Charles University in Prague

Faculty of Sciences

Virus Restriction and Adaptation during Latent and Chronic Infection

Habilitation thesis

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CONTENT

LIST OF PUBLICATIONS

Chapters in monographies

1. Vonka, V., Kanka, J., Hirsch, I., Krcmar, M., Suchankova, A., Zavadova, H., and Jelinek, J. (1985). ***Serono Symposia***. *Herpes and Papilloma Viruses. Their Role in the Carcinogenesis of the Lower Genital Tract, Milano*.
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3. Hirsch, I., Roubal, J., and Vonka, V. (1975). Physical structure of herpes simplex virus type 1 DNA. ***IARC Sci Publ***(11 Pt 1)**,** 155-9.

Original publications

1. Farnault L, Gertner-Dardenne J, Gondois-Rey F, Michel G, Chambost H, Hirsch I, Olive D. (2015). Full but impaired activation of innate immunity effectors and virus-specific T cells during CMV and EBV disease following cord blood transplantation.**Bone Marrow Transplant**. 50(3):459-62. . **IF=3.570**

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3. Farnault L, Gertner-Dardenne J, Gondois-Rey F, Michel G, Chambost H, Hirsch I, Olive D. (2013). Clinical evidence implicating gamma-delta T cells in EBV control following cord blood transplantation. **Bone Marrow Transplant**. 48(11):1478-9. **IF=3.570**

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6. Gondois-Rey F, Granjeaud S, Kieu Sle T, Herrera D, Hirsch I, Olive D. (2012). Multiparametric cytometry for exploration of complex cellular dynamics. **Cytometry A**. 81(4):332-42. **IF=3.066**

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8. Trejbalová K, Blazková J, Matousková M, Kucerová D, Pecnová L, Vernerová Z, Herácek J, Hirsch I, Hejnar J. (2011). Epigenetic regulation of transcription and splicing of syncytins, fusogenic glycoproteins of retroviral origin. **Nucleic Acids Res**. 39(20):8728-39. **IF=9.112**

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Rapports and reviews

REVIEWER

Hepatology, Journal of Hepatology, Journal of Virology, Virus Research, Journal of AIDS, European Journal of Immunology, Vaccine, Gene, Immunological Letters, Archivum Immunologiae et Therapiae Experimentalis, Microbial Pathogenesis, Journal of Immunological Research, BioMed Reseach International

RAPPORTEUR FOR GRANTING AGENCIES

Czech Science Foundation (GAČR)
French Evaluation Agency for Research and Higher Education (AERES)
La Ligue Contre le Cancer (francouzská charitativní organizace)
The AIDS Fonds Netherlands
Australia's National Health and Medical Research Council (NHMRC)
National Institutes of Health (NIH), USA
United States-Israel Binational Science Foundation (BSF)

Invited speaker

Mayo Clinic and Charles University, Faculty of Medicine in Hradec Králové Joint Scientific Conference. Targeting B Cell Receptor-Like Signaling Restores Interferon-Alpha Production In Plasmacytoid Dendritic Cells Exposed To Hepatitis C Virus". Inv. Pavel Boštík

Institute of medical microbiology, Charles University in Prague, Hepatitis B and C infection: role of the innate immunity. 26.6.2013. Inv. Z. Mělková

NIH**,** NIAID Vaccine Research Center (VRC) : Hepatitis C virus and the innate immunity. Bethesda. MD 2.11.2012. Inv. Leonid. B. Margolis

SFR Bioscience, UMS3444/US8, CIRI, Centre International de Recherche en Infectiologie, le CIRI. Hepatitis C virus and the innate immunity. Lyon, 3.7.2012. Inv. Patrick Lecine

CIRI, Oncoviruses and Innate Immunity; INSERM U1111. Hepatitis C virus and the innate immunity. Lyon, 24.5.2012. Inv. Uzma Hasan

Institut de Recherche sur les Maladies Virales et Hépatiques : Hepatitis C virus and the innate immunity. INSERM, Unité 1110, Strasbourg, 23.3.2012. Inv. Thomas Baumert.

Inserm Unit 871, Hepatitis Viruses and Related Diseases, Lyon. Hepatitis C virus and the innate immunity.18.1.2011. Inv. D. Durantel

ÚHKT Praha. Lecture celebrating the 80th birthday of prof. V. Vonky. Hepatitis C virus and the innate immunity. 17.11.2010. Inv. Š. Němečková

19th International Conference "AIDS, Cancer and Public Health" St. Petersburg, Russia, "Regulation of HIV latency" 25/06/2010. Invited by organizers.

St. Petersburg University, Russia: Presentation of L. M. Margolis at the occasion of attribution of the Medal of the St. Petersburg University, Russia, 24/05/2010. Inv. Prof. Kozlov.

Inserm Unit 871, Hepatitis Viruses and Related Diseases, Lyon "Hepatitis C virus and the innate immunity", April 15, 2010. Inv. R. Parent.

Institute of Virology, Strasbourg "Hepatitis C virus and the innate immunity". November 28, 2009. Inv. T. Baumert.

9ème Réunion du Réseau National Hépatites, ANRS, Paris, 23 Janvier 2009. « Hepatitis C virus impairs plasmacytoid dendritic cell-associated production of interferon alpha".

Czech microbiological siciety: "Latence HIV". Konference v rámci mezinárodního kolokvia « Pokrok ve výzkumu HIV », organizováno Českou mikrobiologickou společností, Praha, 4.11.2008.

Institute of medical microbiology, Charles University in Prague, 6.11.2008. « Hepatitis C virus and the innate immunity »,

International Congress "Human papillomavirus in Human Pathology", Neoplastic Diseases Caused by Viruses, Praha, 1-3 květen, 2008.

Grants, principal investigator

Sensing of HIV-1 by plasmacytoid dendritic cells: dichotomy of immunoreceptor signaling. Granted by Czech Science Foundation for a period of 1/01/2014-31/12/2016.

Effects of hepatitis C virus on molecular mechanisms controlling production of IFN-alpha in plasmacytoid dendritic cells. Granted by French National agency for AIDS Research and Viral Hepatitis (ANRS) for a period 01/09/2010 → 31/08/2012.

Alterations of the innate immunity in the course of HCV infection. Co-PI with Daniel Olive. Granted by French National agency for AIDS Research and Viral Hepatitis (ANRS) for a period 01/01/2008-31/12/2009.

Interaction of HCV with the innate immunity and signaling via Toll-like receptors. Granted by French National agency for AIDS Research and Viral Hepatitis (ANRS) for a period 01/09/2007-31/08/2009.

Hepatitis C virus uptake and cross-presentation by human dendritic cells. Granted by French National agency for AIDS Research and Viral Hepatitis (ANRS) for a period 01/06/2009-01/05/2011.

Persistence of HIV: Relation between cellular quiescence and HIV latency in models of reservoirs of infection. Granted by French National agency for AIDS Research and Viral Hepatitis (ANRS) for the period of 01/07/2002-30/06/2004.

Reactivation of HIV latency. Granted by ECS-Sidaction (private charity organization) for the period 2002-2004.

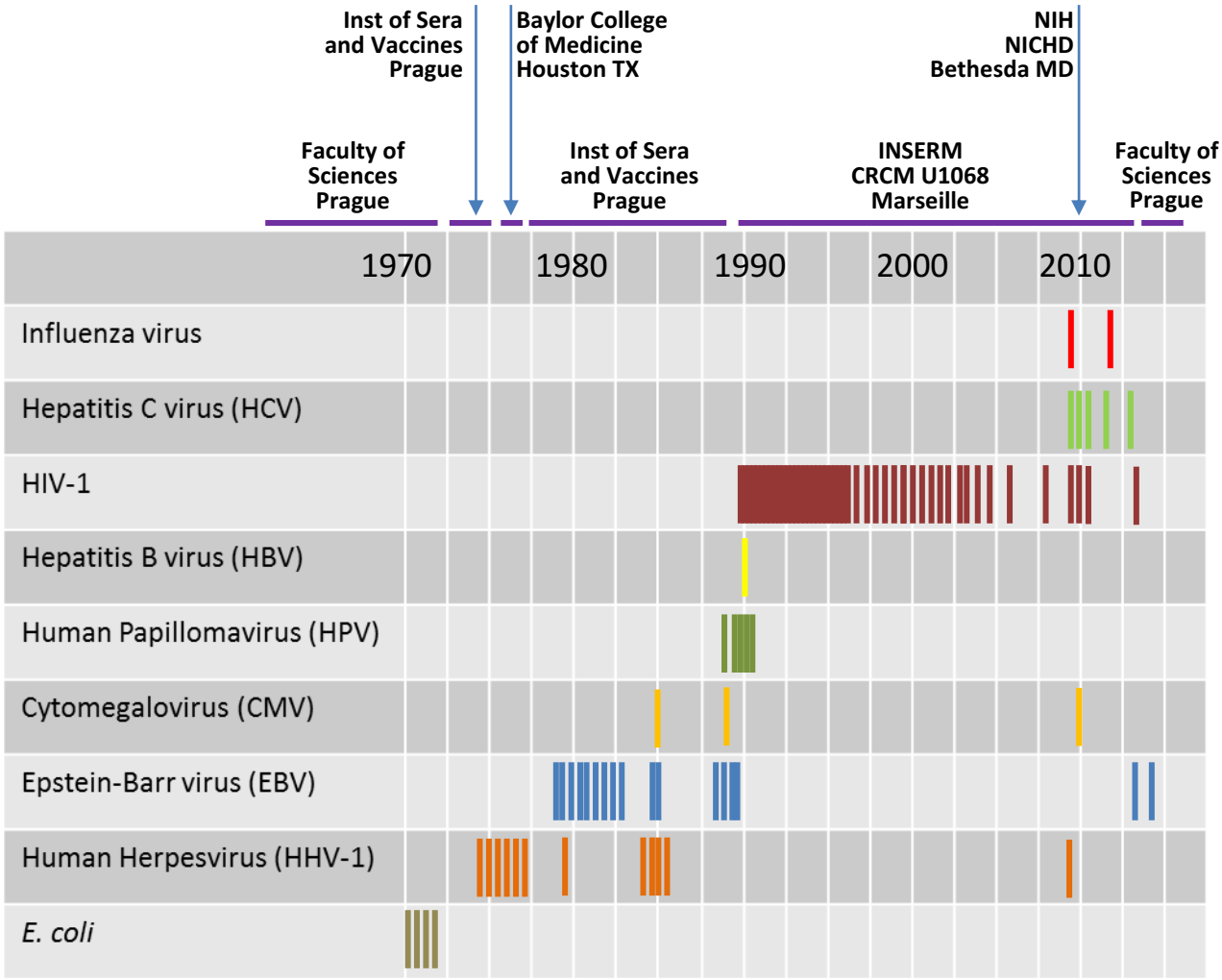
Study of viral genes involved in cytopathogenicity of HIV-1. Granted by ANRS for a period of 01/01/1995-31/12/1998.

Study of infection of intestinal cells with human immunodeficiency virus type 1. Granted by ANRS for a period of 01/01/1995-31/12/1998.

INTRODUCTION

In the present habilitation thesis, I structured my scientific work into thematically different parts related to different mechanism of interaction of viruses with the cell. These parts are connected with my projects, laboratories and investigated viruses. I introduce each part by scientific and organizational context followed by the most representative papers. The overview of my research activity is shown in Table 1.

Table 1. *Structure of scientific activity: viruses, years, publications and laboratories**



**vertical bars indicate papers on a given virus published in indicated year; laboratories are shown by interrupted horizontal blue line*

1973-1980 REPLICATION OF HUMAN HERPES VIRUS TYPE 1 (HHV-1) (8 PAPERS)

Context

My implication in the field of human virology started in 1973 at the Department of Experimental Virology of the Institute of Sera and Vaccines (ÚSOL) in Prague, under direction of Prof. Vladimir Vonka. As a part of a broad project on the etiological role human herpesviruses in cancer, I was involved in the study of molecular mechanisms of replication of Human herpesvirus type 1 (HHV-1), known also as Herpes simplex virus type 1 (HSV-1). I began my scientific career by demonstration of the presence of ribonucleotides linked to DNA of HSV-1 (Hirsch and Vonka, 1974) [37 times cited]. In parallel, I started to investigate molecular topology of HSV DNA, and molecular characteristics of newly synthesized HSV-1 DNA, exemplified by reference (Hirsch et al., 1976) [20 times cited]. I continued in this research direction during my postdoctoral stay at the Department of Virology and Epidemiology, Baylor College of Medicine in Houston, TX, under direction of Prof. Joseph L. Melnick (granted by the International Agency for Research on Cancer (IARC) WHO), exemplified by reference (Hirsch et al., 1977) [36 times cited].

Research outcome

Our study, which demonstrated the presence of ribonucleotides linked to genomic HSV-1 DNA (Hirsch and Vonka, 1974) followed a long lasting discussion about the presence of single stranded interruptions in the double-stranded DNA of HSV-1. The interruptions could be related to repair replication of viral and cellular DNA in HSV-infected cells, or to the presence of ribonucleotides sensible to alkaline conditions in the single stranded interruption detection system (Nishiyama and Rapp, 1981; Roizman, 1979a). In our paper, we infected cells of a continuous cell line derived from rabbit embryo fibroblasts with HSV-1 and maintained in the presence of either [5-^{3}H]uridine or [methyl-^{3}H]thymidine or $^{32}PO_4^{3-}$. Nucleocapsids were isolated from the cytoplasmic fraction, partially purified, and treated with DNase and RNase. From the pelleted nucleocapsids, DNA was extracted and purified by centrifugation in sucrose and cesium sulfate gradients. The acid-precipitable radioactivity of [5-^{3}H]uridine-labeled DNA was partially susceptible to pancreatic RNase and alkaline treatment; the susceptibility to the enzyme decreased with increasing salt concentration. No drop of activity of DNA labeled with [^{3}H]thymidine was observed either after RNase or alkali treatment. Base composition analysis of [5-^{3}H]uridine-labeled DNA showed that the radioactivity was recovered as uracil and cytosine. In the cesium sulfate gradient, the purified [5-^{3}H]uridine-labeled DNA banded at the same position as the ^{32}P-labeled DNA. The presented data tended to suggest that ribonucleotide sequences are present in HSV DNA, that they are covalently attached to the viral DNA, and that they can form double-stranded structures.

Together with the structure of HSV DNA, we studied also its replication (Hirsch et al., 1976). Conclusions of this work were consistent with isomerization of U_L and U_S segments of HSV DNA, and replication of genetic material of HSV *via* the formation of concatemers; it preceded suggestion of the rolling circle mechanism of HSV DNA replication (Roizman, 1979a, b). In addition, we investigated formation of single-stranded interruptions during DNA replication. HSV DNA yielded a heterogeneous sedimentation profile in neutral sucrose gradients, with the main peak occurring at approximately 40S. Components sedimenting slower than virion DNA and a rapidly sedimenting intracellular HSV DNA were also observed. Both the low-molecular weight and the rapidly sedimenting components seemed to be precursors of virion DNA: they almost completely disappeared after a 60-min chase of a 3-min pulse of ^{3}H-thymidine, and were converted into DNA which cosedimented with virion ^{32}P-labeled DNA. However, sedimentation analysis in alkaline sucrose gradients showed that a 60-min period was insufficient for completing the maturation of HSV DNA. No evidence for the formation of covalently closed circles during the replication process was obtained. The presence of single-stranded regions in the replicative form of HSV DNA was revealed. Some of the short-pulse (30 sec) labeled HSV DNA (26.1%) was eluted from hydroxylapatite columns with the properties of single-stranded DNA, and 22% of its trichloroacetic acid precipitability was susceptible to single-strand specific S1 nuclease treatment. Pulse-chase experiments indicated that the life-time of this single-stranded component in nascent DNA was probably not longer than 3 min. A small

proportion of single-stranded regions, however, survived for longer periods. Almost all of the newly synthesized short-pulse-labeled HSV DNA exhibited an affinity for nitrocellulose filters. This affinity, which was S1 nuclease-sensitive, gradually decreased with prolongation of the time of the chase. After chasing the pulse for 1 h, the attachment of newly synthesized DNA was comparable with virion DNA.

Then we analyzed intracellular replicating molecules of HSV-1 DNA by use of restriction endonucleases and electron microscopy (Hirsch et al., 1977) (**Figure 1**). HSV-1 infected rabbit kidney cells were either pulse-labeled with [^{3}H]thymidine ([^{3}H]dT) or density-labeled with 5-bromodeoxyuridine (BUdR). Replicating [^{3}H]dT-labeled HSV-1 DNA (ρ_{CsCl} = 1.725 g/cm^3) or BUdR-labeled HSV-1 DNA HSV (ρ_{CsCl} = 1.750 g/cm^3) were isolated by CsCl density gradient centrifugation. Viral molecules with sedimentation coefficients of 56 to 86 S were isolated, concentrated, mixed with ^{32}P-labeled marker 56 S DNA from purified virions, and cleaved with *Endo*R.*Eco*RI or *Hind* III. The highest ^{3}H to ^{32}P ratio for replicating HSV-1 DNA was found in fragments constituting the joint region of HSV-1 DNA. Electron microscopic observations of replicating molecules revealed the presence of both internal replication loops and lariat-type molecules. Though the origin and function of the lariat molecules is not known, the results presented suggest that the origin of HSV-1 DNA synthesis is located in the joint region of the S arm of the molecule. Our study helped to elaborate the model of HSV DNA replication by the rolling circle mechanism (Mocarski and Roizman, 1982; Roizman, 1979a, b).

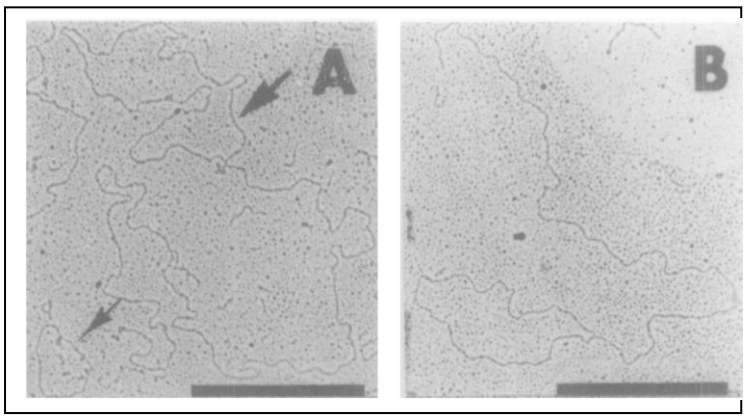


Figure 1. Electron micrographs of HSV DNA molecules extracted from RK cells at 3 hr p.i. (A and B) representative molecules containing terminal lariat structures.

The end loop (large arrow) generally does not exceed 5 μm. A small circular molecule is indicated by the small arrow. The bars represent a distance of 1 μm. *From (Hirsch et al., 1977).*

1984-1985 ETIOLOGICAL ROLE OF HSV-1 CERVICAL CARCINOMA (4 PAPERS)

Context

After my return from postdoctoral stay at the Baylor College of Medicine back to ÚSOL, I participated due to my experience in the field of molecular biology of HSV-1 in a consortium of experts, guided by Prof. Vladimír Vonka, in the prospective study on the relationship between cervical neoplasia and HSV-2, a major project at the Department of Experimental Virology (ÚSOL), exemplified by references (Vonka et al., 1984a; Vonka et al., 1984b) [71 and 85 times cited]. My major role in this prospective study was to develop a type specific test differentiating HSV-1 and HSV-2 antibodies (Suchankova et al., 1984) [22 times cited].

Research outcome

To determine the risk associated with previous HSV-2 infection and possibly other virus infections, a prospective study of cervical neoplasia in more than 10,000 women was performed in the 1975-1983 period (Vonka et al., 1984b). The subjects were selected at random from an alphabetical listing of eligible women living in one district of Prague. At enrollment colposcopy and cervical cytology were performed, a blood sample was taken and data regarding education, socio-economic status, personal habits and sexual and reproduction-associated attributes were obtained from each woman. A total of 10,683 women were enrolled; a complete set of data was obtained in 10,389 women. Women with normal or non-significant findings were invited for further colposcopical and cytological

investigations after 2 years and 4 years, the other women were followed at 3- to 6-monthly intervals. In women with highly significant findings, histological investigation was performed. The total of 150 cases of moderate to severe dysplasia (i.e. cervical intraepithelial neoplasia, grade II, CIN II), 83 cases of carcinoma in situ (CIN III) and 21 cases of invasive carcinoma (INCA) were detected. More than 60% of the patients were ill at enrollment, the other cases developed in subjects with originally slightly suspicious (27 CIN II, 17 CIN III, 3 INCA) or negative findings (30 CIN II, 12 CIN III, 3 INCA). Analysis of the data indicated significantly positive correlation of one or more of these clinical conditions with a number of sexual and reproduction-related attributes of which early age at first intercourse was most consistent. Among the other attributes, the smoking habit was associated with the highest risk of developing the disease. A negative correlation of cervical neoplasia with several attributes was demonstrated; of these diathermoelectrocoagulation of the ectopic epithelium and transformation zone of cervix was the most important single protective factor. On the basis of these findings, control subjects were selected for serological studies.

HSV-1 and HSV-2 antibodies in individuals enrolled in the prospective study were determined by a micro-solid-phase radioimmunoassay with *Helix pomatia* lectin/Sepharose 4B-purified antigens from HSV-1- and HSV-2-infected cells (Suchankova et al., 1984). A low but constant heterotypic reactivity was found with HSV-1 antigen, whereas HSV-2 antigen was type specific, as evidenced by (1) its reactivity with sera from HSV-2- but not with those from HSV-1-immunized animals, (2) its reactivity with monoclonal antibody to glycoprotein C but not with that to other HSV-2 glycoproteins, and (3) the negative results obtained in serum samples from 57 children, of whom 49 possessed HSV-1 neutralizing antibody. Antibody to HSV-2 (anti-HSV-2) was detected in serum samples from 14 of 16 subjects treated for genital herpetic lesions. The prevalence of anti-HSV-2 in women did not differ in the 25-35- and 36-45-year-old age groups but did correlate with the number of sex partners, with only 10% of women reporting one sex partner and 33% of those reporting more than 10 sex partners having anti-HSV-2.

Sera obtained at enrollment in the study from patients suffering from moderate to sever dysplasia (cervical intraepithelial neoplasia grade II), carcinoma in situ (cervical intraepithelial neoplasia grade III) and invasive carcinoma, or developing any of these conditions in the course of the prospective study, and from control subjects, were examined for HSV-2 antibody presence (Vonka et al., 1984a) [85 times cited]. We used *Helix pomatia* lectin-purified antigens from HSV-1- and HSV-2-infected cells for this purpose (Suchankova et al., 1984). The controls were matched with the patients by age, age at first intercourse, number of sexual partners, smoking habits and history of diathermoelectrocoagulation of the ectopic epithelium and transformation zone of cervix. Only those subjects were selected as controls who remained free of pathological colposcopical and cytological findings throughout the observation period, i.e. for at least 4 years after their serum sample was obtained. The microneutralization test (MNT) and type-2-specific solid-phase radioimmunoassay (SPRIA) were used as serological tests. No difference in the prevalence of HSV-2 antibody between the patients and controls was revealed by either test. Various combinations of the results from the two tests also failed to show any difference between patients and controls. Moreover, no significant differences were observed in the prevalence of HSV-2 antibody between patients suffering from the various pathological conditions and those diagnosed at enrollment and later in the course of the study. These results do not provide any support for the hypothesis of the involvement of HSV-2 in cervical neoplasia. Our conclusions paved the way to discovery of papillomaviruses as the etiological agent of cervical neoplasia (zur Hausen, 2009).

1978-1988 ETIOLOGICAL ROLE OF EPSTEIN-BARR VIRUS (EBV) IN OROPHARYNGEAL CARCINOMA (16 PAPERS)

Context

In addition to the study of the role of HSV in cervical carcinoma, we investigated at the Department of Experimental Virology (ÚSOL) together with my PhD student Beda Břicháček also involvement of EBV in oropharyngeal carcinomas based mostly on the research of EBV footprints in oropharyngeal tissues (Brichacek et al., 1984) [44 times cited], (Brichacek et al., 1983) [32 times cited].

Research outcome

In parallel to our study of the etiological role of HSV in cervical carcinoma, we investigated involvement EBV in oropharyngeal carcinoma. While the etiologic role of EBV in Burkitt lymphoma and nasopharyngeal carcinoma was clearly demonstrated, its role in other neoplasia is less clear (Iezzoni et al., 1995; Young et al., 1988). We focused our attention to tissues originating, like nasopharynx, from the Waldeyer´s ring, the palatine tonsils and the supraglottic larynx. The presence of EBV genetic material was demonstrated in thin sections of biopsy specimens in 6 of 7 carcinomas of human palatine tonsil by the *in situ* hybridization test (Brichacek et al., 1984) (**Figure 2**). The biopsy specimens from 4 tonsillar carcinomas exhibited a strong positive reaction; two biopsy specimens were weakly positive. Two tumor-free biopsy specimens were negative. None of the thin sections reacted with ^{3}H-labeled HSV DNA probe. Collectively, our results suggest an association between EBV and tonsillar carcinoma.

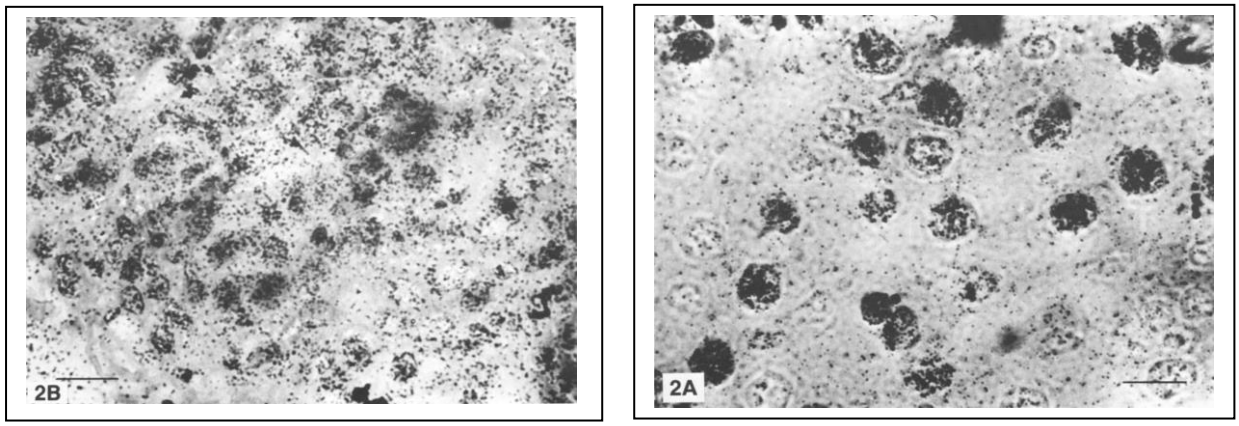


Figure 2. Autoradiogram of an in situ hybridization of 'H-Iabeled EBV DNA with thin section of the (2A) patient #1 *(bar=15 µm)* and (2B, next plate) patient #2 *(bar=20* µm) tonsillar tumors.

The two tissues were tested in two different experiments. *From (Brichacek et al., 1984).*

In another study, thin sections of biopsies from five patients with supraglottic laryngeal carcinoma who possessed antibody to early antigen of EB virus were examined for the presence of EB virus nuclear antigen (EBNA) and EB virus DNA by cytological hybridization *in situ* (Brichacek et al., 1983). Both EBNA and EB virus DNA were found in the tumour cells of three of the patients. The present findings suggest an association between EB virus and supraglottic carcinoma of the larynx.

1990- ACTIVE: HIV-1 LATENCY AND VIRUS RESERVOIRS (14 PAPERS)

Context

A major scientific subject of my research group established in Inserm laboratory in Marseille, France, directed by Jean-Claude Chermann, where I moved in 1988, was investigation of HIV-1 persistence and latency. Our research activity was focused to the study of establishment and eradication of virus reservoir(s) in patients treated by antiretroviral therapy (ART). These projects were supported by Inserm and by the French National Agency for AIDS research and virus hepatitis (ANRS); our results from this period are exemplified by references (Pion et al., 2003a) [32 times cited], (Blazkova et al., 2009) [126 times cited], (Sanchez et al., 1997) [88 times cited], (Biancotto et al., 2004) [51 times cited], (Anastasi et al., 2004) [21 times cited]. In this project I have collaborated intensively with Leonid B. Margolis from NICHD NIH, Bethesda, MD (Biancotto et al., 2004; Biancotto et al., 2008; Brichacek et al., 2010; Gondois-Rey et al., 2002), with Ruth M. Ruprecht from the Dana Farber Cancer Institute at the Harvard Medical School (Pion et al., 2001; Pion et al., 2003b), with Eric Verdin from the Gladstone Institute at UCSF (Blazkova et al., 2009; Pion et al., 2003a), with Carine van Lint from the Université Libre de Bruxelles, Brussels (Blazkova et al., 2009) (Pierard et al., 2010) and with Jiri Hejnar from the Institute of Molecular Genetics in Prague (Blazkova et al., 2009; Pion et al., 2003a).

Research outcome

The current protocols of ART are efficient in decreasing the HIV-1 load below the limit of detection, reducing mortality due to HIV-1 infection. Despite the potency of ART, however, HIV-1 establishes latent infection in several cell-type reservoirs, including that consisting of resting memory CD4+ T cells, which escapes host immune responses and antiretroviral therapy. HIV-1 latency is thus the main obstacle to the eradication of the virus from infected patients (Archin et al., 2014; Eisele and Siliciano, 2012; Ho et al., 2013; International et al., 2012; Palmer et al., 2011; Trono et al., 2010; Virgin and Walker, 2010).

DNA methylation of retroviral promoters and enhancers localized in the provirus 5' long terminal repeat (LTR) is considered to be a mechanism of transcriptional suppression that allows retroviruses to evade host immune responses and antiretroviral drugs. However, the role of DNA methylation in the control of HIV-1 latency has never been unambiguously demonstrated, in contrast to the apparent importance of transcriptional interference and chromatin structure, and has never been studied in HIV-1-infected patients. In contrast to the previously described systems, we and others have shown that transcriptional suppression of wild-type HIV-1 promoter is not accompanied by CpG methylation of the 5´ LTR (Blazkova et al., 2009; Duverger et al., 2009; Pion et al., 2003a).

In our first study on HIV-1 persistency we demonstrated that transcriptional suppression of *in vitro*-integrated HIV-1 does not correlate with proviral DNA methylation (Pion et al., 2003a). We investigated whether the CpG methylation of the HIV-1 promoter can directly influence the expression of the HIV-1 genome and thereby contribute to the persistence and latency of HIV-1. The levels of CpG methylation in the promoter of HIV-1 were studied after bisulfite-induced modification of DNA in five Jurkat clonal cell lines transduced by an HIV-1 LTR-driven retroviral vector and expressing enhanced green fluorescent protein (GFP) and in primary resting memory T cells challenged with HIV-1 or with an HIV-1-derived retroviral vector. We found that basal HIV-1 promoter activities were low or undetectable in three tested HIV-1 LTR-GFP clones, one of which encoded the Tat protein, and they reached medium or high levels in two other clones. The CpG dinucleotide that occurred in a latently infected clonal cell line 240 nucleotides upstream of the transcription start remained methylated after reactivation of HIV-1 transcription with 10 nM phorbol-12-myristate-13-acetate. In two clones showing a medium promoter activity and in resting memory T cells, the HIV-1 LTR was generally not methylated. Our results show that the methylation profiles of the HIV-1 LTR, including those present in latently infected cells, are low and do not correlate with the transcriptional activity. We suggest that, in a noncloned cellular population in which the HIV-1 proviruses are randomly integrated in the human genome, HIV-1 latency is imperfectly controlled by CpG methylation and is inherently accompanied by residual replication.

In our second study on persistent infection with HIV-1, we showed in an *in vitro* model of reactivable latency and in a latent reservoir of HIV-1-infected patients that CpG methylation of the HIV-1 5' LTR is an additional epigenetic restriction mechanism, which controls resistance of latent HIV-1 to reactivation signals and thus determines the stability of the HIV-1 latency (Blazkova et al., 2009). CpG methylation acts as a late event during establishment of HIV-1 latency and is not required for the initial provirus silencing (**Figure 3**). Indeed, the latent reservoir of some aviremic patients contained high proportions of the non-methylated 5' LTR. The latency controlled solely by transcriptional interference and by chromatin-dependent mechanisms in the absence of significant promoter DNA methylation tends to be leaky and easily reactivable. In the latent reservoir of HIV-1-infected individuals without detectable plasma viremia, we found HIV-1 promoters and enhancers to be hypermethylated and resistant to reactivation, as opposed to the hypomethylated 5' LTR in viremic patients. However, even dense methylation of the HIV-1 5' LTR did not confer complete resistance to reactivation of latent HIV-1 with some histone deacetylase inhibitors, protein kinase C agonists, TNF-alpha, and their combinations with 5-aza-2deoxycytidine: the densely methylated HIV-1 promoter was most efficiently reactivated in virtual absence of T cell activation by suberoylanilide hydroxamic acid (SAHA). SAHA could be a part of the “kick and kill” strategy, in which latent proviruses are first reactivated and then killed by efficient ART. Tight but incomplete control of HIV-1 latency by CpG methylation might have important implications for strategies aimed at eradicating HIV-1 infection.

This was the first study in which the level of CpG methylation of HIV-1 promoter was assessed in HIV-1-infected individuals.

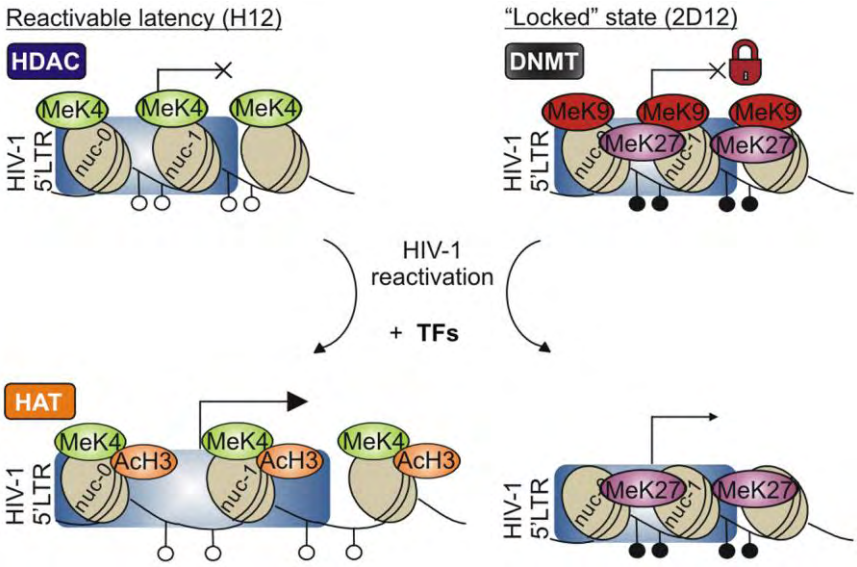


Figure 3. Two-step model of epigenetic control of HIV-1 latency.

In the state of reactivable latency exemplified by the H12 cell line, the HIV-1 promoter is hypomethylated, histone 3 (H3) is methylated on lysine 4 (MeK4), but deacetylated by histone deacetylases (HDAC), nuc-0 and nuc-1 are in proximal position; chromatin is condensed. After reactivation with TNF-α and PMA, the hypomethylated HIV-1 promoter-associated H3 is acetylated by means of histone acetyltransferases (HAT). Nucleosomes nuc-0 and nuc-1 are in distal position. In the "locked" silent state exemplified by the 2D12 cell line, the HIV-1 promoter is methylated by DNMT, and H3 is demethylated on K4 and methylated on K9 (MeK9) and K27 (MeK27). After reactivation of the hypermethylated HIV-1 promoter the HIV-1 LTR-associated H3 remains methylated on K27 but is demethylated on K9; CpG methylation is not changed and the repression is overcome by excess of transcription factors. The percentage of cells harboring reactivated HIV-1 is markedly lower in the clone with the "locked" highly methylated promoter (2D12) than in the clone with hypomethylated promoter (H12). Open circles, nonmethylated CpG residues; closed circles, methylated CpG residues, TFs, transcription factors. *From (Blazkova et al., 2009)*

In addition to reactivable latency, we aimed to assess the extent of non-inducible defective HIV-1 proviruses in infected individuals. HIV-1 genomes present in peripheral blood mononuclear cells (PBMCs) of infected persons or in lymphocytes infected *in vitro* were studied by long-distance PCR (LD-PCR) using primers localized in the HIV-1 LTR (Sanchez et al., 1997). The full-length 9-kb DNA was the only LD-PCR product obtained in peripheral and cord blood lymphocytes from seronegative donors infected *in vitro*. However, a high proportion (27% to 66%) of distinct populations of extensively deleted HIV-1 genomes of variable size was detected in PBMCs of 15 of 16 HIV-1-infected persons. Physical mapping of defective genomes showed that the frequency of deletions is proportional to their proximity to the central part of HIV-1 genome, which is consistent with a deletion mechanism involving a single polymerase jump during reverse transcription. Sequencing of deletion junctions revealed the presence of short direct repeats of three or four nucleotides. The number of defective HIV-1 genomes decreased after *in vitro* activation of PBMCs. Persistence of full-length and deleted genomes in *in vitro* activated PBMCs correlated with isolation of an infectious virus. Our results represent the first quantitative assessment of intragenomic rearrangements in HIV-1 genomes in PBMCs of infected persons and demonstrate that, in contrast to *in vitro* infection, defective genomes accumulate in PBMCs of infected persons.

Finally, to design strategies to purge latent reservoirs of HIV-1, we investigated mechanisms by which a non-tumor-promoting phorbol ester, prostratin, inhibits infection of CD4+ T lymphocytes and at the same time reactivates virus from latency (Biancotto et al., 2004). Our goal was to achieve efficient reactivation of HIV-1 from the latency without undesirable activation and proliferation of the immune system. Prostratin was a candidate agent for the "kick and kill" strategy to eradicate HIV-1 reactivated

from latency by efficient ART. CD4+ T lymphocytes from PBMC and in blocks of human lymphoid tissue were stimulated with prostratin and infected with HIV-1 to investigate the effects of prostratin on cellular susceptibility to the virus. The capacity of prostratin to reactivate HIV from latency was tested in CD4+ T cells harboring preintegrated and integrated latent provirus. Prostratin stimulated CD4+ T cells in an aberrant way. It induced expression of the activation markers CD25 and CD69 but inhibited cell cycling. HIV-1 uptake was reduced in prostratin-stimulated CD4+ T PBMC and tissues in a manner consistent with a downregulation of CD4 and CXCR4 receptors in these systems. At the postentry level, prostratin inhibited completion of reverse transcription of the viral genome in lymphoid tissue. However, prostratin facilitated integration of the reverse-transcribed HIV-1 genome in nondividing CD4+ T cells and facilitated expression of already integrated HIV-1, including latent forms. Thus, while stimulation with prostratin restricts susceptibility of primary resting CD4+ T cells to HIV infection at the virus cell-entry level and at the reverse transcription level, it efficiently reactivates HIV-1 from pre- and postintegration latency in resting CD4+ T cells.

In addition to our effort to develop a "kick-and-kill" strategy to purge HIV-1 reservoir, I participated also in development of directly-acting anti-HIV-1 drugs. In collaboration with Jean-Louis Kraus from the Faculty of Science, Aix-Marseille University, we elaborated a concept of potent nonclassical nucleoside antiviral drugs based on the N,N-diarylformamidine. New formamidine-3TC (3TC = 2',3'-dideoxy-3'-thiacytidine) analogues have been synthesized through various methods, and their antiviral activities (HIV, HBV) have been evaluated *in vitro* (Anastasi et al., 2004) [20 times cited]. Anti-HIV-1 in acutely infected MT-4 cells and PBMCs showed that compounds substituted by N,N-diarylformamidine side chains at the 4-N nucleic base position (compounds 3 and 8-11) had at least equivalent anti-HIV activity as 3TC (EC_{50} = 0.5 and 11.6 microM, respectively). Moreover, the newly synthesized compounds demonstrated higher anti-HBV activity (EC_{50} ranging from 0.01 to 0.05 microM) compared to the parent nucleoside 3TC (EC_{50} = 0.2 microM). It should be underlined that these new promising derivatives inhibited HIV in cells of a macrophage lineage, which are known to be cellular reservoir for HIV. These results were particularly of interest, since the antiviral activities appeared not to be mediated through the formamidine bond hydrolysis and consequently the release of free 3TC. These new analogue series were found to be highly stable to hydrolysis even after prolonged incubation in different biological media (t(1/2) ranged from 48 to 120 h). This enzymatic stability, coupled to the fact that no delay in the antiviral response was observed compared to the free 3TC antiviral response, suggest that this new N,N-diarylformamidine nucleoside series should not be considered as classical prodrugs.

To complement our studies on reactivation of latent replication competent HIV-1, I studied in collaboration with the laboratory of Jiří Hejnar from the Institute of Molecular Genetics of the Academy of Sciences of Czech Republic epigenetic regulation of transcription and splicing of syncytins, fusogenic glycoproteins of endogenous retroviral origin (Trejbalova et al., 2011). Syncytin-1 and -2, human fusogenic glycoproteins encoded by the *env* genes of the endogenous retroviral loci ERVWE1 and ERVFRDE1, respectively, contribute to the differentiation of multinucleated syncytiotrophoblast in chorionic villi. In non-trophoblastic cells, however, the expression of syncytins has to be suppressed to avoid potential pathogenic effects. We studied the epigenetic suppression of ERVWE1 and ERVFRDE1 5'-LTR by DNA methylation and chromatin modifications. Immunoprecipitation of the provirus-associated chromatin revealed the H3K9 trimethylation at transcriptionally inactivated syncytins in HeLa cells. qRT-PCR analysis of non-spliced ERVWE1 and ERVFRDE1 mRNAs and respective *env* mRNAs detected efficient splicing of endogenously expressed RNAs in trophoblastic but not in non-placental cells. Pointing to the pathogenic potential of aberrantly expressed syncytin-1, we have found deregulation of transcription and splicing of the ERVWE1 in biopsies of testicular seminomas. Finally, ectopic expression experiments suggest the importance of proper chromatin context for the ERVWE1 splicing. Our results thus demonstrate that cell-specific retroviral splicing represents an additional epigenetic level controling the expression of endogenous retroviruses.

1990- 2013 HIV-1 PATHOGENESIS AND TROPISM (33 PAPERS)

Context

The second major HIV-1 research program of our group in the Inserm U322 laboratory in Marseille was the study of viral pathogenesis and tropism. As a part of this project, we characterized HIV-1 isolates obtained in the Pasteur institute early after the virus isolation in 1983 (Chermann et al., 1991). We showed that the initial HIV-1 (BRU) isolate, growing strictly in primary T cells, was contaminated during the summer 1983 by HIV-1 (LAI) isolate, able to grow in T cell lines. Contaminated culture was unconsciously provided to NIH, where the HIV-1 LAI was probably selected in the effort to isolate T cell line-adapted virus by complementation. Identification of these isolates was in the center of international debate on priority of HIV-1 discovery between Luc Montagnier and Robert Gallo. Our work on pathogenesis project continued when my group moved to the Marseille Centre for Cancer Research (CRCM) of INSERM, affiliated also to the Aix-Marseille University and to the IPC Cancer Research Hospital. We developed a model system based on the construction of recombinants between highly cytopathic Zairian strain HIV-1 NDK and a prototype HIV-1 virus (Hirsch et al., 1990)[33 times cited], (Rey et al., 1991) [72 times cited], and studied HIV-1 tropism in trophoblasts (Zachar et al., 1991) [62 times cited], macrophages (Schmidtmayerova et al., 1992) [45 times cited], and intestinal cells (Chenine et al., 2002).

Research outcome

We showed that HIV-1 uses flexible replication strategies to infect different cell types and adapts efficiently in a new host cell. Our major model system consisted of a highly cytopathic subtype D Zairian strain HIV-1 NDK and recombinants derived from this and a prototype strain. We demonstrated that typically lymphotrpic HIV-1 NDK is able to infect in addition to primary CD4+ T lymphocytes and CD4+ T cell lines, also macrophages, trophoblasts and intestinal cells by CD4-independent mechanism.

In the first report of this series we howed that differences in replication and cytopathogenicity of HIV-1 are not determined by LTR. The growth properties of molecular clones of a highly cytopathic Zairian HIV1-NDK and prototype viruses were compared to correlate genetic variations with biological changes (Hirsch et al., 1990). The cloned HIV1-NDK retained the highly replicating cytopathic phenotype and formed larger syncytia than the prototype. One of the major differences in the alignment of the nucleotide sequence of the HIV1-NDK and HIV1-BRU prototypes was localized in the negative regulatory element (NRE) of the long terminal repeat (LTR). In a chloramphenicol acetyl transferase (CAT) assay, we failed to detect a significant difference between LTR promoter activity of the prototype and HIV1-NDK, suggesting that the LTR of both phenotypes had a similar function. The complete recombinant provirus DNA molecules bearing HIV1 LTR derived from one phenotype and the rest of the genomes from the other phenotype were constructed and transfected. The high cytopathogenicity of both the original and the chimeric viruses was correlated with the high speed of virus replication. Cytopathogenicity, morphology of syncytia, and replication kinetics of the recombinant viruses were determined by the functions coded within an internal part of HIV1 genome, covering the gag to env region, which were, however, not within LTR.

First, we demonstrated that productive infection of CD4+ cells by selected HIV strains is not inhibited by anti-CD4 monoclonal antibodies. Differential susceptibility of four diverse HIV strains to inhibition of infection of CD4+ CEM cells by anti-CD4 monoclonal antibodies was studied (Rey et al., 1991). The highly cytopathic HIV-1 246 and NDK strains were able to infect CEM cells and undergo several cycles of replication at saturating doses of LEU3-A, OKT4-A, and 13B8-2 monoclonal antibodies, whereas propagation of reference HIV-1 BRU and weakly cytopathic strain HIV-1 PAS was inhibited. Postadsorption treatment by anti-CD4 antibodies had stronger inhibitory effect than did treatment during the virus adsorption period. In parallel experiments, the same monoclonal antibodies successfully blocked syncytium formation between uninfected MT4 cells and CEM cells infected by all four HIV-1 virus strains tested. To explain these seemingly contradictory data we have postulated that anti-CD4 antibodies efficiently inhibit cell-to-cell but not virus-to-cell infection.

By the same approach we demonstrated that human transformed trophoblast-derived cells lacking CD4 receptor exhibit restricted permissiveness for HIV-1. We investigated the nature of interaction of the malignantly transformed cell lines of trophoblast origin BeWo, JAR, and JEG-3 with three different human immunodeficiency virus type 1 (HIV-1) isolates (RF, 3B, and NDK) (Zachar et al., 1991). After inoculation with cell-free virus, the persistence of infection was determined for 1 month by monitoring the presence of viral DNA in the cells by the PCR. Furthermore, the infectious virus in the culture supernatant was assayed with CEM-SS cells, and attempts to rescue the virus by cocultivation with CEM-SS cells were made. Appraised on the basis of the relative amount of viral DNA and the frequency of positive cocultivation. JEG-3 was the most permissive and BeWo was the least permissive cell line. However, when the cells were transfected with two biologically active molecular clones of HIV-1, the BRU and NDK isolates, all three cell lines turned out to support the production of mature virus progeny to the same extent. The abundance of viral DNA sequences in the infected cells varied with the isolate, showing an overall decline from RF to NDK. The amount of viral DNA in the cells and its expression decreased during the period of observation; this decrease was mirrored in an erosion of the virus recovery rate at cocultivation from 71% recovery on day 8 to failure of isolation on day 32. None of the cell lines expressed detectable amounts of cell surface CD4 molecules when assayed by flow microfluorometry and direct radioimmunoassay. Northern (RNA) blot hybridization analysis of both the total RNA and the mRNA did not reveal any CD4-specific message: nonetheless, by using the PCR, sequences specifically related to the CD4 gene were uncovered. The data demonstrate that the trophoblast-derived cell lines are susceptible to infection with HIV and that they support transient viral replication in the initial phases of infection. However, the latent form of infection may persist over a period of several weeks.

Then we described distinctive pattern of infection and replication of HIV-1 strains in blood-derived macrophages (BDM) (Schmidtmayerova et al., 1992). The macrophage-tropic virus HIV1-PAR, isolated from cerebrospinal fluid of HIV1-seropositive man, induced cytopathic effect accompanied by different magnitude of the virus production in BDM obtained from different donors. HIV1-PAR-specific RNA was detected by in situ hybridization in 15 and 66% of BDM producing low and high levels of virus, respectively. In contrast with HIV1-PAR, infection of BDM with two laboratory strains adapted to T-cell lines, HIV1-LAV prototype and HIV1-NDK, a Zairian virus that is highly cytopathic for T-lymphocytes, resulted in a low production of HIV1 p24gag in culture fluid. Expression of HIV1-LAV and HIV1-NDK RNA was detected by in situ hybridization in a maximum of 1% of macrophages. Only HIV1-NDK, and not HIV1-LAV, induced ultrastructural alterations in BDM. In contrast with a striking difference in the production of macrophage-tropic and T-lymphotropic viruses, no significant differences were found in the proportion of macrophages containing retrotranscribed genomes of HIV1-. HIV1 DNA was detected by in situ hybridization in 93, 100, and 80% of macrophages infected with HIV1-PAR, HIV1-LAV, and HIV1-NDK, respectively. A higher level of HIV1 DNA was detected by PCR in the BDM infected with HIV1-PAR than in that infected with HIV1-LAV and HIV1-NDK. The results indicate that both macrophage-tropic as well as T-lymphotropic viruses can enter and retrotranscribe their genomes in a vast majority of macrophages.

2006-ACTIVE: VIRUS RESTRICTION – INTERACTION WITH THE INNATE AND ADAPTIVE IMMUNITY (6 PAPERS)

Context

After relocation to Marseille´s Centre for Cancer Research (CRCM, INSERM U1068) I recentered my interest to the study of mechanisms of chronic virus infections related to cancer. I was mostly interested in interaction of viruses with cellular restriction mechanisms and with natural or innate anti-viral immunity. Among professional immune cells responsible for anti-viral defense, we selected as a major subject of our scientific interest the plasmacytoid dendritic cells (pDCs), which were intensively investigated at CRCM also because of their involvement in non-viral cancer pathogenesis (overviewed in (Hirsch et al., 2010) [47 times cited]. In a project supported by ANRS we investigated interaction of pDCs with hepatitits C virus, as exemplified by references (Gondois-Rey et al., 2009) [26 times cited], (Dental et al., 2012). [9 times cited], and (Florentin et al., 2012) [13 times cited].

Together with my major collaborator in the "pDC-HCV project", Prof. Thomas Baumert from the Institute of virology in Strasbourg (Dental et al., 2012; Florentin et al., 2012; Gondois-Rey et al., 2009), we studied cell signaling in hepatocytes infected by HCV ((Zona et al., 2013) [26 times cited]). In collaboration, with Andrea De Maria from the National Institute for Cancer Research in Genova, Italy, we studied effect of HCV infection on capacity of NK cells to mediate virus clearance and sustained immune response (Bozzano et al., 2011) [30 times cited].

Our group participated in studies of the immune responses against other viruses than HCV involved in persistent infections. Earlier, in collaboration with Marie Suzan, we studied cell signaling triggered in monocytes/macrophages by HIV-1 infection (Suzan et al., 1991) [45 times cited], and more recently, in collaboration with Leonid B. Margolis, we studied the mechanism by which HIV-1 facilitates its own infection in T lymphocytes (Biancotto et al., 2008) [30 times cited]. Finally, in collaboration with René A. W. van Lier from the Academic Medical Center, Amsterdam, The Netherlands, we studied adaptive responses during primary CMV infection (Serriari et al., 2010) [31 times cited]. My present research program continues in this direction.

Research outcome

Viruses are recognized by pDCs through Toll-like receptors 7 and 9 (TLR7/9) localized to the endosomal compartment. A common functional feature of these nucleoside-sensing TLRs is the induction of type I interferon (IFN) and pro-inflammatory cytokines, and the induction of cell differentiation (Gilliet et al., 2008) (**Figure 4**). Although signaling via TLR7 and TLR9 activates a

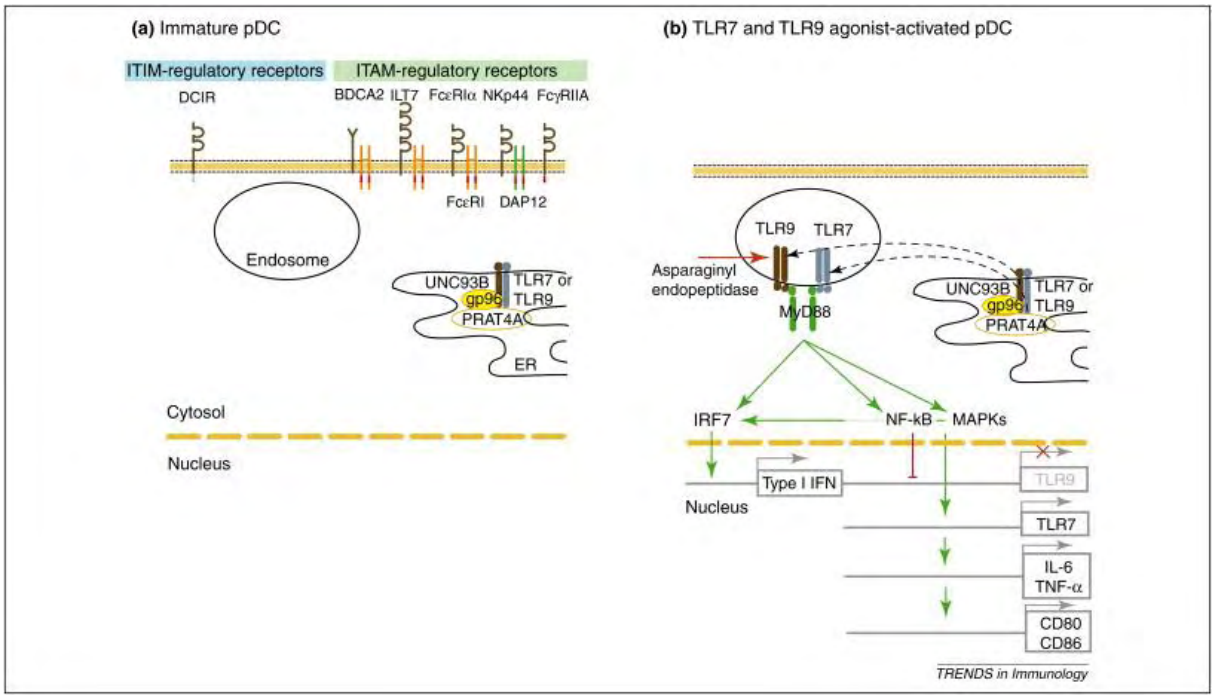


FIGURE 4. Control of TLR9 signaling in immature and TLR7 and TLR9 agonist-activated pDCs.

(a) Immature pDCs express TLR7 and TLR9, which are constitutively expressed and reside predominantly in the ER in association with UNC93B, gp96 and PRAT4A. pDC-surface receptors: DCIR, which contains an ITIM (in blue); BDCA2; ILT7; FcεRIα; and NKp44 associated with the γ-chain of FcεRI (in orange) or with DAP12 (in green), both of which contain an ITAM (in red). **(b)** Following exposure to virus or to synthetic agonists, TLR7 and TLR9 relocate from the ER to the endosomes. After relocation into the endolysosomal compartment, TLR7 and TLR9 are cleaved by lysosomal proteases. Conformational changes in the TLRs lead to the activation of MyD88 and to the propagation of downstream signals, which result in the activation of MAPKs, NF-κB and IRF7, which leads to secretion of type I IFNs. Translocation of NF-κB to the nucleus upregulates TLR7, pro-inflammatory cytokines, IL-6, TNF-α, and co-stimulatory molecules CD80 and CD86, but downregulates expression of TLR9. *From (Hirsch et al., 2010).*

variety of cell types, these TLRs are most commonly known as strong promoters of type I IFN secretion from pDCs. In addition to nucleotide-sensing TLRs, pDCs also recognize pathogens through a battery of cell surface-localized regulatory receptors, including C-type lectin and Fc receptors. The principal function of these regulatory receptors on pDCs is to facilitate antigen capture and to prevent aberrant immune responses by modulating production of type I IFNs and pro-inflammatory cytokines.

HIV-1, hepatitis B virus (HBV), hepatitis C virus (HCV), and human papillomavirus type 16 cause persistent infections that frequently precede cancer development (Hirsch et al., 2010). Virions of these viruses are weak inducers of interferon-α and impair TLR9 function. Loss of TLR9 responsiveness also occurs in tumors without viral etiology such as breast, ovary, and head and neck carcinomas. The principal question was, whether these viruses and cancer cells are invisible for pDCs (stealth viruses), or whether these viruses and cancer cells actively inhibit immune response. Recent reports have suggested that viruses and components of the tumor microenviroment interact with regulatory receptors on pDCs to impair TLR7 and TLR9 signaling, and to downregulate TLR9 gene expression. Thus, impaired Toll-like receptor 7 and 9 signaling: from chronic viral infections to cancer. The limited responsiveness of pDCs might contribute to reduced innate immune responses during chronic viral infections and oncogenesis, and represent a target for new therapeutic approaches based on TLR agonists.

We used HCV and its interaction with pDC as a major model in our studies. Viral elimination by IFN-α-based therapy in more than 50% of patients chronically infected with HCV suggests a possible impairment of production of endogenous IFN-α by pDCs in infected individuals (**Figure 4**). In this study, we investigated the impact of HCV on pDC function. We show that exposure of pDCs to patient serum- and cell culture-derived HCV resulted in production of IFN-α by pDCs isolated from some donors, although this production was significantly lower than that induced by influenza and HHV-1 (Gondois-Rey et al., 2009). Using specific inhibitors we demonstrate that endocytosis and endosomal acidification were required for IFN-α production by pDCs in response to cell culture-derived HCV. HCV and noninfectious HCV-like particles inhibited pDC-associated production of IFN-α stimulated with TLR9 agonists (CpG-A or HHV-1) but not that of IFN-α stimulated with TLR7 agonists (resiquimod or influenza virus). The blockade of TLR9-mediated production of IFN-α, effective only when pDCs were exposed to virus prior to or shortly after CpG-A stimulation, was already detectable at the IFN-α transcription level 2 h after stimulation with CpG-A and correlated with down-regulation of the transcription factor IRF7 expression and of TLR9 expression. In conclusion, rapidly and early occurring particle–host cell protein interaction during particle internalization and endocytosis followed by blockade of TLR9 function could result in less efficient sensing of HCV RNA by TLR7, with impaired production of IFN-α. This finding confirmed also by work from other laboratories (Decalf et al., 2007; Shiina and Rehermann, 2008) is important for our understanding of HCV-DC interaction and immunopathogenesis of HCV infection.

Then we studied differences in recognition of cell-free HCV particles and cell-associated HCV (HCV-infected hepatoma cells) by pDCs. pDCs exposed to HCV-infected hepatoma cells, in contrast to cell-free HCV virions, produce large amounts of IFN-α. To further investigate the molecular mechanism of HCV sensing, we studied whether exposure of pDCs to HCV-infected hepatoma cells activates, in parallel to interferon regulatory factor 7 (IRF7)-mediated production of IFN-α, nuclear factor kappa B (NF-κB)-dependent pDC responses, such as expression of the differentiation markers CD40, CCR7, CD86, and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and secretion of the proinflammatory cytokines TNF-α and interleukin 6 (IL-6) (Dental et al., 2012). We demonstrate that exposure of pDCs to HCV-infected hepatoma cells surprisingly did not induce phosphorylation of NF-κB or cell surface expression of CD40, CCR7, CD86, or TRAIL or secretion of TNF-α and IL-6. In contrast, CpG-A and CpG-B induced production of TNF-α and IL-6 in pDCs exposed to the HCV-infected hepatoma cells, showing that cell-associated virus did not actively inhibit TLR-mediated NF-κB phosphorylation. Our results suggest that cell-associated HCV signals in pDCs via an endocytosis-dependent mechanism and IRF7 but not via the NF-κB pathway. In spite of IFN-α induction, cell-

associated HCV does not induce a full functional response of pDCs. These findings contribute to the understanding of evasion of immune responses by HCV.

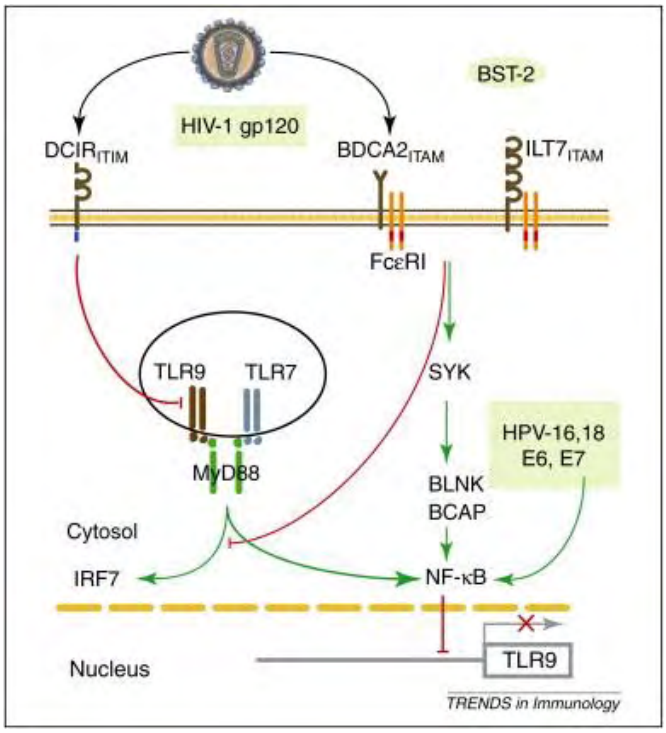


FIGURE 5. Impaired TLR7 and TLR9 signaling in virus-exposed pDCs and in tumor-environment-exposed pDCs.

The interactions that lead to impairments of TLR7 and TLR9 signaling for HIV-1, HPV-16 and BST-2 are illustrated as examples: HIV-1 gp120 associates with DCIR-ITIM and BDCA2-ITAM. Ligation of pDC BDCA2-ITAM-associated regulatory receptors with HIV-1 gp120 activates the BCR-like NF-κB pathway, including SYK, BLNK, and BCAP. Ligation of pDC DCIR-ITIM-associated regulatory receptors with HIV-1 gp120 inhibits activation of TLR9 with its agonists. Engagement of the receptor by the virus can induce blockade of TLR9 signaling just as ligation of the same surface receptor with MAbs does. Expression of HPV-16 proteins E6 and E7 in keratinocytes induces NF-κB, which after translocation into the nucleus downmodulates TLR9 expression. A high concentration of ligands such as BST2 within a tumor tissue can be responsible for a reduced capacity of tumor-associated pDCs to produce IFN-α after stimulation with CpG. *From (Hirsch et al., 2010).*

In our most original report of this series, we showed that HCV glycoprotein E2 is a novel BDCA-2 ligand and acts as an inhibitor of IFN production by pDCs, similarly as was shown for HIV-1 gp120 (Martinelli et al., 2007) (**Figure 5**). The elimination of HCV in > 50% of chronically infected patients by treatment with IFN-α suggests that pDCs, major producers of IFN-α, play an important role in the control of HCV infection. However, despite large amounts of Toll-like receptor 7-mediated IFN-α, produced by pDCs exposed to HCV-infected hepatocytes, HCV still replicates in infected liver. We showed that HCV envelope glycoprotein E2 is a novel ligand of pDC C-type lectin immunoreceptors (CLRs), blood DC antigen 2 (BDCA-2) and DC-immunoreceptor (DCIR) (Florentin et al., 2012). HCV particles inhibit, *via* binding of E2glycoprotein to CLRs, production of IFN-α and IFN-λ in pDCs exposed to HCV-infected hepatocytes (Dolganiuc and Szabo, 2011; Takahashi et al., 2010), and induce in pDCs a rapid phosphorylation of Akt and Erk1/2, in a manner similar to the crosslinking of BDCA-2 or DCIR. Blocking of BDCA-2 and DCIR with Fab fragments of monoclonal antibodies preserves the capacity of pDCs to produce type I and III IFNs in the presence of HCV particles. Thus, negative interference of CLR signaling triggered by cell-free HCV particles with Toll-like receptor signaling triggered by cell-associated HCV results in the inhibition of the principal pDC function, production of IFN. Our results demonstrate that exposure of pDCs to cell-free or cell-associated form of the same virus results in a totally different outcome – activation or suppression of the principal pDC function, IFN production. These disparate effects are controlled by interaction of both viral forms with different types of pDC receptors and illustrate the multitude of mechanisms employed by HCV to escape from immune recognition (Decalf et al., 2007; Dental et al., 2012; Dolganiuc and Szabo, 2011; Florentin et al., 2012; Shiina and Rehermann, 2008; Takahashi et al., 2010).

In collaboration with group of Thomas Baumert we studied also signal events accompanying entry of HCV in hepatocytes. We showed that HRas signal transduction promotes HCV cell entry by triggering assembly of the host tetraspanin receptor complex. It is known that HCV entry is dependent on coreceptor complex formation between the tetraspanin superfamily member CD81 and the tight junction protein claudin-1 (CLDN1) on the host cell membrane. The receptor tyrosine kinase EGFR acts as a cofactor for HCV entry by promoting CD81-CLDN1 complex formation via unknown mechanisms (**Figure 6**). We identify the GTPase HRas, activated downstream of EGFR signaling, as a

key host signal transducer for EGFR-mediated HCV entry (Zona et al., 2013). Proteomic analysis revealed that HRas associates with tetraspanin CD81, CLDN1, and the previously unrecognized HCV entry cofactors integrin b1 and Ras-related protein Rap2B in hepatocyte membranes. HRas signaling is required for lateral membrane diffusion of CD81, which enables tetraspanin receptor complex assembly. HRas was also found to be relevant for entry of other viruses, including influenza. Our data demonstrate that viruses exploit HRas signaling for cellular entry by compartmentalization of entry factors and receptor trafficking.

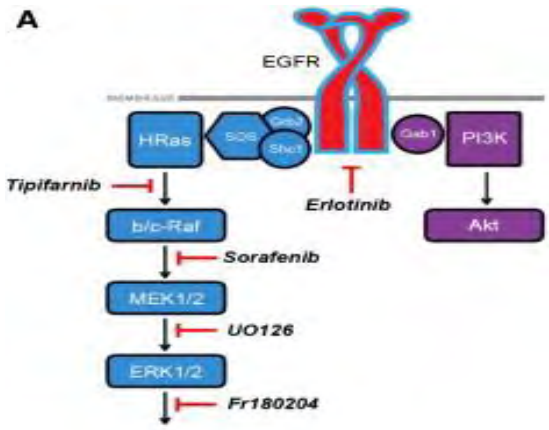


Figure 6. EGFR Signaling Pathways in Human HCV-Permissive Liver Cells, Hepatocytes, and Patient-Derived Liver Tissue

Scheme of the two main canonical EGFR signaling cascades: the MAPK and the PI3K/AKT pathways. Inhibitors targeting members of these pathways are indicated. *From (Zona et al., 2013).*

Efficient NK cell-anti-HCV response is essential for the virus clearance and sustained immune response. Specific NK cell killer inhibitory receptor (KIR):HLA haplotype combinations have been associated with successful clearance of acute and chronic HCV infection. Whether an imbalance of activating NK cell receptors also contributes to the outcome of treatment of chronic HCV infection, however, is not known. In collaboration with Andrea De Maria, we studied peripheral NK cell phenotype and function in 28 chronically viraemic HCV genotype I treatment-naıve patients who underwent treatment with pegylated IFN-α and ribavirin (Bozzano et al., 2011). At baseline, chronically infected patients with sustained virological response (SVR) had reduced CD56brightCD16(+/-) cell populations, increased CD56dullCD161 NK cell proportions, and lower expression of NKp30, DNAM-1, and CD85j. Similarly, reduced NK cell IFN-γ production but increased degranulation was observed among nonresponding (NR) patients. After treatment, CD56brightCD16(+/-) NK cell numbers increased in both SVR and NR patients, with a parallel significant increase in activating NKp30 molecule densities in SVR patients only. *In vitro* experiments using purified NK cells in the presence of rIL-2 and IFN-α confirmed upregulation of NKp30 and also of NKp46 and DNAM-1 in patients with subsequent SVR. Thus, differences in patient NK cell receptor expression and modulation during chronic HCV-1 infection are associated with subsequent outcome of standard treatment. Individual activating receptor expression/function (NKp30, NKp46, DNAM-1) integrates with KIR:HLA genotype carriage to determine the clearance of HCV infection upon combined IFN-α and ribavirin treatment.

Then we showed that NF-κB is induced during monocyte differentiation by HIV type 1 infection. The production of HIV-1 progeny was followed in the U937 promonocytic cell line after stimulation, either with retinoic acid or PMA, and in purified human monocytes and macrophages (Suzan et al., 1991). Electrophoretic mobility shift assays and Southwestern blotting experiments were used to detect the binding of cellular transactivation factor NF-κB to the double repeat-κB enhancer sequence located in the long terminal repeat. PMA treatment, and not retinoic acid treatment of the U937 cells acts in inducing NF-κB expression in the nuclei. In nuclear extracts from monocytes or macrophages, induction of NF-κB occurred only if the cells were previously infected with HIV-1. When U937 cells were infected with HIV-1, no induction of NF-κB factor was detected, whereas high level of progeny virions was produced, suggesting that this factor was not required for viral replication. These results indicate that in monocytic cell lineage, HIV-1 could mimic some differentiation/activation stimuli allowing nuclear NF-κB expression. This work suggests that virus infection in monocytes and

macrophages is directly involved in the initiation of inflammatory reaction, a hallmark HIV-1 infection.

Furthermore, we demonstrate mechanisms by which HIV-1 appears to facilitate its own infection in ex vivo-infected human lymphoid tissue. In this system, HIV-1 readily infects various CD4+ T cells, but productive viral infection was supported predominantly by activated T cells expressing either CD25 or HLA-DR or both (CD25/HLA-DR) but not other activation markers: There was a strong positive correlation (r=0.64, P=.001) between virus production and the number of CD25+/HLA-DR+ T cells. HIV-1 infection of lymphoid tissue was associated with activation of both HIV-1-infected and uninfected (bystanders) (Biancotto et al., 2008) T cells. In these tissues, apoptosis was selectively increased in T cells expressing CD25/HLA-DR and p24gag but not in cells expressing either of these markers alone. In the course of HIV-1 infection, there was a significant increase in the number of activated (CD25+/HLA-DR+) T cells both infected and uninfected (bystander). By inducing T cells to express particular markers of activation that create new targets for infection, HIV-1 generates in ex vivo lymphoid tissues a vicious destructive circle of activation and infection. In vivo, such self-perpetuating cycle could contribute to HIV-1 disease.

Finally we studied the role of immune checkpoint molecules during primary human CMV infections. B and T lymphocyte attenuator (BTLA), like its relative programmed cell death-1 (PD-1), is a receptor that negatively regulates murine T cell activation. However, its expression and function on human T cells is currently unknown. We report in this study on the expression of BTLA in human T cell subsets as well as its regulation on virus-specific T cells during primary human CMV infection. BTLA is expressed on human CD4 T cells during different stages of differentiation, whereas on CD8 T cells, it is found on naive T cells and is progressively downregulated in memory and differentiated effector-type cells (Serriari et al., 2010). During primary CMV infection, BTLAwas highly induced on CMV-specific CD8 T cells immediately following their differentiation from naive cells. After control of CMV infection, BTLA expression went down on memory CD8 cells. Engagement of BTLA by mAbs blocked CD3/CD28-mediated T cell proliferation and Th1 and Th2 cytokine secretion. Finally, in vitro blockade of the BTLA pathway augmented, as efficient as anti–PD-1 mAbs, allogeneic as well as CMV-specific CD8 T cell proliferation. Thus, our results suggest that, like PD-1, BTLA provides a potential target for enhancing the functional capacity of CTLs in viral infections.

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