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). 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 described four spliced peptides, two of which are spliced in the reverse order (3). One of these peptides also contains two additional post-translational modifications, resulting in the conversion of asparagines into aspartic acids, through a process a N-glycosylation/deglycosylation. We also compared the efficiency of peptide splicing by

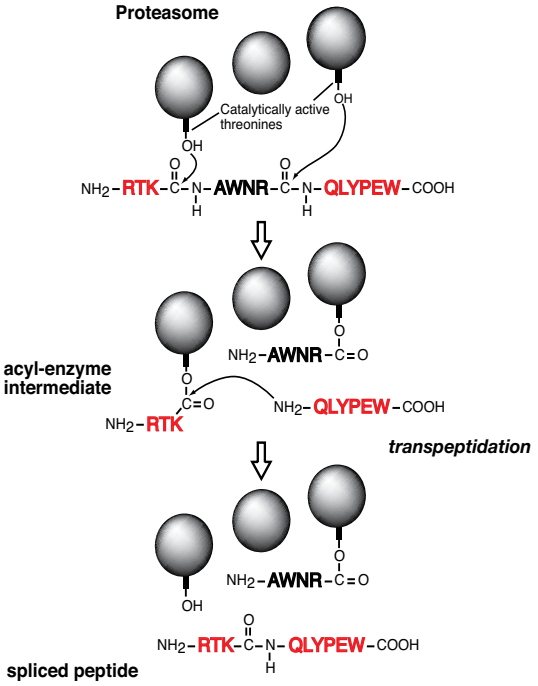

Figure 1. Model of the peptide-splicing reaction in the proteasome. The active site of the catalytic subunits of the proteasome is made up of the side-chain of a threonine residue, which initiates proteolysis by performing a nucleophilic attack on the carbonyl group of the peptide bond. An acyl-enzyme 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 are able to splice peptides. However, their ability to produce a given spliced peptide varies according to their ability to perform the relevant cleavages to liberate the fragments to splice.

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

B. Guillaume, V. Stroobant, A. Busse, E. De Plaen

Using a series of novel antibodies recognizing catalytic subunits of the human proteasome in their native conformation, we identified proteasomes that are intermediate between the standard proteasome and the immunoproteasome (4). 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. Other functional aspects of these intermediate proteasomes are currently evaluated.

ANTIGENIC PEPTIDE PRODUCTION 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 (5). This peptide, with sequence EVDPIGHLY, is presented by HLA-A1 and has been widely used in clinical trials of cancer vaccines. Insulin-degrading enzyme (IDE) 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 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) (6). 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 (7). 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 (8). 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-base drug design. Two promising families were identified.

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 made transgenic mice, in which we can

induce melanoma in 70% of mice injected with tamoxifen (9). These tumors express the tumor antigen encoded by cancer-germline gene P1A. These tumors 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. Current efforts aim to devise therapeutic vaccination approaches able to induce tumor rejection despite this abnormal inflammation.

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. 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.

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

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

In our previous work, we performed high-density transcriptomic studies in synovial biopsies from patients with RA, prior to initiation of therapy with TNF inhibitors. We identified, and confirmed by immunohistochemistry on synovial tissue, several markers of resistance to therapy. Strikingly, we demonstrated that the genes associated with poor response to TNF blockade are induced in synovial fibroblasts by the addition of pro-inflammatory cytokines such as TNF-alpha, IL-1beta or combinations of these cytokines with IL-17, thereby suggesting that these molecules play a role in the mechanisms of resistance to TNF blockade in RA.

IL-7R is one of the genes associated with poor-response to TNF inhibitors in RA. We demonstrated that exposure of RA synovial fibroblasts to pro-inflammatory cytokines induce the production of a soluble form of IL-7R (sIL-7R). sIL-7R can be detected in the sera of patients with RA, and we demonstrated that serum titers are strongly associated with resistance to TNF blockade. We are currently extending these observations in additional cohorts of RA, but also SLE patients. We hypothesize that sIL-7R is a marker of end-organ inflammation (joint, kidney,…), and exposure to pro-inflammatory cytokines.

Finally, we performed high-density transcriptomic studies in synovial biopsies of RA patients prior to and after rituximab (depleting anti-CD20 antibody) therapy. We found that rituximab inhibits IL-17 production in the synovium and induces genes involved in repair mechanisms (10). We also identified synovial markers of response to rituximab therapy, which are different from those predicting response to TNF blockers, thereby demonstrating the specificity of our findings, and their relevance for future clinical applications.

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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 AND CD4 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 tumor-infiltrating T lymphocytes show impaired IFN-γ secretion

Both human CD8 and CD4 tumor-infiltrating T lymphocytes (TIL) were isolated from tumor ascites or solid tumors and compared with T lymphocytes from blood donors. TIL secrete low levels of INF-γ and other cytokines upon non-specific stimulation with anti-CD3 and anti-CD28 antibodies (7-10). TCR were observed to be distant from the co-receptors on the cell surface of TIL, either CD8 or CD4, whereas TCR and the co-receptors co-localized on blood T lymphocytes (Figure 1).

Reversing the anergy of tumor-infiltrating T lymphocytes with galectin ligands

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-

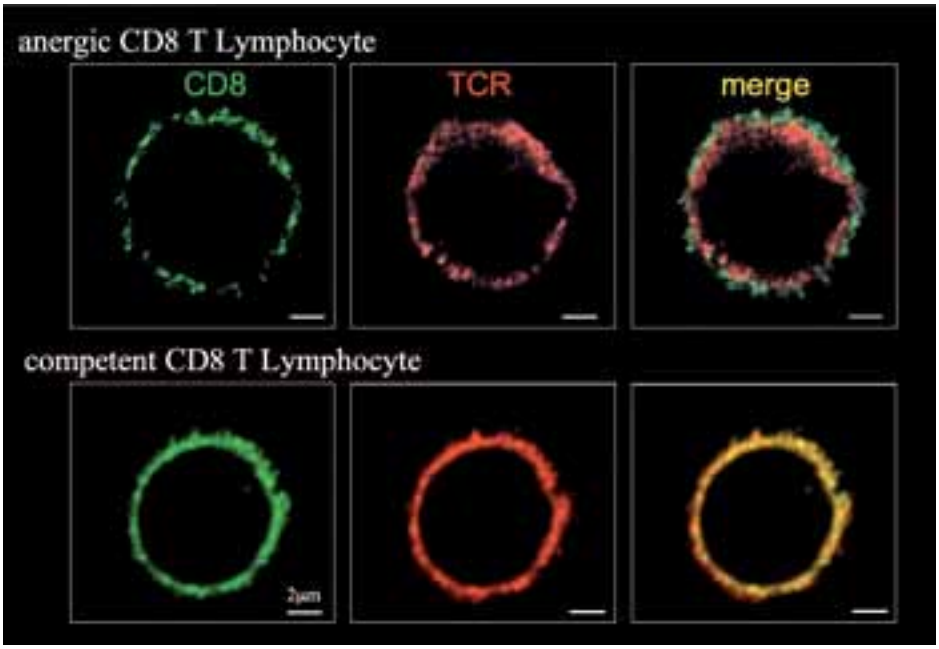

Figure 1. TCR and CD8 do not co-localize on CD8 T cells with impaired functions.

acetyllactosamine (LacNAc), a galectin-competitor ligand, restored this secretion (Figure 2). Our working hypothesis is that TIL have been stimulated by antigen chronically, 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 chronically 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

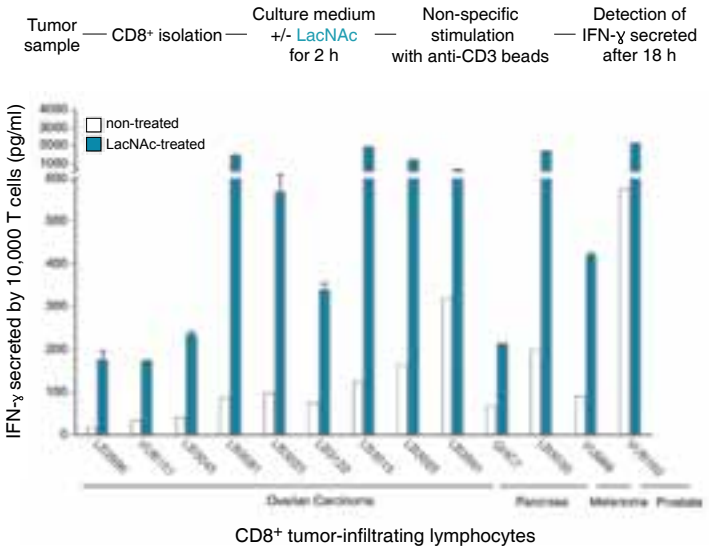

Figure 2. Treatment of tumor-infitrating lymphocytes with a galectin ligand reverses anergy.

restore TCR mobility and boost IFN-γ secretion by TIL. We recently strengthened this hypothesis by showing that both CD4 and CD8 TIL that were treated with an anti-galectin-3 antibody, which could disorganize lattice formation, had an increased IFN-γ secretion compared to untreated cells.

Towards a clinical trial combining vaccination and galectin-binding polysaccharides

Galectin competitor ligands, *e.g.* disaccharides LacNAc, are rapidly eliminated in urine, preventing their use *in vivo*. We recently found that a plant-derived polysaccharide, currently 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. In tumor-bearing mice vaccinated with a tumor antigen, injections of this polysaccharide led to tumor rejection in half of the mice, whereas all control mice died. In non-vaccinated mice, the polysaccharide had no effect by itself. These results suggest that a combination of galectin-3 ligands and therapeutic vaccination may induce more tumor regressions in cancer patients than vaccination alone. Translation of these results to the clinic was unfortunately impossible because the company producing this polysaccharide got bankrupted. We recently identified another plant-derived polysaccharide that binds to galectins and was already used in combination with chemotherapy in phase II clinical trials in colorectal cancer patients. This compound was as effective as LacNAc in boosting the secretion of IFN-γ by treated TIL. A clinical trial with this new compound, in combination with anti-tumoral vaccination, will hopefully be launched in 2011 in clinical centers close to the Brussels Branch. We are currently trying to understand the very early activation events that are defective in TIL.

IS THE SPONTANEOUS ANTI-TUMOR T CELL RESPONSE OF BREAST CARCINOMA PATIENTS A CLINICAL PROGNOSTIC FACTOR ?

Several retrospective studies suggest a correlation between the survival of patients with ovarian or colorectal carcinoma and infiltration of their tumors by immune cells. So far, prospective data validating these observations do not exist. In collaboration with Dr Javier Carrasco (Grand Hôpital de Charleroi) and Dr Jean-Pascal Machiels (Cliniques Universitaires St-Luc), we set out a prospective study aimed at looking for a correlation between the clinical outcome of patients with non-metastatic breast carcinoma and their spontaneous anti-tumor T cell response. Considering our experience in quantitative approaches to detect very weak T cell responses in the blood of melanoma patients, Danièle Godelaine set out the evaluation of the frequencies of anti-tumor CD8 T lymphocytes in the blood of breast cancer patients recruited in the two clinical centers. Frequencies are evaluated by mixed lymphocyte-peptide cultures, carried out with HLA-A2 and A3-restricted peptides derived from HER2/neu and hTERT, two proteins usually overexpressed in breast tumors, followed by detection of specific cells with HLA-peptide tetramers. Blood samples are collected before and after surgery. Tumors removed at surgery are analyzed by immunohistology for infiltration by immune cells, and fragments are frozen for further analysis. This prospective study foresees to include 172 patients whose clinical evolution will be followed up over a 5-year period. So far, 25 patients have been included. Eight of them have a frequency against the targeted antigens ranging from 10^{-5} and 10^{-6} among blood CD8 T cells, whereas the frequency in healthy donors blood was estimated to be lower than $5x10^{-7}$. We hope to identify the patients with a better prognosis in order to offer them an adapted care avoiding unnecessary heavy treatments.

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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, as well as antibodies directed against inhibitory cytokines such as TGFß, 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 of skin grafts 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, F. Brasseur, I. Jacquemart, B. Lethé, C. Lurquin, C. Uyttenhove, 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. Repeated immunizations with male lymphoblasts did not induce rejection of established skin grafts. We tested local approaches to break this tolerance. Repeated local injections of a low dose of IL-12, combined with IFNα, caused graft rejection in all mice. This was also the case when IL-12 was combined with ligands against Toll-like receptors 3, 7 or 9 (TLR). Like IFNα, IL-1α, IL-18 and IL-2 were incapable of inducing rejection on their own, but synergized effectively with IL-12.

We tested combinations of agents that are approved for clinical use. Repeated local injections of a combination of low doses of IL-2, GM-CSF and IFNα with TLR7 ligands gardiquimod or imiquimod caused 100% rejection. The crucial components appear to be IL-2 and gardiquimod. A clinical trial has been launched in which a small number of patients with superficial lesions of metastatic melanoma will receive vaccinations with tumor antigens combined with a local treatment composed of the three cytokines available as registered medicines and Aldara, a cream containing imiquimod.

For this treatment to be therapeutically effective, it would be necessary that the rejection of the locally treated metastasis would result into an antitumoral T-cell expansion that would also eliminate other metastases. In CBA females carrying two male skin grafts, the local treatment of one graft which caused its complete rejection, induced, in a fraction of the mice, a systemic immune response sufficient to cause partial or complete rejection of a distant graft. We are presently exploring additional local and systemic treatments that may improve this distant rejection.

To improve our understanding of the action of the local agents, we follow the fate of naïve CD8 T-lymphocytes endowed with an anti-H-Y-Kk T-cell receptor, collected from transgenic mice and transferred into the grafted mice. With a PCR specific for the α chain gene of this receptor, we were able to assess that these lymphocytes concentrate in the graft as soon as 7 days following the onset of the local treatment with IL-2, GM-CSF, IFNα and imiquimod. In the absence of this treatment, these lymphocytes do not accumulate in the graft.

AMINE-REACTIVE OVA MULTIMERS FOR AUTO-VACCINATION AGAINST CYTOKINES AND OTHER IMMUNE MEDIATORS.

C. Uyttenhove (in collaboration with R. Marillier and J. Van Snick)

We have made significant progress in our efforts to develop auto-vaccines against cytokines as a tool for studying their functions in vivo and develop a panel of monoclonal antibodies of mouse origin against mouse and human cytokines with therapeutic perspectives. Self cytokines linked chemically to a non-self protein or genetically associated to a defined foreign sequence become immunogenic. The proposed rationale underlying this process is that the self-reactive B cell that has captured the complex or fusion protein will present foreign peptides on its MHC Class II membrane proteins and thus attract help from T cells reactive with the non-self structure.

Many gaps still exist in our understanding of the precise mechanisms involved in this auto-vaccination but we recently noted a positive correlation between immunogenicity and immunogen size in a series of anti-IL-12 vaccines that were fractionated according to size. This raised a dilemma, since the larger the complex,

also the greater the risk for structural alterations of the antigen.

To circumvent this problem, we tried a two-step procedure. We first made large ovalbumin (OVA) multimers by treating OVA with glutaraldehyde and, after purifying the polymerized products by size exclusion chromatography, reacted these with the target cytokine before saturating remaining glutaraldehyde sites with a pan-DR epitope peptide (PADRE) to maximize immunogenicity.

Using this procedure, mice were successfully immunized against the chemokine GCP-2/CXCL6, the cytokines GM-CSF, IL-17F, IL-17E/IL-25, IL-27, TGFß-1 and the matrix metalloproteinase-9 MMP-9/gelatinase B. Monoclonal Abs derived from such immunized mice would have obvious advantages for the study and control of the in vivo functions of these proteins. We therefore attempted to generate such reagents from our auto-vaccinated mice (5). Currently, we obtained a mAb specific for mouse and human TFGß-1, MTGFß-1.13A1, that is a potent inhibitor of TGFß-1 bioactivity; an anti- IL-17F mAb, MM17F-8F5, that abrogates the neutrophil chemotactic activity triggered by IL-17F in vitro; a mAb against GCP-2, that provided the first demonstration of the essential role played by this chemokine in rapid neutrophil mobilization after Leishmania major infection; and, finally, a mAb against mouse IL-27, MM27.7B1, that potently inhibited the bioactivity of this cytokine in vitro and is currently used to evaluate the contribution of IL-27 in anti-tumor activities.

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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). 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 their cognate antigen. 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 (Fig. 1). Different immunization modalities, such as vaccination with peptides like MAGE-3.A1 and NA17.A2, or with the MAGE-3 recombinant protein, both with or without adjuvant, or with an 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 (Fig. 2). This frequency is far beyond the reported incidence of spontaneous regressions of melanoma metastases, estimated at 0.2-0.3%, indicating that these regressions are linked to the vaccinations. However, only 5% of the patients experience a true clinical benefit. Some of the remissions have lasted for several years. There is no evidence that one of the vaccines tested is more effective against the tumors than the others. 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 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 local effect in the tumor environment. We are following two different approaches to try to improve these results: find more immunogenic vaccines, and combine vaccines with treatments that modify the tumor environment in favor of effective tumor rejection.

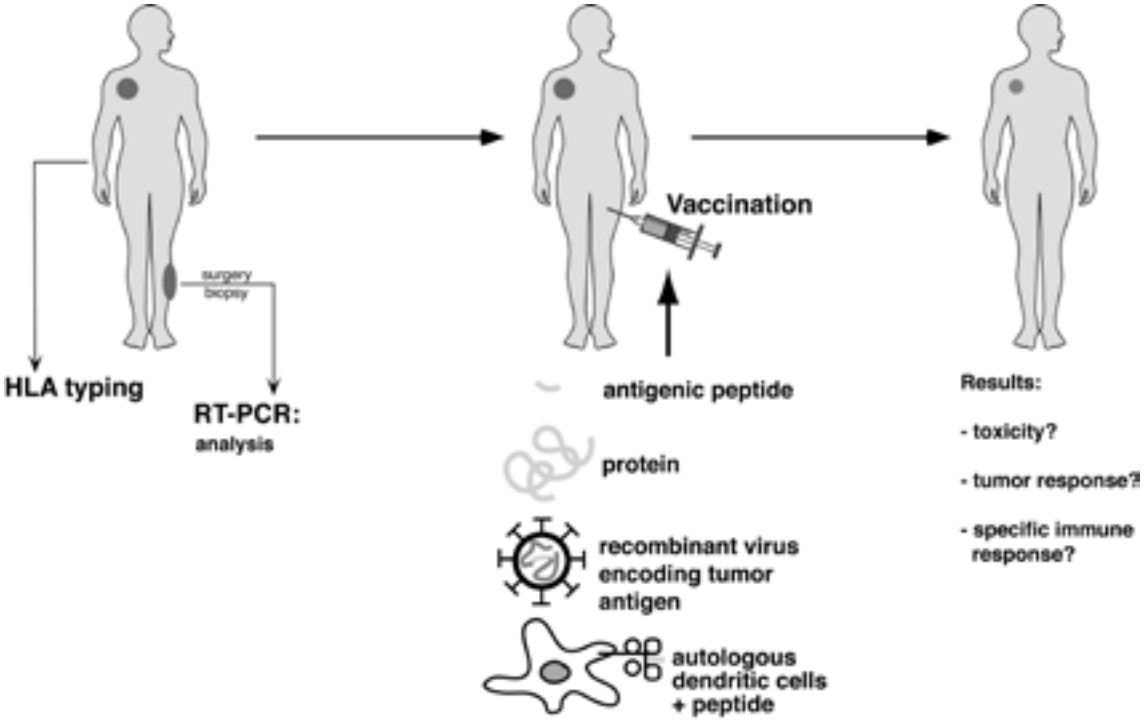

Fig. 1. Principle of anti-tumor vaccination with a defined antigen : The first step is to determine if the patient's tumor cells express the tumor antigen. This can be determined by HLA typing, and by RT-PCR analysis of a tumor sample. Selected patients receive repeated injections of a vaccine with the 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..

VACCINATION OF MELANOMA PATIENTS WITH THERAVAC, A NEW VACCINE CONCEPT

In collaboration with the groups of J.F. Baurain (Centre du Cancer, Cliniques Universitaires St Luc), P. Coulie, B. Van den Eynde, and Cl. Leclerc (Institut Pasteur, Paris France).

In a recently started phase I clinical trial, we are testing the safety, immunogenicity and anti-tumoral effect of a new promising vaccine called Theravac, developed at Institut Pasteur. Theravac is a recombinant chimeric protein vaccine aimed at targeting dendritic cells (DC) in vivo, and force them to express a Tyrosinase. A2 antigen, a peptide derived from the melanocyte and melanoma-specific tyrosinase protein. Theravac is derived from CyaA, a bacterial toxin that binds specifically to CD11b, an adhesion molecule expressed by dendritic cells and macrophages. Upon binding, a portion of the toxin is internalized and neutralizes its target cell in order to turn off innate immunity at the infectious site. In the recombinant vaccine protein, the toxin activity has been inactivated by insertional mutagenesis, and coupled to the Tyrosinase.A2 peptide. Thus, the unique advantage of this vaccine is its ability to target dendritic cells in vivo, with a putative higher immunogenicity as a consequence. Preclinical experiments have shown that Theravac has a very potent capacity to activate Tyrosinase.A2-specific CTL. In our clinical trial, patients with tyrosinase-expressing metastatic melanoma are immunized with repeated injections of Theravac, at increasing doses, in a classical phase I

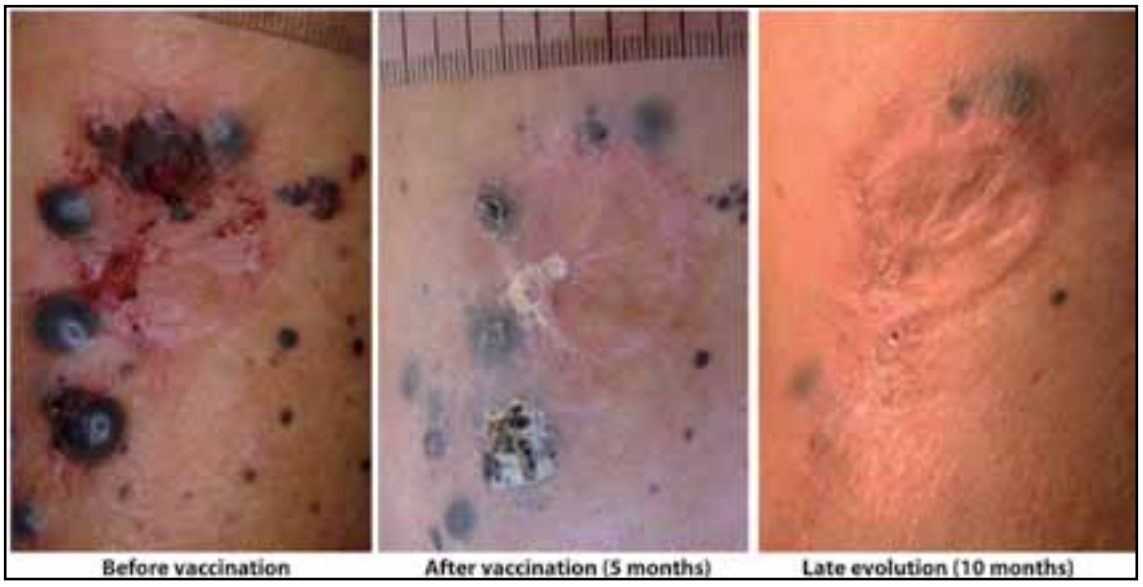

Fig. 2. Example of a complete regression of cutaneous metastases in a melanoma patient after 4 priming vaccinations with an ALVAC recombinant virus expressing the MAGE-3.A1 and MAGE-1.A1 epitopes following by 3 booster vaccinations with the corresponding peptides.

clinical trial design. During the treatment, clinical signs of side effects of the vaccine, including depigmentation occurring as a consequence of anti-melanocyte immune activity, will be recorded, and the size of the metastases will be followed to detect anti-tumoral effect of the vaccine. Blood lymphocytes will be collected before and after the vaccinations to measure the anti-Tyrosinase.A2 immune response. If successful, this new vaccine modality could have a much broader application than in melanoma vaccines.

VACCINATION OF MELANOMA PATIENTS WITH PEPTIDES ASSOCIATED WITH IMMUNOMODULATION OF THE TUMOR ENVIRONMENT

In collaboration with the groups of J.F. Baurain (Centre du Cancer, Cliniques Universitaires St Luc), P. Coulie and T. Boon

In another ongoing clinical trial, melanoma patients with superficial metastases are being vaccinated with a peptide vaccine, either MAGE-3.A1 or NA17.A2, matching the antigenic profile of their tumor. Each of these peptides was previously tested in clinical vaccine trials, and was shown to be well tolerated and associated with tumor regression in some patients. In addition to the vaccine, the patients receive repeated peritumoral injections of a cocktail of pro-inflammatory cytokines and a TLR ligand, in one or two superficial metastases. This local treatment is aimed at inducing a "spark" effect in the tumor environment that could modify it in favor of effective tumor rejection. The same cocktail has been tested in the H-Y mouse model of skin graft rejection, in which it is able to induce effective tissue rejection (see the contribution of T. Boon in this report). As with the other clinical trials run by the group, great attention is given to the collection of biological material (tumor and blood samples), which will allow to study the effect of the treatment on the anti-tumoral immune responses.

VACCINATION OF MELANOMA PATIENTS WITH PEPTIDES ASSOCIATED WITH A GALECTIN-3 INHIBITOR

In collaboration with the groups of J.F. Baurain (Centre du Cancer, Cliniques Universitaires St Luc), P. Coulie and P. van der Bruggen

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 P. 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. In a new clinical trial, in preparation, melanoma patients will receive the same peptide vaccine as in the previous study, in association with repeated infusions of an experimental drug called Davanat®, a plant-extracted oligosaccharide that binds to and inhibits galectins. Galectin-3 is a protein produced by cancer cells that is able to inhibit T cell activation. The group of Pierre van der Bruggen has shown that the anergy that characterizes tumor-associated T cells can be reversed with galectin-3 inhibitors including Davanat®. 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.

STUDY OF THE INFLAMMATORY ENVIRONMENT IN MELANOMA METASTASES

In collaboration with the groups 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 or immunofluorescence analysis of adjacent cryosections, using antibodies directed against tumor cells, T and B cells, macrophages, blood vessels, and various molecules involved in inflammatory reactions (Fig. 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. We are currently characterizing an inflammatory signature that is detected in most tumor samples, and that is associated with T cell activation. We also analyze lymphoid structures present in tumors in which B cell responses seem to occur. The informations gathered from these analyses help us to understand the immune pathways that are active or silent in the tumor environment.

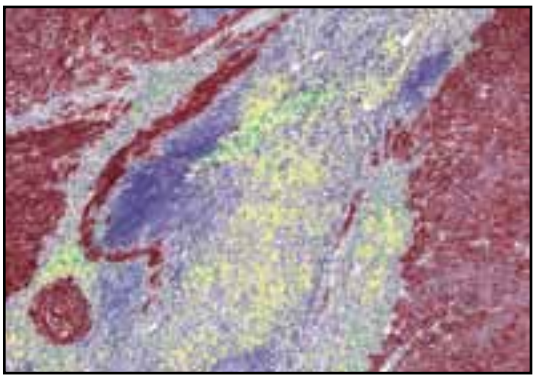

Fig. 3. Example of immune cell infiltration in a melanoma metastasis. The T lymphocytes surround the tumor mass without infiltrating it. This image was reconstructed by superposition of digital microscope images obtained after staining with an antibody directed against melanoma cells (in red) and counterstaining the nuclei with hematoxylin (in blue), followed by elution of antibody and dyes, and by immunofluorescence staining with antibodies directed at CD8+ T lymphocytes (green) and CD4+ T lymphocytes (yellow).

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 para-

sites 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.

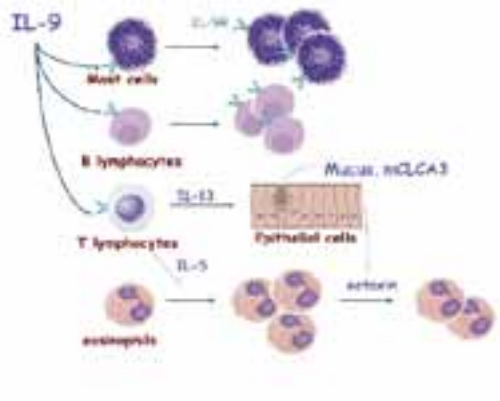

Figure 1. Direct and indirect activities of IL-9 in asthma. IL-9 acts directly on mast cells and B lymphocytes to induce an expansion of these cells and IgE production. IL-9 promotes the proliferation eosinophils indirectly, by upregulating IL-5 production by T cells. Upregulation of IL-13 production 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 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. 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).

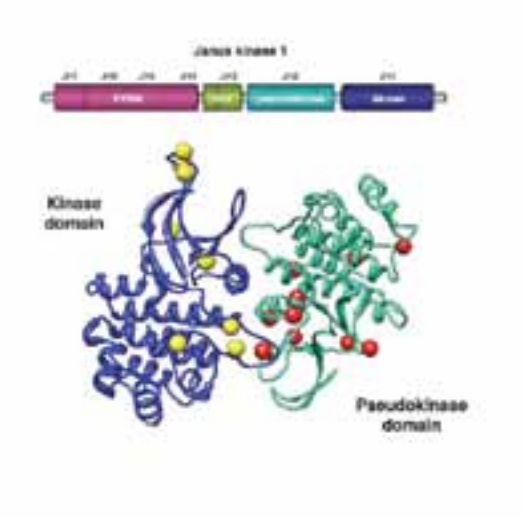

Figure 2. Localization of JAK1 activating mutations in the kinase and pseudokinase domains.

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 deficient 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.

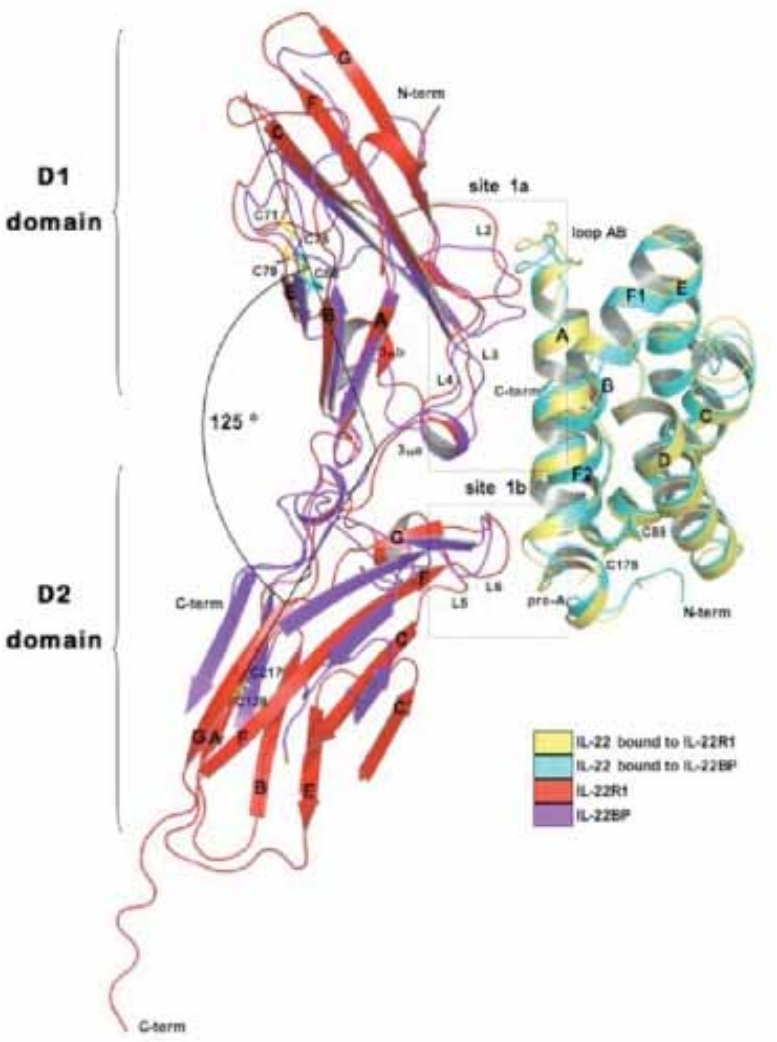

Figure 3. Comparison of IL-22/IL-22BP and IL-22/ IL-22R1 binding interfaces. Superposition of IL-22/IL-22BP (cyan/purple blue) and IL-22/ IL-22R1 (yellow/red) crystal structures shows their binding interfaces outlined by boxes.

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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SIGNAL TRANSDUCTION AND MOLECULAR HEMATOLOGY GROUP
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), which function as homomeric complexes and of heteromeric receptors such as receptors for interleukins (IL) 2 and 9. Activation of these receptors is triggered by cytokine-induced changes in receptor dimerization/oligomerization, which is transmitted via juxtamembrane and transmembrane domains to the cytosolic region and ultimately to members of the Janus family of tyrosine kinases (JAKs).
Our key interests are: i) the structural basis for transmembrane signaling, especially how transmembrane and juxtamembrane sequences switch-on or -off cytokine receptor signaling; and ii) the mechanisms of JAK attachment to receptors, JAK activation and signaling. The laboratory identified constitutively active oncogenic mutants of JAK2, JAK1 and TYK2 and of cytokine receptors, with some being involved in human blood cancers. Specifically the mechanisms by which JAK2 V617F and TpoR W515 mutants induce, in humans, Myeloproliferative Neoplasms (MPNs), such as Polycythemia Vera, Essential Thrombocythemia or Primary Myelofibrosis are actively pursued. Furthermore, a microRNA was recently identified (miR-28) that appears to be a biomarker for a fraction of MPNs without known molecular cause, and that targets the mRNA for TpoR, inhibiting its translation. A major effort will be dedicated to the identification of molecular defects associated with the latter group of MPNs and with acute myeloid leukemia secondary to MPNs. A close collaborative structure has been created with clinicians and clinical biologists at St Luc Hospital for in-depth study of patient-derived cell

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

A. Dusa, C. Pecquet, J.-P.Defour

The JAK-STAT pathway mediates signaling by more than 25 cytokine receptors and is constitutively activated in many cancers. Several mutations in genes coding for JAKs have been identified in the past five years (1). Janus kinases possess two kinase domains, one active and the other, denoted as the pseudo-kinase domain, predicted to be inactive. Four Janus kinases are coded by the human genome JAK1, JAK2, JAK3 and TYK2. JAK2 is crucial for signaling by EpoR, TpoR, the G-CSFR, the interleukin 3 receptor and several other receptors. JAKs are appended to the cytoplasmic juxtamembrane domains of receptors and are switched-on upon ligand binding to the receptors' extracellular domains.

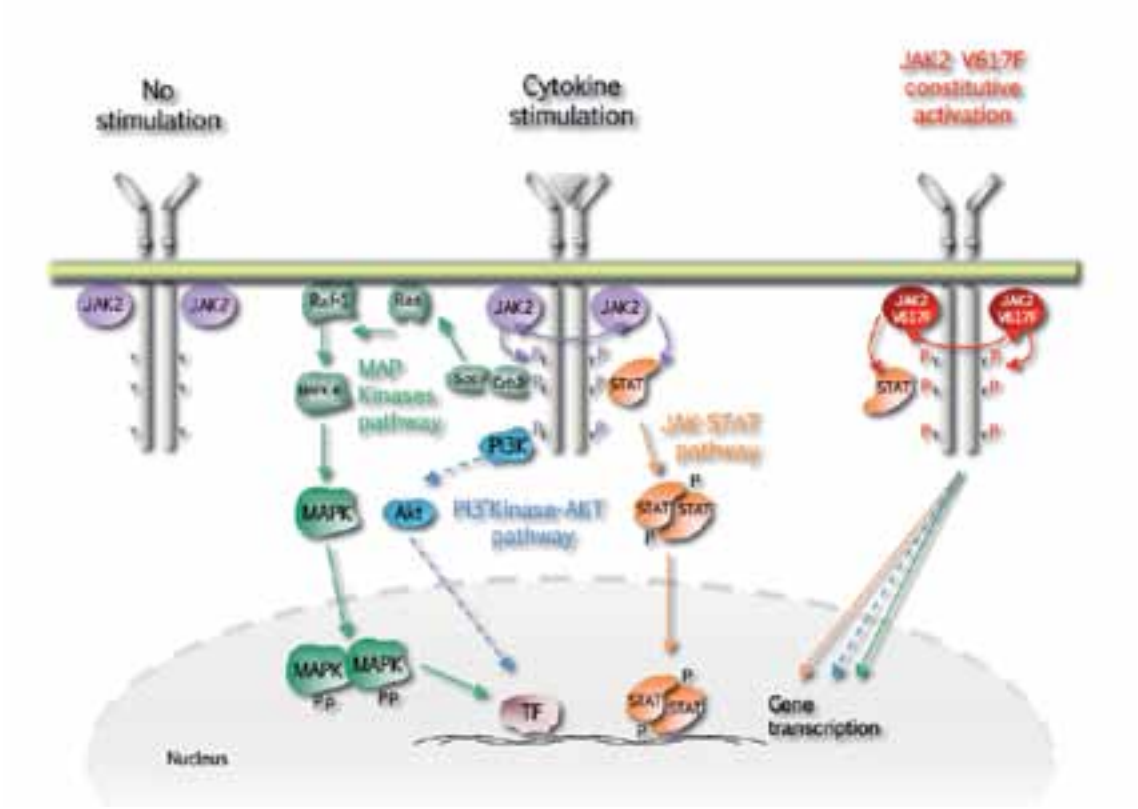

Figure 1. In the absence of cytokine ligands, cytokine receptors (left complex) are preassembled with tyrosine kinases JAK (Janus kinases) in inactive complexes. Cytokine binding to the extracellular domains of receptors (middle complex) induces a conformational change which allows the appended JAKs 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).

Polycythemia Vera (PV), or the Vaquez-Osler disease, is characterized by excessive production of mature red cells and sometimes of platelets and granulocytes. Two other related diseases, Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) are associated with excessive platelet production and fibrosis (scaring) of the marrow due to excessive myeloid cell proliferation, enzyme release and collagen secretion by marrow fibroblasts.

We postulated that JAK2 is involved in the pathogenesis of these diseases because we showed that JAK2 strongly promotes the maturation and cell-surface localization of TpoR (2), a process that was known to be highly defective in PV.

In collaboration with William Vainchenker at the Institut Gustave Roussy in Paris, we have been involved in the discovery of the unique acquired somatic 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 to cytokine receptors (4). Similarly, at least Trp, Leu and Ile can also 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 approximatively 20% of adult T-lymphoblastic leukemia.

A major effort in the group is geared towards the understanding of how a pseudokinase domain mutation can induce kinase domain activation. The aim is to be able to specifically inhibit mutated JAK2 but not the wild type JAK2, which is crucial for several physiologic processes. Towards that end, we identified pseudokinase residue F595 as absolutely required for constitutive activation by V617F, but not for cytokine-induced activation of JAK2/JAK2 V617F (6). A region around F617 and F595, involving the middle of helix C of the JAK2 pseudokinae domain might be a target for specific JAK2 V617F inhibition (Figure 2).

INVOLVEMENT OF TPOR IN MYELOPROLIFERATIVE DISEASES

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

TpoR appears to be central to MPNs: i) most MPN patients strongly down-modulate TpoR levels in megakaryocytes and platelets; ii) mutations in the TpoR intracellular juxtamembrane motif W515 lead to constitutive activation of the receptor, and severe in vivo MPN with myelofibrosis; iii) asparagine mutations in the transmembrane domain of TpoR also activate TpoR and one such mutation has been shown to be associated with ET; iv) alterations of traffic of TpoR to the cell surface can induce thrombocytosis due to insufficient clearance of Tpo and high sensitivity of early megakaryocytes to high Tpo.

We have identified the mechanisms behind the down-modulation of TpoR in MPNs, and showed that JAK2 V617F induces ubiquitinylation, inhibition of recycling and degradation of TpoR (Pecquet et al., submitted). In addition we discovered that Tpo can induce a strong antiproliferative effect in cells that express high JAK2 levels (Pecquet et al., in preparation). This effect can be detected in post-mitotic megakaryocytes (7). We are exploring the precise signaling mediators of this effect and showed that selection against the antiproliferative effect of Tpo occurs in JAK2 V617F

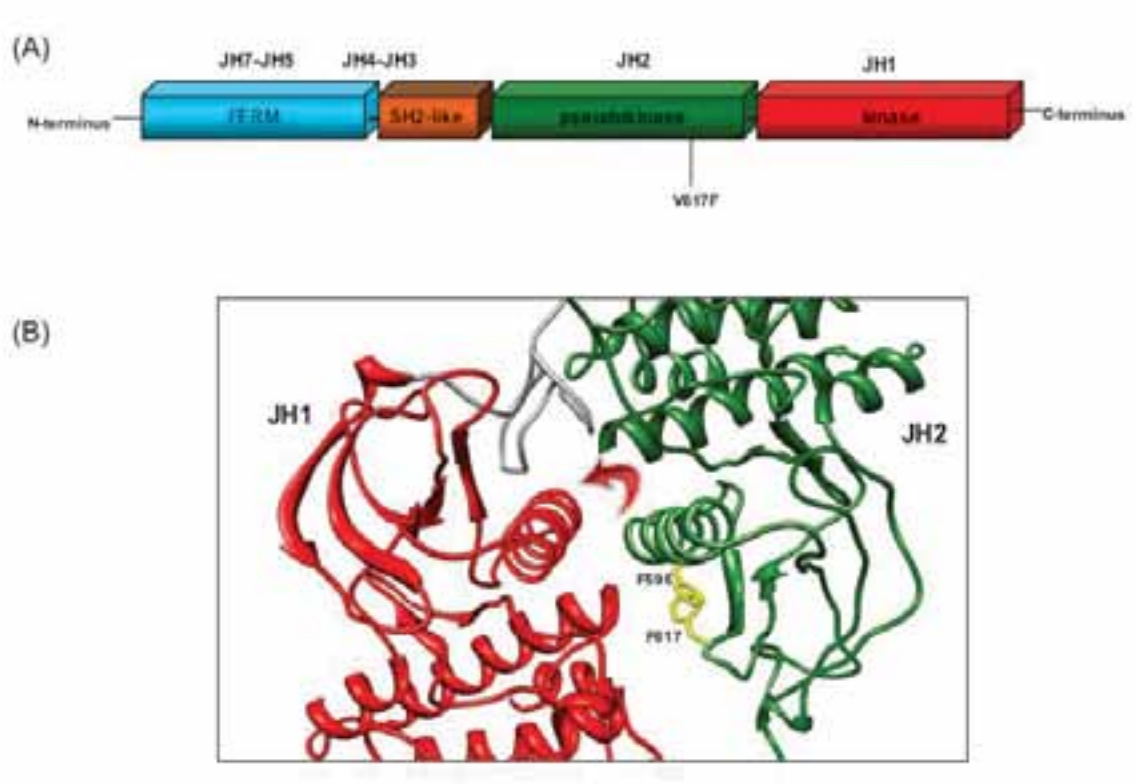

Figure 2. (A) Janus kinase 2 contains several JAK homology domains, JH1, the kinase domain; JH2 the pseudokinase domain; JH3-JH4 the SH2-like domain and JH4-JH7, the FERM (band four point 1, ezrin, radixin, moesin)-like domain. The pesudokinase domain plays a major role in cytokine-dependent activation of the kinase domain, and was implicated in inhibiting the basal activity of the JH1 domain. The V617F mutation is activating the kinase activity of JH1, presumably by preventing the inhibition exerted by JH2 on JH1. The V617F mutation is detected in 98% of PV and approximately 50% of ET and PMF patients. (B) The pseudokinase (JH2) and kinase (JH1) domains of JAK2 are modeled as adopting classical tyrosine kinase structures, interacting with each other and leading to JH1 inhibition. Residue F595 of the helix C of JH2 is required for constitutive activation of JAK2 V617F and of other mutated JAKs proteins, but not for cytokine activation of wild type JAK2. F595 plays a pivotal role in transmitting the conformational change in JH2 to JH1 (red arrow) and eventually in activating the kinase activity of JH1. The region around V617F and the middle of JH2 helix C surrounding F595 could become the target of inhibitors that might specifically decrease constitutive activation of JAK mutants (Alexandra Dusa).

cells, and is partially responsible for TpoR down-modulation in MPN cells, which then continue to proliferate in the presence of Tpo, unlike normal cells.

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 (6). 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 (Figure 3) (7). Why the

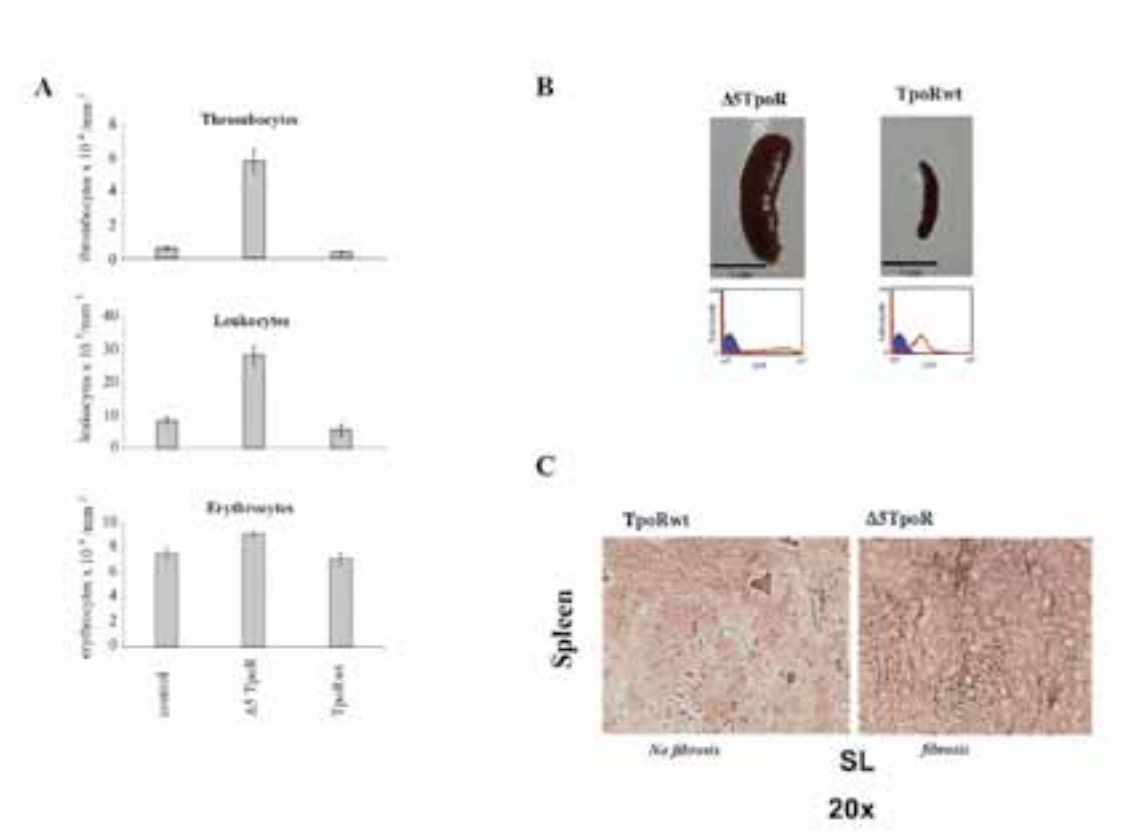

Figure 3. Bone marrow adoptive transfer in lethally-irradiated mice with hematopoietic stem cells expressing the constitutively active Δ5TpoR induces severe myeloproliferative disorder, splenomegaly and fibrosis of the spleen. Δ5TpoR is a mutant where the amphipathic $RW^{515}QFP$ motif is deleted, which 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 (Christian Pecquet and Judith Staerk).

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 (Y626) of TpoR, and appears to involve excessive STAT3 and MAP-kinase signaling (7). Thus, small molecules targeting phosphorylated Y112 (Y626) might be useful in the treatment of myelofibrosis.

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

M. Girardot, J. Van Hees

Cytokine stimulation of cytokine receptors induces transient activation of the JAK-STAT pathway. In contrast, oncogenic forms of receptors or of JAKs (JAK2 V617F) transmit a

continuous signal which results in constitutive activation of STAT proteins. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors or JAKs in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously (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 (Chip) 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 ot be translocated in rare leukemias. LPP is the host gene for miR-28, which we found to down-modulate TpoR translation, impair megakaryocyte differentiation (10). miR-28 is pathologically overexpressed in 30% of MPNs (10). Targets of miR-28, such as E2F6, are critical cell cycle regulators that might influence the phenotype of myeloproliferative disorders. Furthermore, miR-28 is specifically associated with megakaryocyte proliferation and induces a block in differentiation (10). We are studying the mechanisms of pathologic induction of LPP/miR-28 via constitutively active STAT5.

INTERACTION WITH ST LUC HOSPITAL CLINICIANS AND CLINICAL BIOLOGISTS: IDENTIFICATION OF THE MOLECULAR BASES OF MPNS WITHOUT KNOWN MOLECULAR CAUSE

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, Dr. Laurent Knoops), Clinical Biology (Prof. Dominique Latinne, Dr Hélène Antoine-Poirel) and groups of de Duve Institute (Prof. Mark Rider, Prof. Jean-Baptiste Demoulin) our laboratory is performing a large study on the presence and signaling of JAK2, TpoR, and growth factor receptor mutations in patients with myeloproliferative neoplasms. Next generation sequencing will be employed for well-investigated patients, using primary cells that are characterized for functional defects and that do not harbor known mutations in order to unravel novel molecular defects in MPNs and leukemias.

DETERMINATION OF THE INTERFACE AND ORIENTATION OF THE ACTIVATED EpoR, TpoR AND G-CSFR DIMERS

J.-P. Defour, C. Pecquet, E. Leroy

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, 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 α-helices is imposed on the downstream TM α-helix and

intracellular domain.

When each of the seven possible dimeric orientations were imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR, only two fusion proteins stimulated the proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells (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. We have shown that TpoR can signal from several distinct dimeric interfaces, and that besides the normal dimeric interface that leads to formation of platelets, other interfaces promote signaling that leads to myeloproliferative and myelodysplastic disorders (Staerk et al., submitted).

STRUCTURE AND FUNCTION OF JUXTA- AND TRANS-MEMBRANE SEQUENCES OF CYTOKINE RECEPTORS

R.-I. Albu, A. Dusa, J.-P. Defour, 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 and protein ligation assays 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

R.-I. Albu, C. Pecquet

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, γ_c). 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 TpoR, which in turn is essential for clearance of Tpo by circulating platelets. We have identified critical determinants in the extracellular domain of TpoR traffic. While a receptor lacking D1D2 (D3D4-TpoR) is not efficiently localized at the cell surface, introducing the juxtamembrane W515K activating mutation and selection for cytokine-independent growth leads to enhanced expression and detectable cell surface localization of the N-terminally truncated D3D4-TpoR. These results support the notion that cell surface localization is a prerequisite for constitutive signaling by TpoR W515 mutants. The roles of precise extracellular domain glycosylation motifs in TpoR traffic and signaling are also determined.

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LINKS

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