Research at

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(ICP)

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(LICR)

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Ludwig Institute for Cancer Research (LICR),

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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.

Thierry Boon

Faithful to the organizing principles laid down by Mr Ludwig, the Institute conducts its research through ten 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.

Branch staffs vary in size from 30 to over 70, 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 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.

I. TUMOR IMMUNOLOGY AND IMMUNOTHERAPY

OVERVIEW

T lymphocytes are capable of destroying cells that harbour viruses because they recognize viral antigens on their surface. They do so with great efficacy and specificity and, together with the anti-viral antibody response, they usually provide full recovery from viral diseases. For cancer the situation is very different : clearly the immune system does not eliminate most overt cancers. But cancer cells have long be known to differ from normal cells in many respects and it has been a long-standing hope of immunologists that cancer cells express abnormal antigens which could be recognized by the immune system. The existence of tumor-specific antigens was considered to have two major implications : the existence of cancer immunosurveillance and the feasibility of cancer immunotherapy. The hypothesis of cancer immunosurveillance implies that most cancers are eliminated at a very early stage by an immune rejection process. One could add that the immune system, while failing to eliminate some tumors, may nevertheless slow them down at all stages of their progression. The existence of immune surveillance has long been controversial but it is supported by recent evidence obtained with mouse tumors. Cancer immunotherapy is based on the notion that it is possible to artificially improve the response to tumor antigens to make it reach its full potential. Unlike responses directed against viral antigens, anti-tumoral responses may not have been perfected throughout evolution, because escaping cancer probably conferred little or no selective advantage.

Our interest in tumor immunology started with a fortuitous observation made with a mouse tumor which was strictly non-immunogenic. Mice from which this tumor was removed by surgery did not show any protection against a challenge with the same tumor cells. We observed that by treating the tumor cells in vitro with a mutagen we obtained tumor cell mutants that were rejected in the mice by a T lymphocyte mediated process (1). Remarkably the mice that had rejected these "tum-" mutants showed a degree of protection against a challenge with the original non-immunogenic tumor cells (2). The pattern of protection indicated that the tum- mutants owned their phenotype to the acquisition of new strong tumor rejection antigens. The response against these antigens evidently created conditions that facilitated a response against weaker antigens present on the original tumor, a phenomenon which has recently been named "epitope-spreading". These observations were found to apply to all mouse tumors, including spontaneous tumors (3). This led to two conclusions. First, all mouse tumors bear tumor-specific antigens recognized by T cells even though many of them are non-immunogenic.

Second, it is possible to create conditions that favor the T lymphocyte responses against the tumor antigens.

On the basis of these findings we launched an effort to identify the antigens recognized on mouse tumors by T cells. We focused our effort on mouse tumor P815. A first step was to obtain in vitro cytolytic T lymphocytes (CTL) that specifically lysed the P815 cells (4, 5). Then, with considerable help from Jean-Charles Cerrotini and other members of the Lausanne branch, we obtained stable CTL clones directed against tumor antigens of P815. Antigen-loss mutants of P815 were obtained in vitro by selection for resistance to lysis by a CTL clone (6). In addition, we observed that antigen-loss is a mechanism which is used by P815 tumors to escape T cell responses in vivo. The next step was the production of genomic libraries from antigen-bearing cells and the transfection of the DNA into antigen-loss variants. Antigenic transfectants could be detected on the basis of their ability to stimulate the proliferation of the relevant CTL clone and the genes coding for the antigens could be retrieved from these transfectants by using appropriate cosmid technology (7).

About at that time, Alan Townsend showed that influenza virus antigens derived from proteins that remained inside the cells could be detected by T cells. These antigens are recognized by T cells as small peptides presented by class I major histocompatibility complex (MHC) molecules. Our contribution was to show that this also applied to the proteins encoded by the genome of the cell (7, 8). Thus there is a permanent T cell based immune surveillance of the cellular genome and genetic abnormalities can result in the presentation of new antigens leading to elimination by T cells. Our results also demonstrated that there are two major genetic processes that produce tumor-specific antigens. The first is the acquisition of mutations by the cancer cell, which generate peptides which, because of an amino-acid change, either become capable of binding to MHC molecules or contain a new epitope (8). The second is the expression by the tumor of a gene which is not expressed in the normal cells of the adult (9)(Fig. 1).

Around 1985 we began to examine whether the results obtained in mice could be extended to man. We focused our efforts on melanoma because, contrary to most tumors , samples of metastatic melanoma can be converted into stable cell lines fairly frequently. Stimulation of T lymphocytes with autologous melanoma cells produced cytolytic T cells that appeared to lyse the tumor cells specifically. Stable CTL clones were obtained and immunoselection of antigen-loss variants indicated that, like mouse tumors, human tumors express not one but several antigens that are recognized by autologous CTL (10, 11). A cosmid genomic library derived from a tumor cell was then transfected into an antigen-loss variant and this led to the identification of the first gene coding for a human tumor-specific antigen recognized by T cells (12). This previously unknown gene was named *Mage* and it was soon found to be expressed in many melanomas and not in normal cells.

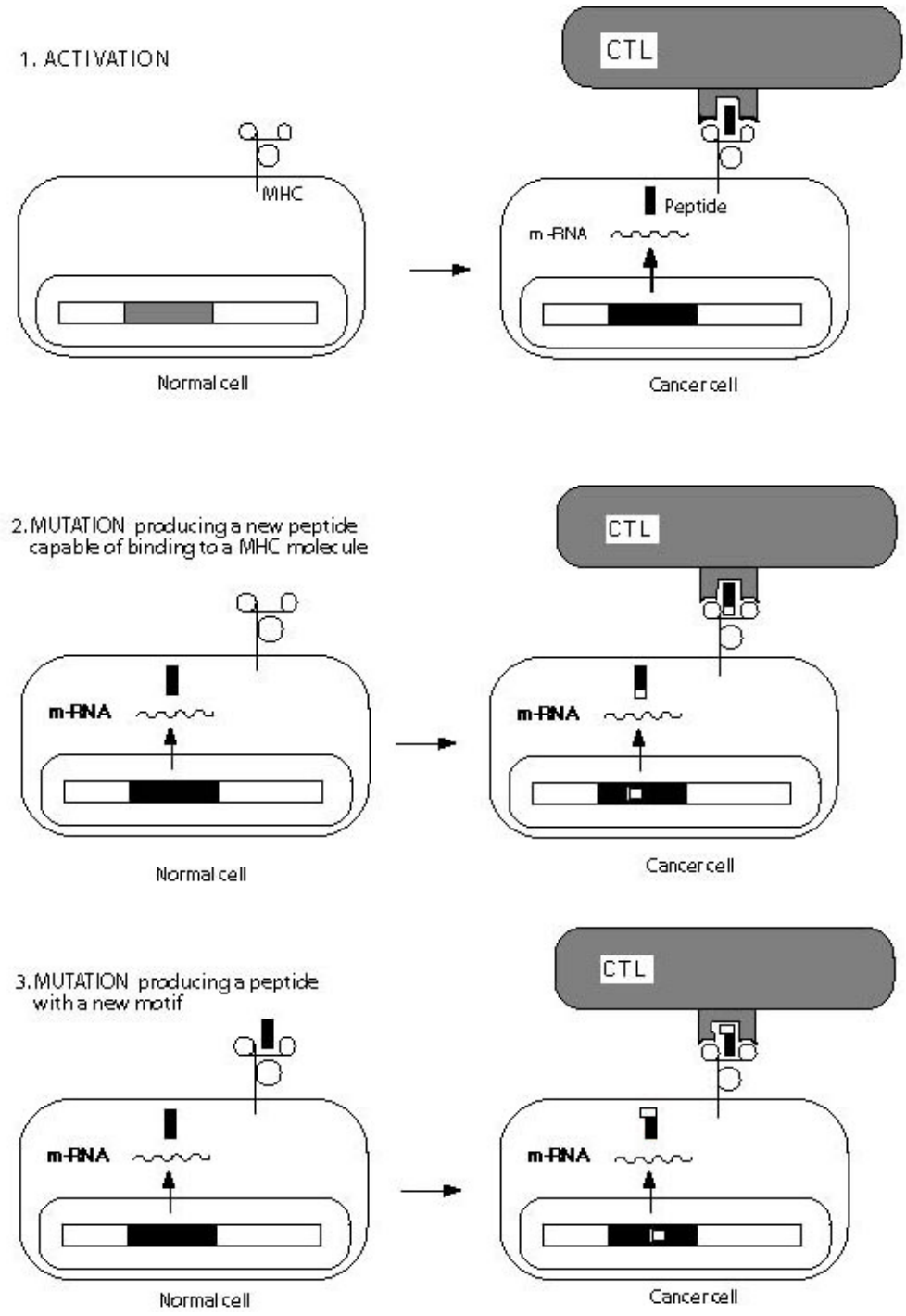

Fig.1

Gene *Mage* belongs to the *Mage-A* gene cluster, which comprises 12 genes (13). It is located on the X chromosome. The homologous families *Mage-B* and *C* are located on the same chromosome. All these genes have the same expression pattern. They are expressed in many tumors of various histological types and not in normal cells with one exception, male germline cells (13). It is therefore

appropriate to refer to these genes by the term cancer-germline genes. It is important to note that the germline cells do not express HLA molecules and therefore cannot present antigens to T cells. The antigens encoded by *Mage* genes appear therefore to be strictly tumor-specific as far as T cell responses are concerned, contrary to what is suggested by the term cancer-testis antigen which is often used to refer to these antigens. The activation of *Mage* genes in cancer and germline cells is not due to the presence of specific transcription factors. It is caused by the demethylation of the promoter in these cells (14). In cancer cells this appears to be a consequence of a global demethylation, which is observed in many cancer cells.

Using similar approaches we identified other families of cancer-germline genes, such as the *Bage* and *Gage* families (15, 16). cDNA enrichment approaches capitalizing on the cancer-specific pattern of expression was used to find additional cancer-germline gene families such as *Lage*, *Sage* and *Hage*. Another approach, Serex, based on antibody responses of cancer patients was initiated by Michael Pfreundschuh. It enabled him to identify the SSX and SCP-1 families. Using this approach, members of the New York Branch identified a second member of the Lage family, named NY-ESO-1, shortly after our discovery of *Lage-1* (17). It is likely that by now most of the cancer-germline genes have been identified.

Antigens encoded by cancer-germline genes ought to be very suitable as therapeutic vaccines for cancer-patients as they are strictly tumor-specific and present on a large proportion of tumors. But genes that are strongly overexpressed in cancer cells relative to normal cells could also be a source of acceptable antigens for cancer immunotherapy. An example is gene *Prame*, which is expressed at a high level in a very large proportion of melanoma and other tumors (18).

Gene mutation was also found to be a major source of human tumor-specific antigens. Interestingly, several mutations that were found to be antigenic also appear to play a role in oncogenesis. One interesting example is a mutation in cyclin-dependent kinase-4 ,which prevents the binding of this protein to P16 (19). As a result CDK4 permanently phosphorylates Rb causing excessive cell cycling. Another example is a mutation in caspase 8, which reduces the sensitivity of cells to pro-apoptotic factors (20). Unfortunately, when a tumor-specific antigen results from a mutation it is expressed on an extremely small proportion of tumors. This precludes the use of this class of tumor-specific antigens as cancer vaccines.

Finally, we observed that CTL of melanoma patients can respond to antigens encoded by melanocytic differentiation genes, such as tyrosinase and Melan-A (also referred to as Mart-1)(21, 22). This is surprising as one would have expected natural tolerance to put a thight block on responses against such self antigens. Other groups proceeded to vaccinate melanoma patients with these antigens and observed tumor regressions in some patients.

The peptides that are presented by MHC molecules at the cell surface result from the degradation of intracellular proteins by the proteasome in the cytosol. The peptides are then transported into the endoplasmic reticulum where they combine with newly synthesized MHC molecules on their way to

the cell surface. The proteasome plays a central role in this pathway, known as the class I antigen processing pathway. Some cells, such as dendritic cells and cells exposed to interferon-gamma, express a different type of proteasome named immunoproteasome, whose catalytic activity is slightly different from that of the standard proteasome. We have shown that a number of human tumor antigens are not produced with the same efficiency by the two proteasome types, some of them are produced only by the standard proteasome whereas others are produced exclusively by the immunoproteasome (23, 24). This means that the peptide repertoire displayed at the cell surface depends not only on the proteins expressed by the cells but also on the type of proteasome they harbor. This parameter should be considered in the context of immunotherapy.

For the vaccination of cancer-patients with a tumor antigen recognized by T lymphocytes, several forms of the antigen can be used. These include antigenic peptides, whole protein, recombinant defective viruses carrying a sequence coding for the antigen, or naked DNA. In addition, dendritic cells derived from blood cells of the patient can be reinfused into the patient after being pulsed with antigenic peptides or protein or after being transfected with encoding cDNA or RNA. The simplest vaccines are the antigenic nona- or decapeptides. However each peptide binds and can be presented only by the protein encoded by one or a few HLA alleles. It is therefore necessary to identify many antigenic peptides to be able to provide an adequate vaccine to the majority of cancer patients. The identification of these antigenic peptides on the basis of the gene sequence involves the obtention of a T cell clone that recognizes this antigen. We have devised approaches to obtain such CD8 T cell clones by stimulating T cells with dendritic cells infected with a recombinant virus carrying the encoding gene (25). CD4 T cell clones have been obtained by stimulation with dendritic cells pulsed with the relevant protein. As a result we have now identified a large number of antigenic peptides encoded by genes *Mage-1* and *Mage-3* (Fig.2).

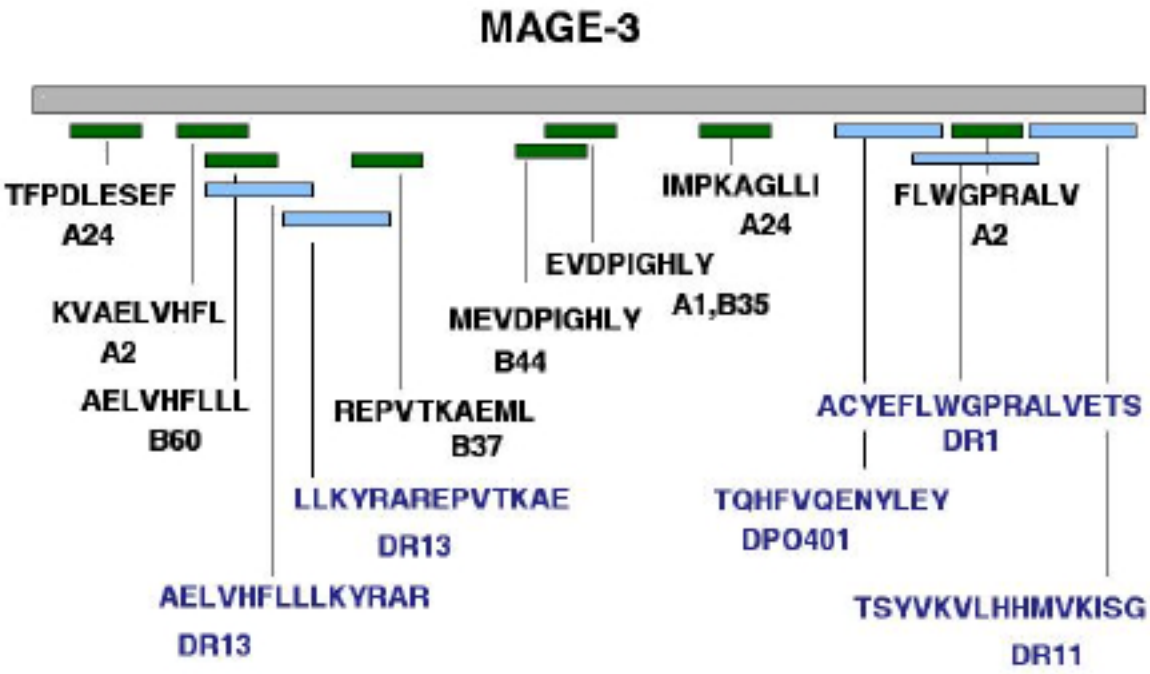

Fig.2

Our first clinical trial involved the vaccination of metastatic melanoma patients with an antigenic peptide which is encoded by *Mage-3* and presented by HLA-A1. Seven patients out of 26 showed evidence of tumor regression (26, 27)(Fig.3). No toxicity was observed. We then examined in a series of small trials whether changes in the vaccine modalities would significantly improve the outcome. We tried more frequent injections of the Mage-3.A1 peptide, combination with an adjuvant or combination with another Mage peptide binding to either a class I or class II HLA molecule. No improvement was observed. Similar results were observed after vaccination with the Mage-3 protein or with an ALVAC recombinant virus coding for *Mage* sequences. In these trials we failed to detect CTL responses against the vaccine. Even though our approach lacked sensitivity, this indicated that the CTL responses were weak at best. To sum up the observations made on about 200 patients, we can say that some evidence of tumor regression is observed on about 20% of the patients with half of them, i.e. 10%, showing complete or partial clinical responses.

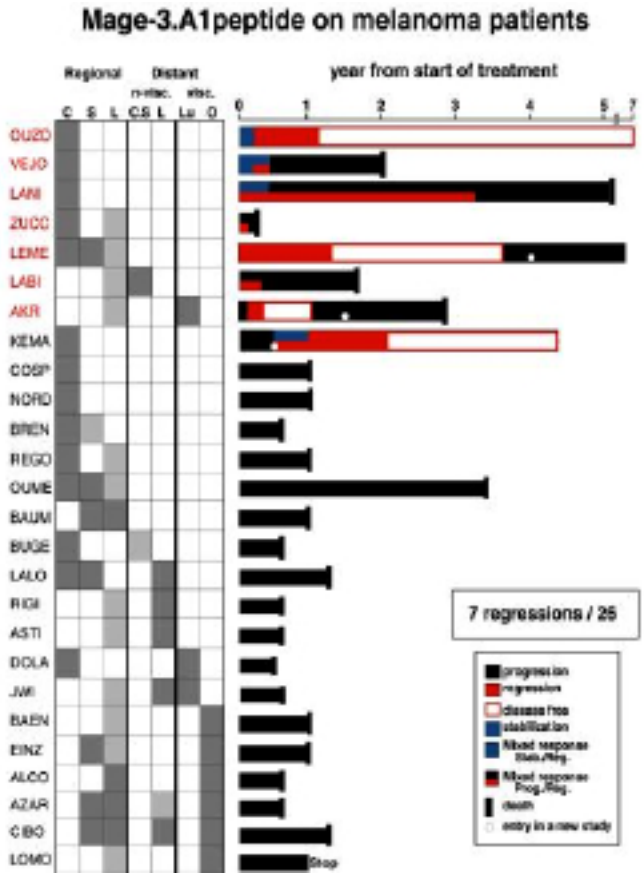

Fig.3

To explain why our vaccinations fail to exert any detectable effect on the tumors of 80% of the patients one can consider two, unfortunately non-exclusive, causes. First, the anti-vaccine CTL response might be inadequate. Second, the tumor might be resistant to immune attack. We set out to analyze the first possibility, considering that if the level of the CTL response to the vaccine was a limiting factor for clinical efficacy, we ought to observe a correlation between the observations of CTL responses ant those of tumor regressions. We therefore developed highly sensitive approaches for the detection of CTL responses combined with an analysis of the T cell receptor diversity of the responding CTL (28). These approaches are beginning to show a correlation between CTL responses and tumor regressions. If this trend is confirmed, it will be crucially important to understand why some patients make CTL responses to the vaccines whereas others do not. When patients vaccinated with antigenic peptides or vaccinia-like recombinant viruses produce a T cell response, it is monoclonal (28). In contrast, patients vaccinated with peptide-pulsed dendritic cells produce either polyclonal responses or no response. We will engage in a systematic study of patients recruited in trials involving dendritic cell vaccination to try to identify factors in their pre-vaccination state that influence their propensity to make a T cell response to the vaccine.

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TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

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

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

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

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

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

Jacques Chapiro, Benoit Guillaume, Sandra Morel, Fanny Piette

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

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

We previously reported that a class-I restricted antigenic peptide derived from an ubiquitous human protein was processed efficiently by the standard proteasome but not by the immunoproteasome. As a result, the relevant epitope is not presented efficiently by mature dendritic cells, which contain immunoproteasomes (1). This could explain how certain potentially autoreactive CTL can escape tolerance induction in the thymus and fail to be activated in the periphery. We have now extended those observations to other epitopes derived from self proteins, and to several antigenic peptides of interest for cancer immunotherapy, including HLA-A2-restricted epitopes derived from tyrosinase, Melan-A^{MART1} and gp100. These results were obtained from in vitro experiments where synthetic peptides of about 20 amino acids, encompassing the epitope, are digested with highly purified preparations of either standard proteasomes or immunoproteasomes. The presence of the antigenic peptide in the digests is tested with the relevant CTL after pulsing on target cells (Fig. 1). It is then confirmed by HPLC and mass spectrometry. Consistent with their poor processing by the immunoproteasome, those epitopes are not presented efficiently to CTL by cells containing immunoproteasomes, such as tumor cells treated with IFNγ for 7 days, or cells transfected with cDNAs encoding the three immunoproteasome subunits, ß1i (LMP2), ß2i (MECL1), and ß5i (LMP7).

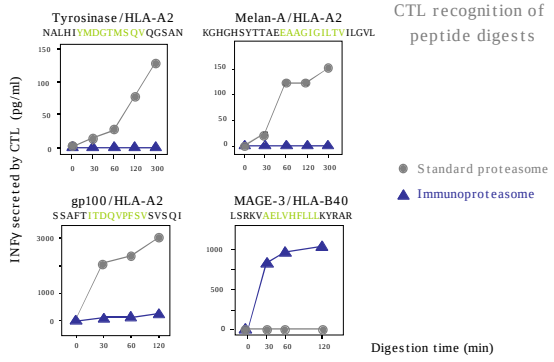

Fig.1. see text for explanations

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

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

Identification of new antigens recognized by autologous CTL on human melanoma

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

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

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

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

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

Catherine Uyttenhove, Dominique Donckers, Luc Pilotte

In order to optimize the vaccination modalities applied in cancer immunotherapy trials, we have continued our systematic assessment of the efficacy of various immunogens to induce CTL responses in mice against defined tumor antigens. In the course of those studies, we immunized mice with antigenic peptide P815E, which is presented by H-2Kd and recognized by tumor-specific CTL raised against P815 tumor cells. This peptide is encoded by the ubiquitously expressed gene MsrA and carries a mutated residue conferring tumor specificity. Unexpectedly, we observed a severe toxicity occurring in the early hours after the third injection, resulting in the death of most mice within 24 h (5). The toxic syndrome was reminiscent of TNF-induced shock, and the sera of ill mice contained high levels of TNF. Toxicity was prevented by injection of neutralizing anti-TNF Abs, confirming the involvement of TNF. Depletion of CD8+ T cells could also prevent toxicity, and ex vivo experiments confirmed that CD8+ lymphocytes were the major cellular source of TNF in immunized mice. Tetramer analysis of the lymphocytes of immunized mice indicated a massive expansion of P815E-specific T cells, up to >60% of circulating CD8+ lymphocytes. A similar toxicity was observed after massive expansion of specific CD8+ T cells following immunization with another P815 peptide, which is encoded by gene P1A and was injected in a form covalently linked to an immunostimulatory peptide derived from IL-1. We conclude that the toxicity is caused by specific CD8+ lymphocytes, which are extensively amplified by peptide immunization in a QS21-based adjuvant and produce toxic levels of TNF upon further stimulation with the peptide. Our results suggest that immunotherapy trials involving new peptides should be pursued with caution and should include a careful monitoring of the T cell response.

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

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

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

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

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

Develoment of a mouse melanoma model for immunotherapy

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

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

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

Six transgenic lines were generated, harboring a construct of respectively the tyrosinase promoter with two enhancer elements, a $CreER^{\Delta D}$ fusion gene flanked by loxP sites, a V12 mutated H-Ras gene, an internal ribosomal entry site and the P815A-antigen encoding gene, P1A. The expression and regulation of the $CreER^{\Delta D}$ fusion gene was analyzed by crossing these mice to a Rosa26 Cre reporter strain. In three transgenic lines specific blue staining could be observed in melanocytes after topical treatment of the ear by 4-hydroxytamoxifen. This implied that the $CreER^{\Delta D}$ fusion gene was expressed specifically in melanocytes and activated upon treatment with its ligand. In order to determine whether the transgene was still intact and functional after Cre-recombination, these transgenic lines were crossed with a CMV-Cre-deleter strain. In one transgenic line a break in the $CreER^{\Delta D}$ gene was observed after recombination. In the other two lines, H-ras and P1A were still detected after Cre-recombination implying that the transgene was arranged in such a way that the tyrosinase-promoter was now driving the expression of genes H-ras and P1A. These lines are now being crossed to a homozygous conditional *Ink4a/Arf* knock-out background in order to induce melanoma formation by applying 4-hydroxytamoxifen to the skin.

Antigen presentation by dendritic cells in Systemic Lupus Erythematosus

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

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

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GENES EXPRESSED IN CANCER AND GERMLINE CELLS

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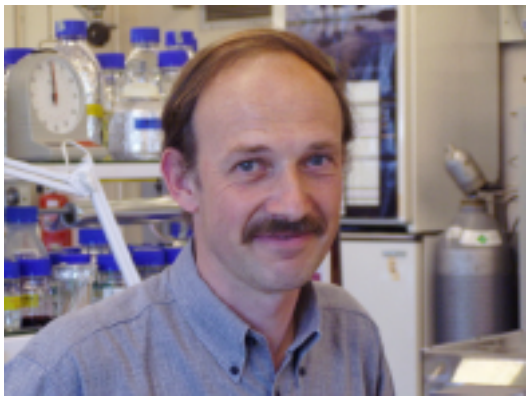

Human tumors express specific antigens arising from the activation of genes, such as MAGE genes, that are normally expressed only in germ cells. As germ cells are not subject to scrutiny by the immune system, antigens encoded by these genes are strictly tumor-specific. We are trying to identify new genes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

Screening procedures based on differential expression profiling allowed the isolation of several genes with cancer and germline specific expression (1 -5). 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 (Fig. 1). The spermatogonia-restricted expression of these genes is likely imposed by their chromosomal location, as the X chromosome becomes inactivated at the onset of meiosis in sperm cells.

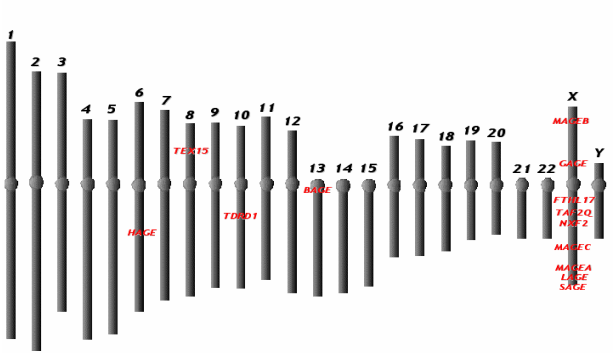

Fig. 1. Chromosome mapping of "cancer-germline" genes

MAGE-A1 belongs to a family of twelve genes located on the X chromosome in region q28 (6-7). To analyze the functions of MAGE-A1, we searched to identify protein partners of this protein. Using yeast two-hybrid screening, we found interaction between MAGE-A1 and transcriptional regulator SKIP. SKIP is an adaptor protein that connects DNA-binding proteins such as Smad3, the Vitamin D receptor, CBF1 or MyoD, to proteins that activate or repress transcription. A repression complex including histone deacetylases (HDAC) is known to bind to SKIP and CBF1. In the presence of the intracellular part of Notch1 (Notch1-IC), the repression complex is detached from SKIP by Notch1-IC and recruitment of an activation complex including histone acetyltransferases (HAT) is facilitated by SKIP. To examine whether MAGE-A1 could interfere with a signalling pathway involving SKIP, we expressed MAGE-A1 in mammalian cells in which Notch1-IC binds to SKIP fused to the Gal4 DNA-binding domain and activates a *CAT* reporter gene containing Gal4-binding sites near its promoter (Fig. 2). We found that MAGE-A1 inhibited the Notch-1/SKIP transcriptional activation. Deletion analysis indicate that binding to SKIP is required to observe MAGE-A1-mediated repression of Notch1-IC transactivation. Moreover, MAGE-A1 was found to actively repress transcription by binding and recruiting histone deacetylase 1. Our results suggest that by binding to SKIP and by recruiting histone deacetylases, MAGE-A1 protein present in the nucleus could repress genes implicated in development and spermatogenesis. We are now trying to identify the genes that are regulated by MAGE-1 by using an inducible transfected *MAGE-A1* gene and the microarray technology.

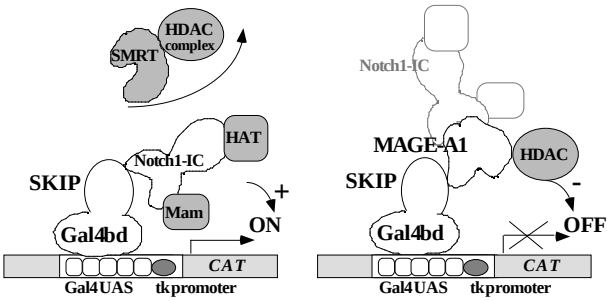

Fig. 2. MAGE-A1 counteracts Notch1-IC transactivation and recruits histone deacetylases

Studies on the transcriptional regulation of cancer-germline genes, such as *MAGE-A1*, showed that DNA methylation is an essential component of their repression in normal somatic tissues (8). The promoters of these genes contain a high density of CpGs, but unlike classical CpG-rich promoters they are heavily methylated in all somatic tissues. In constrast, they are unmethylated in germ cells and in tumors that express these genes. Demethylation and therefore activation of cancer-germline genes in tumors was found to be coincident with overall genome demethylation, a process known to occur in many cancers (9-10). We are currently studying the mechanisms of demethylation of these genes in tumors. This should give insight into the processes leading to genome hypomethylation in cancers. It may also help designing procedures to induce the expressionof specific antigens on tumors, thereby facilitating their elimination by the immune system.

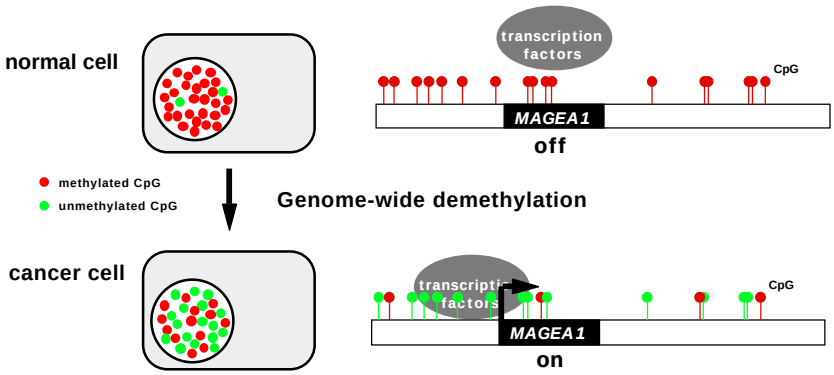

Fig. 3. *MAGEA1* activation as a result of genome demethylation in tumors

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IDENTIFICATION OF HUMAN TUMOR ANTIGENS

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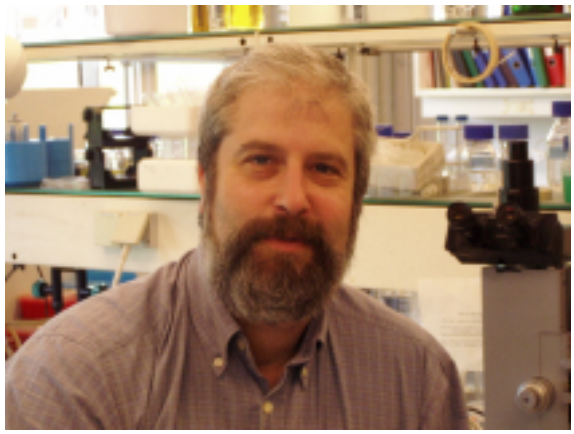

The group led by Pierre van der Bruggen is defining antigenic peptides encoded by genes such as those of the MAGE family. Therapeutic vaccination of cancer patients with MAGE peptides is now in progress, and the identification of additional antigenic peptides is important to increase the range of patients eligible for therapy and to provide tools for a reliable monitoring of the immune response. The group is also involved in designing reliable methods for the monitoring of the CD4$^+$ T cell response to cancer vaccines, and in the study of functional defects of T cells.

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

Yi Zhang, Vincent Stroobant, Christophe Panichelli, Sabrina Ottaviani, Tetsuto Kobayashi

"Cancer germline" genes such as those of the MAGE family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They encode shared tumor specific antigens, which have been used in therapeutic vaccination trials of cancer patients. The first antigens and genes that code for these antigens were identified with anti-tumor cytolytic T lymphocytes obtained from cancer patients (1). A few HLA class I-restricted antigenic peptides were identified by this "direct approach" (2). A large set of additional cancer-germline genes have now been identified by purely genetic approaches (3, 4). As a result, a vast number of sequences are known that can code for tumor-specific shared antigens, but most of the encoded antigenic peptides have not been identified yet. The identification of a large number of antigenic peptides presented by HLA class I and class II molecules is likely to be important for the future of clinical trials with defined antigenic peptides. A large set of peptide/HLA combinations will alleviate HLA restriction and widen the set of eligible patients. It will also facilitate the design of concurrent immunizations against several antigens. Such immunizations could increase the primary anti-tumor efficacy of the vaccine and also decrease the risk of tumor escape by loss of antigen expression.

We have used approaches that we have loosely named "reverse immunology" (5). They aim at identifying antigenic peptides recognized by T cells, using gene sequences as starting point. We have focused this search on the cancer-germline genes, which are expected to code for tumor-specific shared antigens on the basis of their pattern of expression.

Search for antigenic peptides recognized by CD8$^+$ T cells

We stimulated CD8$^+$ T lymphocytes with dendritic cells transduced with viral vectors containing complete MAGE-coding sequences. As this requires the processing of the antigen by the dendritic cells, we surmised that the peptides that would be identified would also be processed in the tumors expressing the MAGE genes. A difficulty of the use of recombinant viruses is the activation of CTL precursors directed against viral antigens. We circumvented this problem by using different vectors for the stimulation of the microcultures, for the lytic assay with the responder T cells, and for the cloning step. This procedure is summarized in Figure 1. Dendritic cells were infected with either an adenovirus or a canarypoxvirus and they were

used to stimulate microcultures of autologous CD8$^+$ T lymphocytes (6). After three weekly stimulations, the responder cells were tested for lysis on autologous EBV-B cells infected with vaccinia-MAGE. Positive microcultures were cloned. To identify the antigenic peptide, the resulting CTL clone was tested for lysis of autologous EBV-B cells pulsed with a complete set of peptides of 16 amino acids that overlap by 12. When a peptide scored positive, shorter peptides were synthesized to identify the shortest optimal peptide. To identify the HLA presenting molecule, the CTL clones were tested for stimulation by cells transfected with the MAGE cDNA together with cDNAs coding for the possible HLA presenting molecules. Finally, relevant tumor targets were used in a lysis assay to ascertain that the antigen was also processed by tumor cells.

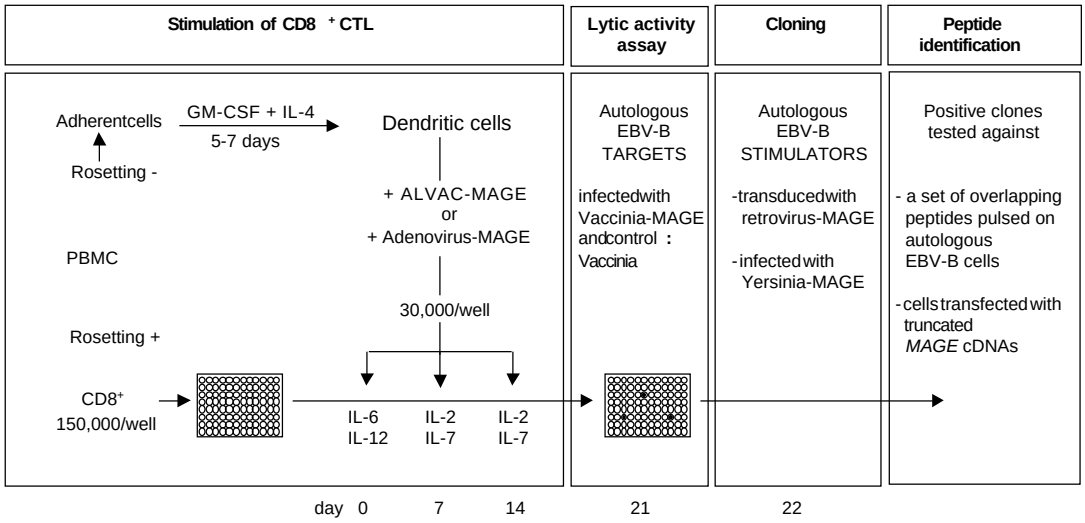

Fig. 1. Overview of the procedure to obtain anti-MAGE CD8$^+$ CTL clones by stimulation with dendritic cells infected with viral vectors carrying a MAGE coding sequence

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

We have identified a MAGE-3.B40 antigen which is the first example of a tumor-specific antigen exclusively presented by tumor cells expressing the immunoproteasome (7). This work was done in collaboration with the group of Benoît Van den Eynde.

We have found that MAGE-1 peptide SAYGEPRKL is recognized by CTL clones that are restricted either by HLA-Cw3, Cw6 or Cw16 (5). The presentation of the same peptide by different HLA molecules may be frequent for HLA-C molecules, because they are more closely related to each other in the peptide-binding region than HLA-A and B molecules. But for HLA-A and B also this may occur more frequently than usually thought. MAGE-3 peptide MEVDPIGLY is presented to different CTL by HLA-B*4402, B*4403, and B*1801 (5). MAGE-1 peptide EADPTGHSY was found to be recognized by different CTL on HLA-A1 and B35 molecules, and to bind to HLA-A29 (5). The same was found for the MAGE-3 homologous peptide EVDPIGHLY.

These results have consequences for the monitoring of the immune response of patients vaccinated with such tumor-specific shared peptides. A number of HLA-A1 patients were injected with MAGE-3.A1 peptide EVDPIGHLY, at a time when we did not know that it could be presented by B35 and A29 (5). The immune response was evaluated with HLA-A1 tetramers folded with the MAGE-3 peptide. Thus, A29 or B35-restricted responses against the peptide may have been missed.

Search for antigenic peptides recognized by CD4$^+$ T cells

Studies in several animal models have demonstrated an important role for CD4$^+$ T cells in inducing and maintaining anti-tumor immunity. It is therefore possible that the addition of antigenic peptides presented by class II to those presented by class I will improve the efficacy of therapeutic anti-tumor vaccination. To identify new HLA-peptide combinations, we used dendritic cells loaded with a recombinant MAGE protein to stimulate autologous CD4$^+$ T lymphocytes (8). After four weekly stimulations, the responder cells were tested for their ability to secrete IFN-γ upon stimulation with the antigen, and the positive microcultures were cloned. The procedure is summarized in Figure 2.

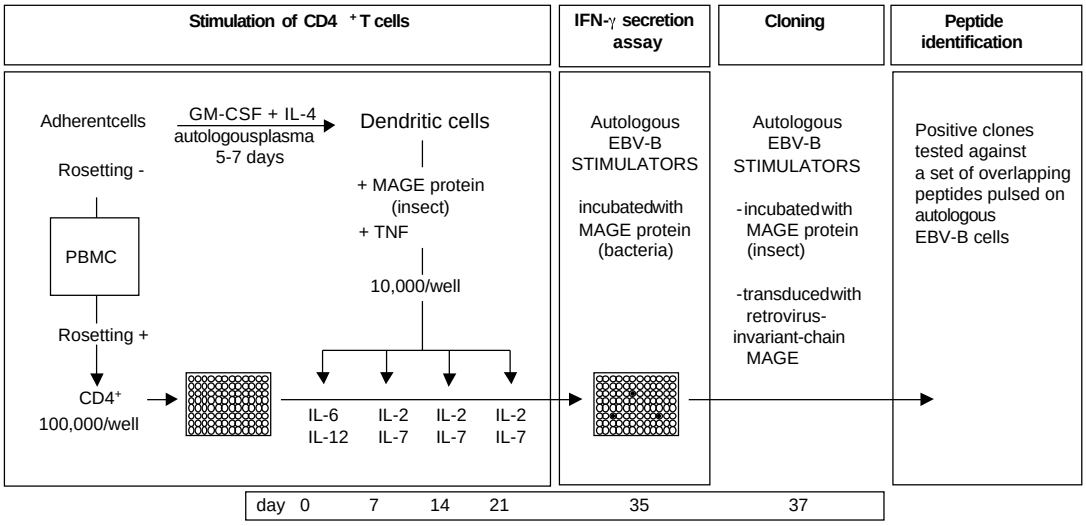

Fig. 2. Overview of the procedure to obtain anti-MAGE CD4$^+$ T cell clones by stimulation with dendritic cells loaded with a whole protein

To identify the antigenic peptide, the positive clones were stimulated with a set of peptides of 16 amino acids that overlapped by 12 and covered the entire MAGE protein sequence. The positive peptide was then tested for recognition on several Epstein-Barr virus immortalized B cell lines (EBV-B cell lines) to identify the HLA presenting molecule. Because a large number of the CD4$^+$ T cells that were obtained in our first experiments appeared to be directed against bacterial contaminants, we chose to alternate the sources of protein used at the various stages of the procedure. For example, to stimulate the lymphocytes, we used a MAGE protein produced in insect cells, and to test the specificity of the responder lymphocytes, we used a protein produced in bacteria. Microcultures that specifically produced IFN-γ after stimulation with the MAGE protein were cloned by limiting dilution using autologous EBV-B stimulator cells either loaded with the MAGE

protein used during the stimulation step, or transduced with a retroviral construct encoding a truncated human invariant chain (Ii) fused with the MAGE protein.

MAGE-1 and MAGE-3 antigenic peptides identified by this procedure are listed in a database (http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm). They include a MAGE-3 peptide presented by HLA-DP4, which is expressed by more than 70% of Caucasians (9). This peptide could not have been found by a peptide stimulation approach because no consensus anchor residue was known for HLA-DP4.

The normal anti-MAGE-3.A1 repertoire

Christophe Lonchay

To estimate the frequency of anti-MAGE-3.A1 CTLp directed against a MAGE-3 peptide (EVDPIGHLY) presented by HLA-A1 in blood from normal donors, groups of about 200,000 purified CD8 cells were stimulated with autologous PHA-activated T cells incubated with peptide MAGE-3.A1, in the presence of IL-6 and IL-12 during the first week followed by two weekly restimulations in the presence of IL-2 and IL-7. After the third week the cultures were tested in a lysis assay against HLA-A1 peptide-pulsed EBV-B cells. On the basis of the fraction of positive groups, the frequency of CTLp directed against the MAGE-3.A1 antigen was estimated to be about 2×10^{-7} in the CD8 lymphocyte population.

Another estimate was obtained recently for an hemochromatosis patient, from whom a large number of blood lymphocytes was collected. Purified blood T cells were incubated with an A1/MAGE-3 tetramer. The very rare cells that were stained by the tetramer and also by anti-CD8 antibodies were sorted at one cell per well and restimulated with irradiated HLA-A1 cells pulsed with the MAGE-3.A1 peptide. On the basis of the number of T cell clones that multiplied and were stained by the tetramer, we estimated the frequency of the naive anti-MAGE-3.A1 CTLp at 6×10^{-7} of the CD8 T cells for. With about five liters of blood containing about 10^6 PBMC/ml with about 15% CD8 T cells, and about 2% of total lymphocytes being located in the blood, the body total number of CD8 cells is approximately 4×10^{10}. Therefore a blood frequency of 3×10^{-7} of the CD8 corresponds to 32,000 anti-MAGE-3.A1 precursors. In the course of this analysis, 15 anti-MAGE-3.A1 T cell clones were obtained, corresponding to 14 different T cell receptors (TCR). Using these numbers to estimate the diversity of the naive T cell repertoire against MAGE-3.A1, one can state with 90% certainty that the naive repertoire directed against MAGE-3.A1 consists of 40 to 400 different TCR.

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

Nathalie Demotte

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

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

Yi Zhang

Patients injected with class II-restricted peptides

For therapeutic vaccination trials, the vaccine can consist of defined antigenic peptides. This approach greatly facilitates the monitoring of the T cell response, because the presumed target of the T cells is completely defined. It allows the use of HLA-peptide tetramers to detect T cell responses in patients. HLA class II tetramers have been more difficult to obtain than HLA class I tetramers, but have already been used to detect low frequencies of CD4 T cells. Didier Colau has recently succeeded in obtaining a DP4.MAGE-3 tetramer, which was produced in insect cells. It stained specifically relevant CD4 clones and we are using this tetramer to estimate the frequency of anti-vaccine CD4 T cells in patients injected with either the MAGE-3.DP4 peptide or dendritic cells pulsed with this peptide.

Patients injected with a protein

For therapeutic vaccination trials, the vaccine can also consist of the entire protein. This has the advantage that antigenic peptides binding to a broad set of HLA molecules can be processed from the vaccine, so that the patients do not have to be selected according to their HLA. However, the detection of the anti-vaccine T cells is more laborious and can not rely on the use of HLA-peptide tetramers. An interesting alternative assay uses bispecific antibodies that bind to the cell surface and capture cytokines immediately after their production. The cells are kept alive, can be cloned, and analyzed further for specificity and TCR expression. We have succeeded to detect anti-MAGE specific T cells using antigen-presenting cells pulsed with a peptide as stimulators. We are now trying to use dendritic cells loaded with a MAGE-3 protein so as to be able to monitor the immune response of patients vaccinated with an entire protein.

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

Therapeutic vaccination with MAGE tumor antigens

We have set up small-scale clinical trials aimed at evaluating the toxicity, the clinical evolution and the immunological response in cancer patients immunized with MAGE vaccines involving either peptides, a recombinant protein or a recombinant viral vector. A total of about 330 patients have been included in these multicentric trials.

Current status of the trials

Twenty-one melanoma patients included in the ongoing study LUD 97-004 have received 9 immunizations with the MAGE-3.A1 peptide, injected intradermally (ID) and subcutaneously (SC) every 10-11 days. Tumor regression was observed in three patients, who had a mixed response. As compared with monthly immunizations with the same peptide, which were associated with 7 regressions among 26 evaluable patients (2), the increase in the vaccination frequency does not seem to improve the clinical benefit. Using in vitro PBL stimulation in limiting dilution conditions followed by HLA/peptide tetramer staining, anti-MAGE-3.A1 CTL responses were detected in 1 of 2 patients with regression, and in none of 4 patients with disease progression. Seven additional patients have received the same peptide associated with the MAGE-3.DP4 peptide, in order to induce simultaneous CD4+ T lymphocyte responses. No regressions were observed, suggesting that no clinical benefit is obtained by the addition of this HLA class II restricted peptide. In a future clinical trial, the MAGE-3.A1 peptide will be mixed with an immunostimulatory CpG-containing oligonucleotide to increase its immunogenicity. In another trial, we will test whether a combination of 4 peptides (MAGE-3.A1, NA17.A2, tyrosinase.A2 and Melan-A.A2) that are individually associated with regression of melanoma metastases will improve the tumor response rate.

The clinical efficacy of the MAGE-3 protein injected ID and SC 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. To date, 5 out of 26 evaluable patients have shown regressions, including 1 complete response lasting for more than 1 year. Thus this vaccine does not seem to induce more regressions than the MAGE-3.A1 peptide, but it does not require that the patient carries a specific HLA type.We will now mix this recombinant protein with adjuvant AS15 containing an immunostimulatory CpG nucleotide, and combine these IM injections with the administration of selected class I or class II peptides by ID and SC routes, which may result in the simultaneous activation of both CD8+ and CD4+ specific T lymphocytes (Study LUD 02-002). Moreover, since a patient with metastatic bladder cancer experienced regression of lymph node metastases upon immunization with the MAGE-3 protein mixed with the adjuvant SB AS-2 (3), simultaneous administration of a MAGE recombinant protein and of some corresponding MAGE peptides will also be tested in the neo-adjuvant setting, in patients with bladder cancer (Study LUD 01-013).

In the LUD 97-005 trial, 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 ID and SC, 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 4 of the 6 patients with regressions, and in 2 of 11 patients with disease progression using our tetramer assay. We plan to investigate in a new trial whether increasing the dose of ALVAC would result in improved immunological and clinical responses.

In study LUD 01-006, patients with completely resected primary or regional metastatic melanoma with a high risk of relapse have been vaccinated with either the MAGE-3.A1 or MAGE-10.A2 peptide injected ID and SC every 2 weeks on 6 occasions. The objective of this ongoing trial is to analyze whether vaccination of melanoma patients with less advanced disease in the adjuvant setting improves the immunological response to a peptide vaccine. Up to now, no CTL response has been detected by our tetramer assay in the 13 patients who have received the complete treatment, including 7 patients with a resected tumor that did not express the appropriate antigen and who are assumed to be immunologically naive.

Relevant observations

Immunization with MAGE peptides, the MAGE-3 recombinant protein or the ALVAC recombinant viral vector, is devoid of significant toxicity.

A minority of melanoma patients (about 20 %) show regression of metastatic lesions following immunization, whatever the MAGE vaccine used. About 10% of the patients show complete or partial clinical responses. Some of them lasted for several years (4). 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 (5). However, more patients need to be analyzed before statistically significant conclusions can be drawn.

No increased immunogenicity of peptide vaccines was observed in disease-free patients with less advanced melanoma, as compared with patients with active, antigen-bearing metastases.

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ANALYSIS OF T CELL RESPONSES OF VACCINATED CANCER PATIENTS *

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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 occured 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.

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

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

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

To detect low-level responses, blood lymphocyte microcultures were stimulated with the antigenic peptide in limiting dilution conditions (Mixed Lymphocyte Peptide Culture, MLPC), followed by tetramer analysis, cloning of the tetramer-positive cells, and T-cell receptor (TCR) sequence analysis of the cytolytic T lymphocyte (CTL) clones that showed strict specificity for the vaccine antigen (for method, see P. Coulie, ICP report).

Christophe Lurquin and Bernard Lethé focused their efforts on detailed analysis of frequencies of characterized T-cell clones in blood, metastases and non tumoral tissue samples, using 'clonotypic' polymerase chain reaction (PCR) amplifications specific for the Vα and Vβ rearrangements of relevant TCR. These PCR amplifications on cDNA were sensitive enough to detect one CTL expressing a given TCR mixed with $3x10^7$ PBMC

of normal donor and they were highly specific for the given TCR insofar as no product was amplified with cDNA prepared from $3x10^7$ PBMC of 5 unrelated donors.

A melanoma patient vaccinated with a tumor-specific antigenic peptide encoded by gene MAGE-3

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 (patient CP64, see P. Coulie).

Since all the $CD8^+$ blood lymphocytes directed against the vaccine antigen presented the same TCR, quantitative evaluation of the overall anti-MAGE-3 CTL response could be easily carried out on blood lymphocytes by reverse transcription-PCR amplification (RT-PCR). This study indicated a frequency of T cells expressing the TCR 48 of 1/20,000 among $CD8^+$ cells in two postimmunisation blood samples collected respectively 5 and 6 months after the onset of vaccination, whereas this TCR was not found among $2.5x10^6$ $CD8^+$ in blood collected before vaccination (table 1).

These results prove that the vaccination induced at least a 100-fold amplification of anti-MAGE-3.A1 CTL clone 48 and that vaccines containing only a tumor-specific antigenic peptide can elicit a CTL response (3).

We have also examined a non-regressing metastasis for the presence of CTL 48. Sections that were 7 μm thick and ~1 cm in diameter were divided in 12 smaller regions and the RNA of each of them was tested by the clonotypic RT-PCR amplification of TCR 48. An average of 3/12 positive areas was obtained. The analysis of immediately adjacent sections showed that there was no correlation between the location of the positive areas in different sections, suggesting that the positives represent single cells. By using CD8β immunostaining on adjacent sections to evaluate the number of $CD8^+$ T cells, we obtained a frequency of ~1/5,000 $CD8^+$ for CTL 48 in this nonregressing metastasis, without significant difference with the postimmune blood frequency.

Our experiments also suggest that the absence of immune attack on this metastasis is not due to a lack of expression of the MAGE-3.A1 antigen by the tumor cells since the melanoma cells were stained by a conformational antibody directed against functional MHC class I molecules and expressed MAGE-3 and HLA-A1 genes as demonstrated by RT-PCR.

Table 1.Detection of TCR 48 by RT-PCR in groups of PBMCs

PBMCs	Number of PBMCs/group	Proportion of $CD8^+$	Positive groups/ Tested groups	Frequency (among $CD8^+$)
Preimmunization	10^7	5%	0/5	$<4x10^{-7}$
Postimmunization I	$7.5x10^4$	6%	4/20	$5x10^{-5}$
Postimmunization II	10^5	9%	11/30	$5x10^{-5}$

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 during two years and the patient was still free of detectable melanoma.

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 : CTL 35 found in more than 95% of the independent microcultures which were analyzed and CTL 103, suggesting that this MAGE-3.A1 response was essentially monoclonal.

PCR amplifications specific for the TCR 35 Vα and Vβ 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. The blood frequency of CTL 35 stayed between 1.5×10^{-6} and 3.3×10^{-6} during one year of vaccination with peptide. Afterwards, a set of five weekly injections of MAGE-3.A1 peptide given therefore at short intervals did not increase the frequency (1.4×10^{-6}) whereas four weekly injections of recombinant ALVAC given five months later boosted the anti-MAGE-3.A1 response with a frequency of CTL 35 increasing to ~1/75,000 $CD8^+$. The frequency did not change signifiantly after new series of peptide and virus injections : values ranging from 4.7×10^{-6} after a boost with peptide to 1.1×10^{-5} after injections of ALVAC were observed by the clonotypic RT-PCR amplification of TCR 35 (4).

By stimulating blood lymphocytes from melanoma patient EB81 with autologous tumor cells (MLTC), a series of CTL clones that specifically lysed autologous melanoma cells were isolated. Some of these CTL clones appeared to recognize peptides presented by HLA-A2 and encoded by gene MAGE-C2, an other cancer-germline gene which is expressed at high level by the melanoma cells of patient EB81. We analyzed the TCR Vβ 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 Vα and Vβ 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 µm thick and ~80 mm^2, fragments of such sections and groups of cells excised from sections with laser microdissection were tested by RT-PCR for the presence of TCR 35 and TCR 16. We obtained a frequency of ~1/32,500 CD8 for anti-MAGE-3 CTL, which represents a 20-fold enrichment relative to blood frequency at the time of the metastasis resection. Moreover, the anti-MAGE-C2 CTL was at least 275-times enriched in the resected sample with a frequency higher than 1/100 CD8 in the lymph node tissue and a frequency >1/5 CD8 in tissue regions strongly invaded by tumoral cells (Figure 1).

Our results suggest that tumor-specific CTL others than those directing against the vaccine antigen can be stimulated after vaccination and may have a function in the tumor rejection process that follows vaccination.

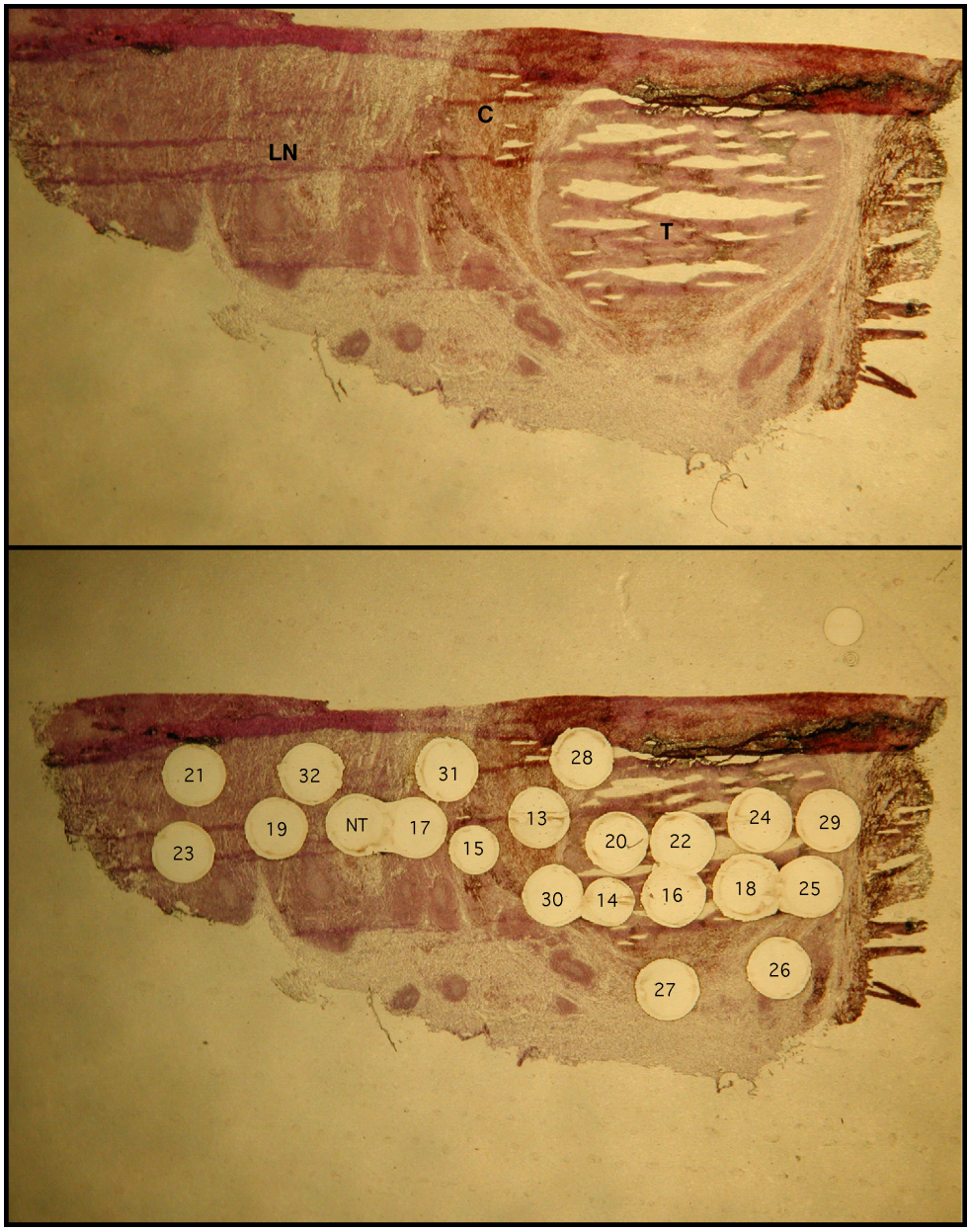

Fig. 1. Section of invaded lymph node from patient EB81
Upper panel : hematoxylin-stained cryosection before laser microdissection ; T, encapsulated tumor ; LN, lymph node tissue ; C, cicatricial area with the presence of melanin.
Lower panel : the same cryosection after excision of microdissected areas (1 mm in diameter) used for determination of CTL 16 frequencies among the $CD8^+$. cDNA of each area was tested by clonotypic PCR amplifications of TCR 16 Vα and Vβ rearrangements and by CD8β quantitative PCR (Taqman method). The number of all CTLs in each area was calculated considering a previously defined estimation of the number of CD8β transcripts in one CTL.

Patients vaccinated with dendritic cells pulsed with a single MAGE-3 peptide presented by HLA-A1

Eleven far advanced patients received 5 vaccinations at 14 days intervals. Regressions of individual metastases were evidenced in 6 of them (7). We monitored the anti-vaccine cytolytic T lymphocyte (CTL) response in 3 regressing patients and in 1 progressing patient, resorting to two parallel approaches. The first approach was a MLPC- A1/MAGE-3 tetramer analysis. In a second approach (*ex vivo*-tetramer), blood lymphocytes were assayed immediately with the A1/MAGE-3

tetramer, the labeled cells were cloned and their diversity analyzed by TCR sequencing. A polyclonal CTL response against the MAGE-3.A1 antigen was observed in the 3 patients who showed regression of some of their metastases after vaccination (# 04, 06 and 09) (see Figure 2) . The frequency estimated after vaccination ranged from $4x10^{-6}$ to $1x10^{-3}$ among $CD8^+$ blood lymphocytes, corresponding to an amplification factor of 10 to 500 above the value found before immunization.

Among the various clonotypes found in each patient, two or three of them were found repeatedly and represented about 60% of the CTL response. Such response was not found in the patient who didn't show any regression of his metastases (# 11).

For patient # 04, clonotypic PCR were set up for clonotypes 1 and 5 and used to test cDNAs derived from freshly thawed groups of lymphocytes. Frequencies in line with those obtained by the MLPC-tetramer and *ex vivo*-tetramer approaches were obtained, indicating that our culture conditions allowed the survival and amplification of most of CTL precursors (CTLp)(8).

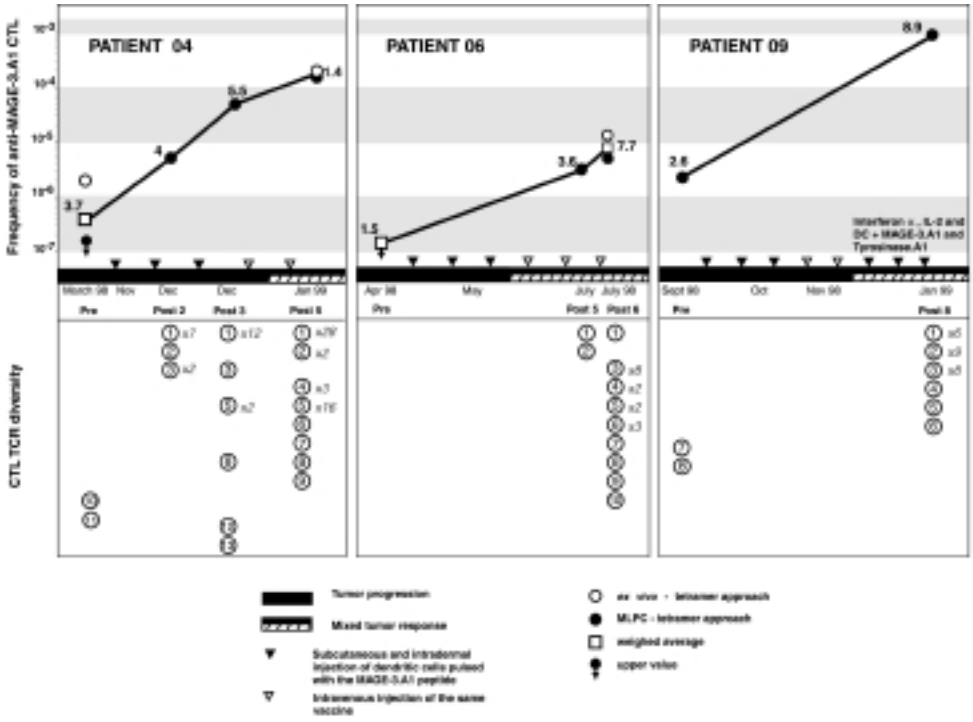

Fig. 2. Frequency and diversity of anti-MAGE-3.A1 CTL.
Bottom panels represent CTL clones, with different numbers for each TCR sequence, and the occurence of repeated clones. TCR numbering starts at 1 for each patient. Similar numbers assigned to CTL of different patients do not represent TCR similarity.

Patients vaccinated with dendritic cells pulsed with multiple peptides.

A new protocol was established by the group of G. Schuler, using dendritic cells pulsed with at least three different tumor epitopes. While HLA-A1 patients received dendritic cells pulsed with the MAGE-1.A1 and the MAGE-3.A1 peptides, HLA-A2 patients were vaccinated with autologous dendritic cells pulsed with the MAGE-4.A2 and the MAGE-10.A2 peptides (9, 10).

We analyzed the T cell response of one of these HLA-A2 patients (A2-R-12), using the MLPC-tetramer approach. As shown in figure 3, no CTLs directed against the MAGE-4.A2 epitope were obtained after the 5 vaccinations. On the contrary, the frequency of anti-MAGE-10.A2 CTLp was evaluated to be $2.35x10^{-5}$ after vaccination. As in the first study with dendritic cells pulsed with the MAGE-3.A1 peptide, the TCR analysis of the CTL clones obtained evidenced a polyclonal response. No CTLs were isolated from the blood sample collected before vaccination, leading to a frequency estimate below $5.3x10^{-7}$ of CD8 T cells. The CTL

responses of other patients included in this protocol are currently being examined to determine if, as suggested for patient A2-R-12, some peptides immunize better than others.

Even though our data provide no information about the effector mechanisms responsible for the observed regressions in these patients, they suggest that DC vaccinations can induce CTL responses that differ from that induced by vaccination with peptide alone in term of their higher frequency and polyclonality.

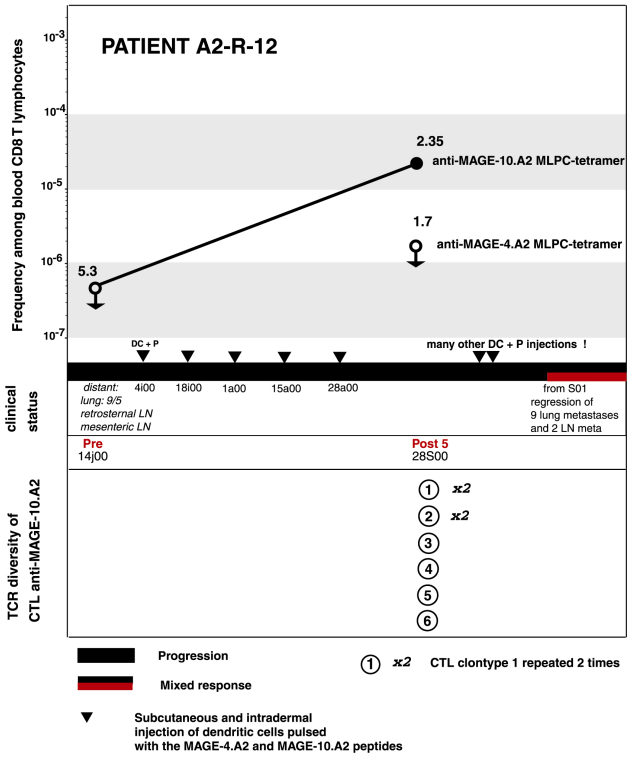

Fig. 3.

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II. CYTOKINE FUNCTION

OVERVIEW

Our interest in cytokines stemmed from the study of various murine cell lines whose in vitro growth was found to be dependent on supernatant from activated T cells. By purifying the active factor from such supernatants, we identified a mouse protein that stimulates the growth of B-cell hybridomas (1). This factor, now designated Interleukin 6 (IL-6), turned out to play a pivotal role as an accessory signal in the early steps of immune responses. IL-6 is also a growth factor for many mouse plasmacytomas and human myelomas, and seems to play a pathogenic role in myeloma patients (2).

Another cytokine, IL-9, was similarly identified at the branch as a growth factor for mouse T Helper clones (3). In vitro, IL-9 exerts activities on T cells, B cells, mast cells, erythroid and myeloid progenitors, as well as immature neurones. However, its major biological activities have been mainly enlightened by the analysis of transgenic mice that overexpress this cytokine.

First, IL-9 transgenic mice, that have a high level of this cytokine in all tissues, are characterized by a high susceptibility to the development of T cell lymphomas. Indeed, approximately 5% of these mice spontaneously develop thymic lymphomas (4). In addition, they show a very high sensitivity to the oncogenic effect of very small doses of chemical mutagens or irradiation. By contrast, normal T cell subsets do not seem to be affected by IL-9 overexpression.

Another major aspect of IL-9 biology is its growth and differentiation activity on mast cells. IL-9 transgenic mice show increased numbers of mast cells in the gut and airways (5). As a result, these animals are particularly resistant to infection by intestinal nematodes such as *Trichinella spiralis* or *Trichuris muris*. The effect of IL-9 on pulmonary mast cells might be related to genetic data pointing to *IL9* as a candidate susceptibility gene for asthma.

Finally, a puzzling activity of IL-9 is a selective increase in the peritoneal B1b cell subpopulation (6). Although the specificity of these cells is far from clear, they might be related to some auto-immune processes.

In line with the oncogenic activity of IL-9 in transgenic mice, this cytokine was shown to be a potent anti-apoptotic factor for T cell lymphomas (7). Interestingly, in the same anti-apoptotic model, we observed that the I-309 chemokine also inhibits some apoptotic processes (8). Further studies have now allowed for the characterization of the I-309 receptor and provide evidence that a distinct activation pathway, namely the Ras-MAP-kinase pathway is responsible for this I-309 activity .

By contrast, the anti-apoptotic effect of IL-9 does not involve MAP-kinases but is mediated by the JAK/STAT pathway. Studies of the mode of action of IL-9 at the molecular level showed that, upon IL-9 binding, the IL-9R associates with a co-receptor protein called γc. This induces the phosphorylation of the JAK1 and JAK3 tyrosine kinases, which are associated with IL-9R and γc,

respectively. A single tyrosine residue of the IL-9R is then phosphorylated and acts as a docking site for 3 transcription factors, 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 known biological activities. For IL-9, we demonstrated that activation of the STAT transcription factors is crucial for all the known effects of IL-9 (9). STAT3 seems to be the main mediator of IL-9-induced differentiation of T lymphocytes and mast cells, and STAT5 is mainly involved in proliferative responses of B and T lymphocytes. Both STAT3 and STAT5 can confer protection against corticoid-induced apoptosis, however. Finally, activation of the three STAT transcription factors was required for optimal cell growth, suggesting additive or synergistic effects in the regulation of gene expression by these proteins (10).

Therefore, we are now focusing on the characterization of genes whose expression is regulated by IL-9 through the activation of STAT transcription factors.

Two of these genes, *bcl3* and *M-ras*, reflect a secondary layer of signal transduction modulation by IL-9. Bcl-3 is a member of the IκB family and favors the nuclear translocation and DNA binding of p50/p50 homodimers of the NF-κB transcription factors. Upregulation of *bcl3* expression by IL-9 thus represents a new and unexpected mechanism of NF-κB activation by cytokines (11).

M-ras is another example of a signal transduction-related gene regulated by IL-9. This new member of the Ras family is induced by IL-9 in mouse T cells and exerts some transforming activities since it can (i) mediate transformation of NIH-3T3 fibroblasts, (ii) promote cytokine-independent proliferation of lymphocyte cell lines, and (iii) protect T lymphomas against corticoid-induced apoptosis (12).

The search for IL-9-induced genes led us to characterize a new gene encoding a 180 amino acid protein, that shows a weak but significant sequence homology with IL-10. This protein, originally designated IL-TIF for IL-10-related T-cell-derived Inducible Factor, is induced by IL-9 in thymic lymphomas, T cells and mast cells. This new cytokine has now been renamed IL-22 and has become a major research topic in our group (13, 14).

In 2001, the group of Stefan Constantinescu joined the Institute and reinforced and extended our focus on signal transduction mechanisms by cytokine receptors such as the receptors for erythropoietin (EPO) and thrombopoietin (TPO). The main topic of this group is to elucidate the mechanisms by which the different chains of cytokine receptors associate between each other and with JAK kinases, including the orientation of the transmembrane and cytosolic domains, and the consequences of these interactions for receptor traffic and signalling.

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CYTOKINES IN IMMUNITY AND INFLAMMATION

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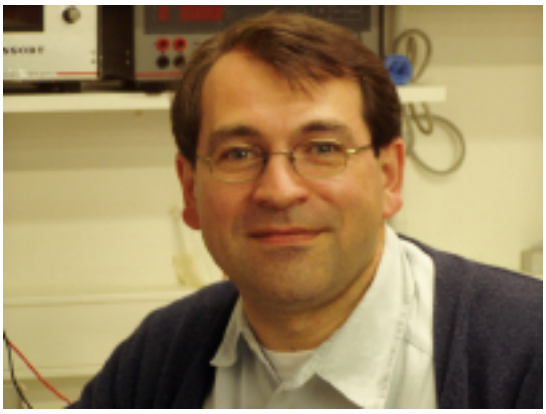

The cytokine group studies the biology of Interleukin-9 (IL-9) and IL-22, two cytokines discovered at the Branch. IL-9 is a TH2 cytokine that plays a role in immune responses against intestinal parasites and asthma. IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, upregulates 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, David Darcaigne, 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 Renauld, Laurent Knoops, Diane Lejeune, 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 γc. This induces the phosphorylation of the JAK1 and JAK3 tyrosine kinases, which are associated with IL-9R and γc, respectively. A single tyrosine residue of the IL-9R is then phosphorylated and acts as a docking site for 3 transcription factors of the STAT family, STAT-1, -3 and -5, which become phosphorylated and migrate to the nucleus, where they activate the transcription of a number of genes. This pathway is common to many cytokines but is often dispensable for their biological activities. For IL-9, our group demonstrated that activation of the STAT transcription factors is crucial for all the effects of IL-9 studied on various cell lines, including positive and negative regulation of cell proliferation, as well as inhibition of corticoid-induced apoptosis in T cell lymphomas. Further analysis demonstrated that STAT-1, -3 and -5 play specific, redundant and synergistic roles in the different activities of IL-9 in vitro (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.

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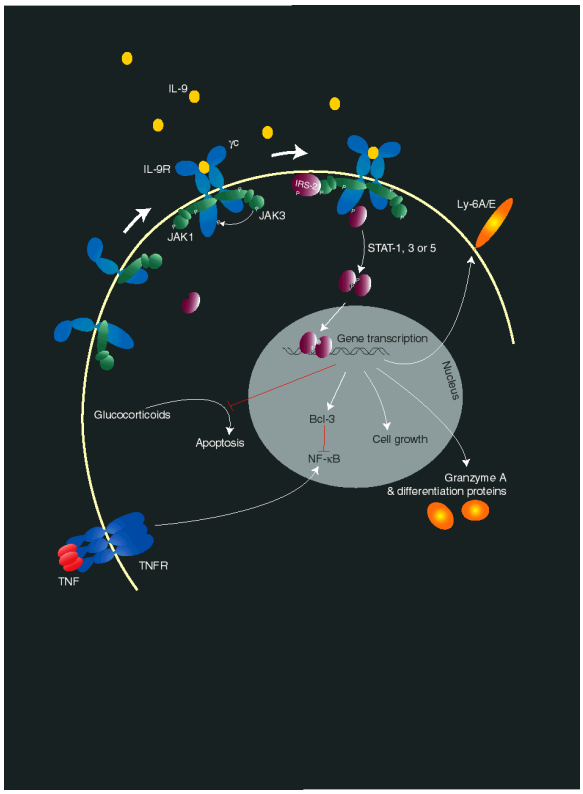

Fig. 1

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

Jean-Christophe Renauld, Jacques Van Snick, Jamila Louahed

Incidentally, our studies of this particular model of the regulation of cell death by cytokines, lead them to purify another protein called I-309, originally described as a human chemotactic factor, and that turned out to exert a significant anti-apoptotic activity for thymic lymphomas (6). However, I-309 and IL-9 trigger completely different pathways and it was shown that the I-309

anti-apoptotic activity was dependent on the activation of G-proteins and the Ras/MAPKinase pathway, whereas the IL-9-mediated effect was not. More recently, we showed that a viral protein related to human chemotactic factors (vMIP-I), and isolated from Herpes viruses that induce T cell tumors, has the same anti-apoptotic activity by binding to the I-309 receptor

IL-9-induced genes

Jean-Christophe Renauld, Jacques Van Snick, Laure Dumoutier, Jamila Louahed, Laurent Knoops, Diane Lejeune, 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 crystallograhic structure solved. Despite its structural homology with IL-10, IL-22 fails to recapitulate any of IL-10 biological activities.

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

Although IL-22 does not share any biological activity with IL-10, these 2 cytokines share a common component of their respective receptor complex, IL-10Rß. Anti-IL-10Rß 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-10Rß is a typical short chain component, with only 76 amino acids in the cytoplasmic domain, whose main function seems to consist in recruiting the Tyk2 tyrosine kinase. In addition to IL-10R ß, IL-22 signalling requires the expression of a long chain protein, called IL-22R and comprising a 319 amino acid long cytoplasmic domain. This chain associates with Jak1, and is responsible for the activation of cytoplasmic signalling cascades such as the JAK/STAT, ERK, JNK and p38 MAP kinase pathways.

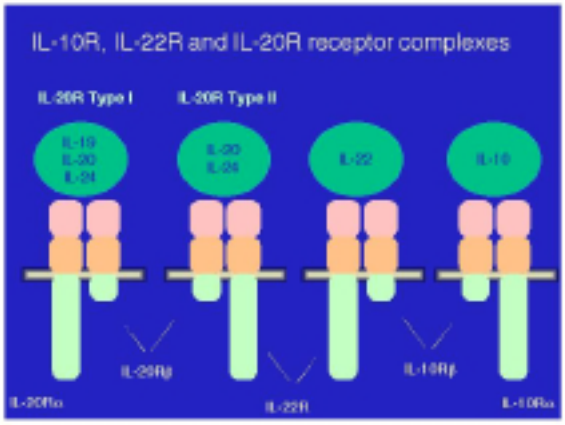

Fig. 2

In addition to its role in IL-22 binding and signalling, the IL-22R chain also forms a functional heterodimeric receptor complex by associating with IL-20R ß, the second short chain member of the IL-10R-related receptor family. This complex mediates STAT-1 and –3 activation by IL-20 and IL-24, but not by IL-22 (11). In addition, IL-20 and IL-24 can also bind to other complexes consisting of IL-20Rα and IL-20Rß. This promiscuity in cytokine receptor usage is illustrated in Fig 2.

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STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS

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Cytokines and their receptors are critical for 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). Activated JAK proteins phosphorylate downstream proteins, like receptors themselves, signal transducers and activators of transcription (STAT) proteins and a variety of other signaling proteins. Phosphorylated STAT proteins dimerize in the cytosol and are translocated to the nucleus where they bind to specific promoter sequences and regulate transcription. 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). The assembly of cell-surface receptor complexes, the structure and orientation of the transmembrane (TM) and cytosolic juxtamembrane (JM) domains, and the regulation by JAK kinases of receptor traffic are major focuses. We also study the mechanisms by which STAT proteins become constitutively activated and how they function in transformed hematopoietic or patient-derived leukemia cells.

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

Nadine Seubert, Yohan Royer, Katharina Kubatzky, Nicole El-Najjar

Epo binding to the erythropoietin receptor (EpoR) results in survival, proliferation and differentiation of erythroid progenitors into mature red blood cells (1). We have shown that, in the absence of Epo, the cell-surface EpoR is dimerized in an inactive conformation which is stabilized by interactions between the TM sequences (2). Epo binding to the extracellular EpoR domain induces a conformational change of the receptor which results in the activation of cytosolic JAK2 proteins. The α-helical orientation of the (TM) and cytosolic JM domains is crucial for receptor activation (3). Specifically, in collaboration with Lily Huang and Harvey Lodish, Whitehead Institute, Cambridge, MA, USA, we have identified a number of key residues in the EpoR cytosolic JM domain which are required for switching on the activity of JAK2 and initiate signaling (4).

To identify the residues that form the interface between the receptor monomers in the activated EpoR dimer we have replaced the EpoR extracellular domain with a coiled-coil dimer of α-helices. Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (1), d (4), and a (7), 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 position (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. 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. At present we are characterizing the signaling events induced by the two active coiled-coil-EpoR fusion proteins and we are performing Cys scanning of the EpoR TM domain and cross-linking assays in order to prove that the dimeric active interface we have identified can be demonstrated in the wild type receptor dimer

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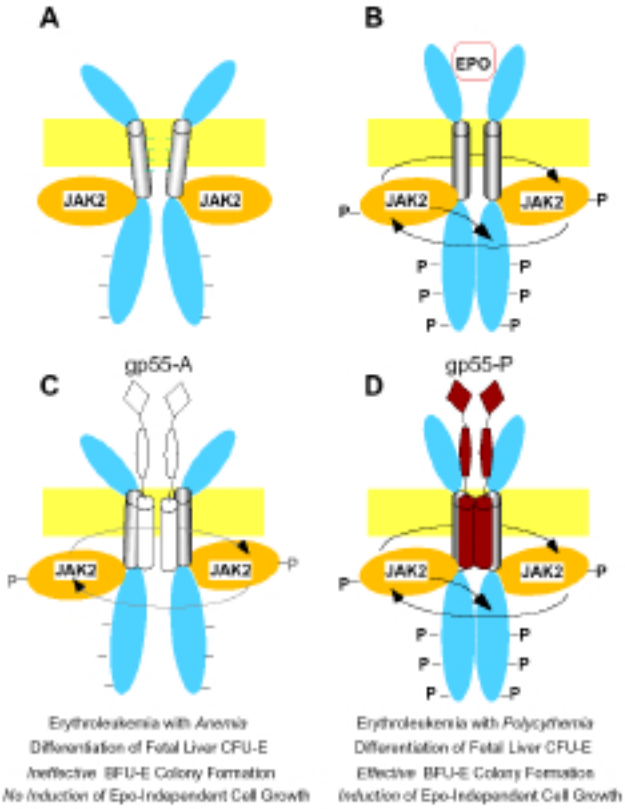

(A) The erythropoietin receptor (EpoR) is an inactive dimer on the cell-surface in the absence of ligand due to interactions between the transmembrane (TM) domains (interrupted line). Cytosolic Janus kinase 2 (JAK2) is bound to the receptor juxtamembrane domain and stimulates receptor folding and traffic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

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

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

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

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

Katharina Kubatzky, Nicole El-Najjar

The structure of the transmembrane and cytosolic domains of cytokine receptors remains a mystery. Due to our previous work showing that the junction between the EpoR TM and intracellular domain is rigid (3) we hypothesized that the important structured segments of the cytosolic domain must contain the cytosolic JM segment. We have cloned the cDNAs coding for the seven coiled-coil-EpoR fusion proteins in the pET31b vector in order to produce recombinant fusion proteins in quantities amenable for biophysical and structural studies. In collaboration with the group of Steven Smith, SUNY, Stony Brook, NY, USA, we will determine the NMR structure of the EpoR TM and cytosolic JM domains in the active and inactive coiled-coil-EpoR fusion proteins. The structural studies will be facilitated by the already known structure of the coiled-coil part of the fusion proteins. Comparing the structure and biophysical properties of the active and inactive coiled-coil-EpoR fusion proteins we aim to determine the conformational requirements of the activated state of the receptor. The information obtained by studying the model coiled-coil-EpoR fusion proteins will be then tested on the wild type EpoR. In addition, the recombinant form of the two active coiled-coil-EpoR fusion proteins will be used as baits in order to identify receptor binding proteins in cellular lysates.

Traffic of cytokine receptors to the cell surface

Yohan Royer

Traffic and cell-surface expression of the EpoR critically depend on the ability of EpoR to bind JAK2 intracellularly (4). In the absence of JAK2 the receptor does not get transported from the endoplasmic reticulum (ER) to the Golgi aparatus and does not acquire EndoH resistance. Strikingly, chimeric proteins that contain the EpoR intracellular domain and the extracellular domains of IL9Rα, IL2Rβ or of the common γ chain, are expressed on the cell-surface as a function of coexpressed JAK2. Moreover, we have observed that in hematopoietic cells overexpressing JAK proteins several cytokine receptors are expressed at significant higher levels on the cell-surface. Particularly, the IL9Rα which requires JAK1 for signaling is expressed at higher levels on the cell-surface when JAK1 but not JAK2 or JAK3 is overexpressed. 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.

Structure and function of the extracellular domain of the EpoR (Katharina Kubatzky, Yohan Royer). Recombinant Epo is the leading drug of modern biotechnology and is widely used in the clinic for treatment of anemias of different causes, such as chronic renal failure, anemia induced by cancer chemotherapeutic agents, anemia of AIDS patients receiving AZT, or anemias of prematurity, rheumatoid arthritis and myelodisplasia. Apart from being expressed in erythroid progenitors, megakaryocytes and endothelial cells, the EpoR is expressed in neurons where it can protect from apoptosis induced by hypoxia. The present challenge is to design a small molecule mimetic or partial mimetic of Epo which should be non-toxic and non-immunogenic. The mechanism by which Epo binding to the extracellular domain changes the conformation of the EpoR is not known but is relevant for the efforts to isolate small molecule activators of EpoR. Epo binds to three loops on each monomer of the EpoR extracellular domain (EpoR-ECD). The same loops are involved in binding of a mimetic peptide and in contacts between un-liganded extracellular domains. Using insect cell produced EpoR-ECD we have shown that Cys181 of the ECD can be used to cross-link two ECD monomers in the presence of ligand. Using mutagenesis and recombinant production of the EpoR-ECD in insect cells we will investigate the conformation of the EpoR-ECD in the presence and absence of ligand as well as the conformation of a mutant constitutively active form of the EpoR-ECD (EpoR R129C) .

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 (5). 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 receptors for IL2 and IL9 via the common γ chain (γ-c)

Yohan Royer

γ-c is a cytokine receptor that is shared by the receptor complexes of several cytokines, such as IL2, IL4, IL7, IL9 and IL15. γ-c binds and activates JAK3. Humans that lack the γ-c or have mutations in JAK3 develop severe combined immunodeficiency. We are investigating the assembly of IL9Rα and IL2Rβ with the common γ chain. We have employed PCR-directed mutagenesis to test the involvement of a conserved cytokine receptor JM hydrophobic motif (3) in IL9Rα and IL2Rβ signaling. It appears that IL9Rα has quite different sequence requirements than IL2Rβ for signaling, although both utilize γ-c. While IL9Rα is rather similar to the EpoR, IL2Rβ does not require hydrophobic residues at positions –1, -2 and –6 from Box 1. We are also investigating the sequence requirements of γ-c JM domain for activating JAK3 and interacting with JAK1. In collaboration with Jean-Christophe Renauld we attempt to isolate novel IL9Rα and γ-c mutants that would reveal the precise domains of IL9Rα and γ-c that trigger activation of JAK1 and JAK3.

Sequence–specific interactions between transmembrane domains

Nicole El-Najjar

Two transmembrane viral enevelope proteins (gp55-P and gp55-A) belonging to the polycythemic (P) and anemic (A) Spleen Focus Forming Viruses (SFFV) strains, can activate the EpoR when co-expressed in the same cell (6). In collaboration with Yoav Henis, Tel-Aviv University, Israel, we have shown that both the gp55-A and gp55-P TM domains specifically interact with the TM domain of the EpoR. gp55-A weakly activates the receptor leading to erythroleukemia with low number of red blood cells (*anemia*). However, gp55-P fully activates the EpoR to stimulate proliferation and differentiation of erythroid progenitors leading to both erythroleukemia and massive red blood cell production (*polycythemia*). The activation of EpoR by gp55-P results from a highly specific interaction between the membrane spanning sequences of the two proteins: Ser238 of the murine EpoR TM and Met390 of the gp55-P TM are critical determinants of this interaction (7). Taking advantage of this specific interaction we are constructing a genetic system where the TM sequence of gp55-P is randomized or replaced with short sequences derived from a lymphoid cDNA library in order to select for novel sequences capable of functioning as TM domains (transmembrane domain trap), of binding EpoR and of activating the EpoR. Several genetic approaches exist to probe the ability of a particular TM sequence to homodimerize/homo-oligomerize but no assay has been reported for selection of hetero-interactions between TM sequences. In our system activation of EpoR signaling will result in cell survival and proliferation which represent a powerful selection.

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

Virginie Moucadel, Yohan Royer, Judith Staerk

Cytokine stimulation of cytokine receptors, induces transient activation of the JAK-STAT pathway. In contrast several mutant forms of cytokine receptors (i.e. EpoR R129C or TpoR S498N) have been isolated that signal constitutively (reviewed in (1, 8, 9)). Such receptors are permanently dimerized in an activated state and induce the biologic effects of the wild type receptors as well as leukemic cell transformation. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously, in the absence of any cytokine. In the transformed cells many of the transient signaling events induced by cytokines are detectable permanently, i.e. ligand-independent phosphorylation of JAK and STAT proteins or high levels of nuclear activated STATs especially STAT5 and STAT3. A similar picture has been noted in patient derived leukemia cells, where constitutively active STAT proteins have been reported in a majority of myeloid and lymphoid leukemia patients. 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.

First, by using bicistronic retroviral vectors and cell sorting we have generated hematopoietic cells that express 5-10 fold higher levels of JAK proteins (such as JAK1, JAK2 and JAK3) or STAT5. Pools of cells that overexpress JAK or STAT proteins but remained cytokine-dependent for growth and survival were isolated. Like the parental hematopoietic cells these JAK/STAT overexpressing cells can be transformed to growth-factor independence by expression of constitutively active forms of cytokine receptors. Because the levels of expression of JAK and STAT proteins in these cells are amenable to detailed biochemical analysis we are using immunoprecipitation of the overexpressed JAK-STAT proteins and mass spectrometry (in collaboration with the laboratory of Mark Rider) in order to map novel phosphorylation sites and to isolate and identify associated proteins.

In a second approach 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. Control, ligand-activated or transformed cells are treated with 1% formaldehyde to cross-link genomic DNA with protein. Cells are lysed, chromatin is sonicated to a certain average length (i.e. 600 bp) and then purified. The isolated DNA- protein complexes are immunoprecipitated with antibodies directed against STAT proteins or with control antibodies. After elution and reversal of cross- linking genomic DNA will be linked to adapters and PCR amplified and sequenced. The isolated genomic fragments will be screened for the presence of STAT-binding sites and tested for the ability to regulate transcription of reporter genes. Newly identified genes regulated by such genomic sequences will be expressed in bicistronic retroviral vectors that allow wide expression of cDNAs at physiologic levels (10). In preliminary experiments we have shown that using chromatin immunoprecipitation and PCR amplification we could isolated several genomic sequences that were bound by STAT5 after ligand addition.

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