CS-17 project : Investigating the EBV replication activation by measuring the circulating Epstein-Barr Virus transcription factor ZEBRA, as predictor of pejorative events in HSC transplant patients (viral syndromes, GvHD, PTLD).

As part of its mission, CRYOSTEM provides scientists from all over the world with a collection of biological resources that is unique in Europe, to accelerate the development of research and medical innovation projects on bone marrow transplantation and its complications.

Interview with Dr. Emmanuel DROUET, leader of the CS-17 research project: "Investigating the EBV replication activation by measuring the circulating Epstein-Barr Virus transcription factor ZEBRA, as predictor of pejorative events in HSC transplant patients (viral syndromes, GvHD, PTLD)."[1]

Question 1: Could you introduce yourself and tell us about your research project?

I teach microbiology to students at the Faculty of Pharmacy and the Faculty of Science in Grenoble (UGA, Université Grenoble Alpes). Attached to the Structural Biology Department of the Unité Mixte de Recherche (UMR UGA-CNRS), I lead a team working on human persistent viruses (RNA and DNA viruses) and their balance with the host immune system.

[1] Projet CS-17 presentation: https://www.cryostem.org/en/research/supported-projects/

Most of our research work has been carried out over the past 10 years within the framework of our affiliation with Grenoble University Hospital. In 2022, the results of a study conducted in collaboration with Strasbourg and Nantes Hospitals, the CRYOSTEM Association and the Microbiology Department of UMC Utrecht in the Netherlands were published in the journal Pathogens[2].

We've been working on the Epstein-Barr virus (EBV) since the early 90s, and more specifically on the pathophysiology associated with the virus' lytic cycle. As a preamble, it's important to remember that, like all Herpesviruses, EBV exists in two forms: a relatively neutral latent form, and a lytic form in which the infected cell produces virions and thus a large repertoire of proteins. I often explain to my students that in the latent phase, the virus restricts its expression to around ten proteins, a figure multiplied by 8 in the lytic phase when it expresses its entire genetic repertoire. These two forms are closely linked to a specific stage of pathology: the induction of cellular transformation during the latent cycle and tumor progression during the lytic cycle.

Another highly problematic feature of EBV is its persistence: once contracted in early childhood or adolescence, it is impossible to eradicate.

Question 2: Is there a vaccine against EBV?

EBV has been known since the 1960s, and the first vaccine trials began very soon after the virus was discovered. Today, there are many vaccine trials underway, but no vaccine is effective against EBV-associated diseases. One of the reasons for this is the difficulty encountered by researchers and clinicians in targeting the pathology to be controlled, and more specifically the markers of viral expression, which are not the same for inflammatory diseases, autoimmune diseases or cancer. We know how to protect patients against the severity of the disease, but we haven't found anything effective to control the activity of the virus in the body.

Question 3: What scientific approach do you intend to use to limit the spread of the virus?

The starting point for any approach to vaccinology is to target the virus' envelope proteins, which enable it to attach to target cells. In the case of EBV, there are 6 envelope proteins, some of which facilitate entry of the virus into the B lymphocyte, for example, but there are others in epithelial cells, which complicates the vaccine strategy[3].

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[2] Pathogens 2022, 11(8), 928; https://doi.org/10.3390/pathogens11080928

[3] Drouet, Emmanuel. 2021. 'Epstein-Barr Virus: Should We Still Invest in Vaccines or Focus on Predictive Tests?' Infectious Diseases. IntechOpen. doi:10.5772/intechopen.101094

"Our research targets a protein involved in the replication cycle of the virus in lytic form."

It is technically very simple to replicate the virus in vitro in specific cell lines that will maintain it in its latent form. If we stimulate the activation of lytic viral replication and the EBV lytic cycle, 100% of the cell population will be in the lytic cycle. To activate this transformation, we use the ZEBRA protein as a switch to change from one form to the other.

In real-life or in vivo conditions, our observations are far more complex. Indeed, it is common to observe at the same time, in all individuals, the presence of populations of cells in the latent phase, at the level of memory B lymphocytes circulating in the blood or present in lymphoid nodes, and that of populations of virus-infected cells in the lytic phase at the level of the tonsils, for example.

"In the case of patients managed with immunosuppressive treatments, as in marrow transplantation, the risks of developing a form of lymphoma are significant."

EBV infects 95-99% of the world's population. In affected individuals, the two forms of the virus described above are present. In its latent form, the virus expresses oncoproteins, i.e. proteins with transforming (oncogenic) power: these oncoproteins transform "host" B lymphocytes into lymphoblasts expressing a small repertoire of proteins (around ten nuclear and membrane antigens), which are fortunately controlled by immunity.

In the case of bone marrow transplants, on the other hand, for patients treated with immunosuppressants, the situation is more problematic, as the risk of developing lymphoma is very high.

Question 4: Why targeting the ZEBRA protein?

"ZEBRA is a very early protein that acts as a transcription factor. It is considered a virulence factor, demonstrated by its role in the expression of strategic genes, but also by its presence in circulating blood."

ZEBRA is a transcription factor involved in tumor progression. In our recent work[4], we were able to show that this protein does not remain in the cells, a situation which is quite similar to that observed in the case of the HIV virus (Tat protein): Tat and ZEBRA are viral transcription factors with the capacity to be excreted into the circulating bloodstream, where they act as a toxin (toxoid proteins). It is important to note that it is then possible to assay them, making them biomarkers of interest, as we shall see later[5].

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[4] Germini D, Sall FB, Shmakova A, Wiels J, Dokudovskaya S, Drouet E, Vassetzky Y. Oncogenic Properties of the EBV ZEBRA Protein. Cancers (Basel). 2020 Jun 5;12(6):1479. doi: 10.3390/cancers12061479. PMID: 32517128; PMCID: PMC7352903

[5] Habib M, Buisson M, Lupo J, Agbalika F, Socié G, Germi R, Baccard M, Imbert-Marcille BM, Dantal J, Morand P, Drouet E. Lytic EBV infection investigated by detection of Soluble Epstein-Barr virus ZEBRA in

ZEBRA follows exactly this pattern. By crossing cell membranes without being degraded, it can penetrate the cytoplasm of B lymphocytes or epithelial cells before crossing the nuclear membrane to activate strategic genes: these include genes coding for the B lymphotropic cytokines IL-6 or IL-10, as well as for metalloproteinases, all of which reinforce immunosuppression in transplant patients and lead to tumor angiogenesis.

"It's as if ZEBRA were preparing the ground for a pool of B lymphocytes by facilitating their infection by the virus."

Described as a very early stage protein in the lytic cycle, it would be interesting to be able to use ZEBRA either as a therapeutic target[6][7] to block viral progression, or as a predictive biomarker to assess the risk of complications in patients.

Question 5: What benefits have you derived from the biological resources made available by CRYOSTEM?

We were given access to the CRYOSTEM collection by Professors Gérard Socié and Régis Peffault de Latour, hematologists in the transplant department at Hôpital Saint-Louis. Initially, we tested the characteristics of the ZEBRA protein using a technique developed on the basis of serum and then plasma. We now hope to be able to carry out the same work using whole blood and strip tests.

Thanks to these biological resources, we have undertaken to validate our hypotheses on the interest of this biomarker by mobilizing an international network to accelerate the development of a new drug candidate. Our clear aim is to offer a robust alternative to the current practices of clinicians, who rely essentially on viral load studies to prevent lymphoma in the context of bone marrow transplants, for example.

In most cases, this involves performing a PCR (Polymerase Chain Reaction) on the patient's whole blood to detect EBV DNA present in memory B lymphocytes. However, as we explained earlier, this data represents a fairly low predictive value, as EBV is a persistent virus affecting 95-99% of the world's population. Moreover, we found that some transplant patients could develop severe, even fatal, forms of lymphoma with undetectable viral loads, while other

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the serum of patients with PTLD. Sci Rep. 2017 Sep 5;7(1):10479. doi: 10.1038/s41598-017-09798-7. PMID: 28874674; PMCID: PMC5585268

[6] Bilger A, Plowshay J, Ma S, Nawandar D, Barlow EA, Romero-Masters JC, Bristol JA, Li Z, Tsai MH, Delecluse HJ, Kenney SC. Leflunomide/teriflunomide inhibit Epstein-Barr virus (EBV)- induced lymphoproliferative disease and lytic viral replication. Oncotarget. 2017 Jul 4;8(27):44266-44280. doi: 10.18632/oncotarget.17863. PMID: 28574826; PMCID: PMC5546479

[7] Drouet, Emmanuel. 2020. 'The Role of the Epstein-Barr Virus Lytic Cycle in Tumor Progression: Consequences in Diagnosis and Therapy'. Human Herpesvirus Infection - Biological Features, Transmission, Symptoms, Diagnosis and Treatment. IntechOpen. doi:10.5772/intechopen.88607

patients with viral loads of over 100,000 copies per milliliter showed no symptoms at all. While the average viral load in asymptomatic patient populations is low, in transplant patients the situation is more complex. In the context of bone marrow transplantation, viral loads are generally correlated in 60-70% of cases with the occurrence of lymphoma, whereas the ZEBRA protein assay appears to be much more precise, with correlations of the order of 95% or more.

It's also important to remember that the use of Rituximab[8] as a preventive treatment for lymphoma in the event of increased viral load in transplant patients can lead to dramatic situations for individuals who are not necessarily predisposed to developing the disease. This treatment can lead to lymphopenia (with hypogammaglobulinemia), thus multiplying the risk of superinfection[9] [10].

To demonstrate the value of ZEBRA as a predictive biomarker, we conducted an ancillary study with samples from GvH patients, the results of which we shared at the EBV congress[11] in 2020. We will be publishing these data in the coming months, but we have already been able to establish correlations between the dosage of this ZEBRA protein and the occurrence of late-onset lymphoma after solid organ transplantation.

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[8]Rituximab - https://www.has-sante.fr/jcms/p_3145051/fr/mabthera-rituximab

[9] Pavanello, F.; Zucca, E.; Ghielmini, M. Rituximab: 13 Open Questions after 20 years of Clinical Use. Cancer Treat Rev. **2017**, 53, 38–46.

[10] Barmettler, S.; Ong, M.-S.; Farmer, J.R.; Choi, H.; Walter, J. Association of Immunoglobulin Levels, Infectious Risk, and Mortality With Rituximab and Hypogammaglobulinemia. JAMA Netw. Open **2018**,

[11] Lupo J et a. (2021) Prognostic value of the soluble ZEBRA (Zta) protein in transplant patients with PTLD and Graft versus Host Disease (GVHD). 19th international Symposium on EBV and associated diseases. Tokyo, (Japan)

About CRYOSTEM

Launched by the French National Research Agency (ANR) as part of its "Investissements d'Avenir" program, the CRYOSTEM project is at the heart of the French government's Cancer Plan, and is supported by the French National Cancer Institute (INCa), the CRYOSTEM project was initiated in 2011 under the aegis of the Société Francophone de Greffe de Moelle et de Thérapie Cellulaire (SFGM-TC) to accelerate research into the complications of hematopoietic stem cell transplantation (HSCT). Today, CRYOSTEM brings together all French transplant units, 28 Biological Resource Centers (BRCs) and over 400 French players in research and care in the field of severe blood disorders.