

Ludwig Institute for Cancer Research
Brussels Branch

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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 91 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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Ludwig Institute - Brussels Branch

B1.74.12

Avenue Hippocrate 74
1200 Bruxelles, Belgium

[T] +32 (2) 764 74 59

[F] +32 (2) 762 94 05

Tumor immunology and antigen processing

Benoît Van den Eynde

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 preclinical melanoma 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.

An antigenic peptide produced by reverse splicing in the proteasome and double asparagine deamidation

A. Dalet, V. Stroobant, N. Vigneron, in collaboration with P. Robbins, K.-I. Hanada and S. Rosenberg, NCI, NIH, Bethesda, USA

Tumor antigens relevant for cancer immunotherapy consist of peptides presented by MHC class I molecules and derived from intracellular tumor proteins. They are recognized by cytotoxic T lymphocytes (CTL) and 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. We have now described four spliced peptides, two of which are spliced in the reverse order. 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 [3]. It is derived from tyrosinase and recognized by tumor-infiltrating lymphocytes isolated from a melanoma patient. The peptide is made of two noncontiguous tyrosinase fragments that are spliced together in the reverse order. In addition, it contains two aspartate residues that replace the asparagines encoded in the tyrosinase sequence. We confirmed that this peptide is naturally presented at the surface of melanoma cells, and we showed that its processing sequentially requires translation of tyrosinase into the endo-

plasmic reticulum and its retrotranslocation into the cytosol, where deglycosylation of the two asparagines by peptide-N-glycanase turns them into aspartates by deamidation. This process is followed by cleavage and splicing of the appropriate fragments by the standard proteasome and additional transport of the resulting peptide into the endoplasmic reticulum through the transporter associated with antigen processing (TAP) (Figure 1).

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 ($\beta 5i$) or two ($\beta 1i$ and $\beta 5i$) 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 proteasome $\beta 5i$ or by intermediate proteasome $\beta 1i$ - $\beta 5i$. Other functional aspects of these intermediate proteasomes are currently evaluated.

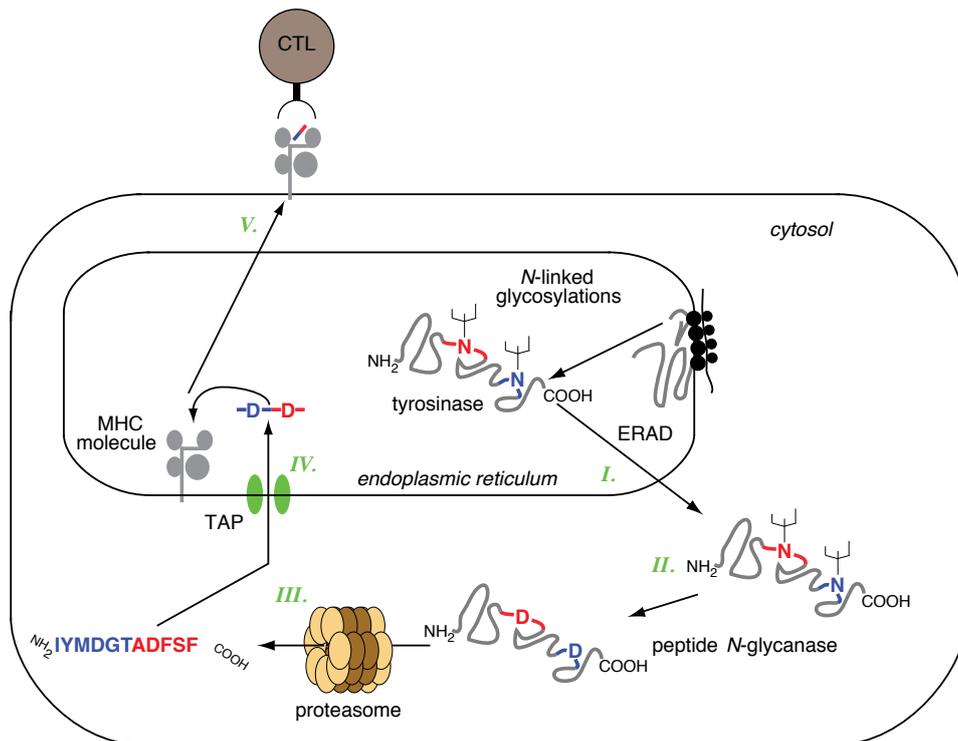


Figure 1. Production of the deamidated reverse-spliced antigenic peptide derived from tyrosinase. Misfolded N-glycosylated tyrosinase proteins are retrotranslocated from the endoplasmic reticulum (ER) into the cytosol (I), where peptide N-glycanase converts two asparagines into aspartates upon removal of the asparagine-associated sugars (II). This is followed by cleavage and reverse splicing of the deamidated protein by the proteasome (III), leading to production of the final antigenic peptide NYMDGTADFSF, which is then transported back into the ER by the TAP transporter (IV) (from reference 3).

Antigenic peptide production by insulin-degrading enzyme

N. Parmentier, V. Stroobant

We studied a proteasome-independent peptide derived from 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.

Inefficient exogenous loading of a tapasin-dependent peptide onto HLA-B*44:02 can be improved by acid treatment or fixation of target cells

N. Vigneron, V. Stroobant, W. Ma, A. Michaux, in collaboration with N. Demotte and P. van der Bruggen, LICR, Brussels, and with RM Leonhardt and P. Cresswell, Yale University, New Haven, CT, USA

We recently identified a new antigenic peptide, which is derived from the MAGE-A1-encoded protein and presented to cytotoxic T lymphocytes (CTLs) by HLA-B*44:02 [6]. Although this peptide is encoded by MAGE-A1, processed endogenously and presented by tumor cells, the corresponding synthetic peptide is hardly able to sensitize target cells to CTL recognition when pulsed exogenously. We observed that endogenous processing and presentation of this peptide is strictly dependent on the presence of tapasin, which is believed to help peptide loading by stabilizing a peptide-receptive form of HLA-B*44:02. In line with this, we showed that exogenous loading of the peptide can be dramatically improved by paraformaldehyde fixation of surface molecules or by peptide loading at acidic pH, two strategies presumably generating or stabilizing a peptide-receptive, empty conformation of the HLA. Altogether, our results indicate a potential drawback of

short peptide-based vaccination strategies and offer possible solutions regarding the use of such problematic epitopes.

A MAGE-C2 antigenic peptide processed by the immunoproteasome is recognized by cytolytic T cells isolated from a melanoma patient after successful immunotherapy

W. Ma, N. Vigneron, J. Chapiro, V. Stroobant, in collaboration with P. Coulie, de Duve Institute

We have pursued our analysis of a melanoma patient who showed almost complete tumor regression following vaccination with MAGE-A1 and MAGE-A3 antigens. We previously described high frequencies of tumor-specific CTL precursors in blood samples collected after but also before vaccination. A set of CTL clones were derived that recognized antigens different from those of the vaccine. Two of these antigens were peptides encoded by another MAGE gene, MAGE-C2. Here we describe the antigen recognized by another tumor-specific CTL clone [7]. It proved to be a third antigenic peptide encoded by gene MAGE-C2, ASSTLYLVF. It is presented by HLA-B57 molecules and proteasome-dependent. Tumor cells exposed to interferon-gamma (IFN- γ) were better recognized by this anti-MAGE-C2 CTL clone. This mainly resulted from a better processing of the peptide by the immunoproteasome as compared to the standard proteasome. Mass spectrometric analyses showed that the latter destroyed the antigenic peptide by cleaving between two internal hydrophobic residues. Despite its higher "chymotryptic-like" (posthydrophobic) activity, the immunoproteasome did not cleave at this position, in line with the suggestion that hydrophobic residues immediately downstream from a cleavage site impair cleavage by the immunoproteasome. We previously reported that one of the other MAGE-C2 peptides recognized by CTL from this patient was also better processed by the immunoproteasome. Together, these results support the notion that the tumor regression of this patient was mediated by an antitumor response shaped by IFN- γ and dominated by CTL directed against peptides that are better produced by the immunoproteasome, such as the MAGE-C2 peptides.

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-1 β leads to a 4- to 10-fold decrease in the level of Microphthalmia-associated transcription factor (MITF-M) (Kholmanskikh et al, 2010). This effect is NF- κ B 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-1 β reduced by 40-100% their ability to activate such anti-melanoma CTL.

Minimal tolerance to a tumor antigen encoded by a cancer-germline gene

I. Huijbers, C. Uyttenhove, D. Colau, L. Pilotte, C. Powis de Tenbosche, in collaboration with AM Schmitt-Verhulst and S. Soudja, CIML, Marseille, France

Cancer-germline genes such as MAGE and NY-ESO1 encode the most relevant antigens for cancer immunotherapy. Some of these genes are expressed at low levels in the thymus, raising the possibility of central immune tolerance that might reduce the immunogenicity of such antigens. We created a mouse line knocked-out for cancer-germline gene P1A, and tested whether P1AKO mice develop stronger immune responses to P1A antigens as compared to wild type mice [8]. We found only slightly stronger P1A-specific immune responses in P1AKO mice, even though these responses were sufficient to induce tumor rejection in defined experimental settings. These results indicate only minimal immune tolerance to antigens encoded by cancer-germline genes and fully confirm their immunogenicity.

Tumoral immune resistance through tryptophan degradation by indoleamine 2,3-dioxygenase

L. Pilotte, P. Larrieu, V. Stroobant, D. Colau, C. Uyttenhove in collaboration with U. Rohrig, V. Zoete and O. Michielin, LICR, University of Lausanne, Switzerland

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 [9]. 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 used computational structure-based methods to design new compounds able to inhibit IDO with a high potency (Röhrig et al, 2012). This approach yielded highly efficient low-molecular weight inhibitors, the most active being of nanomolar potency both in an enzymatic and in a cellular assay, while showing no cellular toxicity and a high selectivity for IDO1 over tryptophan 2,3-dioxygenase (TDO). These triazole compounds will be further optimized with the goal of developing drug candidates.

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.

Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase

L. Pilotte, P. Larrieu, V. Stroobant, D. Colau, E. De Plaen, C. Uyttenhove, in collaboration with E. Dolusic, R. Frédérick, J. Wouters and B. Masereel, NAMEDIC, University of Namur, Belgium

Besides IDO, we recently uncovered the role of another, unrelated, tryptophan-degrading enzyme named tryptophan-dioxygenase (TDO) in tumoral immune resistance [10]. TDO is highly expressed in the liver and regulates systemic tryptophan levels. We found TDO to be expressed in a high proportion of human tumors. We showed that TDO-expressing mouse tumors are no longer rejected by immunized mice. Moreover, we developed a new TDO inhibitor, which, upon systemic treatment, restored the ability of mice to reject tumors. These results describe a mechanism of tumoral immune resistance based on TDO expression and establish proof-of-concept for the use of TDO inhibitors in cancer therapy. This concept will be further developed in the frame of a new spin-off company that we recently launched, named iTeos Therapeutics.

A preclinical melanoma model for cancer immunotherapy

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

We made transgenic mice developing melanomas with a 70-80% incidence after tamoxifen injection (Huijbers et al, 2006). These tumors express the tumor antigen encoded by cancer-germline gene P1A. Initially highly pigmented and indolent, they later dedifferentiate in unpigmented highly aggressive tumors. Mice bearing aggressive tumors show exacerbated systemic inflammation associated with disruption of secondary lymphoid organs, accumulation of immature myeloid cells and immunosuppression (Soudja et al, 2010). Current efforts aim at characterizing this immunosuppression and devising effective therapeutic approaches.

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Benoît Van den Eynde, Member

Ludwig Institute for Cancer Research
B1.74.12
Av. Hippocrate 74-75
B - 1200 Brussels

[T] +32 02 764 75 72
[F] +32 02 764 75 90
[E] Benoit.Vandeneynde@bru.licr.org
[W] http://www.deduveinstitute.be/tumor_imunology.php

Staff members

Etienne **De Plaen**, Senior Investigator • Vincent **Stroobant**, Senior Investigator • Stefania **Cane**, Postdoctoral Fellow • Wenbin **Ma**, Postdoctoral Fellow • Nathalie **Vigieron**, Postdoctoral Fellow • Luc **Pilotte**, Research Associate • Nathalie **Arts**, Graduate Student • Juliette **Lamy**, Graduate Student • Alexandre **Michaux**, Graduate Student • Nicolas **Parmentier**, Graduate Student • Céline **Powis de Tenbossche**, Graduate Student • Florence **Schramme**, Graduate Student • Rui **Cheng**, Research Assistant • Aline **Depasse**, Research Assistant • Thérèse **Aerts**, Technician • Bénédicte **Tollet**, Technician • Marc **Hennequart**, Student • Julie **Klein**, Secretary

Regulation of T lymphocyte function in tumors

Pierre van der Bruggen

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). To that purpose, we have used various approaches that we have loosely named "reverse immunology", because they use gene sequences as starting point (4).

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, 5). 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 (6-9). 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-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, as we have recently shown for CTL clones (10). 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- 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- secretion compared to untreated cells.

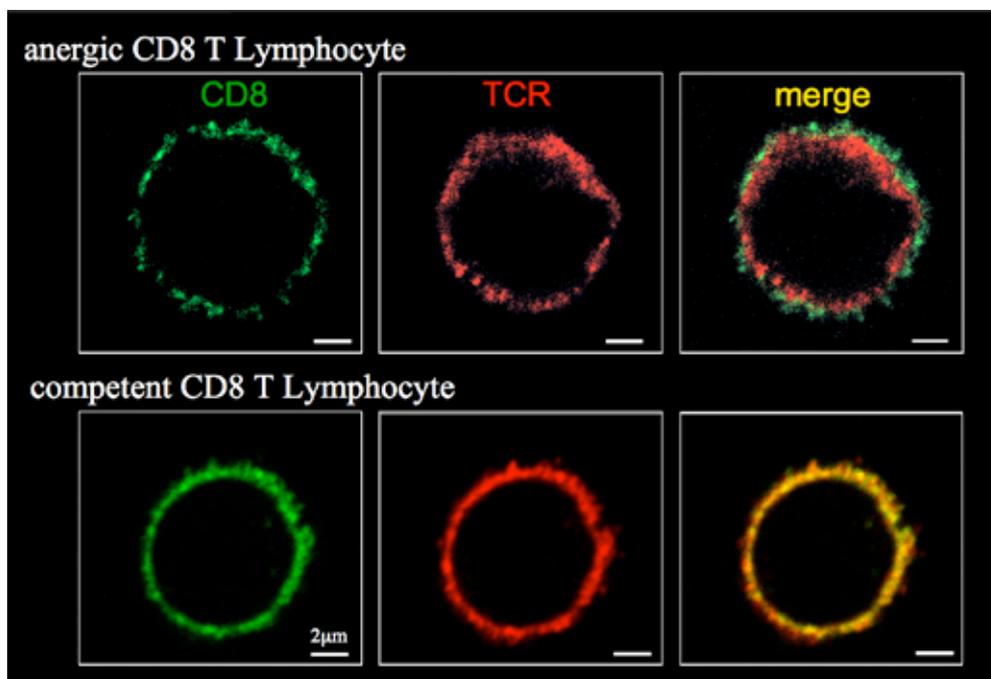


Figure 1. TCR and CD8 do not co-localize on CD8 T cells with impaired functions

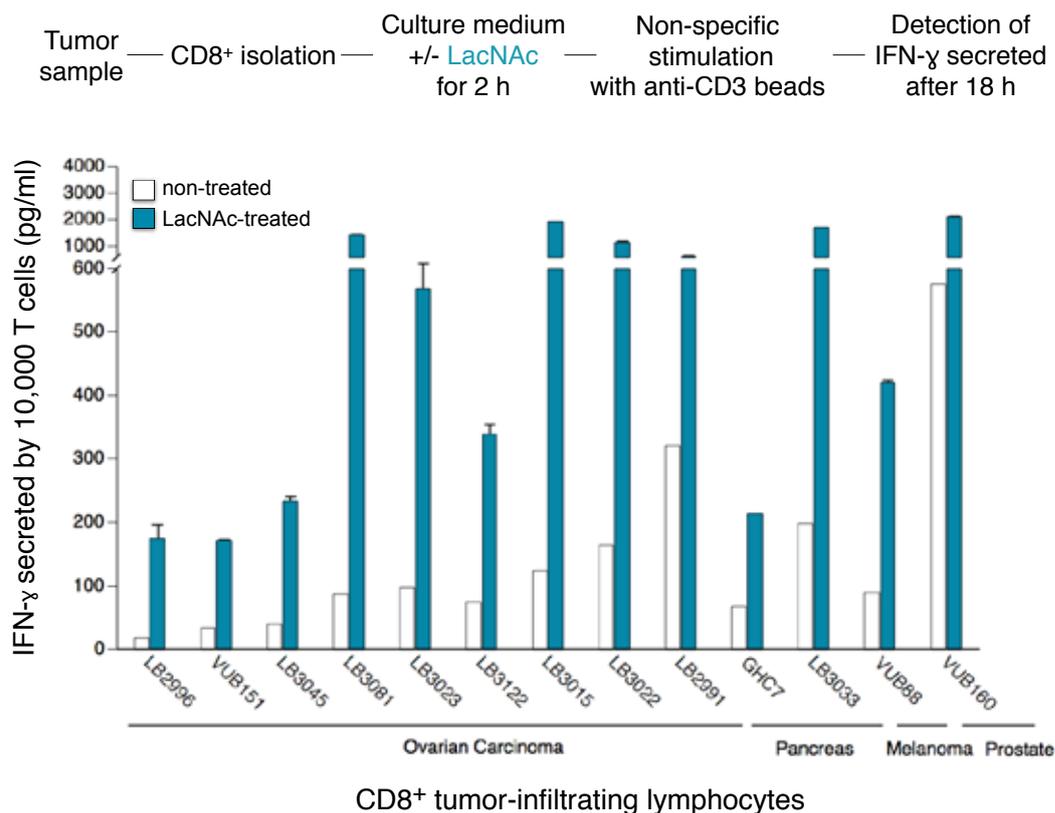


Figure 2. Treatment of tumor-infiltrating lymphocytes with a galectin ligand reverses anergy

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- secretion. Importantly, we observed that not only CD8⁺ TIL but also CD4⁺ TIL that were treated with this polysaccharide secreted more IFN- 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- by treated TIL. A clinical trial with this new compound, in combination with anti-tumoral vaccination, will start in 2012 in different clinical centers. 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?

D. Godelaine and V. Ha Thi, in collaboration with Dr J. Carrasco (Grand Hôpital de Charleroi) and Dr J.P. Machiels (Cliniques Universitaires St-Luc)

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. 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, D. Godelaine set out to evaluate the frequencies of anti-tumor CD8 T lymphocytes in the blood of non-metastatic breast cancer patients prospectively recruited in several clinical centers. Blood samples are collected before and after surgery. Frequencies are evaluated by mixed lymphocyte-peptide cultures, carried out with HLA-A2- and A3-restricted HER2/neu and hTERT peptides, followed by detection of specific cells with HLA-peptide tetramers. Tumors removed at surgery are analyzed by immunohistochemistry for infiltration by immune

cells, and fragments are frozen for further genetic analysis of the T cell receptor repertoire. The prospective follow-up of 172 patients will extend over a 5-year-period. So far, 69 patients have been included and 33 have been screened for frequencies of specific CD8 T lymphocytes. Thirty percent of the screened patients have a frequency against the targeted antigens in the range of 3×10^{-6} among blood CD8 T cells, whereas the mean value in healthy donors is 3×10^{-7} . We hope to identify patients with a better prognosis in order to offer them an adapted care avoiding unnecessary heavy treatments.

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Pierre van der Bruggen, Member

Ludwig Institute for Cancer Research
B1.74.12
Av. Hippocrate 74-75
B - 1200 Brussels

[T] +32 02 764 74 31
[F] +32 02 762 94 05
[E] Pierre.vanderbruggen@bru.licr.org
[W] http://www.deduveinstitute.be/regulation_lymphocyte.php

Staff members

Danièle **Godelaide**, Senior Investigator • Nathalie **Demotte**, Postdoctoral Fellow • Monica **Gordon-Alonso**, Postdoctoral Fellow (since July 2011) • Claude **Wildmann**, Research Associate • Anne-Elisabeth **Petit**, Graduate Student • Grégoire **Wieërs**, Graduate Student (until September 2011) • Débora **Piccolo**, Research Assistant • Vinh **Ha Thi**, Technician • Laurie **Vanbiervliet**, Technician • Nathalie **Krack**, Secretary

Immunotherapy analysis group

Thierry Boon

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-like receptor agonists, as well as antibodies directed against inhibitory cytokines such as TGF- β , 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. This approach is proving to be effective in a mouse skin graft model. A small clinical trial involving tumor bearing melanoma patients is under way.

Inducing rejection of normally tolerated grafts in the H-Y mouse model

F. Brasseur, I. Jacquemart, B. Lethé, C. Lurquin, C. Uyttenhove, T. Boon

A mouse model of skin grafts was developed that recapitulates what happens in cancer patients, where T lymphocytes often infiltrate the tumor without rejecting it. The group tests various approaches to overcome the anergy of such infiltrating T cells. These approaches involve cytokines and Toll-like receptor ligands, as well as antibodies directed against inhibitory cytokines such as TGF- β .

Female CBA mice do not reject male skin grafts, even though they are able to mount a cytolytic T cell response against H-Y, a male specific minor histocompatibility antigen. To break this tolerance, repeated local injections of a low dose of IL-12, combined with IFN- α , caused graft rejection in all mice. Like IFN- α , IL-1 α , IL-18 and IL-2 were incapable of inducing rejection on their own, but synergized effectively with IL-12. One finding of importance for the clinical application of this procedure is that several weekly cycles of cytokine treatments are necessary for complete rejection of the grafts.

We tested combinations of agents that are approved for clinical use. We observed that repeated local injections of a combination of low doses of IL-2 (300 ng), GM-CSF (300 ng) and IFN α (10^5 U) with TLR7 ligand gardiquimod (20 μ g) caused 100% rejection. The crucial components appear to be IL-2 and gardiquimod. However, the same doses of IL-2 and IFN- α , without GM-CSF nor gardiquimod, were also effective when combined with both anti-TGF- β and anti-IL-10 antibodies, whereas anti-CTLA-4 antibody needed the additional presence of GM-CSF to be fully effective. Additional molecules in the process of clinical agreement, such as IFN γ , anti-PDL1 and anti-CD40 agonist antibodies, are presently under evaluation. Vaccinations with intra-peritoneal injections of male lymphoblasts aimed at enhancing anti-H-Y responses synergize effectively with the local cytokine treatment.

Nicolas van Baren presents elsewhere updated results of a clinical trial involving a local treatment composed of IL-2, GM-CSF, IFN- α and Aldara, a cream containing imiquimod, a TLR7 ligand of the same family as gardiquimod.

Amine-reactive OVA multimers for auto-vaccination against cytokines and other immune mediators

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

Using our amine-reactive OVA multimers, recently described, we have produced a series of monoclonal antibodies inhibiting murine GCP-2/CXCL6, cytokines GM-CSF, IL-17F, IL-17E/IL-25, IL-27, TGF β -1 and matrix metalloproteinase-9 [5]. As these monoclonal antibodies were of mouse origin, they can be administered *in vivo* for long periods without inducing any immune reactions against a foreign protein as observed with rabbit or rat antibodies. The mAb against GCP-2 provided the first demonstration of the essential role played by this chemokine in rapid neutrophil mobilization after *Leishmania major* infection [5]. In collaboration with the group of Jo Van Damme (Rega Institute, KUL), we showed that neutralization of GCP-2 inhibited growth and metastasis of a melanoma cell line by decreasing angiogenesis [6]. Our mAbs against mouse IL-27, MM27.7B1, and against TGF β 1, 13A1, that potently inhibited the bioactivity of these cytokines *in vitro* are currently used to evaluate the contribution of IL-27 or TGF β 1 in anti-tumor activities, skin graft rejection and in a model of GVHD. We also succeeded to isolate the first and unique monoclonal antibody directed against the IL-12p35 chain, that contrarily to the existing anti-p40 mAbs, the common chain to IL-12 and IL-23, selectively blocks the IL-12 bioactivity, allowing for the fine dissection of the respective roles of these two cytokines in various *in vivo* models.

Using the same OVA multimers, we immunized mice against periostin/OSF-2, an extracellular matrix protein present in the stroma of many tumors, in mice and humans, that was recently implicated in metastasis development [7]. The first mAbs we isolated are currently being tested (in collaboration with P. Jat and S. Fields, LICR Oxford) to evaluate their ability to block periostin interaction with integrins α v β 3 and α v β 5, one of the mechanisms implicated in cancer cell migration and metastasis establishment.

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Thierry Boon, Emeritus Member

de Duve Institute
B1.74.72
Av. Hippocrate 74-75
B - 1200 Brussels

[T] +32 02 764 75 72
[F] +32 02 764 75 90
[E] Thierry.Boon@bru.licr.org
[W] http://www.deduveinstitute.be/tumor_immunology.php

Staff members

Francis **Brasseur**, Senior Investigator • Bernard **Lethé**, Senior Investigator • Christophe **Lurquin**, Senior Investigator • Catherine **Uyttenhove**, Senior Investigator • Isabelle **Jacquemart**, Postdoctoral Fellow • Marie-Claire **Letellier**, Research Assistant • Maria **Panagiotakopoulos**, Research Assistant • Dominique **Donckers**, Technician • Julie **Klein**, Secretary

Therapeutic vaccination and tumor expression profiling group

Nicolas van Baren

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.

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 an ongoing 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

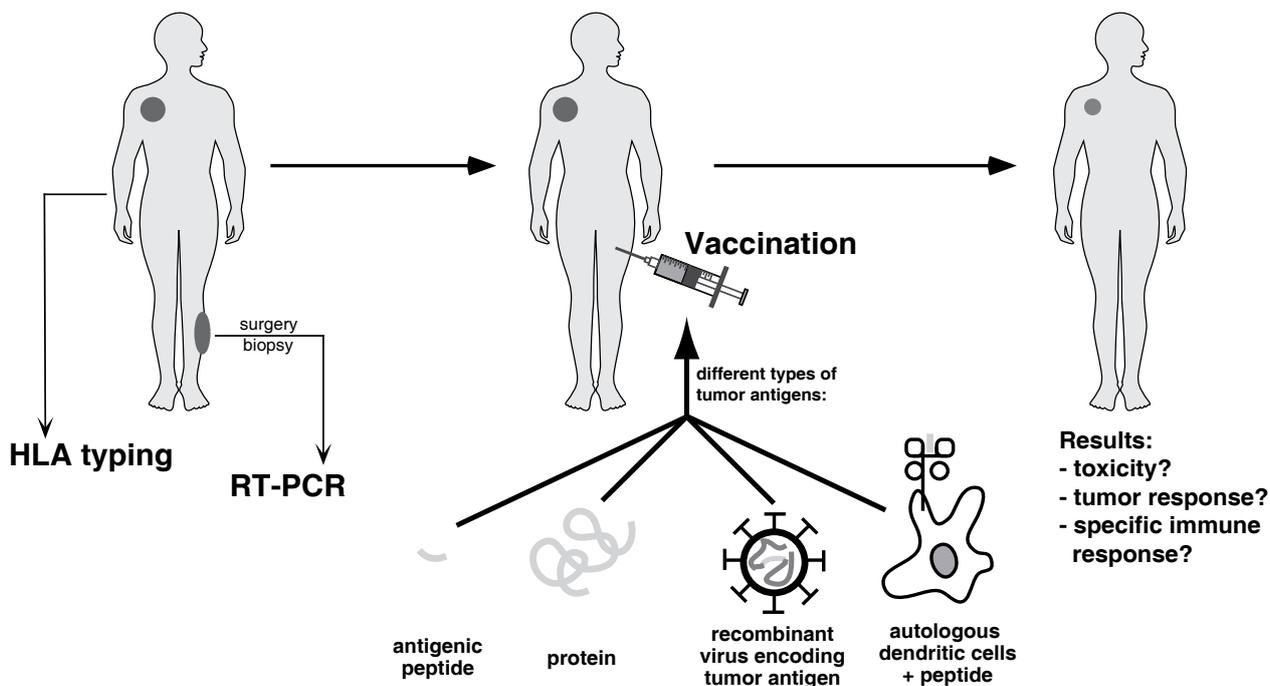


Figure 1. Principle of anti-tumor vaccination with a defined antigen : The first step is to determine if the patient's tumor cells express the tumor antigen. This can be determined by HLA typing, and by RT-PCR analysis of a tumor sample. Selected patients receive repeated injections of a vaccine with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

advantage of this vaccine is its ability to target dendritic cells in vivo, with an expected 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 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, are assessed, and the size of the metastases is followed to detect anti-tumoral effect of the vaccine. Blood lymphocytes are 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 peri-tumoral 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 recently started clinical trial, melanoma patients receive the same

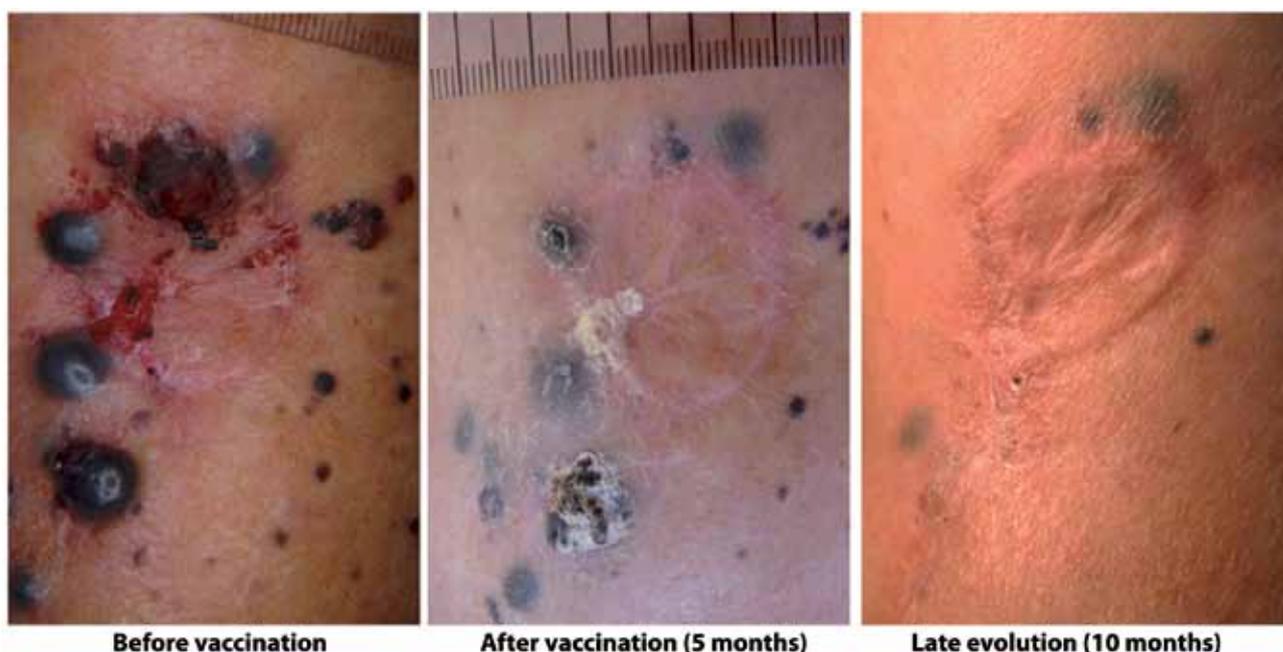


Figure 2. Example of a complete regression of cutaneous metastases in a melanoma patient after 4 priming vaccinations with an ALVAC recombinant virus expressing the MAGE-3.A1 and MAGE-1.A1 epitopes followed by 3 booster vaccinations with the corresponding peptides.

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 inhibitors including Davanat®. We hope that this combined treatment will favor a synergistic interaction between new anti-tumoral CTL responses induced by the vaccine and the inhibition of tumor resistance by the galectin inhibitor.

Study of the inflammatory environment in melanoma metastases

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 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.

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.

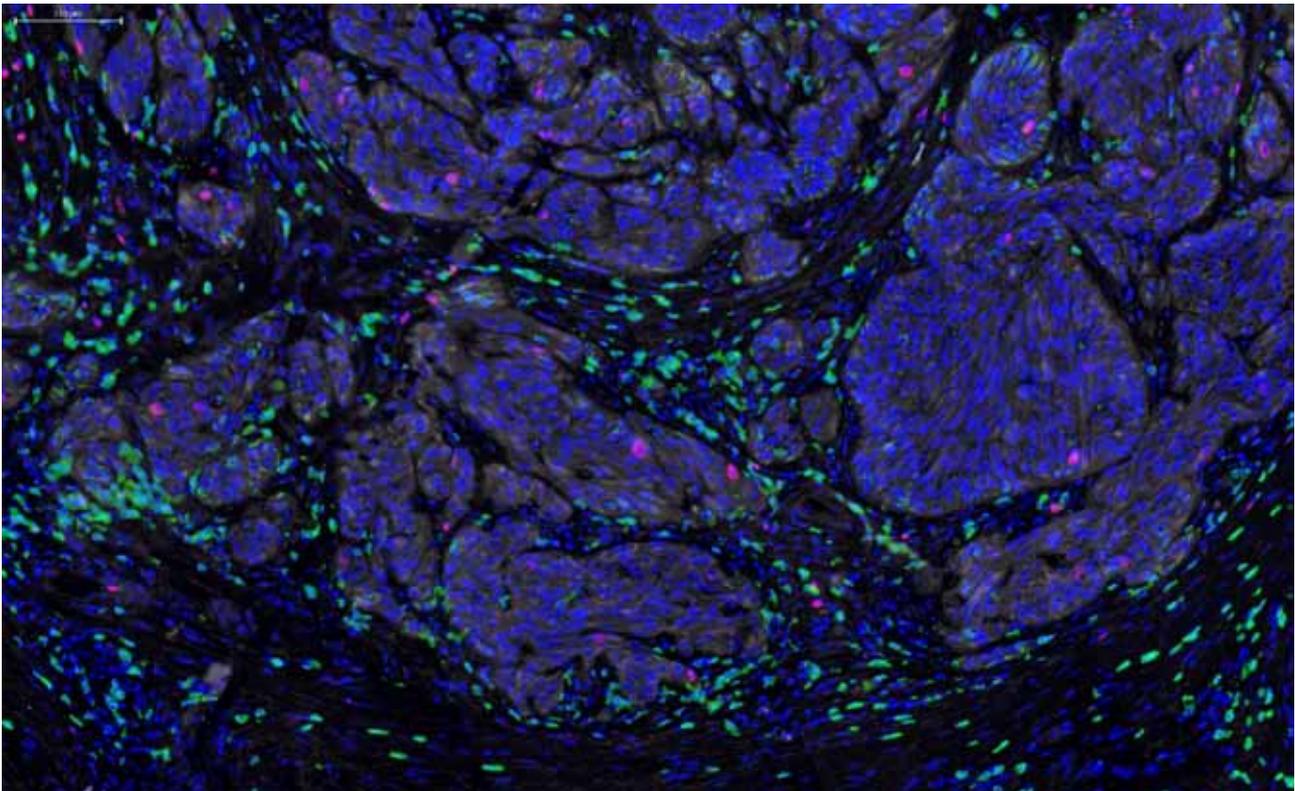


Figure 3. A melanoma metastasis infiltrated by cytolytic T lymphocytes. A tissue section of the tumor was stained with three antibodies coupled to a different fluorescent dye. Melanoma cells and T cells appear in brown and green, respectively. Some melanoma nuclei express the proliferation marker Ki67 (in red). All cell nuclei are stained with a blue fluorescent dye. This image shows that the tumor continues to grow in the presence of an abundant infiltration by T lymphocytes.

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Nicolas van Baren, Senior Investigator

Ludwig Institute for Cancer Research
B1.74.12
Av. Hippocrate 74-75
B - 1200 Brussels

[T] +32 02 764 75 08
[F] +32 02 764 65 65
[E] nicolas.vanbaren@bru.licr.org
[W] http://www.deduveinstitute.be/therapeutic_vaccination.php

Staff members

Jérôme **Degeldre**, Clinical Research Associate • Marjorie **Mercier**, Research Assistant • Madeleine **Swinarska**, Research Assistant

Cytokines in immunity and inflammation

Jean-Christophe Renaud
Laure Dumoutier

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 in our laboratory. 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, 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.

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

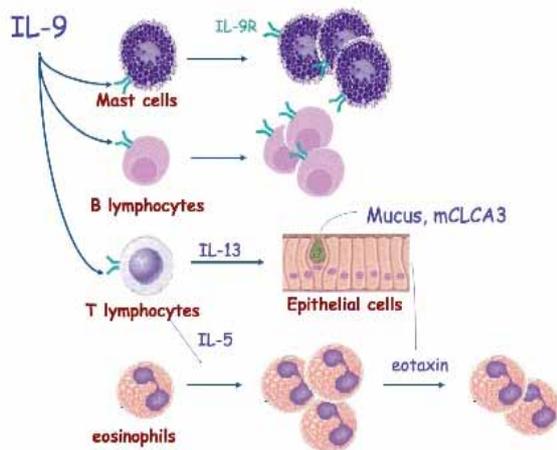


Figure 1. Direct and indirect activities of IL-9 in asthma. IL-9 acts directly on mast cells and B lymphocytes to induce an expansion of these cells and IgE production. IL-9 promotes the proliferation of eosinophils indirectly, by upregulating IL-13 production by T cells. Upregulation of IL-13 production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

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 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 γ_c . This induces the phosphorylation of the JAK1 and JAK3 tyrosine kinases, which are associated with IL-9R and γ_c , respectively. A single tyrosine residue of the IL-9R is then phosphorylated and acts as a docking site for 3 transcription factors of the STAT family, STAT-1, -3 and -5, which become phosphorylated and migrate to the nucleus, where they activate the transcription of a number of genes. This pathway is common to many cytokines but is often dispensable for their biological activities. For IL-9, our group demonstrated that activation of the STAT transcription factors is crucial for all the effects of IL-9 studied on various cell lines, including positive and negative regulation of cell proliferation, as well as inhibition of corticoid-induced apoptosis in T cell lymphomas. Further analysis demonstrated

that STAT-1, -3 and -5 play specific, redundant and synergistic roles in the different activities of IL-9 in vitro. 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 JAK1 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).

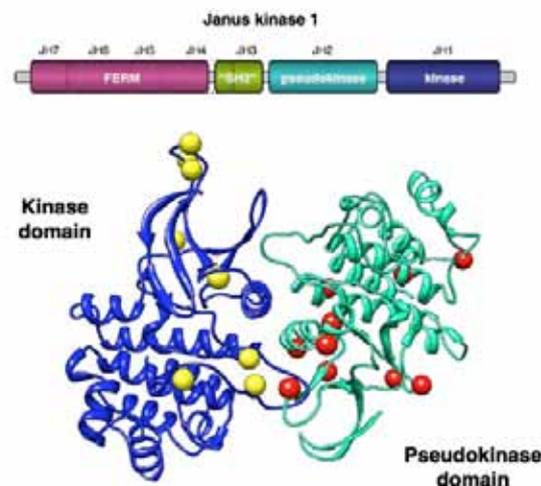


Figure 2. Localization of JAK1 activating mutations in the kinase and pseudokinase domains.

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 preferentially produced by TH17 cells, which are associated with several autoimmune and inflammatory processes. We assessed the role of IL-22 in a mouse model where psoriasiform skin inflammation is triggered by topical application of the TLR7/8 agonist imiquimod. At the macroscopic level, scaly skin lesions induced by daily applications of imiquimod in wild-type mice were almost totally absent in IL-22-deficient mice or in mice treated with a blocking anti-IL-22 Ab. At the microscopic level, IL-22-deficient mice showed a dramatic decrease in the development of pustules and neutrophil infiltration and a partial decrease in acanthosis. At the molecular level, the absence or inhibition of IL-22 strongly decreased the expression of chemotactic factors such as CCL3 and CXCL3 and of biomarkers such as S100A8, S100A7, and keratin 14, which reflect the antimicrobial and hyper-proliferative responses of keratinocytes. Contrasting with this proinflammatory effect of IL-22 in skin inflammation, asthma models showed that IL-22 can have a protective anti-inflammatory activity in lungs. This protective effect of IL-22 has been attributed to an inhibition of IL-13 activity on lung epithelial cells either for CCL17/TARC induction or for IL-25 production. Inhibiting IL-22 in vivo, through antibody treatment or by gene targeting, increased expression of

these inflammatory mediators, infiltration by eosinophils and broncho-hyperresponsiveness.

Both in the psoriasis and asthma models have challenged the dogma that IL-22 is mainly produced by TH17 lymphocytes, and $\gamma\delta$ T cells as well as innate lymphoid cells turned out to be the major producers of this cytokine. We characterized the cells responsible for IL-22 production in response to TLR agonists such as LPS or flagelin. We identified a new innate lymphoid spleen cell population expressing CD25, CCR6 and IL-7R representing 1% of spleen cells from recombination activating gene (Rag2)-deficient mice. This population comprises 60–70% CD4⁺ cells, which produce IL-22, and are still present in common γ chain-deficient mice; the CD4⁺ subset coexpresses IL-22 and IL-17, and is common γ chain-dependent. These cells share a transcriptional program with NKp46⁺ ROR γ t⁺ cells found in intestinal mucosae and involved in antibacterial responses. The importance of IL-22 production for the LPS-triggered response is highlighted by the fact that IL-22-deficient mice are more resistant to LPS-induced mortality, pointing to the pro-inflammatory activity of this cytokine.

Although IL-22 does not share any biological activity with IL-10, these 2 cytokines share a common component of their respective receptor complex, IL-10R β . Anti-IL-10R β antibodies indeed block the IL-22-induced acute phase response in HepG2 cells (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-10R β is a typical short chain component, with only 76 amino acids in the cytoplasmic domain, whose main function seems to consist in recruiting the Tyk2 tyrosine kinase. In addition to IL-10R β , IL-22 signalling requires the expression of a long chain protein, called IL-22R and comprising a 319 amino acid long cytoplasmic domain. This chain associates with JAK1, and is responsible for the activation of cytoplasmic signalling cascades such as the JAK/STAT, ERK, JNK and p38 MAP kinase pathways. An unexpected feature 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-10R β , 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

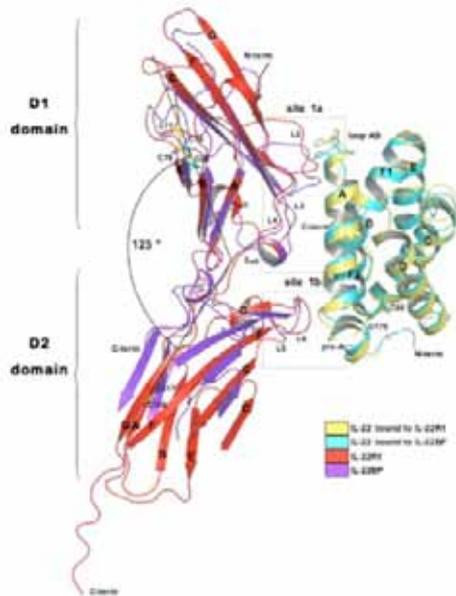


Figure 3. Comparison of IL-22/IL-22BP and IL-22/IL-22R1 binding interfaces. Superposition of IL-22/IL-22BP (cyan/purple blue) and IL-22/IL-22R1 (yellow/red) crystal structures shows their binding interfaces outlined by boxes.

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

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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Jean-Christophe Renauld, Member

Ludwig Institute for Cancer Research [T] +32 02 764 74 64
de Duve Institute [F] +32 02 762 94 05
MEXP - B1.74.05 [E] Jean-Christophe.Renauld@bru.licr.org
Av. Hippocrate 74-75 [W] [http://www.deduveinstitute.be/cytokines_in_](http://www.deduveinstitute.be/cytokines_in_immunity_and_inflammation.php)
B - 1200 Brussels immunity_and_inflammation.php



Laure Dumoutier, Assistant Member

Ludwig Institute for Cancer Research [T] +32 02 764 74 65
de Duve Institute [F] +32 02 762 94 05
MEXP - B1.74.05 [E] Laure.Dumoutier@bru.licr.org
Av. Hippocrate 74-75 [W] [http://www.deduveinstitute.be/cytokines_in_](http://www.deduveinstitute.be/cytokines_in_immunity_and_inflammation.php)
B - 1200 Brussels immunity_and_inflammation.php

Staff members

Jacques **Van Snick**, Member • Reece **Marillier**, Postdoctoral Fellow • Magali **de Heusch**, Postdoctoral Fellow
• Laurent **Knoops**, Postdoctoral Fellow • Perrine **Cochez**, Graduate Student • Muriel **Lemaire**, Graduate
Student • Amandine **Saumet**, Graduate Student • Lorraine **Springuel**, Graduate Student • Astrid **Van Belle**,
Graduate Student • Isabelle **Bar**, Technician • Pamela **Cheou**, Technician • Emilie **Hendrickx**, Technician •
Monique **Stevens**, Technician

Signal transduction and molecular hematology group

Structure and function of cytokine receptors

Stefan Constantinescu

Cytokines and their receptors govern formation of blood cells and development and function of the immune system. Our broad interest is the understanding of the mechanisms that control assembly in the membrane of cell surface receptors that respond to extracellular cues. We study in detail the structure, function and orientation 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 several interleukins.

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). We aim to identify: i) the structural basis for transmembrane signaling, especially how transmembrane and juxtamembrane sequences switch-on or -off cytokine receptor signaling; ii) what are the general rules by which hydrophobic membrane-spanning domains interact in the membrane in a sequence-specific manner and how is this dimerization/oligomerization process regulated; and iii) the mechanisms of JAK attachment to receptors, and their subsequent activation, especially the role of pseudokinase domains in JAK kinase domain activation.

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) are actively pursued. The mutants we identified induce constitutive STAT activation, and we are interested in determining the gene targets of constitutive STAT5 activation, since we identified several novel target genes induced specifically by constitutively active STAT5, and that are involved in MPN pathogenesis. A close interaction structure has been created with clinicians and clinical biologists at St Luc Hospital for in-depth study of patient-derived cells.

The mechanisms by which the mutant JAK2 V617F induces Polycythemia Vera and other myeloproliferative neoplasms in humans

C. Pecquet, E. Leroy, V. Gryshkova, J.-P. Defour, M. Swinarska, D. Colau

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 pseudokinase 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 others. 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-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, granulocyte production and fibrosis (scarring) of the marrow due to excessive myeloid cell proliferation, enzyme release and collagen secretion by marrow fibroblasts.

We have been involved in the discovery of the unique acquired somatic JAK2 V617F mutation in collaboration with William Vainchenker at the Institut Gustave Roussy in Paris. JAK2 V617F responsible for >98% of Polycythemia Vera and for >50% of Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) cases (2). The mutation in the pseudokinase domain activates the kinase domain and constitutive signaling (3) in complexes between JAK2 V617F and cytokine receptors such as EpoR, TpoR and G-CSFR (Figure 1). The homol-

ogous V617F mutations in JAK1 and Tyk2 also enable these kinases to be activated without ligand-binding to cytokine receptors (3). These results suggested that point mutations in JAK proteins might be involved in different forms of cancers (1), with the already reported example of JAK1 mutations in T-lymphoblastic leukemia.

We are interested to understand how a pseudokinase domain mutation can induce kinase domain activation. The aim is to be able to specifically inhibit mutated JAK2 in myeloproliferative neoplasm patients, but not the wild type JAK2, which is crucial for red blood cell and platelet formation. We identified pseudokinase residue F595 as absolutely required for constitutive activation by V617F, but not for cytokine-induced activation of JAK2/JAK2 V617F (4). A region around F617 and F595, involving the middle of helix C of the JAK2 pseudokinase domain might be a target for specific JAK2 V617F inhibition (Figure 2). Expression of segments of JAKs is pursued in different cellular systems for structural studies. Furthermore, protein fragment complementation and protein ligation as-

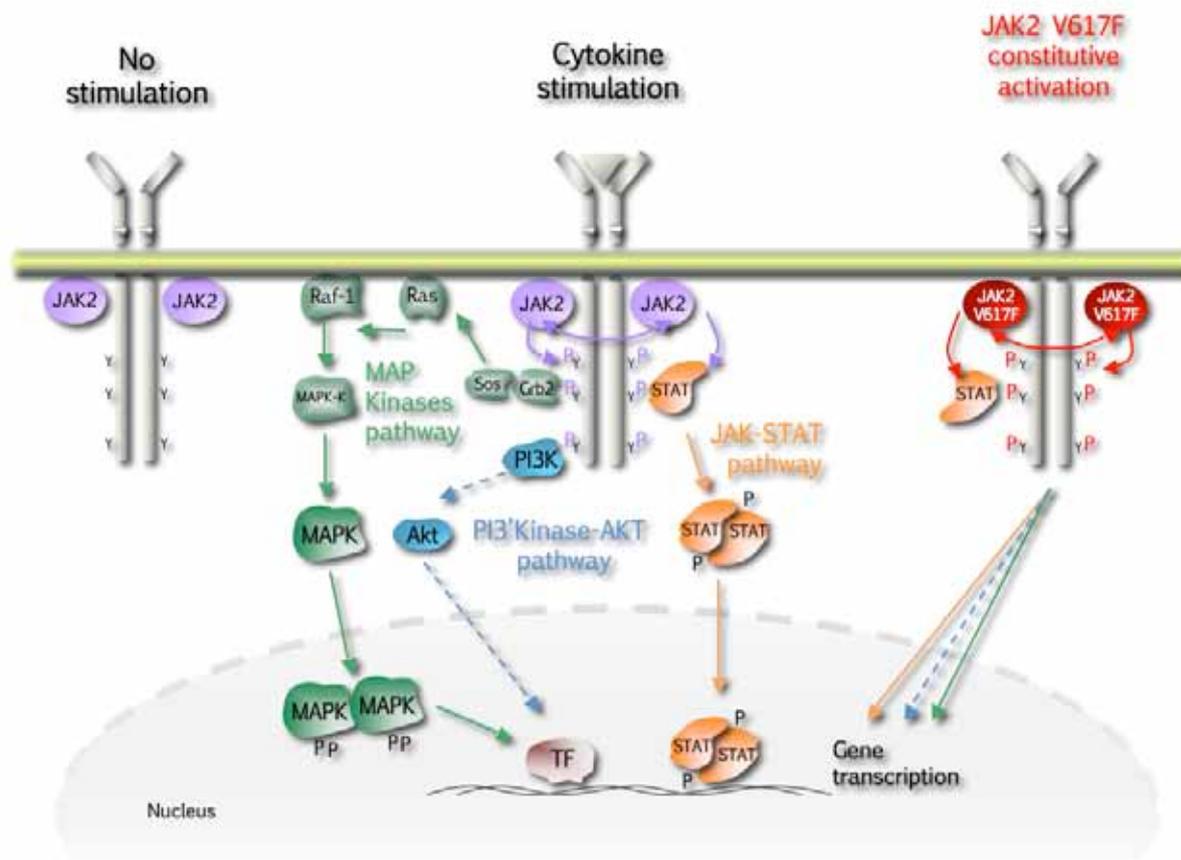


Figure 1. In the absence of cytokine ligands, cytokine receptors (left complex) are preassembled with tyrosine kinases JAK (Janus kinases) in inactive complexes. Cytokine binding to the extracellular domains of receptors (middle complex) induces a conformational change which allows the appended JAKs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

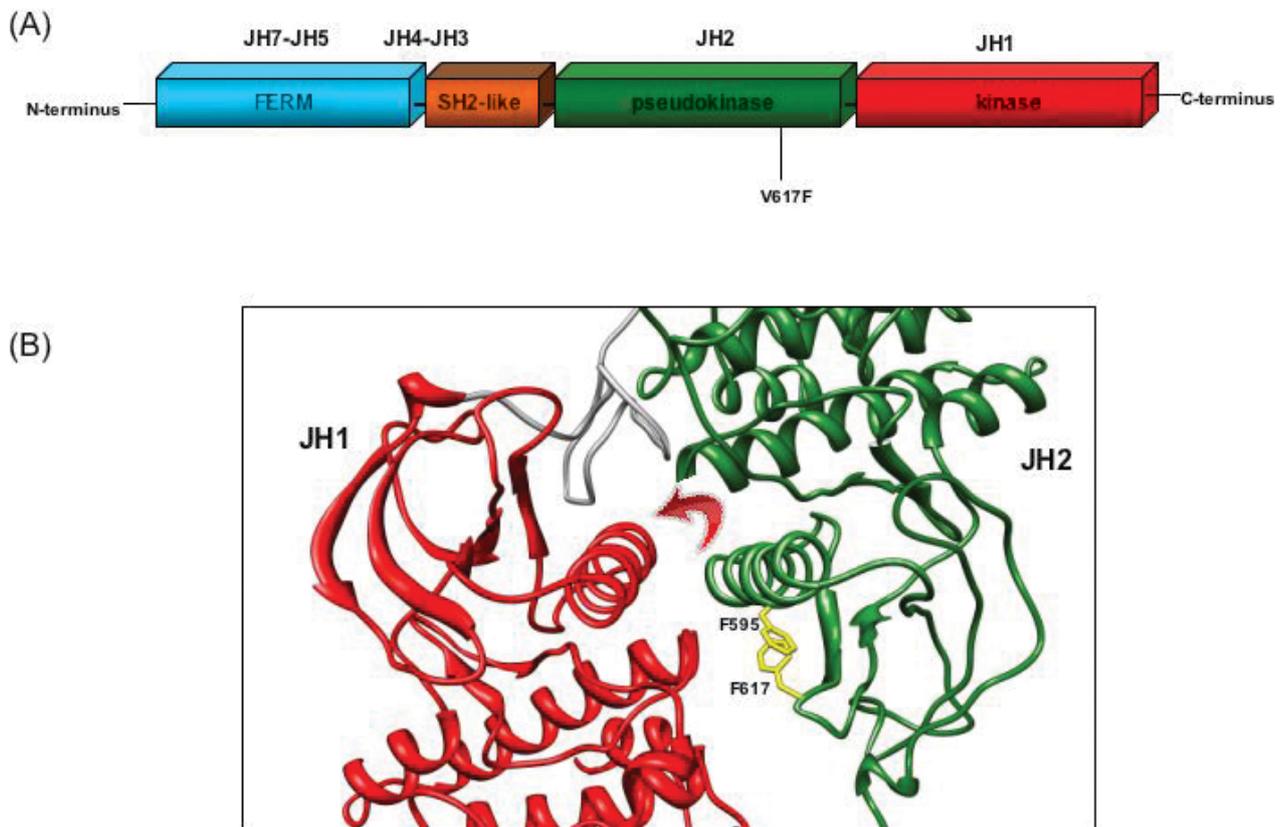


Figure 2. (A) Janus kinase 2 contains several JAK homology domains, JH1, the kinase domain; JH2 the pseudokinase domain; JH3-JH4 the SH2-like domain and JH4-JH7, the FERM (band four point 1, ezrin, radixin, moesin)-like domain. The pseudokinase domain plays a major role in cytokine-dependent activation 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.

says are employed in order to determine the dimerization state and proximity of interaction between mutated JAKs or wild type JAKs themselves and with cytokine receptors.

Involvement of pathologic TpoR signaling in myeloproliferative neoplasms

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

Thrombopoietin (Tpo) is a cytokine produced by the liver that is critical for regulation of the formation of platelet cells, the small enucleated blood cells that derive from the cytoplasm of megakaryocytes and that prevent bleeding upon injury. Tpo also regulates the numbers of hematopoietic stem cells and other myeloid cells.

TpoR appears to be central to MPNs. First, observations of Jerry Spivak from Johns Hopkins University indicated that

most MPN patients strongly down-modulate TpoR levels in megakaryocytes and platelets. Second, mutations in the TpoR intracellular juxtamembrane motif W515 led to constitutive activation of the receptor, and severe in vivo MPN with myelofibrosis. Third, asparagine mutations, which induce dimerization of the transmembrane domain of TpoR also activate TpoR and one such mutation has been shown to be associated with familial ET. Fourth, alterations of TpoR traffic 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 ubiquitylation, inhibition of recycling and degradation of TpoR (5). In addition we discovered that Tpo can induce a strong antiproliferative effect in cells that express high JAK2 levels (5). This effect can be detected in postmitotic mega-

karyocytes (Plos Biol 2010, 8, e1000476). We showed that selection against the antiproliferative effect of Tpo occurs in JAK2 V617F 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.

Biochemically, co-expression of TpoR and JAK2 V617F leads to strong ubiquitinylation of K540 and K544 of TpoR cytosolic domain and proteasome-mediated degradation of TpoR. In the same time, JAK2 itself is ubiquitinylated. Using the Ubi-Scan approach coupled to mass spectrometry we identified a number of proteins that acquire either ubiquitin or ISG15 or other ubiquitin-like molecules in a Tpo-dependent manner and we are establishing the roles of such post-translational modifications in Tpo-signaling and JAK2 V617F-mediated proliferation.

A novel mechanism by which a tryptophan residue regulates TpoR activation

J.-P. Defour, V. Gryshkova, C. Pecquet

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 (Δ 5TpoR) 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). Biophysical experiments performed in collaboration with Dr. Steve Smith at SUNY Stony Brook, and fluorescence complementation studies showed that W515 actually regulates the orientation, tilt and dimerization of the upstream transmembrane helix, and prevents receptor activation (Defour et al., manuscript in preparation). In vivo, in bone marrow reconstituted mice, the Δ 5TpoR and TpoR W515A mutant induce massive expansion of platelets, neutrophils and immature erythroid progenitors and myelofibrosis by day 45 (Figure 3). 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.

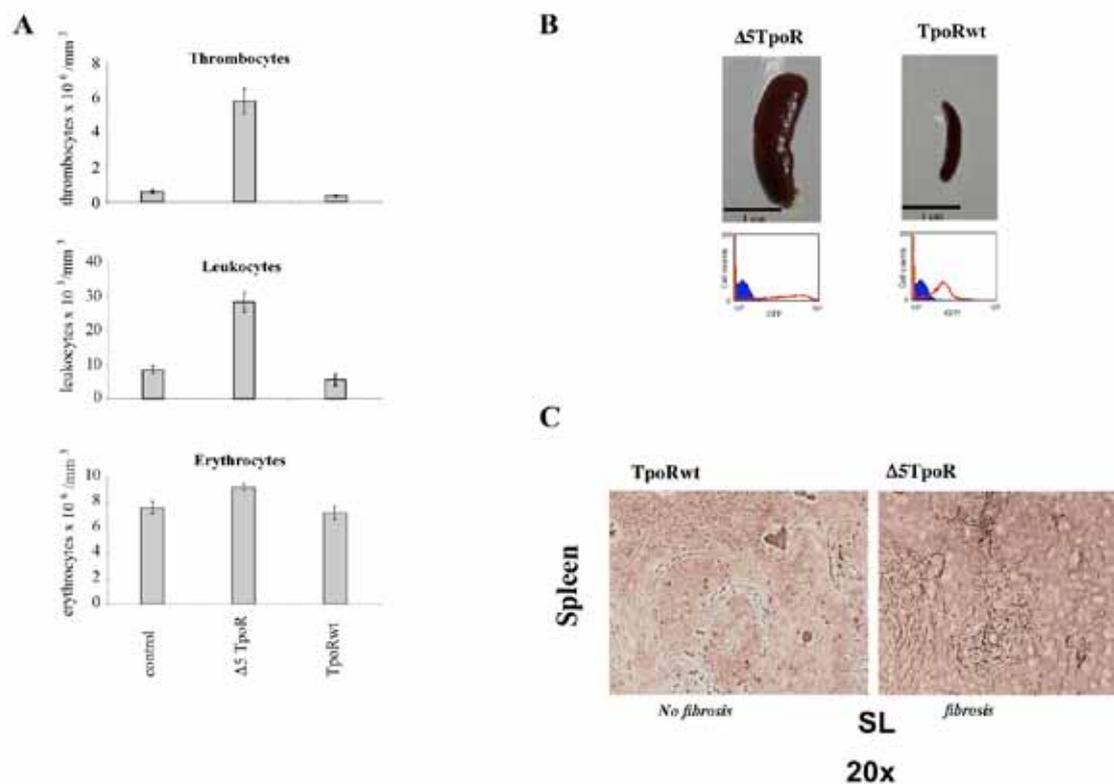


Figure 3. Bone marrow adoptive transfer in lethally-irradiated mice with hematopoietic stem cells expressing the constitutively active Δ 5TpoR induces severe myeloproliferative disorder, splenomegaly and fibrosis of the spleen. Δ 5TpoR is a mutant where the amphipathic RW515QFP motif is deleted, which results in constitutive activation of receptor signaling. (A) Peripheral cell counts recorder 45 days after reconstitution indicates leukocytosis and thrombocytosis induced by Δ 5TpoR. (B) Splenomegaly was induced by Δ 5TpoR at day 45 post reconstitution. The spleen size in mice reconstituted with bone marrow transduced with wtTpoR 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 Δ 5TpoR 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 Δ 5TpoR mice.

Determination of the interface and orientation of the activated cytokine receptors, such as EpoR and TpoR

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 of the receptor, which results in the activation of cytosolic JAK2 proteins. 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 (8).

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 was imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR, only two fusion proteins out of seven stimulated the proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells (8). 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.

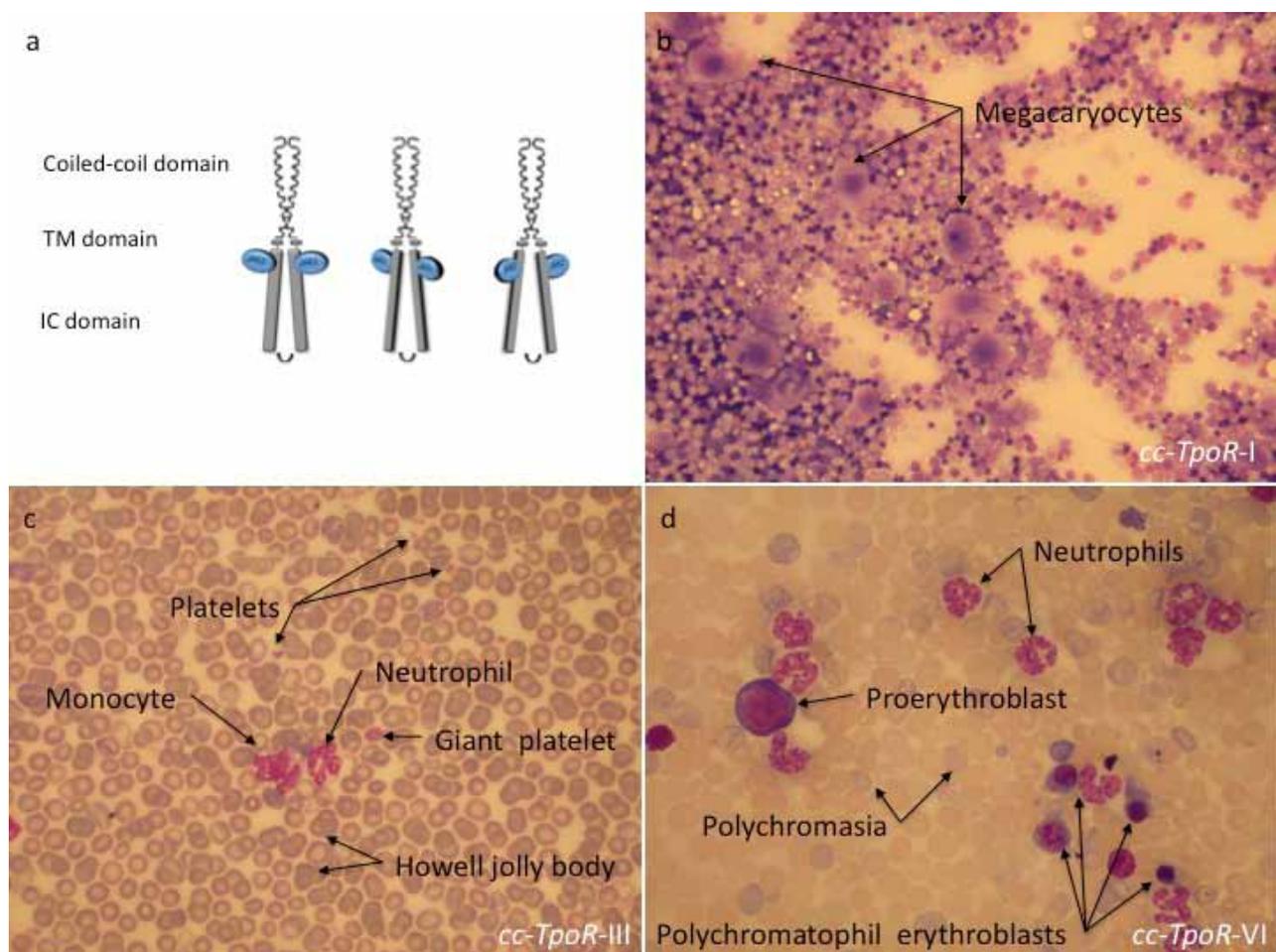


Figure 4. Different dimeric orientations of the thrombopoietin receptor lead to distinct phenotypes in the bone marrow and in the peripheral blood in bone marrow reconstituted mice. (A) The fusion of the coiled coil protein to differently engineered transmembrane domains of the thrombopoietin receptor (TpoR) imposes distinct dimeric orientations to the receptor. The different effects induced by three such chimeric receptors (cc-TpoR-I, cc-TpoR-III and cc-TpoR-VI) could be observed in the bone marrow and in the peripheral blood of lethally irradiated mice reconstituted with bone marrow cells retrovirally transduced with the indicated cc-TpoR fusion proteins. IC: intracellular, TM: transmembrane. (B) cc-TpoR-I induced a strong megakaryocytic response in the bone marrow with normal cellularity and a correct maturation of the myeloid lineages (May Grunwald stained bone marrow smear X 50, and normal platelet formation). (C) cc-TpoR-III induces granulocytosis, monocytosis and a very weak erythroblastosis in the peripheral blood (May Grunwald stained peripheral blood smear X 50) and weak dysplasia for the megakaryocytic and erythroid lineages in the marrow (not shown). (D) cc-TpoR-VI induced a stronger erythroblastosis and granulocytosis in the peripheral blood than cc-TpoR-III (May Grunwald stained peripheral blood smear X 50). (Jean-Philippe Defour).

Similar studies are undertaken for the related TpoR (Figure 4a). 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 (Figure 4b), other interfaces promote signaling that leads to myeloproliferative and myelodysplastic disorders (Figure 4c and d) (9). One orientation (cc-TpoR-II) corresponds to the inactive receptor state, and another (cc-TpoR-I) induces megakaryocyte differentiation and physiologic platelet formation (Figure 4b). The dimeric conformations represented by cc-TpoR-III and cc-TpoR-VI induce a combination of myeloproliferation and dysplasia of the erythroid lineage (9). Interestingly, the dimer orientation that induces the highest levels of JAK2 activation, cc-TpoR-IV, also induces strong cell-to-cell adhesion and expansion of early myeloid progenitors, as well as immortalization of myeloblasts. Our hypothesis is that in this dimeric orientation the receptor signals to maintain progenitors and possibly hematopoietic stem cells in the niche, and that signals induced by this dimer orientation might be recapitulating the quiescence-inducing effects of TpoR in HSCs. We will use this set of seven differently oriented TpoR dimers to dissect the signals induced by TpoR at the different stages of hematopoietic development.

Structure and function of juxtamembrane and transmembrane sequences of transmembrane 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 immunofluorescence co-patching of differentially epitope tagged receptors along with protein ligation and protein fragment complementation 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 col-

laboration with the groups of Profs. Jean-Noel Octave in our university and Steven O. Smith (SUNY Stony Brook, NY). We identified three Gly-X-X-X-Gly motifs in the juxtamembrane and transmembrane domain of APP and showed that these consecutive motifs promote transmembrane helix dimerization and amyloidogenic processing of APP (J. Biol. Chem. 2008 283, 7733, Proc. Natl. Acad. Sci. USA 2009, 106, 1421).

Constitutive activation of JAK-STAT signaling pathways 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, 8). 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 (TTCNNNGAA) 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 Preferred 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 (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

J.-P Defour, J. Van Hees

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, 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) 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.

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Links

Group

Ludwig Institute for Cancer Research Ltd. NewsLink Sept 2005 of our group (<http://www.licr.org/12124501528/news-link/0509/>)

European Commission Marie Curie Research Training Network ReceptEUR (www.recepteur.org)

Research:

Whitehead Institute for Biomedical Research, MIT, Lodish Lab (<http://www.wi.mit.edu/lodish/>)

SUNY Stony Brook, Structural Biology, Smith Lab

<http://sos.bio.sunysb.edu/lab/homepage>

<http://csb.sunysb.edu/structuralbiology/homepage>

<http://csb.sunysb.edu/bsb/graduate-program-in-biochemistry-and-structural-biology>

European Hematology Association (EHA)

Scientific Program Committee EHA16 (London), EHA17 (Amsterdam)

Hematology (American Society of Hematology Education Book)

<http://www.asheducationbook.org/>

Learning:

Biologie moléculaire de la cellule (Molecular Cell Biology H.F. Lodish) French Edition

Vainchenker W, Delhommeau F, Constantinescu SN, Bernard OA.

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Blood. 2011 Aug 18;118(7):1723-35. Epub 2011 Jun 7. Review.

Bioinformatics:

Institute of Bioinformatics Bangalore, India

<http://www.ibioinformatics.org/>



Stefan Constantinescu, Member

Ludwig Institute for Cancer Research
de Duve Institute
SIGN - B1.75.07
Av. Hippocrate 74-75
B - 1200 Brussels

[T] +32 02 764 75 40
[F] +32 02 764 75 66
[E] Stefan.Constantinescu@bru.licr.org
[W] http://www.deduveinstitute.be/signal_transduction_and_molecular_hematology.php

Staff members

Christian **Pecquet**, Postdoctoral Fellow • Michael **Girardot**, Postdoctoral Fellow • Vitalina **Gryshkova**, Postdoctoral Fellow • Roxana-Irina **Albu**, PhD Student • Emilie **Leroy**, PhD Student • Jean-Philippe **Defour**, PhD Student • Céline **Mouton**, Research Assistant (Attachée de recherche) • Joanne **Van Hees**, Research Assistant (Attachée de recherche, Lab Manager) • Madeleine **Swinarska**, Research Assistant • Didier **Colau**, Senior Investigator • Julien **Doornaert**, Administrative Assistant

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