

In a second approach, a large number of

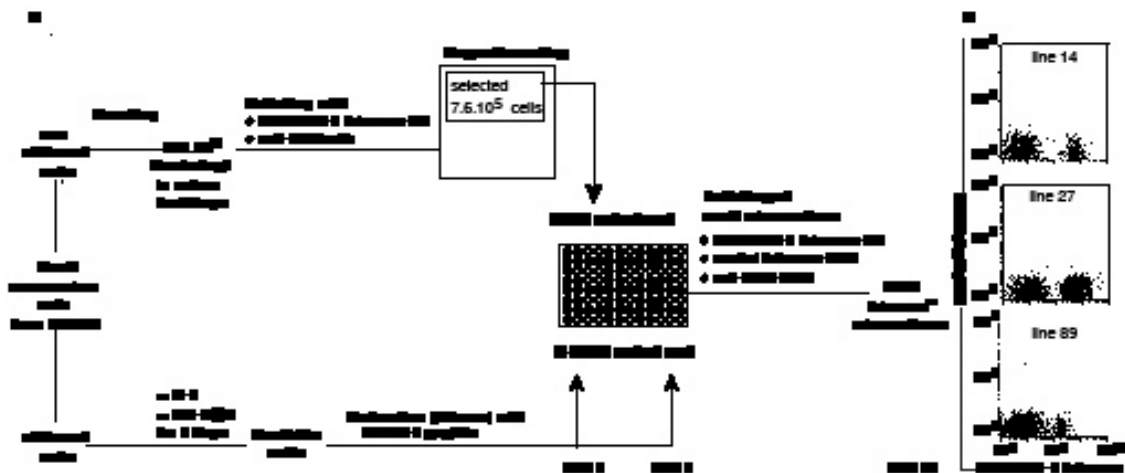


Figure 2. Overview of the procedure using HLA-peptide fluorescent multimers to isolate rare anti-MAGE-1 CD8⁺ CTL clones.

T cells are incubated with HLA/peptide multimers conjugated to phycoerythrin (PE), and with anti-PE antibodies coupled to magnetic beads. The multimer-positive cells are first enriched by magnetic sorting. The selected cells are distributed in microwells and stimulated with peptide-pulsed autologous mature dendritic cells on days 0 and 7. The microcultures are screened on day 19 for the presence of cells specifically labeled with multimers (Fig.2) (5).

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 (6). After four weekly stimulations, the responder cells were tested for their ability to secrete IFN- γ upon stimulation with the antigen, and the positive microcultures were cloned.

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

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

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 (7).

Detection of CD4 T cell response in vaccinated cancer 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. Although the production of soluble MHC class I multimers has rapidly expanded since their first use in 1996, the development of MHC class II complexes has proved to be more difficult probably due to the intrinsic structural instability of soluble class II molecules. We have developed a very sensitive approach using fluorescent HLA class II/peptide multimers to detect antigen specific CD4 T cells in vaccinated cancer patients. We have produced HLA-DP4 multimers loaded with the MAGE-3₂₄₃₋₂₅₈ peptide and used them to stain *ex vivo* peripheral blood lymphocytes from melanoma patients injected with dendritic cells pulsed with several HLA class I and class II tumor anti-

genic peptides, including the MAGE-3₂₄₃₋₂₅₈ peptide (8). The multimer positive CD4 T cells were sorted and amplified in clonal conditions. Specificity of the clones was assessed by their ability to secrete IFN- γ upon contact with the MAGE-3 antigen. Using this approach, low frequencies of about 1×10^{-6} anti-MAGE-3.DP4 CD4 cells among CD4 cells could be detected. A detailed analysis of one patient showed an anti-MAGE 3.DP4 CD4 T cell amplification of at least 3,000-fold upon immunization. T cell receptor analysis of the clones from this patient demonstrated a polyclonal response against the MAGE-3 peptide. One advantage of the multimer staining approach is that antigen-specific CD4 T cells are detected independently of their effector functions. We have started to analyze several patients injected with different vaccines containing the MAGE-3.DP4 peptide: peptide with or without adjuvant, dendritic cells pulsed with peptides. We intend to correlate the type of vaccine and the functional phenotype of the anti-vaccine T cells isolated from the vaccinated cancer patients.

Patients injected with a protein

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

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

ning of these cells, and evaluation of the number of T cell clones that secrete IFN- γ upon stimulation with the antigen (9). An important criterion is that T cell clones must recognize not only stimulator cells loaded with the protein but also stimulator cells transduced with the MAGE-3 gene, so as to exclude the T cells that recognize contaminants generated by the protein production system. We analyzed the frequencies of anti-vaccine CD4 T cells in five metastatic melanoma patients, who have been injected with a MAGE-3 protein without adjuvant and showed evidence of tumor regression. Anti-MAGE-3 CD4 T cells were detected in one out of the five patients. 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 of one anti-MAGE-3 CD4 T cell clonotype was confirmed by PCR analysis on blood lymphocytes. The 13 anti-MAGE-3 clones, which corresponded to five different TCR clonotypes, recognized the same peptide presented by HLA-DR1.

This monitoring procedure has the potential to detect the complete set of T lymphocytes that recognize the vaccine-derived peptides on various HLA molecules. Frequencies as low as 1 per million specific CD4 T cells can be measured with frozen samples corresponding to less than 50 ml of blood, whereas other techniques, such as *ex vivo* ELISPOT assays, reach their limit of detection at 1/25,000 of the CD4 T cells. The availability of T cell clones ensures a strict assessment of the specificity of the T cells, including their ability to recognize both cells loaded with the protein and cells transduced with the protein coding sequence. It also makes it possible to define the T cell receptor sequence of the anti-vaccine T cells and, therefore to analyze the TCR diversity and establish the presence of repeated clonotypes, an essential criterion to assess the occurrence of a response when the frequency is low (5). In addition, a direct quantitative evaluation of the frequency of certain clonotypes can be obtained by PCR performed on RNA extracted

from blood lymphocytes and tumor samples.

A limitation of our experiments is that only those anti-MAGE-3 CD4 T cells that produce IFN- γ can be detected. However, our approach could easily be extended by using a “cytokine secretion assay” for the detection of cells producing another cytokine, such as IL-10. It could also be extended to the detection of specific CD8 T cells, provided stimulator cells are used that are able to activate both CD8 and CD4 T cells. Various autologous cells, either electroporated with RNA constructs or infected with recombinant viral vectors, are currently tested.

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. The labeling of these inactive CTL by an HLA-peptide tetramer was strongly reduced, even though the amount of T cell receptor (TCR) at their surface is similar (10).

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

Our results indicate that tetramers may fail to reveal some CD8 and CD4 specific T

cells bearing the relevant TCR, even when such functionally arrested T cells retain the potential to participate in immune responses. They also suggest the existence of a new type of functional defect of T cells.

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. 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;101:14631-8.
6. 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.
7. 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** 2005;19:279-85.
8. Zhang Y, Renkvist N, Sun Z, Schuler-Thurner B, Glaichenhaus N, Schuler G, Boon T, van der Bruggen P, Colau D. *A polyclonal anti-vaccine CD4 T cell response detected with HLA-DP4 multimers in a melanoma patient vaccinated with MAGE-3.DP4-peptide-pulsed dendritic cells.* **Eur J Immunol** 2005;35:1066-75.
9. Zhang Y, Sun Z, Nicolay H, Meyer RG, Renkvist N, Stroobant V, Corthals J, Carrasco J, Eggermont AM, Marchand M, Thielemans K, Wolfel T, Boon T, van der Bruggen P. *Monitoring of anti-vaccine CD4 T cell frequencies in melanoma patients vaccinated with a MAGE-3 protein.* **J Immunol** 2005;174:2404-11.
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

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Tumor cells carry antigens such as MAGE antigens that are absent from normal tissues, and that can be targeted by cytolytic T lymphocytes (CTL) (1). 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 time points 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 380 patients have been included in these multicenter trials.

Clinical trials with the MAGE-3.A1 peptide

In a pilot study, the synthetic Mage-3.A1 peptide was administered to 45 HLA-A1 patients with MAGE-3 expressing melanoma, by subcutaneous (s.c.) and intradermal (i.d.) in-

jections 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 at 4-week intervals to 5 patients, without any evidence of tumor regression. A combination of the MAGE-3.A1 and MAGE-1.A1 peptides was administered s.c. and i.d. every 3 weeks to 11 patients. Two of them experienced tumor regression (1 CR, 1 MxR). MAGE-3.A1 was injected s.c. and i.d. every 10-11 days instead of

every 3-4 weeks to analyze whether vaccination at higher frequency could improve the clinical response rate. Among 21 patients treated, three had regressions of tumor lesions (3 MxR). The same peptide was associated with the HLA class II-restricted MAGE-3.DP4 peptide, in order to induce both CD8 and CD4 T cell responses, hoping for an improved immunological and antitumoral effectiveness. None of the 7 patients evaluable after 9 i.d. and s.c. vaccinations given every 10-11 days had tumor regression.

Initial assessment of the CTL responses induced by vaccination with the MAGE-3.A1 peptide was hampered by the lack of sensitivity of available CTL monitoring techniques. More recently, a new approach with improved sensitivity, involving lymphocyte-peptide culture and the use of HLA/peptide tetramers, was used to document a significant increase in CTLp frequency in a patient who showed tumor regression following vaccination with this peptide at high frequency. This method also showed that the CTL response was monoclonal. It was extended to 19 other patients who received this peptide without adjuvant. None had a detectable CTL response, indicating that this vaccine is weakly immunogenic (4).

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

In an ongoing study, the MAGE-3.A1 peptide is mixed with an immunostimulatory CpG-containing oligonucleotide to try to increase its immunogenicity (Study LUD

02-001). This new and promising adjuvant activates antigen presenting cells after binding to Toll-like receptor 9, and is thought to enhance CTL responses. Likewise, CpG 7909 is mixed with 8 HLA-A2 peptides in an other ongoing study (LUD 03-007). Results of both studies will be compared in terms of therapeutic efficacy and immune response.

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 partial response and 4 mixed responses. Thus this vaccine does not seem to induce more regressions than the MAGE-3.A1 peptide, but it does not require that the patient carries a specific HLA type (6). We 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 10 to 20 %) show regression of metastatic lesions following immunization, whatever the MAGE vaccine used. About 5 to 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., Beauduin 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.
6. Kruit W., van Ojik H., Brichard V., Escudier B., Dorval T., Dréno B., Patel P., van Baren N., Avril M-F, Piperno S., Khammari A., Stas M., Ritter G., Lethé

B., Godelaine D., Brasseur F., Zhang Y., van der Bruggen P., Boon T., Eggermont A. and Marchand M. *Phase I/II study of subcutaneous and intradermal immunization with a recombinant MAGE-3 protein in patients with detectable non-visceral metastatic melanoma.* **Int J Cancer**, in press, 2005.

ANALYSIS OF T CELL RESPONSES OF VACCINATED CANCER PATIENTS *

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*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 res-

ponses 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 tetrame-

ric 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 3×10^7 PBMC of normal donor and they were highly specific for the given TCR insofar as no product was amplified with cDNA prepared from 3×10^7 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 10^7 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 3.6×10^{-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, which remained stable during the peptide vaccinations, rose to 1.1×10^{-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 (9). 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 $\sim 3.5 \times 10^{-5}$ 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, ~ 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 $\sim 1/32,500$ 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 (10).

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** 2000;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 D, Carrasco J, Lucas S, Karanikas V, Schuler-Thurner B, Coulie PG, Schuler G., Boon T, Van Pel A. *Polyclonal cytolytic T lymphocyte responses observed in melanoma patients vaccinated with dendritic cells pulsed with a MAGE-3.A1 peptide.* **J Immunol** 2003;171:4893-97.

9. Germeau C, Ma W, Schiavetti F, Lurquin C, Henry E, Vigneron N, Brasseur F, Lethé B, De Plaen E, Velu T, Boon T, Coulie PG. *High frequency of antitumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens.* **J Exp Med** 2005;201:241-248.
10. Lurquin C, Lethé B, De Plaen E, Corbière V, Théate I, Van Baren N, Coulie PG, Boon T. *Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen.* **J Exp Med** 2005;201:249-257.

CYTOKINES IN IMMUNITY AND INFLAMMATION

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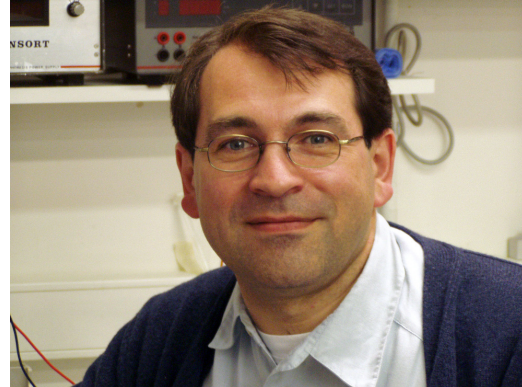
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The cytokine group studies the biology of Interleukin-9 (IL-9) and IL-22, two cytokines discovered at the Branch. IL-9 is a TH2 cytokine that plays a role in immune responses against intestinal parasites and asthma. IL-22, originally identified as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

Interleukin 9

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 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. 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.

IL-9 receptor and signal transduction

Jean-Christophe Renault, 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

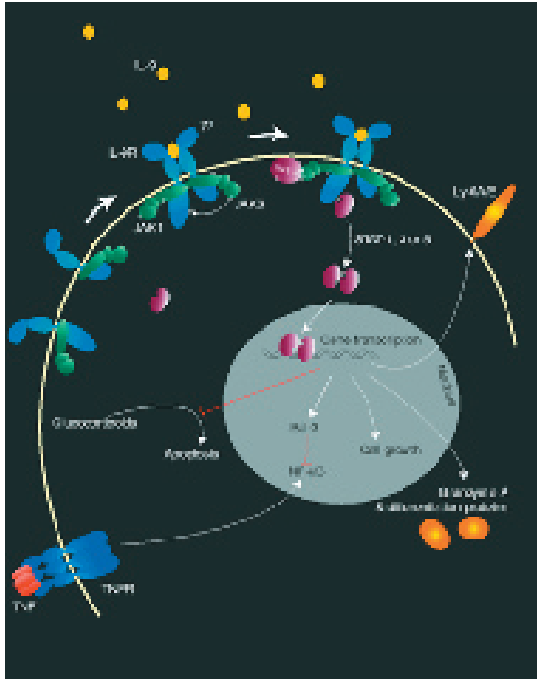


Figure 1.

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

Jean-Christophe Renauld, Jacques Van Snick, Jamila Louabed

Incidentally, our studies of this particular model of the regulation of cell death by cytokines, led us to purify another protein called I-309, originally described as a human chemotactic factor, and that turned out to exert a significant anti-apoptotic activity for thymic lymphomas (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 Renauld, Jacques Van Snick, Laure Dumoutier, Jamila Louabed, Laurent Knoops, 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-κB

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

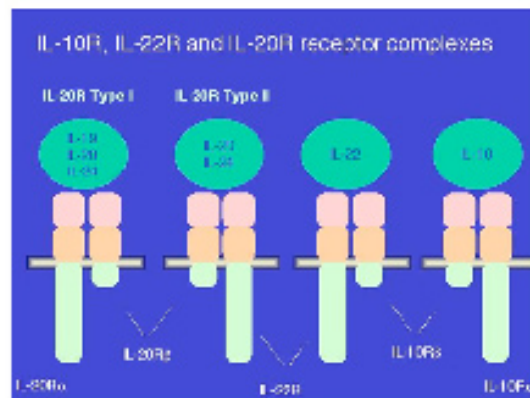


Figure 2.

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.

Selected publications

1. Renauld 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, Renauld 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, Renauld JC, Van Snick J. Anti-IL-9 vaccination prevents worm expulsion and blood eosinophilia in *Trichuris muris*-infected mice. **Proc Natl Acad Sci U S A** 2000;97:767-72.
 4. Renauld JC, Druez 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, Renauld JC. Distinct roles for *STAT1*, *STAT3*, and *STAT5* in differentiation gene induction and apoptosis inhibition by interleukin-9. **J Biol Chem** 1999;274:25855-61.
 6. Van Snick J, Houssiau F, Proost P, Van Damme J, Renauld 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, Renauld JC. Interleukin-9 regulates NF- κ B 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, Renauld 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, Renauld JC. Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor. **Proc Natl Acad Sci U S A** 2000;97:10144-9.
 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.

SIGNAL TRANSDUCTION GROUP: STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS

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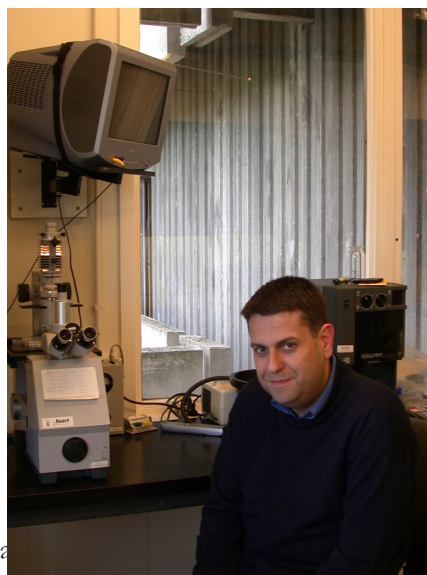
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Cytokines and their receptors are critical for the formation of mature blood cells and for the function of the immune system. Signaling by cytokine receptors is triggered by ligand-induced changes in receptor dimerization/oligomerization, which induces the activation of cytosolic Janus tyrosine kinases (JAK). 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. The laboratory is actively investigating the mechanisms by which a JAK2 point mutant induces Polycythemia Vera and myeloproliferative diseases in humans.

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

Judith Staerk

Janus kinases possess two kinase domains,

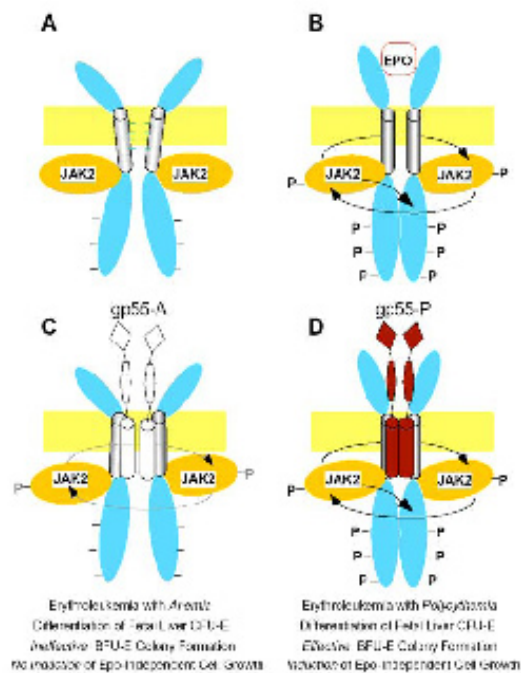
one active and the other, denoted as the pseudokinase domain, inactive. JAK2, one of the four known JAKs (JAK1, JAK2, JAK3 and Tyk2) is crucial for signaling by several cytokine receptors, such as the erythropoietin receptor (EpoR), the thrombopoietin receptor (TpoR), the interleukin 3 receptor and the growth hormone receptor. JAKs are appended to the cytoplasmic juxtamembrane domains of recep-

tors and are switched-on upon ligand binding to the receptors' extracellular domains (1). In addition to their role in signaling, JAKs appear to play chaperone roles for promoting traffic of cytokine receptors to the cell-surface (2).

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

ted), the very process that is defective in PV.

In collaboration with Prof. William Vainchenker and his INSERM unit at the Institut Gustave Roussy in Paris, we have been involved in the discovery of a novel point mutant of JAK2, where a unique V617F mutation in the pseudokinase domain renders the enzyme constitutively active (3). The mutation alters a physiologic inhibition exerted by the pseudokinase domain on the kinase domain (3). This mutant is found in >80% of PV patients and in 50% of Essential Thrombocythemia and Idiopathic Myelofibrosis (IMF), two other diseases that belong to the myeloproliferative syndromes. Current projects include the determination of downstream signaling proteins activated by the mutant JAK2, and the characterization of cytokine receptor signaling in the



(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).

presence of the mutant and wild type JAK2. These results suggest that point mutations in JAK proteins may be involved in different forms of cancers and autoimmune diseases.

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

Katharina Kubatzky, Judith Staerk, Yohan Royer, Virginie Moucadel

The determination of the interface of the active erythropoietin receptor (EpoR) dimer has been a priority during the last year (4). 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 (5). 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 (1).

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 (4). 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 (4). 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.

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 (6). 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 (6). The same residues were found to be in the interface of the active coiled-coil-EpoR fusion proteins (4). 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. 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 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 activate 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 in the cell is totally different from the one of the IL9R or the EpoR. The γ_C is localized in big intracellular patches (colocalizing with LAMP1), whereas the IL9R and the EpoR exhibit a normal diffuse distribution, as we have shown in collaboration with Pierre Courtoy.

We have observed that, in hematopoietic cells, over-expressing 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 (7). 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 Moucade, 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). 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 transformed cells many of the

transient signaling events induced by cytokines are detectable permanently, i.e. ligand-independent phosphorylation of JAK and STAT proteins or high levels of nuclear activated STATs especially STAT5 and STAT3. A similar picture has been noted in patient-derived leukemia cells. The critical questions we would like to answer concern the mechanisms by which the JAK-STAT remain permanently activated in transformed cells and which genes are regulated by constitutively active STAT proteins in leukemic cells. Using chromatin immunoprecipitation and sequencing of native promoters bound by STAT5 we noted that in transformed cells STAT5 can also bind to low affinity N4 sites not only to N3 sites, which is characteristic of ligand-activated STAT5 (9). 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 (9).

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 (10) that allow wide expression of cDNAs at physiologic levels.

Selected publications

1. 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
2. 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.
3. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, Garcon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villeval JL, Constantinescu SN, Casadevall N, Vainchenker W. *A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera.* **Nature** 2005;434:1144-1148.
 4. 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.
 5. 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 USA** 2001;98:4379-84.
 6. Kubatzky, KF, Liu, W., Goldgraben, K., Simmerling, C, Smith, SO, Constantinescu, SN. *Structural requirements of the extracellular to transmembrane domain junction for erythropoietin receptor function.* **J Biol Chem** 2005;280:14844-14854.
 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. Moucadel, V., Constantinescu, SN. *Differential STAT5 signaling by ligand-dependent and constitutively active cytokine receptors.* **J Biol Chem** 2005;280:13364-73.
 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.* **DNAandCellBiol**2004;23:355-65.