Research

at the

de Duve Institute

and

Brussels Branch of the Ludwig Institute for Cancer Research

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LUDWIG INSTITUTE FOR CANCER RESEARCH

BRUSSELS BRANCH

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

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

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

Branch staffs vary in size from 30 to over 90, and internationally the Institute employs some 800 scientists, clinicians and support personnel. The quality of the research is monitored on an ongoing basis by the Institute's Scientific Committee and by an external peer review process.

The Brussels Branch of the Institute was created in 1978. It is composed of 93 members and was headed by Thierry Boon until 2009. The Branch is now headed by Benoît Van den Eynde, the current Branch Director.

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TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

The group follows three main lines of research. The first focuses on the processing of tumor antigens, studying the role of the proteasome and other proteases in the production of tumor antigenic peptides. The second studies mechanisms whereby tumors resist immune rejection. The third develops new preclinical models for cancer immunotherapy. The long term goal of these projects is to better understand the interaction of tumors with the immune system and devise strategies to improve the efficacy of cancer vaccines.

PEPTIDE SPLICING BY THE PROTEASOME

A. Dalet, V. Stroobant, N. Vigneron

Tumor antigens relevant for cancer immunotherapy consist of peptides presented by MHC class I molecules and derived from intracellular tumor proteins. They result from the degradation of these proteins, which is mainly exerted by the proteasome. We have described a new mode of production of antigenic peptides by the proteasome, which involves the splicing of peptide fragments, either in the normal or the reverse order (1, 2). In the two cases we initially described, we showed that splicing occurs in the proteasome catalytic chamber through a reaction of transpeptidation involving an acyl-enzyme intermediate (Figure 1). We have now demonstrated that the same mechanism accounts for the splicing of a third spliced peptide, derived from FGF5, despite the fact that the fragments to splice are distant from each other by 40 amino acids (3). We also compared the efficiency of splicing by

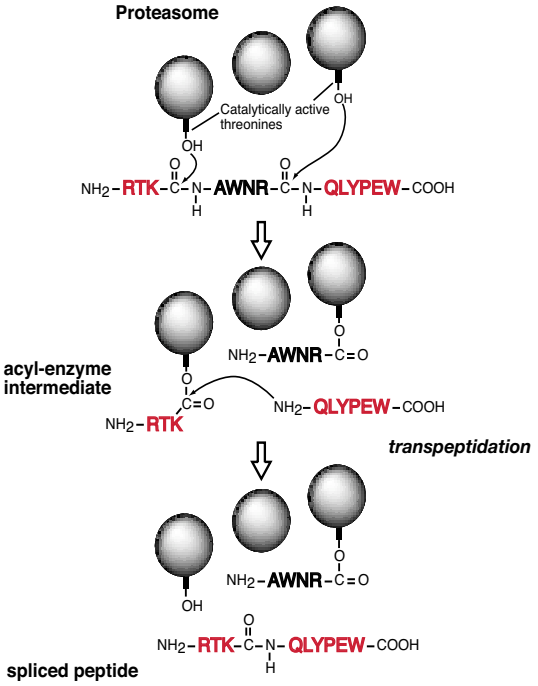

Figure 1. Model of the peptide-splicing reaction in the proteasome. The active site of the catalytic subunits of the proteasome is made up of the side-chain of a threonine residue, which initiates proteolysis by performing a nucleophilic attack on the carbonyl group of the peptide bond. An acyl-enzyme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

the standard proteasome and the immunoproteasome, which is found in antigen-presenting cells and cells exposed to interferon-gamma, and contains three inducible catalytic subunits ß1i, ß2i and ß5i instead of the standard catalytic subunits ß1, ß2 and ß5. We found that both proteasomes were able to splice peptides, but their relative efficiency was different for each peptide, depending on the major cleavage sites. This is consistent with the transpeptidation model of splicing.

NEW PROTEASOME TYPES THAT ARE INTERMEDIATE BETWEEN THE STANDARD PROTEASOME AND THE IMMUNOPROTEASOME

B. Guillaume, V. Stroobant, A. Busse

Using a series of novel antibodies recognizing catalytic subunits of the human proteasome in their native conformation, we also identified proteasomes that are intermediate between the standard proteasome and the immunoproteasome. They contain only one (ß5i) or two (ß1i and ß5i) of the three inducible catalytic subunits of the immunoproteasome. These intermediate proteasomes represent 30-54% of the proteasome content of human liver, colon, small intestine and kidney. They are also present in human tumor cells and dendritic cells. We studied the processing of a series of antigenic peptides by these intermediate proteasomes, and identified two tumor antigens that are processed exclusively either by intermediate proteasomes ß5i or by intermediate proteasomes ß1i-ß5i.

PRODUCTION OF AN ANTIGENIC PEPTIDE BY INSULIN-DEGRADING ENZYME

N. Parmentier, V. Stroobant

We studied a proteasome-independent peptide derived form tumor protein MAGE-A3, and we identified insulin-degrading enzyme as the protease producing both the C-terminus and the N-terminus of this peptide (4). This peptide, with sequence EVDPIGHLY, is presented by HLA-A1 and has been widely used in clinical trials of cancer vaccines. Insulin-degrading enzyme is a cytosolic metallopeptidase not previously known to play a role in the class I processing pathway. Cytotoxic T lymphocyte recognition of tumor cells was reduced after metallopeptidase inhibition or IDE silencing.

Separate inhibition of the metallopeptidase and the proteasome impaired degradation of MAGE-A3 proteins, and simultaneous inhibition of both further stabilized MAGE-A3 proteins. These results suggest that MAGE-A3 proteins are degraded along two parallel pathways that involve either the proteasome or IDE and produce different sets of antigenic peptides presented by MHC class I molecules.

MODULATION OF TUMOR ANTIGEN EXPRESSION BY INFLAMMATORY CYTOKINES

E. De Plaen, O. Kholmanskikh

We recently observed that treating some melanoma cell lines with the inflammatory cytokine IL-1ß leads to a 4- to 10-fold decrease in the level of Microphtalmia-associated transcription factor (MITF-M) (5). This effect is NF-kB and JNK-dependent. MITF-M regulates the expression of melanocyte differentiation genes such as Melan-A, tyrosinase and gp100, which encode antigens recognized on melanoma cells by autologous cytolytic T lymphocytes (CTL). Accordingly, treating some melanoma cells with IL-1ß reduced by 40-100% their ability to activate such anti-melanoma CTL.

TUMORAL IMMUNE RESISTANCE THROUGH TRYPTOPHAN DEGRADATION

L. Pilotte, P. Larrieu, V. Stroobant

An important factor limiting the efficacy of immunotherapy is the development of mechanisms allowing tumors to resist or escape immune rejection. Immune resistance mechanisms often involve modulation of the tumoral microenvironment resulting in local immunosuppression. We described one such mechanism, based on the expression by tumor cells of Indoleamine 2,3-dioxygenase (IDO), a tryptophan-degrading enzyme inducing a local tryptophan depletion that severely affects T lymphocyte proliferation (6). Our data in a preclinical model indicate that the efficacy of therapeutic vaccination of cancer patients could be improved by concomitant administration of an IDO inhibitor. In collaboration with the group of Olivier Michielin in Lausanne, we described new compounds able to inhibit IDO in the micromolar range, not only in enzymatic assays but also in cellular assays (7). These compounds will be further optimized with the goal of developing drug candidates. In parallel, a large effort was launched in collaboration with academic and industrial partners to identify IDO inhibitors by high-throughput screening of a chemical library and by structure-based drug design.

We have produced a monoclonal antibody against human IDO, which we used to characterize IDO expression in normal and tumoral tissues. Although others reported high expression of IDO in dendritic cells of murine tumor-draining lymph nodes, our results in humans indicate that a subset of mature human dendritic cells express IDO but these cells are present in normal lymph nodes and not enriched in tumor-draining lymph nodes. However, we observed expression of IDO in a high proportion of human tumors, confirming our initial observation.

NEW PRECLINICAL MODELS FOR CANCER IMMUNOTHERAPY

C. Powis de Tenbossche, (in collaboration with C. Uyttenhove, de Duve Institute and A.-M. Schmitt-Verhulst, CIML, Marseille)

We have devised a mouse melanoma model, in which we can induce melanoma in 70% of mice injected with tamoxifen (8). These tumors express the tumor antigen encoded by cancer-germline gene P1A. They can be either highly

pigmented and indolent, or unpigmented and highly aggressive. We observed a correlation between aggressive tumor progression and the occurrence of exacerbated systemic inflammation, involving disruption of secondary lymphoid organs, extramedullary hematopoiesis and accumulation of immature myeloid cells, which may contribute to tumoral immune resistance (9).

Cancer-germline genes, which encode tumor antigens of the MAGE-type, are expressed at a low level in the thymus, possibly inducing some level of central immune tolerance that may explain the poor immunogenicity of many of the antigens encoded by these genes. To address this issue, we produced mice that are knockout for cancer-germline gene P1A. These mice are normal and fertile. Their ability to develop an immune response against the P1A-encoded antigen is slightly higher than the wild-type mice, resulting in a better ability to reject P1A-expressing tumors spontaneously. Analysis of the repertoire of TCR genes revealed some differences in Vß gene usage. This result is consistent with the deletion of high affinity T cells recognizing P1A-encoded antigens in wild-type mice. We conclude that there is a limited central tolerance towards antigens encoded by cancer-germline genes.

TRANSCRIPTOMIC STUDIES IN SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) AND RHEUMATOID ARTHRITIS (RA)

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

SLE is a systemic autoimmune disorder of unknown etiology, characterized by the activation of autoreactive CD4 T and B cells directed against constituents of the chromatin and the production of pathogenic antinuclear antibodies. Recently, several groups identified a characteristic interferon signature in PBMC from SLE patients, i.e. the over-expression of genes induced by type I interferons. We compared SLE synovitis and the synovitis of osteoarthritis (OA) and RA using high-density oligonucleotide spotted microarrays. Our results indicate that SLE arthritis is characterized by a very specific molecular signature that is distinct from that of OA and RA, with up-regulation of interferon (IFN)–inducible genes and down-regulation of genes involved in extracellular matrix (ECM) homeostasis. The latter observation is probably associated with the less destructive character of SLE compared to RA and OA. These results have immediate clinical applications for the differential diagnosis of arthritis.

We also performed global gene expression studies on synovial biopsies from RA patients treated with TNF blockers. We identified gene signatures in pre-treatment synovial tissue that predict the absence of response to TNF blockade. Not surprisingly, these genes can be induced in synovial cells by other inflammatory cytokines (such as IL-1b or IL-17), alone or in combination with TNF-a (10). These observations can be useful to guide therapeutic decisions.

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REGULATION OF T LYMPHOCYTE FUNCTION IN TUMORS

The identification of tumor-specific antigens recognized by T lymphocytes on human cancer cells has elicited numerous vaccination trials of cancer patients with defined tumor antigens. These treatments have induced T cell responses but have shown a low clinical efficacy in tumor-bearing melanoma patients. We believe that progress depends on unraveling the different blockages for efficient tumor destruction. The analysis of the T cell responses of melanoma patients vaccinated against tumor antigens has led us to consider the possibility that the limiting factor for therapeutic success is not the intensity of the anti-vaccine response but the degree of anergy presented by intratumoral lymphocytes. We aim at a better understanding of dysfunctions of the immune system in tumors and more precisely T lymphocyte dysfunctions.

PREVIOUS WORK IN OUR GROUP: IDENTIFICATION OF TUMOR ANTIGENS RECOGNIZED BY T CELLS

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

Genes such as those of the MAGE family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They are therefore referred to as "cancer-germline genes". They encode tumor specific antigens, which have been used in therapeutic vaccination trials of cancer patients (2). A large

set of additional cancer-germline genes have now been identified by different approaches, including purely genetic approaches. As a result, a vast number of sequences are known that can code for tumor-specific shared antigens. The identification of a larger set of antigenic peptides, which are presented by HLA class I and class II molecules and recognized on tumors by T lymphocytes, could be important for therapeutic vaccination trials of cancer patients and serve as tools for a reliable monitoring of the immune response of vaccinated patients (3-4). To that purpose, we have used various approaches that we have loosely named "reverse immunology", because they use gene sequences as starting point (5).

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

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

The identification of specific tumor antigens recognized by T lymphocytes on human cancer cells has elicited numerous clinical trials involving vaccination of tumor-bearing cancer patients with defined tumor antigens. These treatments have shown a low clinical efficacy. Among metastatic melanoma patients, about 5% show a complete or partial clinical response following vaccination, whereas an additional 10% show some evidence of tumor regression without clear clinical benefit. We believe that progress depends on unraveling the different blockages for efficient tumor destruction.

The tumors of the patients about to receive the vaccine, already contain T cells directed against tumor antigens. Presumably these T cells are exhausted and this impaired function is maintained by immunosuppressive factors present in the tumor. The T cell response observed in some vaccinated patients reinforce an hypothesis proposed by Thierry Boon and Pierre Coulie: anti-vaccine CTL are not the effectors that kill the tumor cells but their arrival at the tumor site containing exhausted anti-tumor CTL, generates conditions allowing the reawakening of the exhausted CTL and/or activation of new anti-tumor CTL clones, some of them contributing directly to tumor destruction (2, 6). Accordingly, the difference between the responding and the non-responding vaccinated patients is not the intensity of their direct T cell response to the vaccine but the intensity of the immunosuppression inside the tumor. It is therefore important to know which immunosuppressive mechanisms operate in human tumors.

Human CD8 tumor-infiltrating T lymphocytes show impaired IFN-γ secretion

Human CD8 tumor-infiltrating T lymphocytes (TIL) were isolated from tumor ascites or solid tumors and compared with T lymphocytes from blood donors. They were unable to secrete INF-γ or other cytokines after non-specific stimulation with anti-CD3 and anti-CD28 antibodies (7). TCR were observed to be distant from CD8 on the cell surface of TIL, whereas TCR and CD8 co-localized on blood T lymphocytes (Figure 1).

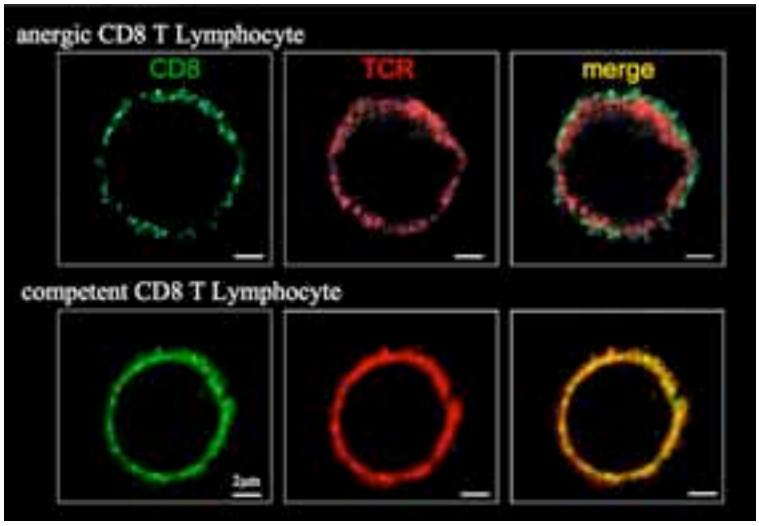

Figure 1. TCR and CD8 do not co-localize on recently stimulated CTL without effector functions.

Why do galectin-3 ligands improve human TIL function?

We have attributed the decreased IFN-γ secretion to a reduced mobility of T cell receptors upon trapping in a lattice of glycoproteins clustered by extracellular galectin-3. Indeed, we have shown that treatment of TIL with N-acetyllactosamine (LacNAc), a galectin-competitor ligand, restored this secretion (Figure 2). Our working hypothesis is that TIL have been stimulated by antigen recently, and that the resulting activation of T cells could modify the expression of enzymes of the N-glycosylation pathway, as shown for murine T cells. The recently activated TIL, compared to resting T cells, could thus express surface glycoproteins decorated with a set of glycans that are either more numerous or better ligands for galectin-3. Galectin-3 is an abundant lectin in many solid tumors and carcinomatous ascites, and can thus bind to surface glycoproteins of TIL and form lattices that would thereby reduce TCR mobility. This could explain the impaired function of TIL. The release of galectin-3 by soluble competitor ligands would restore TCR mobility and boost IFN-g secretion by TIL. We recently strengthened this hypothesis by showing that $CD8^+$ TIL treated with an anti-galectin-3 antibody, which could disorganize lattice formation, had an increased IFN-γ secretion.

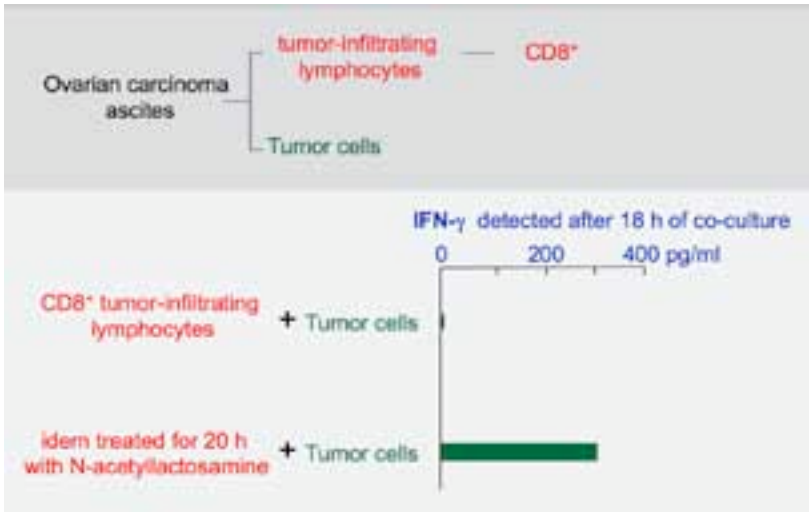

Figure 2. Treatment of tumor-infiltrating lymphocytes with a galectin ligand reverses anergy.

Towards a clinical trial combining vaccination and galectin-binding polysaccharides

These observations indicate that ex vivo human tumor-infiltrating lymphocytes can recover their effector functions with galectin-competitor ligands and suggest that treatment of cancer patients with galectin-competitor ligands could correct the anergy of TIL. It is possible that peptide vaccination combined with local injection of a galectin-competitor ligand will be more effective at producing tumor regression than vaccination alone. Galectin competitor ligands, e.g. disaccharides lactose and LacNAc, are rapidly eliminated in urine, preventing their use in vivo. Other compounds that could block interactions between galectin-3 and glycoproteins are under development by several groups. We found that a plant-derived polysaccharide, which is in clinical development, detached galectin-3 from TIL and boosted their IFN-γ secretion. Importantly, we observed that not only $CD8^+$ TIL but also $CD4^+$ TIL that were treated with this polysaccharide secreted more IFN-γ upon ex vivo re-stimulation. We therefore intend to pursue clinical trials involving the use of these polysaccharides in combination with anti-tumoral vaccination.

DETECTION OF ANTI-MAGE-A3 REGULATORY T CELLS IN VACCINATED MELANOMA PATIENTS

The presence of regulatory T cells, which have the ability to dampen immune responses, could participate in an immunosuppressive environment in tumors. We have analyzed the blood T cells from 14 vaccinated melanoma patients who carried the HLA-DP4 allele and whose tumor expressed MAGE-A3 (8). The vaccines involved various antigens present on melanoma cells and all contained the MAGE-$A3_{243-258}$ peptide presented to T cells by HLA-DP4. The vaccines were either a mix of peptides without adjuvant, autologous mature dendritic cells loaded with peptides, or a MAGE-A3 protein mixed with adjuvant and combined with peptides. Our approach, outlined in Figure 3, involved the ex vivo selection of $CD4^+$ T cells that were labeled by DP4 MAGE-A3 tetramer and amplified under clonal conditions. A total of 197 $tetramer^+$ stable clones were isolated from 10 out of the 14 patients. Each of the 197 $tetramer^+$ $CD4^+$ T cell clones recognized the MAGE-A3.DP4 antigen. Anti-MAGE-A3.DP4 cells were found in 1 out of 2 patients injected with a mix of peptides without adjuvant, in 3 out of 6 patients injected with peptide-loaded dendritic cells, and in each of the 6 patients injected with a MAGE-A3 protein mixed with adjuvant and peptides. The frequencies in the blood samples collected after at least four vaccines ranged from $2x10^{-6}$ to $2x10^{-3}$ among the $CD4^+$ blood T lymphocytes. We found no correlation between the frequencies of anti-MAGE-A3.DP4 T cells and the clinical evolution of the patients, but the very small number of patients and the diversity of the vaccines preclude any conclusion.

Interestingly, 12 out of 197 clones expressed CD25 in resting state. This $CD4^+CD25^+$ phenotype was evocative of T cells with suppressive activity, known as regulatory T cells. Because the antigen recognized by our $CD4^+$ T cell clones was known, we designed a suppression assay where the potential regulatory T cell clone and an indicator T cell clone are each stimulated with their specific antigen presented by irradiated EBV-B cells. These twelve $CD25^+$ clones had a high capacity to suppress in vitro the proliferation of another T cell clone. Eleven of them had an high FOXP3 expression at rest and an unmethylated *FOXP3* gene. They secrete upon stimulation no IFN-γ, IL-2, IL-4, IL-5 or IL-10, but they produce active TGF-β. Their suppressive activity in vitro seems partly attributable to their secretion of active TGF-β. These regulatory T cell clones represent about 5% of the anti-MAGE-A3.DP4 T

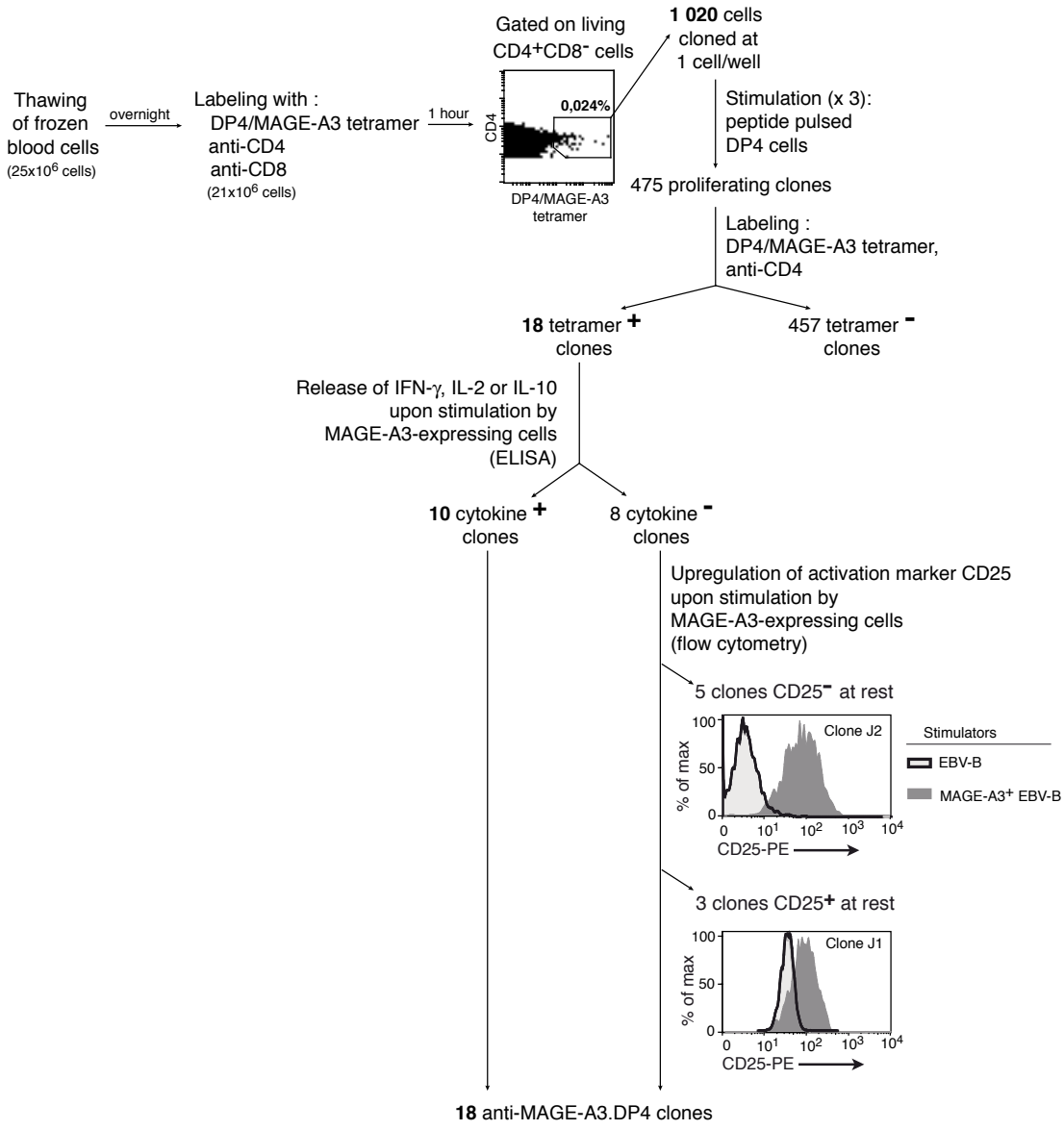

Figure 3. Example of the procedure used to obtain anti-MAGE-A3.DP4 T cell clones from blood cells of vaccinated patients. The numbers indicated correspond to an experiment performed with blood cells of patient EB97 collected after the sixth vaccination.

cell clones that we have isolated. In addition to these clones with an unmethylated *FOXP3* gene, several CD25 clones had a significant but lower suppressive activity, expressed *FOXP3* in the resting state but *FOXP3* demethylation was not observed.

This work is the first to describe the presence of anti-vaccine regulatory T cells not only on the basis of markers such as FOXP3, but also on the basis of their suppressive activity in vitro. FOXP3 seems of doubtful value as unique marker for regulatory T cells, due to its transient expression in some activated non-

regulatory T cells and also in some of our resting T cells without suppressive activity. Exclusive to T cells with a suppressive activity and a stable expression of FOXP3 is the demethylation of an intronic sequence of FOXP3. Thus, a quantitative DNA methylation analysis of FOXP3 based on RT-PCR could become a routine technique to identify what, in our opinion, are the best regulatory T cell candidates.

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IMMUNOTHERAPY ANALYSIS GROUP

The identification in the early 1990's of human tumor-specific antigens that are recognized by T cells led to widespread attempts at vaccinating cancer patients with these antigens to induce tumor regression (1). Vaccination of metastatic melanoma patients with MAGE peptides resulted in evidence of tumor regression in about 15% of the patients, with complete and partial clinical responses in only 7% of the patients (2). Why did most patients fail to respond? A plausible hypothesis was that the anti-MAGE T cell response was too weak. However, none of the numerous attempts to boost the efficacy of the vaccines, for instance with adjuvants or by the use of dendritic cells, resulted in improvement of the clinical efficacy.

Our analysis of a few responding patients led us to a different hypothesis. Several groups reported a long time ago that human tumors contain tumor-infiltrating lymphocytes (TILs). These T lymphocytes could be extracted from the tumors and were capable of destroying tumor cells in vitro after short-term cultivation in the presence of IL-2. However, inside the tumor, they must have become inactive ("anergic") at one point, since the tumor is progressing. We made the paradoxical observation that, when vaccination causes complete tumor regression, the T lymphocytes directed against the vaccine antigen are present in the tumor in very small numbers, clearly insufficient to cause rejection. But they reactivate the "anergic" tumor infiltrating T lymphocytes that are present in the tumor in large numbers as a result of a past spontaneous immune response of the patient. It is these reactivated TILs which are capable of destroying the bulk of the tumor cells (3, 4). Our new hypothesis is that what differentiates the non-regressing and the regressing patients is not their direct response to the vaccine but the severity of the anergy of their TILs.

Accordingly, our new strategy to improve anti-tumoral vaccination is to supplement it with a local treatment of the tumor with various cytokines and Toll receptor agonists effectors, to reduce the immunosuppression in the tumor. This should facilitate the action of the anti-vaccine T lymphocytes which provide the "spark" firing the regression response. We are exploring this approach in a mouse model and we hope that positive results will lead to new small-scale clinical trials.

INDUCING REJECTION OF NORMALLY TOLERATED GRAFTS IN THE H-Y MOUSE MODEL.

C. Bourdeaux, I. Jacquemart, B. Lethé, C. Lurquin, C. Uyttenhove, A. Van Pel, T. Boon

Female CBA mice do not reject male skin grafts, even though they are able to mount a specific anti-H-Y cytolytic T cell response. We tested several approaches to break this tolerance. Immunization with irradiated male lymphoblasts did not induce rejection of established skin grafts, but repeated local injections of a low dose of IL-12, combined with IFN_, caused rejection in all mice. This was also the case when IL-12 was combined with ligands of Toll-Like Receptors 3, 7 or 9. IL-1_, IL-18 and IL-2 were incapable of inducing rejection on their own, but synergized effectively with IL-12.

To follow lymphocyte infiltration into the graft after cytokine injections, we used adoptive transfer of transgenic T cells bearing a receptor specific for a H-Y antigen. When transferred to a female CBA before grafting, naïve transgenic CD8 T cells did not infiltrate or reject the graft. But a low dose of IL-12 injected locally stimulated infiltration of the graft, whereas IFN_ had no effect. Our results suggest that local delivery of cytokine combinations may enhance the efficacy of anti-tumoral vaccination.

Contrary to naïve transgenic anti-HY CD8 T cells, adoptively transferred activated transgenic CD8 T cells infiltrated the grafts. Interestingly, skin grafts were rejected when the transferred T cells had been stimulated in vitro with H-Y peptide pulsed B-blasts, but were not rejected when the CD8 T cells had been stimulated in vitro with antibodies against CD3 and CD28. We tried to identify the critical difference between the CD8 T cells stimulated with the specific antigen and those stimulated with anti-CD3 antibodies. Comparison of the expression levels of cell surface markers by FACS analysis revealed that a CD8 T cell population stimulated with the H-Y peptide is composed of a larger proportion of CD62L-negative effector memory T lymphocytes, known to circulate in the periphery, whereas the population stimulated with anti-CD3 is composed of more CD62L-positive T lymphocytes, known to home to secondary lymphoid tissues. The functional avidity of the two activated CD8 T cell populations was tested by their capacity to secrete IFNg in response to various concentrations of H-Y peptide. No apparent avidity difference between the two populations was observed.

TGF-SS2 IN MELANOMA CELLS.

B. Lethé, C. Lurquin, in collaboration with J. Stockis

To understand the anergy of tumor-infiltrating lymphocytes in many patients, we have analyzed tumor samples of a series of metastatic melanoma patients, who were vaccinated with the MAGE-3.A1 tumor-specific antigen, for their expression of a series of genes coding for tumor-specific antigens, differentiation antigens and genes putatively associated with immunosuppression. Tumor samples for 26 patients who did not show any tumor regression were compared with 14 patients who showed significant tumor regression. For the gene coding for transforming growth factor beta 1 (TGF-b1) the ratio of non-regressors to regressors was 1.6, for TGF-b2 it was 2.4 and for TGF-b3 it was 1.1. One tumor cell line that was analyzed similarly produced a high amount of the TGF-b2 polypeptide (latent form) and showed SMAD2 phosphorylation, indicating that TGF-b2 is active. Strikingly, this cell line was exceptional being unable to support proliferation of autologous anti-tumoral cytolytic T cells. These results suggest that TGF-b1 and b2 may participate to an immunosuppressive environment protecting melanoma tumors against immune rejec

CONTROL OF IL-9 PRODUCTION BY T HELPER LYMPHOCYTES.

C. Uyttenhove, J. Van Snick

Since its discovery, IL-9 has been considered a TH2 cytokine but recent analyses involving intracellular cytokine staining of naïve CD4 T cells activated in vitro indicated that IL-9 did not fit the TH2 paradigm. In a publication by Marc Veldhoen and Brigitta Stockinger (5), to which we participated by producing the first antibody suitable for intracellular IL-9 detection, it was indeed shown that IL-9 producing cells were selectively induced when CD4 T cells were stimulated in the presence of TGF-b and IL-4, suggesting the existence of a novel T helper subset designated "TH9" (5). We have now provided evidence that these TH9 cells also develop when in vivo primed T cells are restimulated in vitro with the priming antigen in the presence of TGF-b and IL-4, indicating that this cytokine milieu can completely orient an established immune response to selective IL-9 production.

We then started a search for factors other than IL-4 that could stimulate IL-9 production in the presence of TGF-b. We found that TGF-b combination with IL-1a, IL-1b, IL-18, and IL-33 had equivalent IL-9 stimulating activities in all mouse strains tested, including IL-4- and IL-4-R-deficient animals. As IL-9 levels were much lower in TH2 cultures (IL4 + anti-TGF-b) or TH17 cultures (TGF-b + IL-6), these results identify TGF-b/IL-1 and TGF-b/IL-4 as the main control points of IL-9 synthesis.

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THERAPEUTIC VACCINATION AND TUMOR EXPRESSION PROFILING GROUP

Cancer cells express tumor-specific antigens that can be targeted by cytolytic T lymphocytes (CTL). Our group has developed small scale clinical immunotherapy trials in which patients with advanced cancer, often metastatic melanoma, have been treated repeatedly with a vaccine containing one or several defined tumor antigens that are expressed by their tumor. Whilst these vaccines have shown no toxicity and have been associated with evidence of tumor regression in some patients, their overall anti-tumoral effect is poor. It is thought that this limited effectiveness is a consequence of the acquisition and selection by the tumor of immunosuppressive features, that allow it to resist immune mediated rejection. We are now focusing on new therapeutic approaches that combine a vaccine and an immunomodulatory treatment that is aimed at reverting the immunosuppressive tumor environment.

THERAPEUTIC VACCINATION WITH MAGE TUMOR ANTIGENS

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

Many if not all human cancers express tumor antigens that can be recognized by T cells. These antigens are small peptides derived from endogenous proteins presented at the surface of tumor cells by HLA molecules. In vitro, cytolytic T lymphocytes (CTL) lyse selectively tumor cell lines that express the cognate antigen. MAGE antigens are examples of tumor-specific antigens. They are encoded by MAGE genes, which are expressed in many different tumor types, such as melanoma, non-small cell lung cancer, bladder cancer, head-and-neck cancer and multiple myeloma. These genes are not expressed in normal somatic tissues. They are expressed in germline cells such as spermatogonias, which are devoid of surface HLA class I molecules, and thus can not present MAGE antigens on their surface. Thus, MAGE antigens are good candidates for cancer vaccines, because they are strictly tumor-specific, and are shared by various cancers.

Based on these findings, we have launched phase I/II clinical trials in which patients with advanced cancer, mainly melanoma, were repeatedly immunized with one or more tumor-specific antigens (Figure 1). 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 res-

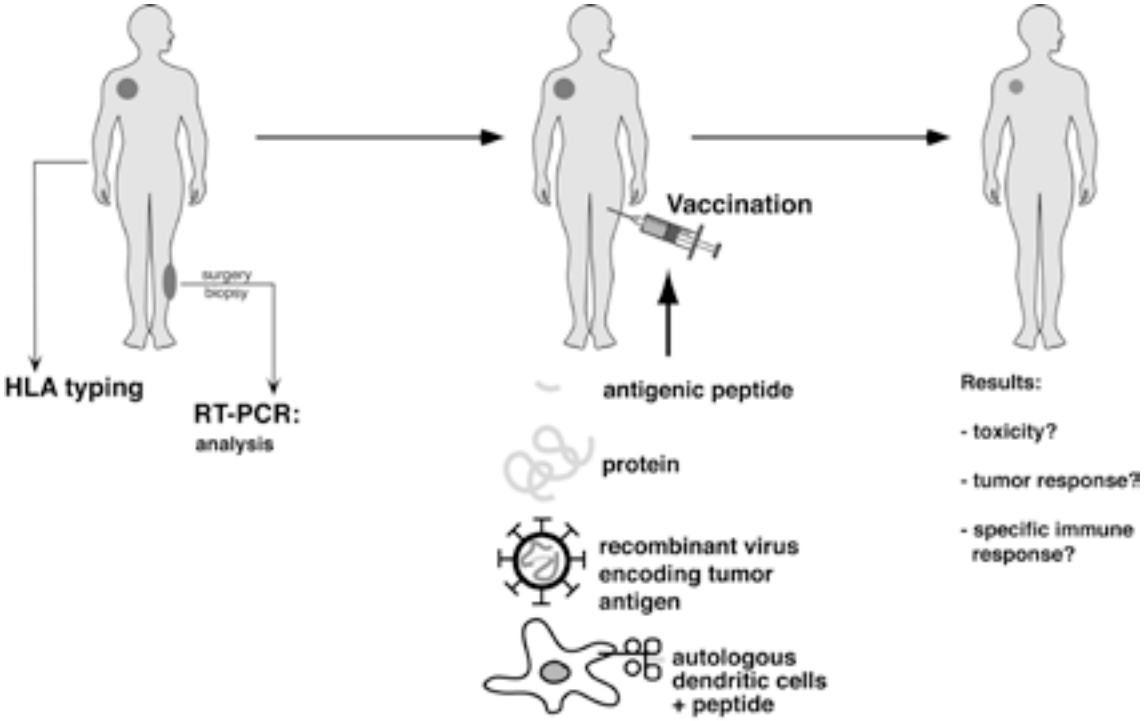

Figure 1. Principle of anti-tumor vaccination with a defined antigen : The first step is to determine if the patient's tumor cells express the tumor antigen. This can be determined by HLA typing, and by RT-PCR analysis of a tumor sample. Selected patients will receive repeated injections of a vaccine with the antigen. Usually this vaccine is a synthetic peptide, a recombinant protein, a recombinant virus coding for the antigen, or dendritic cells derived from the patient's blood and forced to express this antigen. The effect of vaccinations on tumor progression is then assessed. Their immunogenicity is analyzed by comparing the frequency of anti-vaccine CTL in the pre and post-immune blood.

ponse to the vaccine antigen occurred, and by determining whether immunological and clinical responses are correlated. Secondly, these trials allow crucial biological material to be collected from vaccinated patients. Blood samples provide T cells, which allow to analyze the spontaneous and vaccine-induced immune response against tumor antigens. Tumor samples allow to analyze the interaction between cancer cells and immune cells in the tumor environment.

Different immunization modalities, such as immunization with peptides, or with the MAGE-3 recombinant protein, both with or without adjuvant, or with the ALVAC recombinant viral vector, have already been tested. They are all devoid of severe toxicity. A minority of vaccinated melanoma patients (about 10 to 20%) showed regression of metastatic lesions. This frequency is far beyond the reported incidence of spontaneous regressions of melanoma metastases, estimated at 0.2-0.3%, indicating that these regressions are linked to the vaccinations (Figure 2). However, only 5% of the patients experience a true clinical benefit. Some of the remissions have lasted for several years. There is no evidence that one of the vaccines tested is more effective against the tumors than the others. CTL responses were detected in a minority of patients vaccinated either with peptides or with the ALVAC virus. These responses were often weak, and, in the case of the MAGE-3.A1 antigen, were observed mostly in patients who had tumor regressions.

The most likely explanation for the poor effectiveness of cancer vaccines is the fact that tumors have acquired the ability to resist destruction by anti-tumoral T cells, following repetitive in vivo challenge with spontaneously

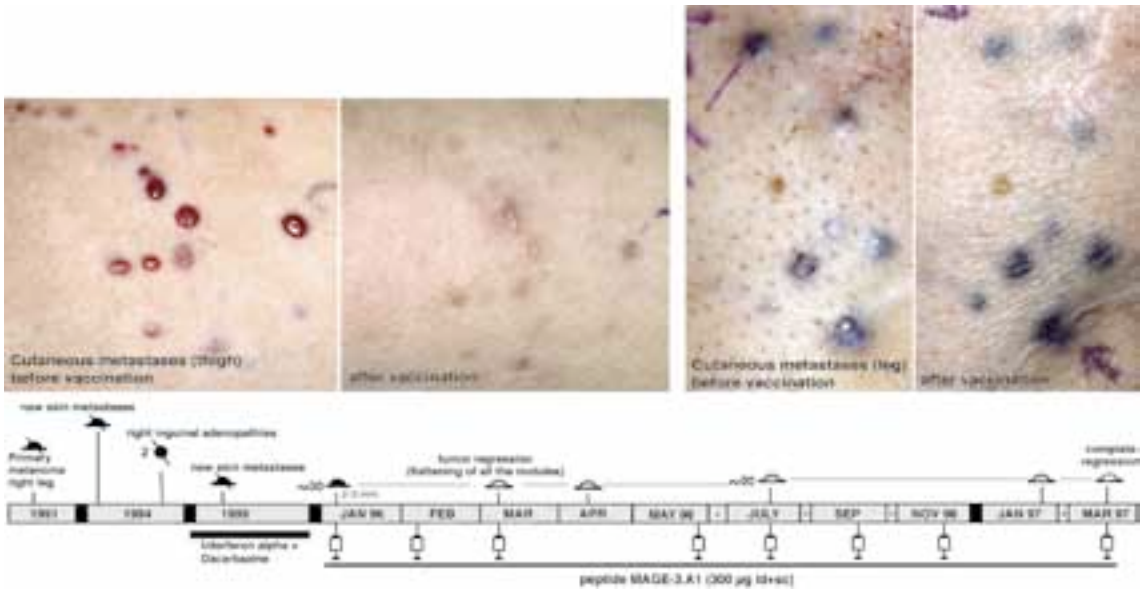

Figure 2. Example of a complete regression of cutaneous metastases in a melanoma patient after repeated vaccinations with the MAGE-3.A1 peptide given without immunological adjuvant.

occurring immune responses. The molecular mechanisms of tumor resistance remain largely unknown, despite the many candidates that have been proposed. Importantly, we have observed that tumor-infiltrating lymphocytes (TIL) purified from melanoma metastases can rapidly recognize and kill autologous tumor cells in vitro, indicating that tumor resistance is a consequence of local immunosuppression in the tumor environment.

We are now setting up a new clinical trial in which patients with advanced melanoma, including superficial metastases, will receive repeated peptide vaccinations, as previously, but now combined with a local immunomodulatory treatment. The latter will associate immunostimulatory cytokines and/or TLR ligands, administered in or close to 1 or 2 superficial metastases. The precise treatment will be chosen on the basis of skin graft rejection experiments performed in a murine model that mimics the situation observed in tumors. The vaccine is aimed at inducing new anti-tumoral T lymphocyte responses, and the local treatment is aimed at modifying the tumor environment in favor of effective tumor rejection.

Recent work in the laboratory has shown that the state of anergy that characterizes tumor-associated T cells can be reversed pharmacologically (see the contribution of Pierre van der Bruggen in this report). Inhibitors of galectin-3, a protein produced by cancer cells that is able to interfere with effective T cell activation, have been able to reactivate anergic T cells in vitro. We are currently developing a new clinical trial, in which patients with advanced melanoma will receive a treatment combining a peptide vaccine and an experimental drug that inhibits galectin-3. We hope that this combined treatment will result in the induction of anti-tumoral CTL responses by the vaccine, in synergy with the inhibition of tumor resistance by the galectin-3 inhibitor.

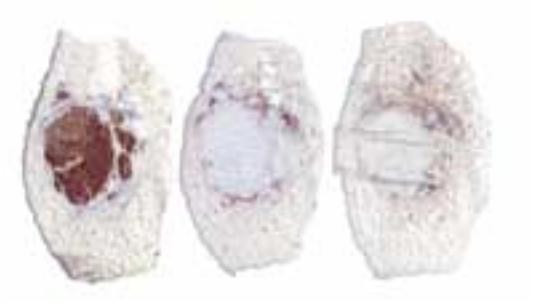

Figure 3. Cryosections obtained from a cutaneous melanoma metastasis were stained with antibodies directed against either melanoma cells (left), T lymphocytes (middle) and macrophages (right), and counterstained with hematoxylin. The corresponding cells appear in red. These images show clearly that the inflammatory cells do not infiltrate, but rather surround the tumor mass.

EXPRESSION PROFILING OF TUMOR SAMPLES FROM VACCINATED PATIENTS

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

Using the microarray technology, we have established the gene expression profile of a series of tumor samples, mainly cutaneous metastases, obtained from melanoma patients. This approach is combined with systematic immunohistological analysis of adjacent cryosections, using antibodies directed against tumor cells, T and B cells, macrophages, blood vessels, and various molecules involved in inflammatory reactions (Figure 3). In addition, adjacent cryosections are analyzed by performing laser capture microdissection of selected areas, e.g. T cell rich areas, followed by RT-qPCR analysis of T cell, macrophage, melanoma cell and inflammation associated genes. These complementary approaches help us to characterize the inflammatory events that take place inside the metastases, and to understand the interaction between the tumor cells and the inflammatory cells at the tumor site.

ANALYSIS OF MELANOCYTE-DERIVED TUMORS BY NON-LINEAR OPTICS TECHNIQUES

Our group collaborates with several other European groups in a project aimed at developing innovative imaging microscopy and endoscopy approaches that might improve cancer diagnosis. These approaches are based on spectroscopical analysis of tissue sections or samples illuminated with one or several laser beams of selected frequencies, using so-called Raman and Coherent Anti-Stokes Raman Spectroscopy (CARS) microscopes. The Raman and CARS effects involve light reflection that depends on the molecular bonds present in the illuminated sample. The objective is to identify spectral signatures associated with tumor cells, which would allow to detect and quantify these cells in conventional microscope preparations without staining. Eventually, this technique coupled to an endoscope might allow to detect the presence of cancer cells in vivo. The current project is focused on melanoma and benign naevus samples, and is at an early, proof-of-feasibility stage of development.

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CYTOKINES IN IMMUNITY AND INFLAMMATION

The cytokine group studies the biological activities of cytokines in inflammatory and tumoral processes, as well as the molecular mechanisms underlying these activities. Our work focuses on Interleukin-9 (IL-9) and IL-22, two cytokines discovered at the Branch. IL-9 is produced by a particular T lymphocyte population, called TH9, and plays a role in immune responses against intestinal parasites and asthma. Dysregulation of IL-9 signalling is also implicated in tumoral transformation and this process has been studied in an in vitro tumorigenesis model, leading to the identification of oncogenic mutations of the JAK1 gene. IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, upregulates the production of acute phase reagents and antibacterial proteins in the liver, the lung and intestinal mucosae, and in the skin. IL-22 appears to play a key role in wound healing and skin inflammation processes such as psoriasis. The role of these cytokines in inflammation is currently being investigated using transgenic and gene-targeted mice for these cytokines and their receptors, and by using an original strategy of anti-cytokine vaccination.

INTERLEUKIN 9

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. 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 two essential properties of IL-9: its activity on mast cells and eosinophils with consecutive implications in asthma, and its tumorigenic potential in T lymphocytes.

IL-9-transgenic mice : parasite infections and asthma

Although IL-9 overproduction is viable and IL-9 transgenic mice did not show any major abnormality at the first look, they 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. Conversely, blocking IL-9 activity resulted in a failure to expel T. muris parasites and in decreased eosinophilic responses against the parasite (1).

The other side of the coin was the discovery that IL-9 overexpression, such as that characterizing the IL-9 transgenic animals, resulted in bronchial hyperresponsiveness upon exposure to various allergens. Our observations showed that IL-9 promotes asthma through both IL-13-dependent and IL-13-independent pathways (2), as illustrated in Figure 1. The potential aggravating role of IL-9 in asthma was confirmed by genetic analyses performed by others and pointing to both IL-9 and the IL-9 receptor genes as major candidate genes for human asthma. In addition, we found that asthma patients produce increased amounts of IL-9. Phase II clinical trials using anti-IL-9 antibodies produced in our laboratory have been initiated in collaboration with Medimmune.

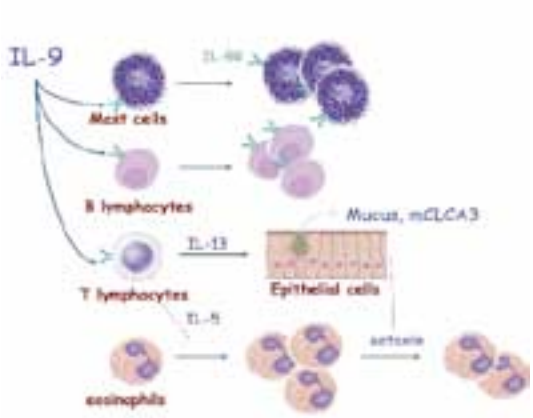

Figure 1. Direct and indirect activities of IL-9 in asthma. IL-9 acts directly on mast cells and B lymphocytes to induce an expansion of these cells and IgE production. IL-9 promotes the proliferation eosinophils indirectly, by upregulating IL-5 production by T cells. Upregulation of IL-13 production by T cells meadiates IL-9 activities on lung epithelial cells, including mucus production and secretion of eotaxin, which is required to recruit eosinophils into the lungs (2).

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 (3). 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 RECEPTOR AND SIGNAL TRANSDUCTION

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 gc. This induces the phosphorylation of the JAK1 and JAK3 tyrosine kinases, which are associated with IL-9R and gc, 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. 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.

ROLE OF JAK OVEREXPRESSION AND MUTATIONS IN TUMOR CELL TRANSFORMATION

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

Recently, we elucidated the mechanism responsible for the second step of this tumoral transformation process, as we found that the majority of the cytokine-independent tumorigenic clones acquired an activating mutation in the kinase or in the pseudokinase domain of JAK1 (Figure 2).

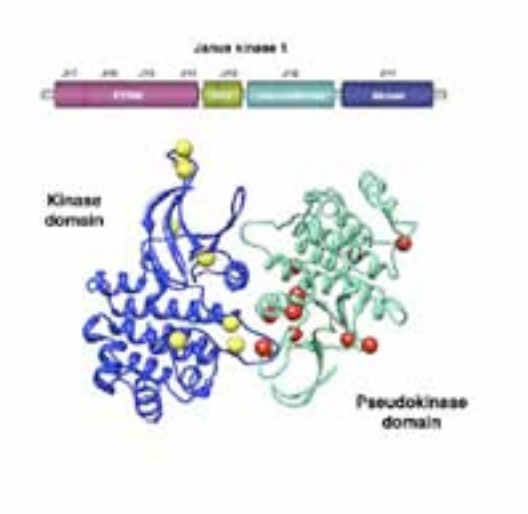

Figure 2. Localization of JAK1 activating mutations in the kinase and pseudokinase domains.

In parallel to these observations, in collaboration with Prof. Marco Tartaglia (University of Rome), we identified activating mutations in JAK1 in 20% of T-cell acute lymphoblastic leukemia (T-ALL) and in 3% of B-ALL patients, confirming the relevance of our in vitro model-derived JAK1 mutations for human malignancies. Further analysis of human ALL samples showed that JAK1-mutated leukemias share a type I IFN transcriptional signature, suggesting that these mutants do not only activate growth-promoting pathways, but also antiviral pathways. Expression of these activating JAK1 mutants in murine hematopoietic cell lines recapitulated this signature in the absence of IFN, but also strongly potentiated

the in vitro response to IFN. Finally, we also showed in an in vivo leukemia model that cells expressing mutants such as JAK1(A634D) are hypersensitive to the anti-proliferative and anti-tumorigenic effect of type I IFN, suggesting that type I IFNs should be considered as a potential therapy for ALL with JAK1 activating mutations (6).

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

Searching for genes specifically regulated by IL-9 in lymphomas, we identified 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. Despite its structural homology with IL-10, IL-22 fails to recapitulate any of IL-10 biological activities. Biological activities of IL- 22 include the induction of acute phase proteins in liver (7) and protection against experimental hepatitis and colitis (L. Dumoutier, unpublished results). Among the different T cell subset, IL-22 was found to be preferentialy produced by TH17 cells raising some speculations about its potential role in autoimmune processes (8). Experiments are in progress to establish the role of this cytokine in skin inflammatory processes such as psoriasis, by using mice defiicent either in IL-22 or in its receptor.

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 (7). All receptor complexes for IL-10-related cytokines include a long chain and a short chain, based on the length of the cytoplasmic domain of these transmembrane proteins. IL-10Rß is a typical short chain component, with only 76 amino acids in the cytoplasmic domain, whose main function seems to consist in recruiting the Tyk2 tyrosine kinase. In addition to IL-10R ß, IL-22 signalling requires the expression of a long chain protein, called IL-22R and comprising a 319 amino acid long cytoplasmic domain. This chain associates with JAK1, and is responsible for the activation of cytoplasmic signalling cascades such as the JAK/STAT, ERK, JNK and p38 MAP kinase pathways. An unexpected featur of the IL-22R chain is the fact that the C-terminal domain of this receptor is constitutively associated with STAT3, and that STAT3 activation by this receptor does not require the phosphorylation of the receptor, in contrast to the mechanism of STAT activation by most other cytokine receptors (9).

Beside this cell membrane IL-22 receptor complex composed of IL-22R and IL-10Rß, we identified a protein of 231 amino acid, showing 33 % amino acid identity with the extracellular domains of IL-22R, respectively, but without any cytoplasmic or transmembrane domain. This soluble receptor has been named IL-22 binding protein (IL-22BP), because it binds IL-22 and blocks its activities in vitro, demonstrating that this protein can act as an IL-22 antagonist.

The crystal structure of IL-22, alone and bound to its cellular receptor IL-22R or to its soluble receptor IL-22BP has been characterized in collaboration with Prof. Igor Polikarpov (University of Sao Paulo) and is illustrated in Figure 3.

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

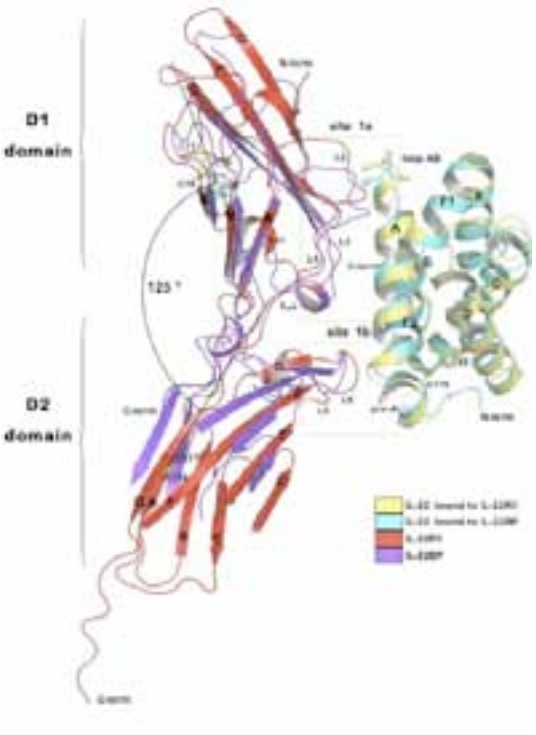

Figure 3. Comparison of IL-22/IL-22BP and IL-22/IL-22R1 binding interfaces. Superposition of IL-22/IL-22BP (cyan/purple blue) and IL-22/IL-22R1 (yellow/red) crystal structures shows their binding interfaces outlined by boxes.

ANTI-CYTOKINE VACCINATION

Beside conventional gene targeting strategies, that were used in our lab to generate mice deficient in the IL-9R, in IL-22 or in IL-22R, we developed a new strategy of anti-cytokine vaccination leading to the production in vaccinated mice of anti-cytokine autoantibody that block the biological activities of endogenous cytokines. Neutralizing auto-antibodies against cytokines such as IL-9, IL-12 and IL-17 have been induced upon vaccination with the autologous cytokines chemically coupled with OVA (IL-9, IL-17) or with the Pan DR T helper epitope PADRE (IL-12). This strategy contributed to demonstrate the role of IL-9 in an intestinal helminth infection (1), of IL-12 in atherosclerosis and of IL-17 in experimental autoimmune encephalomyelitis. More recently, we developed a new procedure of anti-cytokine vaccination by taking advantage of tumor cells as a vaccine against peptides presented at their surface in fusion with a human transmembrane protein. These vaccination methods represent simple and convenient approaches to knock down the in vivo activity of soluble regulatory proteins, including cytokines and their receptors, and are currently validated with additional targets in inflammatory models.

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STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS

Cytokines and their receptors are critical for the formation of mature blood cells and for the function of the immune system. We study the structure and function of several cytokine receptors, such as those for erythropoietin (Epo), thrombopoietin (Tpo), Granulocyte Colony Stimulating Factor (G-CSF), and interleukins (IL) 2 and 9. Activation of these receptors is triggered by cytokine-induced changes in receptor dimerization/oligomerization, which lead to the activation of cytosolic Janus tyrosine kinases (JAK). Regulation by JAK kinases of receptor traffic, the assembly of cell-surface receptor complexes, the mechanisms of dimerization of transmembrane (TM) and cytosolic juxtamembrane (JM) domains, and mechanisms of JAK catalytic activation are major points of interest. The laboratory identified constitutively active mutants of JAK2, JAK1 and Tyk2 and of thrombopoietin receptor and is actively investigating the mechanisms by which JAK2 V617F and thrombopoietin receptor W515 mutants induce, in humans, Myeloproliferative Neoplasms, such as Polycythemia Vera, Essential Thrombocythemia or Primary Myelofibrosis

THE MECHANISMS BY WHICH THE MUTANT JAK2 V617F INDUCES POLYCYTHEMIA VERA AND OTHER MYELOPROLIFERATIVE NEOPLASMS IN HUMANS

A. Dusa, C. Pecquet

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

Polycythemia Vera (PV), or the Vaquez disease, is characterized by excessive production of mature red cells and sometimes of platelets and granulocytes. Erythroid progenitors in PV are hypersensitive to Epo or independent of

erythropoietin (Epo) for proliferation and differentiation. Strikingly, the traffic of TpoR is defective in myeloid progenitors from PV. A hint that JAK2 or JAK2-binding proteins may be involved in PV came when we showed that the wild type JAK2 strongly promotes the maturation and cell-surface localization of TpoR, the very process that is defective in PV (2).

In collaboration with William Vainchenker at the Institut Gustave Roussy in Paris, we have been involved in the discovery of the JAK2 V617F mutation, that is responsible for >98% of Polycythemia Vera and for >50% of Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) cases (3, 4). The mutation in the pseudokinase domain alters a physiologic inhibition exerted by the pseudokinase domain on the kinase domain and allows the mutated JAK2 to bind and activate EpoR, TpoR and G-CSFR in the absence of cytokines (Figure 1). Saturation mutagenesis at position V617 showed that not only Phe, but also Trp, Leu, Ile and Met can activate JAK2, although Trp is the only mutation that exhibits comparable activity with V617F (5). The homologous V617F mutations in JAK1 and Tyk2 also enable these kinases to be activated without ligand-binding

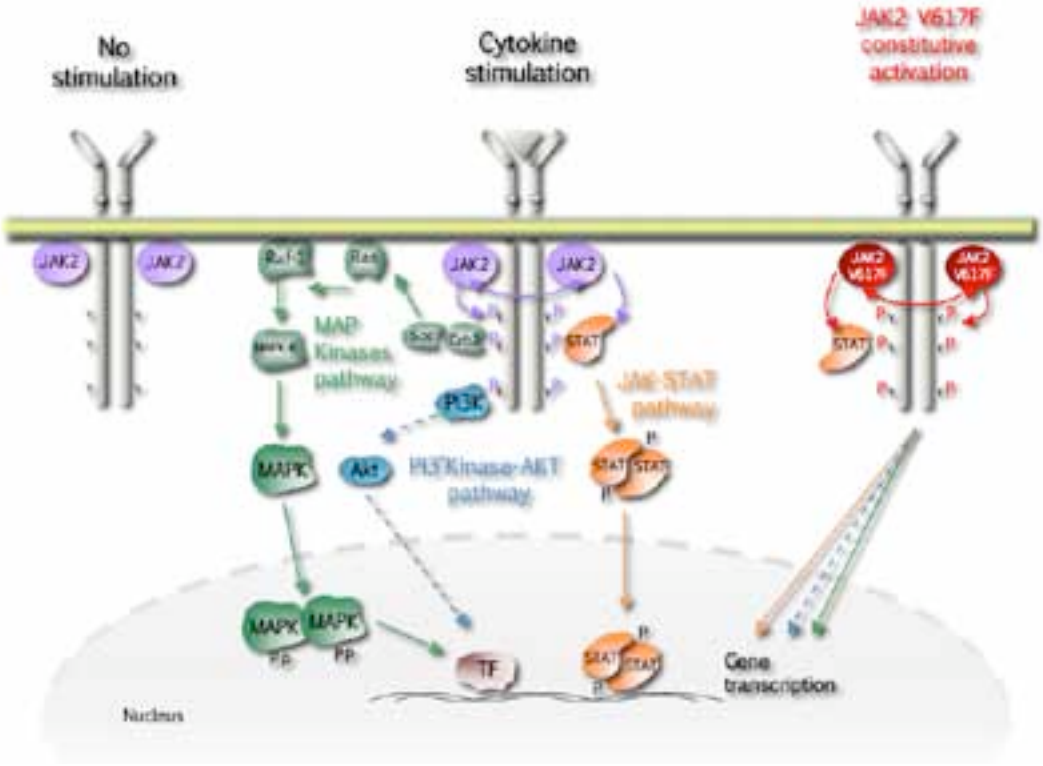

Figure 1. In the absence of cytokine ligands, cytokine receptors (left complex) are preassembled with tyrosine kinases JAK (Janus kinases) in inactive complexes. Cytokine binding to the extracellular domains of receptors (middle complex) induces a conformational change which allows the appended JAKs to cross-phosphorylate and activate each other. In turn, JAKs phosphorylate tyrosine residues (Py) on the cytosolic regions of receptors, which attract SH2- and PTB-containing signaling proteins. These proteins become themselves phosphorylated and either translocate to the nucleus to regulate gene expression (such as STATs, Signal Transducers and Activators of Transcription) or initiate kinase signaling cascades (such as Mitogen Activated Protein-Kinases, MAPK, phosphatydylinositol-3-kinase, PI3K, and Akt). The mutant JAK2 V617F binds to the cytosolic domains of receptors and can trigger signaling in the absence of any cytokine binding to the extracellular receptor domain (right complex). As a consequence, signaling is induced permanently and myeloid progenitors survive, proliferate and differentiate in an uncontrolled manner. (Jean-Michel Heine)

to cytokine receptors (4). Similarly, at least Trp, Leu and Ile also can activate JAK1, besides Phe, at the homologous V658 position. These results suggested that point mutations in JAK proteins might be involved in different forms of cancers (1). An example of such involvement is the identification of mutants in the pseudokinase domain of JAK1 in ~ 20% of adult T-lymphoblastic leukemia.

Involvement of TpoR in myeloproliferative diseases

C. Pecquet, M. Girardot, J.-P. Defour

When the protein sequences of TpoR and the closely related EpoR were aligned, we realized that the TpoR contains a unique amphipathic motif (RWQFP) at the junction between the transmembrane and cytosolic domains. Deletion of this motif (delta5TpoR) results in constitutive activation of the receptor (6), suggesting that these residues maintain the receptor inactive in the absence of TpoR. Mutagenesis of the RWQFP motif showed that W515 (W508 in the murine) is the key residue maintaining human TpoR normally inactive. In vivo, in bone marrow reconstituted mice, the delta5TpoR and TpoR W515A induce massive expansion of platelets, neutrophils and immature erythroid progenitors and myelofibrosis by day 45 (7) (Figure 2). We predicted that mutations in the amphipathic motif W515 may exist in patients with myelofibrosis (6). Indeed, residue W515 has been found to be mutated to either leucine or lysine by the groups of D. G. Gilliland and A. Tefferi. Why the phenotype induced by TpoR W515 mutants is much more severe than that of JAK2 V617F is under investigation in our group. We recently established that the myelofibrosis phenotype induced by TpoR W515 mutants depends on cytosolic Y112 of TpoR, and appears to involve excessive MAP-kinase signaling (7). Thus, small molecules targeting phosphorylated Y112 might be useful in the treatment of myelofibrosis.

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

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

N. Caceres, A. Dusa, J.-P. Defour

Epo binding to the erythropoietin receptor (EpoR) results in survival, proliferation and differentiation of erythroid progenitors into mature red blood cells. In the absence of Epo, the cell-surface EpoR is dimerized in an inactive conformation, which is stabilized by interactions between the TM sequences. Epo binding to the extracellular EpoR domain induces a conformational change of the receptor, which results in the activation of cytosolic JAK2 proteins (8).

To identify the residues that form the interface between the receptor monomers in the activated EpoR dimer we have replaced the EpoR extracellular domain with a coiled-coil dimer of α-helices (9). Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (one), d (four), the register of the coiled-coil a-helices is imposed on the downstream TM a-helix and intracellular domain.

When each of the seven possible dimeric orientations were imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR, only two fusion proteins stimulated the proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells (9). Since the predicted dimeric interfaces of the two active fusion proteins are very close, a unique dimeric EpoR conformation appears to be required for activation of signaling. In this active conformation TM residues L241 and L244 and JM residue W258 are predicted to be in the interface.

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

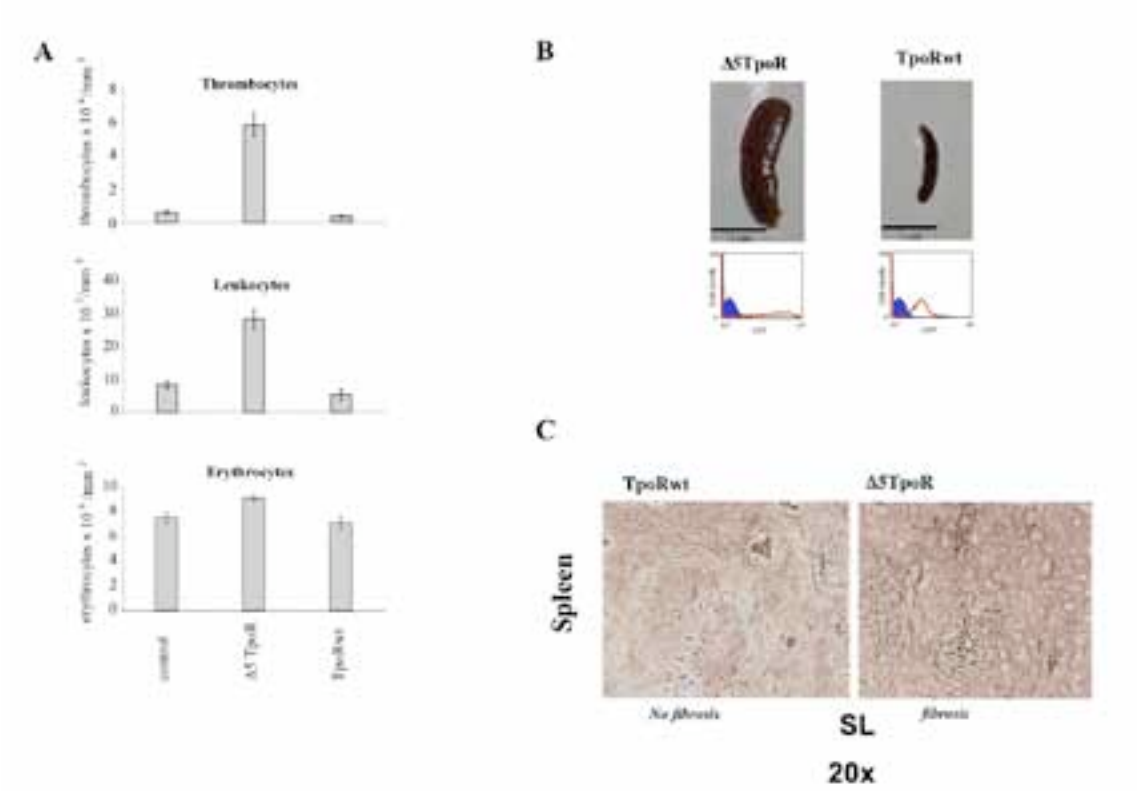

Figure 2. Bone marrow adoptive transfer in lethally-irradiated mice with hematopoietic stem cells expressing the constitutively active Δ5TpoR induces severe myeloproliferative disorder, splenomegaly and fibrosis of the spleen. Δ5TpoR is a mutant where the amphipathic RW515QFP motif is deleted, which results in constitutive activation of receptor signaling. (A) Peripheral cell counts recorder 45 days after reconstitution indicates leukocytosis and thrombocytosis induced by Δ5TpoR. (B) Splenomegaly was induced by Δ5TpoR at day 45 post reconstitution. The spleen size in TpoRwt mice was equivalent to that in control healthy mice. Green fluorescence protein (GFP) levels were equal after transduction, but enhanced migration to the spleen and proliferation explain the high GFP levels in Δ5TpoR spleens. (C) Histology of spleen sections of mice reconstituted with the indicated constructs. Silver staining (SL) for reticulin indicates fibrosis of the spleen in the Δ5TpoR mice (C. Pecquet and J. Staerk).

STRUCTURE AND FUNCTION OF JUXTAMEMBRANE AND TRANSMEMBRANE SEQUENCES OF CYTOKINE RECEPTORS

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

We have previously shown that the EpoR as well as a fraction of IL2/IL9 receptors exist on the cell surface as a preformed ligand-independent inactive dimers (homomeric and heteromeric in the case of IL2/IL9 receptor complexes). For the EpoR, transmembrane domain interactions stabilize the inactive dimer at the surface and the EpoR TM sequence is an example of TM dimer based on purely hydrophobic sequences (Proc. Natl Acad. Sci USA 2001, 98, 4379-84; EMBO J., 1999, 18, 3334-47). We study potential transmembrane interactions in the context of other transmembrane proteins, such as TpoR, G-CSFR. We use cell surface immunofluorecence co-patching of differentially epitope tagged receptors in order to determine the ligand-independent state of cell surface complexes. Preformed cytokine receptor oligomers might be important for supporting signaling by mutated JAKs in the absence of ligand. In addition to cytokine receptors, we study the role of transmembrane dimerization in the amyloidogenic processing of Amyloid Precursor Protein (APP) in collaboration with the group of Prof. Jean-Noel Octave. We identified three Gly-X-X-X-Gly motifs in the juxtamembrane and transmembrane domain of APP and showed that these motifs promote amyloidogenic processing of APP (J. Biol. Chem. 2008 283, 7733).

TRAFFIC OF CYTOKINE RECEPTORS TO THE CELL-SURFACE

C. Pecquet, R.-I. Albu

We have observed that, in hematopoietic cells, over-expression of JAK proteins leads to enhanced cell-surface localization of cytokine receptors (i.e. EpoR TpoR, IL9R, IL2R, gc). For some receptors, the effect of the cognate JAK is to promote traffic from the endoplasmic reticulum (ER) to the Golgi apparatus, while for others, such as the TpoR, JAK2 and Tyk2 also protect the mature form of the receptor from degradation by the proteasome, and thus JAKs enhance the total amount of cellular receptor (2). In collaboration with Pierre Courtoy, we are employing confocal microscopy of epitope tagged receptors in order to define the precise intracellular compartments where receptors and JAKs interact. Our working hypothesis is that the N-terminus FERM domain of JAK proteins exerts a generic pro-folding effect on cytosolic domains of cytokine receptors. Furthermore, the extracellular fibronectin type III modules of TpoR (D1, D2, D3 or D4) appear to be critical for efficient cell surface localization of the receptor. The W515K activating mutation was introduced in TpoR mutants that lack segments of the extracellular domain; these mutants are normally impaired in their traffic. Selection in the absence of Tpo leads to enhanced cell surface localization of N-terminally truncated TpoR mutants that also posses the activating W515K mutation. Microarray experiments are determining which chaperones or signaling proteins are overexpressed in selected cells, that might stimulate TpoR traffic.

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

M. Girardot

Cytokine stimulation of cytokine receptors, induces transient activation of the JAK-STAT pathway. In contrast, oncogenic forms of re-

ceptors or of JAKs (JAK2 V617F) transmit a continuous signal which results in constitutive activation of STAT proteins. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors or JAKs in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously (1, 9). A similar picture has been noted in patient-derived leukemia cells. The critical question is which genes are specifically regulated by constitutively active STAT proteins in leukemic cells. Using chromatin immunoprecipitation and sequencing of native promoters bound by STAT5 we noted that, in transformed cells, STAT5, and mainly STAT5B, can also bind to low affinity N4 (TTCNNNNGAA) DNA sites, not only to the high affinity N3 sites, which are characteristic of ligand-activated STAT5. We are attempting to identify the promoters actually bound by STAT proteins in living cells in physiologic and pathologic situations. We identified one specific target gene of constitutive active STAT5B signaling in megakaryocytes of MPN patients, namely Lipoma Prefered Partner (LPP) (10), a gene found to be translocated in rare leukemias. LPP is the host gene for miR-28, which we found to down-modulate TpoR translation, impair megakaryocyte differentiation. miR-28 is pathologically overexpressed in 30% of MPNs (10). Tagets of miR-28, such as E2F6, are critical cell cycle regulators that might influence the phenotype of myeloproliferative disorders (10), thus linking spcific gene induction by constitutive STAT signaling to phenotype of disease.

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Links

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