



HAL
open science

Modulation of the innate immune response by the oncoviruses EBV and HPV

Peggy Parroche

► **To cite this version:**

Peggy Parroche. Modulation of the innate immune response by the oncoviruses EBV and HPV. Human health and pathology. Université Claude Bernard - Lyon I, 2011. English. NNT : 2011LYO10270 . tel-01137747

HAL Id: tel-01137747

<https://theses.hal.science/tel-01137747>

Submitted on 31 Mar 2015

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

THESE

Présentée devant l'UNIVERSITE CLAUDE BERNARD – LYON I

Pour l'obtention

du DIPLOME DE DOCTORAT

par

Peggy Parroche

**MODULATION OF THE INNATE IMMUNE
RESPONSE BY THE ONCOVIRUSES EBV
AND HPV**

Président du jury : Pr Jean Francois Nicolas

Directeur de thèse : Dr Massimo Tommasino

Co-directeur de thèse : Dr Uzma Hasan

Examineur : Dr Alexander Weber

Rapporteur : Pr Vincent Marechal

Rapporteur : Dr Ivan Hirsch

REMERCIEMENTS:

Dans un premier temps je souhaite remercier mes directeurs de thèse les docteurs Massimo Tommasino et Uzma Hasan. Merci de m'avoir donné la chance d'effectuer une thèse sous votre supervision. Je tiens aussi à remercier le docteur Jacqueline Marvel qui m'a accueillie dans son unité durant mes dernières années de thèse et m'a aidée pour l'obtention du financement de ma 4^{ème} année auprès de la FRM.

Je tiens également à remercier le professeur Jean-François Nicolas et le docteur Alexander Weber, respectivement président et examinateur de mon jury de thèse, pour leur présence aujourd'hui. Je veux remercier plus particulièrement le docteur Ivan Hirsch et le professeur Vincent Maréchal qui ont accepté d'être rapporteur de ma thèse et, de par leur suggestions intéressantes, ont permis d'améliorer mon manuscrit. Mes remerciements vont aussi aux docteurs Christophe Caux et Alexander Weber, membres de mon comité de thèse pour leurs conseils ainsi que pour des discussions très intéressantes et pertinentes au long de ces 4 années de thèse. Merci aux docteurs Evelyne Manet, Henry Gruffat, Nadège Goutagny, Daniele Viariso, Lutz Gissman, Florence Calvez-Kelm et James McKay pour leur collaboration dans mes projets de recherche.

Un grand merci au docteur Nadège Goutagny pour sa disponibilité, ses conseils techniques, scientifiques et pour ses nombreux commentaires qui m'ont permis d'améliorer mon manuscrit.

Je voudrai également remercier le docteur Christine Bain qui m'a formée, m'a donnée goût à la science et m'a permis de développer mon sens critique. Merci au docteur Geneviève Inchauspé qui m'a donné l'opportunité de débiter dans le monde de la recherche au sein de son équipe et de partir à l'étranger.

Mes remerciements vont également aux membres de l'équipe ICB du CIRC et d'I2V à Lyon Sud, merci pour vos conseils et votre soutien. Merci à Marine, Claudia et Ikbal pour les moments de détente partagés au CIRC. Merci à Karine, Karen Gaëlle qui m'ont tenue compagnie dans la pièce de culture. Merci à François pour son zen et sa gentillesse. Merci à Anne-Marie qui m'a servie de guide mes premiers mois aux CIRC.

Merci à Dou, ClairM, Pikachu, Choukette, My, Yvan, Catwoman et les bikeuses pour avoir réussi ponctuellement à me faire oublier le stress et la pression. Merci à Estelle qui a partagée avec moi la dernière semaine avant l'envoi de mon manuscrit et me parle toujours, enfin je l'espère.

Merci à ma moitié, qui a toujours été là pour me soutenir, me changer les idées et me motiver quand il le fallait. Merci à ma mère qui a toujours cru en moi et fait le maximum pour que je réalise mes souhaits.

Je remercie les personnes qui se sont déplacées pour ma soutenance et tous ceux que j'ai oublié.

TABLE OF CONTENTS

Remerciements.....	3
Table of Abbreviations.....	9
List of figures and tables.....	11

I. GENERAL INTRODUCTION13

A. ONCOVIRUSES 16

1. EPSTEIN-BARR VIRUS.....16

a) Clinical aspects.....	16
(1) Natural history.....	16
(2) Chronic stage of the virus: EBV associated malignancies.....	17
b) Molecular aspects and viral cycle.....	17
(1) Viral cycle	17
(2) Molecular aspects	19
(a) EBV genome	19
(b) EBV latent genes	21
(i) <i>The EBV nuclear antigen (EBNA)</i>	21
(ii) <i>The latent membrane proteins (LMP)</i>	21
(iii) <i>EBV encoded RNA (eber)</i>	2
(iv) <i>EBV miRNAs</i>	2
(c) EBV lytic phase genes.....	22
c) Models of study.....	23

2. HUMAN PAPILLOMA VIRUS.....23

a) Clinical aspects.....	23
b) Molecular aspects and viral cycle.....	25
(1) Molecular aspects.....	25
(2) Viral cycle.....	26
c) HPV models of study.....	27
(1) Organotypic cultures	27
(2) Animal papillomavirus	27
(3) Transgenic Mouse models	27
d) HPV therapeutic and vaccinal approaches.....	28

B. MECHANISMS OF ONCOGENESIS 31

1. CELL CYCLE Deregulation31

a)	Cell cycle.....	31
	(1) Cell cycle phases	31
	(2) Cell cycle regulation by cyclins and CDK	33
	(3) DNA Damage Response	35
	(a) p53: guardian of the genome	35
	(b) p21.....	39
	(4) G1 and G2 checkpoints.....	40
	(5) Association of cell cycle actors to tumors development.....	40
b)	EBV cell cycle deregulation.....	42
c)	HPV cell cycle deregulation.....	43

2. IMMUNE RESPONSE TO ONCOVIRUSES AND ESCAPE..... 46

a)	Immune response to oncoviruses.....	46
	(1) Innate response.....	46
	(a) Toll like receptors	48
(i)	<i>Introduction</i>	49
(ii)	<i>Structure and signaling.....</i>	49
(iii)	<i>Ligand and expression.....</i>	49
(b)	<i>Toll-like recognition of oncoviruses.....</i>	53
(i)	<i>Cell surface recognition of oncoviruses.....</i>	53
(ii)	<i>Endosomal sensing of oncoviruses.....</i>	55
(iii)	<i>Cytosolic virus sensing.....</i>	55
	(2) Adaptive immune response.....	63
	(a) Introduction.....	63
	(b) Adaptive Immune response to HPV.....	64
	(c) Adaptive Immune response to EBV.....	65
b)	Escape to immune responses.....	66
	(1) EBV.....	66
	(a) Impairment of innate immunity.....	66
	(b) Adaptive Immune escape.....	68
	(2) HPV.....	69
	(a) General mechanisms of immune escape.....	69
(i)	<i>Low profile.....</i>	69
(ii)	<i>Codon usage.....</i>	69
(iii)	<i>Molecular mimicry.....</i>	70
	(b) Modulation of innate immunity by HPV.....	70
(i)	<i>Interference with type I IFN.....</i>	70
(ii)	<i>Modulation of PRRs</i>	71
	(c) Modulation of adaptive immunity.....	71
(i)	<i>Modulation of Antigen presentation.....</i>	71
(ii)	<i>Modulation of chemokines and cytokines profiles.....</i>	71
(iii)	<i>Modulation of APCs.....</i>	73
c)	TLR modulation and cancer association.....	73
	(1) Association of TLR SNPs to cancer.....	73
	(2) TLRs modulation and cancer.....	74
	(a) Direct effect on cancer cells.....	74
(i)	<i>Expression on cancer cells.....</i>	74
(ii)	<i>Apoptosis.....</i>	74
(iii)	<i>TLR9 pathway and tumorigenesis</i>	76
	(b) Indirect effect of tumor micro-environment.....	77
(i)	<i>Angiogenesis.....</i>	77
(ii)	<i>TLRs modulation on Immune cells.....</i>	77
(iii)	<i>Use of TLRs agonist as anti-cancer therapeutic.....</i>	78

II. PRESENTATION OF THE THESIS PROJECT.....	81
III. RESULTS.....	85
A. PAPER 1: HUMAN PAPILLOMAVIRUS TYPE 16 E6 INHIBITS P21WAF1 TRANSCRIPTION INDEPENDENTLY OF P53 BY INACTIVATING P150SAL2.....	87
<hr/>	
B. PAPER 2: RESTORATION OF TOLL-LIKE RECEPTOR 9 INDUCES A SLOWDOWN IN CELL PROLIFERATION: INVOLVEMENT OF THE DOWN-REGULATION OF TLR9 IN CARCINOGENESIS.....	95
<hr/>	
C. PAPER 3 : EBV LATENT MEMBRANE PROTEIN 1 IS A NEGATIVE REGULATOR OF TLR9.....	111
<hr/>	
IV. CONCLUSION AND PERSPECTIVES.....	125
A. NEW MECHANISM E6 DEPENDENT P21 DEREGULATION.....	127
<hr/>	
B. TLR9 AND CELL PROLIFERATION.....	129
<hr/>	
C. INNATE IMMUNE RESPONSE AND ONCOVIRUSES.....	131
<hr/>	
V. REFERENCES.....	139

TABLE OF ABBREVIATIONS

APC	Professional antigen presenting cells
BARTs	Bam HI-A region rightward transcripts
BL	Burkitt's lymphoma
CAE	Chronic active EBV
cDCs	Conventional dendritic cell
CDKs	Cyclin-dependent kinases
CIN	Cervical intraepithelial neoplasia
CTL	Cytotoxic T cells
DAMPs	Danger Associated Molecular Patterns
DDR	DNA damage response
EBERs	Epstein Barr encoded RNAs
EBNA	Epstein Barr nuclear antigen
EBV	Epstein Barr virus
GC	Gastric carcinoma
HBV/HCV	Hepatitis C / Hepatitis B Virus
HD	Hodgkin's lymphoma
HIV	Human Immunodeficiency Virus
HNSCC	Head and neck squamous cell carcinomas
HPV	Human papillomavirus
HSIL	High grade squamous intraepithelial lesion
HSV-1	Herpes simplex virus type 1
HTLV-1	Human T lymphotropic virus type 1
IM	Infectious mononucleosis
IRFs	Interferon regulatory factors
KSHV	Kaposi sarcoma-associated herpesvirus
LC	Langerhans Cells
LCL	Lymphoblastoid cell lines
LMP	Latent membrane proteins
LRR	Leucine rich repeat
MAP kinases	Mitogen activated proteins kinases
MCPyV	Merkel cell polyomavirus
mDCs	Myeloid dendritic cells
MHC	Major Histocompatibility Complex
NF κ B	Nuclear factor kappa b
NLRs	NOD-like receptors
NPC	Nasopharyngeal carcinoma
ODNs	Oligonucleotides
OHL	Oral hairy leukoplakia
PAMPs	Pathogen Associated Molecular Patterns
PD	Phosphodiester
pDCs	Plasmacytoid dendritic cells
PRR	Pattern recognition receptors
PS	Phosphorothioate
PTLD	Post-transplant lymphoproliferative disease
RLR	RigI-like receptors
ROS	reactive oxygen species

SIL	Squamous intraepithelial lesion
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphisms
T/NKL	T or NK lymphomas
TLRs	Toll-Like Receptors
VSV	Vesicular stomatitis virus

LIST OF FIGURES AND TABLES

Figure 1: Percentage of infectious cancer among worldwide total cases of cancer 2002.....	14
Figure 2: Schematic presentation of the primo-infection by EBV.....	18
Figure 3 : Organization of the EBV genome.....	20
Figure 4: Structure of HPV16 genome.....	24
Figure 5: HPV productive life cycle.....	24
Figure 6: Cell cycle phases.....	30
Figure 7: Cell cycle transition from G1 to S.....	32
Figure 8: Cell cycle transition from G2 to M.....	34
Figure 9: The DNA damage response.....	36
Figure 10: p53 guardian of the genome.....	37
Figure 11: induction of p21 and effectors functions.....	38
Figure 12: Changes in protein expression during neoplastic progression.....	44
Figure 13: Type I IFN signal transduction.....	46
Figure 14: Cell surface recognition of oncoviruses.....	54
Figure 15: Endosomal recognition of oncoviruses.....	56
Figure 16: Cytosolic recognition of DNA oncoviruses.....	58
Figure 17: Cytosolic recognition of RNA oncoviruses.....	60
Figure 18: Induction of IL1 β by cytosolic sensors.....	62
Figure 20: Thesis projects.....	82
Supplementary figures	
1: IL1 β production induced by HPV16 virus in keratinocytes.....	132
2: IL1 β production induced by HPV16 virus in THP1.....	132
3: IL1 β production induced by HPV16 virus in mBM-Macrophages.....	134
4: IL1 β reporter assay in Hek expressing HPV16E6E7.....	134
5: Induction of IL1 β upon stimulation with Nalp3 or Aim2 inflammasome.....	136
Table 1: Human oncoviruses, related malignancies and possible mechanisms of action.....	16
Table 2: EBV associated malignancies. The percentage indicates the frequencies of EBV carrying tumors.....	17
Table 3: EBV gene expression during the different latency programs and correlation with diseases.....	19
Table 4: Principal type of cancers associated with HPV persistence.....	23
Table 5: Alteration of cell cycle regulators by EBV proteins.....	42
Table 6: Alteration of cell cycle regulators by HPV proteins.....	43
Table 7: Cellular expression and ligands for TLRs.....	51
Table 8: Structural properties of CpGs ODN.....	51
Table 9: Activities of the different classes of CPG-ODN.....	52
Table 10: Recognition of oncoviruses by membrane bound TLRs.....	53
Table 11: Cellular expression and ligands for RLRs.....	57
Table 12: Cellular expression and ligands for NLRs and pythin family members involved in virus recognition.....	61
Table 13: Immunomodulatory activities of EBV latent and lytic proteins.....	67
Table 14: Immunomodulatory activities of HPV proteins.....	70

I. GENERAL INTRODUCTION

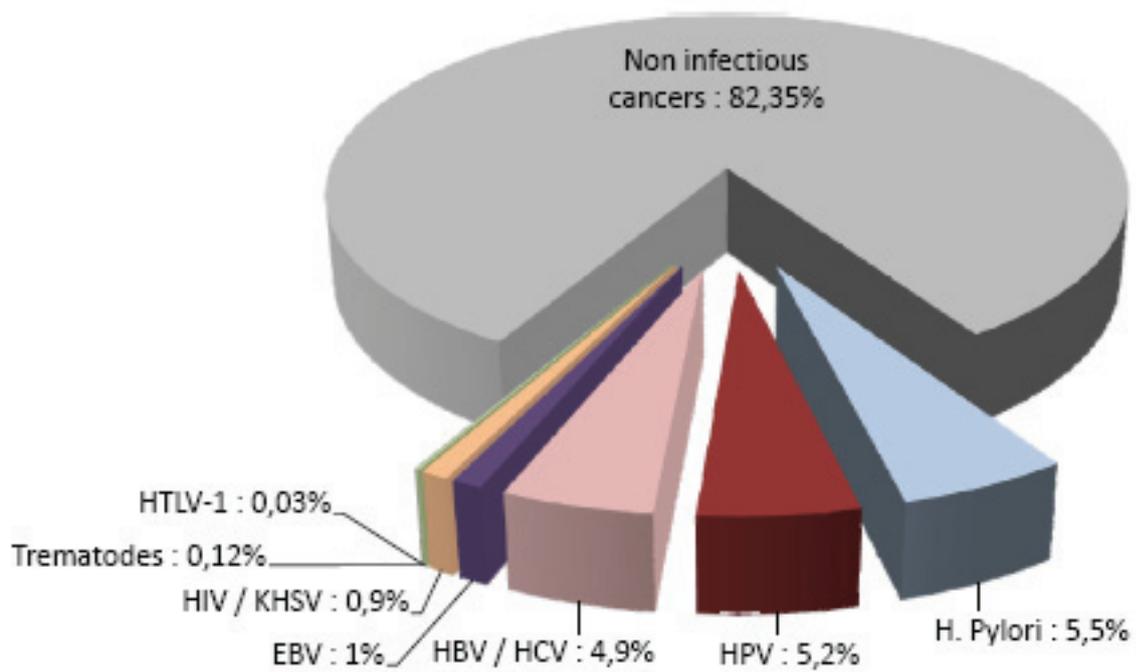


Figure 1 : Percentage of infectious cancer among total cases of cancer in 2002 (from Parkin et al 2006). KHSV: Kaposi sarcoma-associated herpesvirus, HTLV1: Human T lymphotropic virus type 1, HIV: Human Immunodeficiency Virus).

Cancer represents the second most common cause of death in industrialized countries. Epidemiological and biological studies have now conclusively proved that a variety of infectious agents constitute one of the main causes of cancer worldwide. It has been pointed out that more than 20% of cancers are from infectious origin [1] (Figure 1). Both DNA and RNA viruses have been shown to be the primary cause of cancer in humans. Among those, almost 60% are attributed to infection by Human papillomavirus (HPV), Epstein - Barr virus (EBV) and Hepatitis C / Hepatitis B Virus (HBV/HCV) [2], the 40% percent left are principally attributed to *H. pylori* infection (Figure 1).

Cervical cancer is the second most common cancer causing the death of approximately 300,000 women per year worldwide [3]. HPV high-risk mucosal types are associated to 98% of all cervical cancer cases. HPV16 is the most carcinogenic type, being present in approximately 50% of cervical cancers. In contrast to acute infection, persistent infection can last decades in the host and occurs when the immune system fails to clear the virus. It is now widely accepted that a persistent infection with a high risk HPV type is mandatory for the development of cancer. Regarding EBV, over 90% of the world's population is infected. Like all the viruses of the *Herpesviridae* family, it persists in the host under a latent form. EBV chronic infection is also mandatory in order to give rise to malignancies, such as Burkitt lymphoma or Hodgkin disease, but must be coupled with host or environmental factors. Cancer will develop if the oncovirus persists and progress to induce cellular transformation.

Failure of the immune response is a key component for the development of virus and non-virus induced cancers [4]. The ability of the virus to deregulate the first line of immune defense, the innate immune response, would permit cellular transformation of the host cell and cancer initiation. Immune and non-immune cells bear a set of molecules called pattern recognition receptors (PRR). They are well conserved throughout evolution and sense molecular motifs that are conserved among broad groups of microorganisms (PAMPs Pathogen Associated Molecular Patterns) or released by injured or stressed cells (DAMPs: Danger Associated Molecular Patterns). There are four families of PRRs: the TLRs (Toll-Like Receptors), the NLRs (NOD-like receptors), the RLRs (RIG-like receptors) and the pythrin family members. DNA viruses such as EBV and HPV are sensed by various PRRs. However they may have developed strategies to avoid PRR recognition in order to persist in the host.

Furthermore, the ability of TLRs to also regulate cell proliferation, survival and/or apoptosis may play a role in the control of inflammatory responses and tissue repair processes [5-8]. These two functions of TLRs are likely to be also important for cancer initiation and progression. First, as cancer is an abnormal and uncontrolled tissue repair process, TLR signaling could play a key role in regulating or controlling cancer progression. Second, deregulated TLR signaling leading to exacerbated inflammation would participate to cancer development. Furthermore genetic variations of TLR genes have been shown to be associated with increased risk of various cancers and other infection and non-infection related diseases [9-11].

The purpose of this project is to study how HPV and EBV deregulate the innate immune response. I will first introduce clinical and molecular aspects of EBV and HPV infection and depict the mechanisms leading to oncogenesis and more precisely the immune escape mechanisms and the subversion of the cell cycle used by these viruses in order to persist. Then the results obtained during my PhD will be exposed in the form of articles published or in the process of submission. In the last part I will discuss the results obtained in the light of what is currently known.

The viruses causing cancers, known as oncovirus, belong to different taxonomic group of DNA or RNA viruses. The first virus to be widely acknowledged as responsible of tumor was the Rous Sarcoma Virus (RSV) in chicken in 1910. However two years before Ellerman and Bang discovered that a virus-containing filtrate could transmit leukemia to chickens. In human five DNA oncoviruses exist, EBV , HPV [14], HBV, Kaposi sarcoma-associated herpesvirus (KSHV) [12] and Merkel cell polyomavirus (MCPyV) [13] and two RNA oncoviruses Human T lymphotropic virus type 1 (HTLV-1) and HCV. Table 1 depicts the oncovirus associated diseases and the mechanism of oncogenesis that lead to cancer development.

Oncovirus	Related diseases	Mechanism of oncogenesis
EBV	Nasopharyngeal carcinoma, burkitt's lymphoma, immune-suppression-related non-Hodgkin lymphoma, extranodal NK/T-cell lymphoma, Hodgkin's lymphoma, Gastric carcinoma, lympho-epithelioma-like carcinoma	Cell proliferation, inhibition of apoptosis, genomic instability, cell migration
HBV	Hepatocellular carcinoma, Cholangiocarcinoma, non Hodgkin lymphoma	Inflammation, liver cirrhosis, chronic hepatitis
HCV	Hepatocellular carcinoma, Cholangiocarcinoma	Inflammation, liver cirrhosis, chronic hepatitis
KSHV	Kaposi's sarcoma, primary effusion lymphoma, multicentric Castleman's disease	Under debate
HPV mucosal high risk type	Carcinoma of the cervix, vulva, vagina, penis, anus, oral cavity, oropharynx, larynx and tonsil	Immortalization, genomic instability, inhibition of DNA damage response, anti-apoptotic activity
HTLV-1	Adult T-cell leukaemia and lymphoma	Immortalization and transformation of T cells
MCPyV	Merkle cell carcinoma (needs to be validated)	Unknown

Table 1: Human oncoviruses, related malignancies and possible mechanisms of action. Adapted from [14].

From now on I will focus on EBV and HPV and will introduce the clinical and molecular aspect for each of them.

1.EPSTEIN-BARR VIRUS

a) Clinical aspects

(1) Natural history

EBV, initially called human herpes virus 4, is one of the eight known human herpes viruses. It was isolated for the first time by Anthony Epstein, Bert Achong and Yvonne Barr in 1964 from Burkitt lymphoma clinical samples [15]. It belongs to the *Herpesviridae* family and the *Gammaherpesvirinae* subfamily.

The majority of the population (over 90%) is infected with this virus. Most of the primo-infection happens during childhood by oral transmission. While in young children EBV acute infection is asymptomatic, during adulthood it may give rise in half of the cases to a self-limiting lymphoproliferative disorder called infectious mononucleosis (IM) [16]. The intensity of the disease varies but can last weeks or month before resolving. Complications are rare, however some reports showed a possible role for EBV in promoting autoimmunity and central nervous system tissue damage [17].

While the major target of EBV is B cells, it also infects epithelial cells [18], T/NK cells [19] and might target monocytes/macrophages/LCs (Langerhans Cells) [20].

(2) Chronic stage of the virus: EBV associated malignancies

The tumors associated with EBV are mainly lymphomas such as Burkitt's lymphoma and Hodgkin's lymphoma or carcinomas from epithelial origin such as gastric carcinoma and nasopharyngeal carcinoma EBV is linked to a wide range of malignancies in immunodeficient individuals. Table 2 lists the major malignancies as well as the percentage of which are associated to EBV infection. The lymphomas are mainly from B cell origin however rarely EBV infects T or NK cells and lead to the development of a T/NK lymphomas with a high frequency. The event leading to lymphomas development is the inability of the host immune system to control EBV infection. Indeed, immunosuppressive drugs following transplantation especially in children have been associated with the development of EBV lymphomas (PTLD).

Malignancies	EBV association (%)
Burkitt's lymphomas	95-100 – Endemic 20-30 - Sporadic
Hodgkin's lymphomas	40 – Western world 90 – Children in central America
Post-transplantation lymphomas	80
Aids—associated b-cell lymphoma	100 - Primary central nervous system lymphoma 30-50 - Burkitt's lymphomas 90-100 – Primary effusion lymphomas 30 – Diffuse large cell lymphomas (centroblastic) 90 - Diffuse large cell lymphomas (immunoblastic)
T cell lymphomas	10-30
Gastric cancers	10
Nasopharyngeal carcinoma undifferentiated	100

Table 2: EBV associated malignancies. The percentage indicates the frequencies of EBV carrying tumors.

In the case of Burkitt's lymphomas, the endemic form occurring in equatorial Africa is associated with chronic malaria that might dampen the host immune system. The sporadic variant occurring outside of Africa is also associated with an impairment of the immune system usually associated with HIV (Human Immunodeficiency Virus) infection or immunosuppressive drugs. Key factors in the development of Burkitt's lymphomas are the translocation of the c-myc oncogene into one of the immunoglobulin loci leading to its constitutional activation and the inability of the host to eliminate the EBV positive cells.

b) Molecular aspects and viral cycle

(1) Viral cycle

EBV is transmitted to a new host *via* salivary secretions to the oropharynx and must cross the epithelium to reach B cells, its main target (Figure 2). It is not known to what extent or whether epithelial cells of the oropharynx become infected during EBV primo infection. It is possible that EBV might infect directly the epithelial cells. Actually EBV has been shown to infect epithelial cells in AIDS patients with oral hairy leukoplakia and the mRNA of CD21 the receptor of the virus has been detected in some tonsillar epithelial cells [21]. It is also possible that the virus would cross the epithelium to access B cells perhaps *via* wounds or as a result of inflammation induced leakiness. The model of infection during primo infection by EBV has been

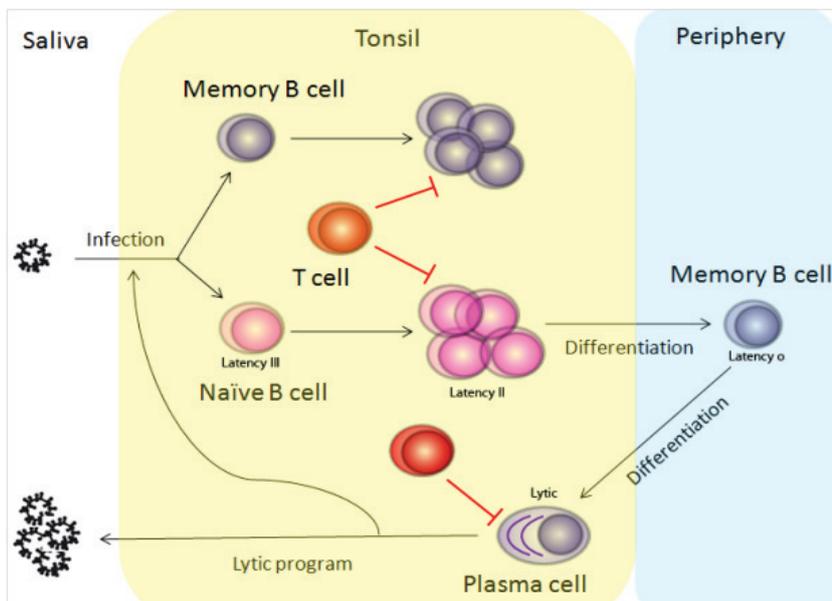


Figure 2: Primo-infection by EBV.

extrapolated from the model of EBV infection proposed by Thorley-Lawson *et al* [22]. Attachment to B cells is mediated by protein-protein interaction between the envelope glycoprotein gp350/220 and the complement receptor type 2 (CR2 or CD21)[23]. Fusion and endocytosis is triggered by the interaction of the EBV glycoprotein, gp42 with HLA class II [24], and is thereafter mediated by the core fusion complex, gH/gL/gp42 [25-27]. The nucleocapsid is then released in the endosome. The linear genome of the virus circularizes and the virus persists in the cell as episome. The newly infected B cells express all the latent EBV genes known as latency III (Table 3), characteristic of newly infected B cells *in vitro* [28]. The dogma is that EBV will first infect naïve B cells since they were the only one found to express the latency III genes [22, 28-30] (Figure 2). The EBV infected cells proliferate and some of them move to the tonsillar germinal center. The clonal expansion in the germinal center increase the pool of infected B cells where they express a more limited EBV expression pattern known as latency II (Table 3) [22].

Program	Genes /RNA expressed	Infected B cell type	Function	Disease
Latency III	EBNA1,2,3A,3B,3C,LP, LMP1,2A,2B,EBER, BARTs	Naive	B cells activation	IM, CAE, PTLD
Latency II	EBNA1, LMP1, LMP2A,2B	Germinal center	B cells Differentiation into memory	HD, NPC, T/NKL, GC
Latency I	EBNA1, EBER, BARTs	Dividing peripheral memory	Cell division	BL
Latency 0	EBER	Peripheral memory	Life time persistence	
Lytic	All lytic genes	Plasma cells	Virus replication	OHL

Table 3: EBV gene expression during the different latency programs and correlation with diseases. IM: infectious mononucleosis, CAE: chronic active EBV, PTLD: post-transplant lymphoproliferative disease, HD: Hodgkin’s lymphoma, BL: Burkitt’s lymphoma, NPC: nasopharyngeal carcinoma, GC: gastric carcinoma, OHL: oral hairy leukoplakia, T/NKL: T or NK lymphomas.

The B cells will then differentiate into memory B cells. The majority of EBV-infected B cells will be eliminated by cytotoxic T cells. The few remaining EBV positive memory B cells will be the reservoir of EBV-infected B cells, in which no EBV genes are expressed (latency 0) (Table 3 and Figure 2). EBV is found only in memory B cells in peripheral blood [31]. Upon activation, memory B cells differentiate into plasma cells. The virus enters then into a lytic program and the viral genome linearizes to replicate. New viral particles will be produced, and then they could re-infect naïve B cells (Figure 2) [32, 33]. Naïve B cells might not be the only target of EBV infection. Indeed, *in vitro* naïve and memory B cells show the same susceptibility to EBV infection [34] and it is possible that *in vivo* EBV doesn’t need to express the latency III program to establish viral latency, so *in vivo* memory B cells might be infected by EBV. EBV exploits the physiology of normal B cell to establish (activation and germinal center differentiation), to persist (memory B-cell) and to replicate (plasma cell differentiation) in immunocompetent host. Then a balance state is achieved between EBV reactivation and host immune surveillance. Indeed under normal circumstances EBV is not an oncogenic virus but break of this balance might lead to cancer development.

(2) Molecular aspects

(a) EBV genome

EBV is an enveloped virus with an icosahedral capsid and a double-stranded DNA genome of about 172 Kb in size. In virus particles the genome is linear. However after infection of B cells, the DNA circularizes and persists as an episome in the nuclei of infected cells. The genome contains terminal repeats (TR), few other small

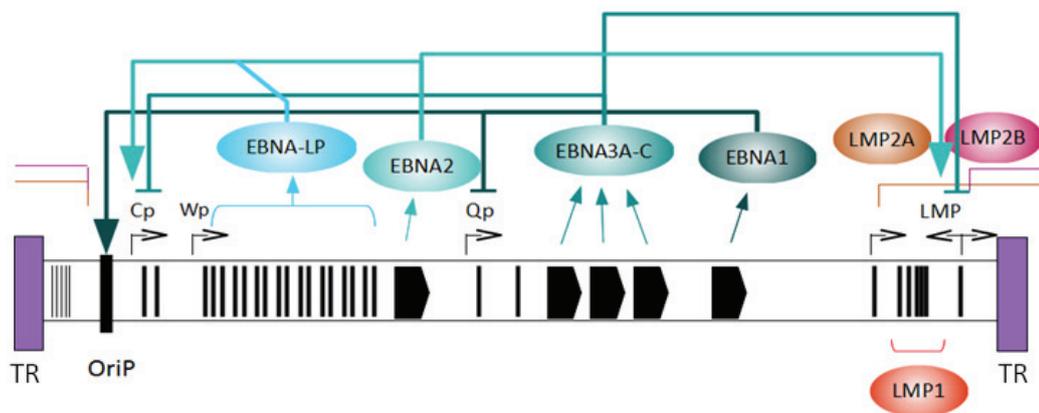


Figure 3: Organization of the EBV genome. The EBNA and LMPs proteins and promoters are localized on the genome. The regulation of the promoters by the EBNA genes products is depicted.

repeat and a large tandemly repeated DNA sequence the major internal repeat (IR1) (Figure 3). Terminal repeats (TR) are found at the extremity, they allow the circularization of the genome. IR1 divide the genome into short and long unique sequences (U_s and U_L) that contain EBV genes (Figure 3). The study of the open reading frame allowed the determination of almost hundred coding sequences. Only the major proteins will be described. Four different latency (1-4) programs exist and are tightly coupled to the state of the infected B cells (Table 3). The genome contains several promoters, named Cp, Wp, Qp and LMP promoters that are differentially activated in the different latency phases and lytic phase (Figure 3). Promoter activity is regulated by Epstein–Barr nuclear antigen (EBNA) proteins (Figure 3) and allow the virus to first establish (latency I,II,III) then either maintain a persistent infection (latency 0) or replicate and lyse the infected cells (lytic phase). Depending on the latency program, EBV expresses the EBNA and latent membrane proteins (LMP), and the Epstein–Barr encoded RNAs (EBERs) and the Bam HI-A region rightward transcripts (BARTs) (Table 3). In the latency I and II the only EBNA gene expressed is EBNA1, transcribed from the Qp promoter. In Latency II, in addition to the EBNA1, the LMP genes are also expressed from the LMP promoters. The Latency III involves the expression of all the EBNA proteins from the Cp or Wp promoters and the LMPs proteins by alternative 3' processing and alternative splicing. Transcription of the EBNA and LMP genes is auto regulated by the EBNA genes products. During the lytic cycle a specific set of genes are expressed: the immediate early proteins, the early proteins and the late proteins. The function of the main proteins and transcripts generated during latency and lytic phases will be presented below.

(b) EBV latent genes

(i) The EBV nuclear antigen (EBNA)

EBNA proteins are a group of nuclear proteins which modulate viral and cellular transcription.

EBNA1 is expressed in all virus infected cells and is required for replication, maintenance and segregation of the episomal EBV genome *via* OriP binding [35]. As shown in Figure 3 EBNA1 activates the transcription from Cp, Wp and LMPs promoters and inhibits Qp activity. It might mediate inhibition of apoptosis [36]. EBNA1-derived peptides are not presented by MHC Class I molecules. Therefore B cells only expressing EBNA1 (latency I) are not recognized by cytotoxic T cells [37]. **EBNA2** is the first viral protein to be expressed after infection of B cells *in vitro*. It is the main viral transactivator; it activates the promoters necessary for the production of all the latent proteins and several cellular genes such as c-myc and CD2[38]. It can drive the infected cell through G1 as detailed later [39]. EBNA2 inhibit RBP-Jk, a transcriptional receptor of the Notch signaling pathway, allowing the transcription of genes contributing directly to B cell immortalization. Recombinant viruses carrying an EBNA2 gene that lacks the RBP-j interaction domain are not able to immortalize B cells [40, 41]. **EBNA-LP** strongly and specifically potentiates EBNA2 mediated transcription [42, 43]. The **EBNA3** family members are antagonizing EBNA2 effect on RBP-Jk by competing for the binding to this protein [44, 45]. Through the modulation of RBP-jk, EBNA2/EBNALP/EBNA3 modulate the Notch pathway.

(ii) The latent membrane proteins (LMP)

LMP1 is essential for EBV mediated transformation. It is EBV main oncogene [46]. Transgenic mice expressing this gene in B cells develop lymphoma [47](Kulwichit, Edwards et al. 1998). Interference with LMP1 functions

by mutation impedes B cell transformation *in vitro* [48]. This protein acts as a constitutively active CD40 receptor. The binding between CD40 and CD40 ligand on Th cells delivers a rescue signal to the B cells [49]. The signal induces survival and proliferation *via* NF- κ B, AP-1 and JAK/STAT [50]. This protein regulates pro and anti-apoptotic genes. LMP1 is up-regulated during lytic cycle, probably to provide anti-apoptotic and immune evasion signals [51]. This protein is detectable in most EBV associated tumors but not in non tumoral tissue [52-54]. Because of its pro-oncogenic role the cellular level of LMP1 is finely tuned by several cellular mechanisms such as autophagy [55], ubiquitin proteasomal degradation [56] and by post-translational blocking *via* miRNA [57]. Exosomes are vesicles derived from the endosomal pathway of many cell types. LMP1 can be found into exosomes, which would participate to the suppression of immune responses against EBV-associated tumors [58, 59]. Exosomes deriving from APC contain molecules such as MHC Class I and II, CD86 and ICAM-1. Therefore they are able to stimulate CD4+/CD8+ T and B lymphocyte proliferation [60-63]. **LMP2** delivers a constitutive, ligand independent BCR signal [64]. The BCR induces two types of signal, however LMP2a is able to provide only one, the signal ensuring the survival of resting B cells [65]. Furthermore LMP2a inhibits the BCR signaling and the induction of the EBV lytic cycle thus maintaining EBV in a non-replicative latent state [66-68].

(iii) *EBV encoded RNA (eber)*

EBERs are two small poly A non-coding nuclear RNAs. In cells latently infected with EBV the EBERs are the most abundant viral transcript and are transcribed by RNA polymerase III [71, 72]. The EBERs are critical for efficient transformation of EBV-infected B lymphocytes. EBER deficient EBV viruses are 100 fold less efficient to transform B cells than EBER positive [73]. Different hypothesis exist to explain this EBER dependent enhancement of cell growth. EBER1 has been shown to associate with L22 (component of the 60S ribosomal subunit) and this association correlates with enhancement of cell growth [74]. EBER2 was also shown to play a role in EBV induced B cell transformation *via* IL6 production [75]. They bind Lupus erythematosus-associated antigen, La, however the significance of this binding is unknown.

(iv) *EBV miRNAs*

miRNAs are small non-coding RNAs that bind to fully or partially complementary sequences from mRNAs. This binding usually impairs translation and reduces the stability of the targeted mRNAs [69]. EBV was the first virus shown to encode microRNA. They map to the BHRF1 and BART region of the genome [70] The BHRF1 miRNA cluster appears to strongly potentiate the transforming properties of EBV by inhibiting genes that antagonize B cell growth and promote apoptosis [71, 72].

(c) *EBV lytic phase genes*

Reactivation of lytic EBV infection from latently infected cells is a process tightly regulated that begins with the expression of the immediate-early (IE) genes, followed by the expression of the early viral genes and finally the late genes. *In vivo* antigen mediated activation of B cells may be a physiologic stimulus for lytic reactivation of EBV [73]. Other extracellular signals may be important for EBV reactivation such as TGF β [74] or CD4+ T cell interaction [75] The IE genes are BZLF1 (Zebra EB1) and BRLF1 (Rta). Their expression is triggered by host cell transcription factors. They are transcriptional transactivators that trigger the replication of EBV [76, 77] and initiate a cascade of lytic viral gene expression [78]. Beside this role BZLF1 and BRLF1 affect

a variety of cellular proteins function and pathway. The early gene products include proteins that regulate transcription, RNA transport and stability, proteins which inhibit cellular apoptosis and help immune evasion and protein involved in the viral replication. Late viral gene expression occurs following viral replication, allowing the expression of structural proteins such as nucleocapsid proteins and viral membrane associated glycoproteins. EBV viral DNA is then packaged within the capsid and the mature virion is released.

c) Models of study

EBV-infected cells are rare in healthy people, only one to fifty EBV + cells per million of B cells [79]. So their study *in vivo* in humans is impossible. It is however possible to infect *in vitro* B cells purified from peripheral blood mononuclear cells (PBMCs). EBV will transform those B cells in lymphoblastoid cell lines (LCL) that will express mainly a latency III program, although low level of lytic EBV infection is detectable too [28].

Gamma herpes viruses are highly species specific, thus animal models for EBV are not available. Murine gammaherpesvirus 68 (MuGHV) is a natural pathogen of bank voles and wood mice and has been shown to infect laboratory strain mice [80]. Experimental infections of mice with MuGHV share many features of EBV infections in human [81]. MuGHV and EBV both show epithelial and B-cell tropism, virus-driven B-cell activation and proliferation, and a syndrome of acute infectious mononucleosis [81, 82]. However MuGHV lacks homologs of EBV latency-associated and transforming proteins and lymphomas occurs only in a low percentage of MuGHV-infected mice [83].

The best primate model available to mimic EBV infection and pathogenesis is based on the infection of rhesus monkeys with the rhesus lymphocryptovirus (LCV). Naïve animals can be inoculated by oral route and the acute and latent infections reproduce many aspects of EBV [84]. However lymphoma developments in immunosuppressed animals require intravenous injection of LCV infected B cells [85].

2. HUMAN PAPILLOMA VIRUS

Human Papillomavirus is the most common sexually transmitted disease in the world. Most of the HPV infections are benign. They infect cutaneous and mucous epithelia and induce papillomas or warts which generally regress. However when HPV persist, it might progress into cancer. Indeed HPV has been shown to be the etiologic agent of cervical cancer [86]. Cancers due to HPV are a public health issue since it is the second most common cancer in women worldwide with an estimated 493,000 new cases and 274,000 deaths in 2002 [3].

Types of Cancer	Main HPV type	% of association with HPV
Cervical cancer	HPV16 and 18	100%
Anogenital cancer	HPV16	50%
Head and Neck Cancers	HPV16	25-30%
Skin Cancer (EV and transplant patients)	HPV5, 8 and 20	Needs to be assessed

Table 4 : Principal type of cancers associated with HPV persistence [14].

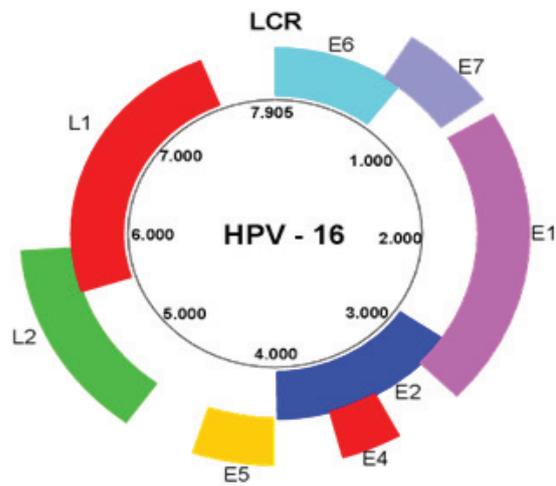


Figure 4: Structure of the HPV 16 genome
 (From http://accres.inrp.fr/acces/ressources/sante/epidemies-et-agents-infectieux/comprendre/cancer_viro_induits/le-virus-hpv)

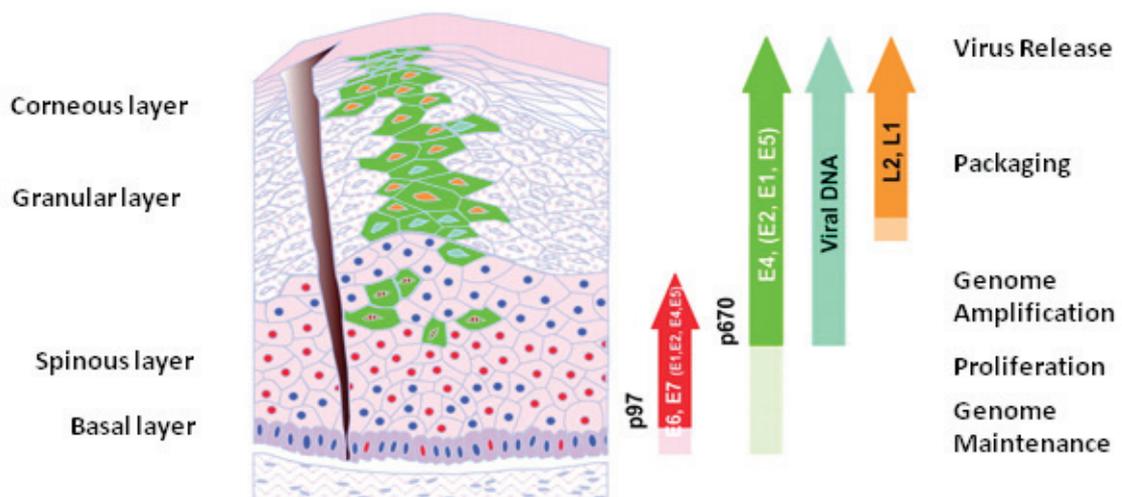


Figure 5: HPV productive life cycle. The different layers of the epithelium are on the left and the HPV life cycle and the proteins involved are on the right. E7 expression are shown in red. (From MIDDLETON, PEH ET AL. 2003.)

a) Clinical aspects

The Human Papillomavirus belong to the *Papillomaviridae* family. Over 100 papillomas virus types have been identified.

HPV show a high degree of tissue tropism. Different HPV types infect specific anatomic regions. The viruses of the alpha genera infect the mucosa and the epithelium of the genital tract. These HPV type from alpha genus are subdivided into two groups: the low risk HPV type (e.g. HPV6) that are mainly associated with genital warts and non-metastasizing tumors and the high risk HPV type associated with high-grade dysplasia especially cervical intraepithelial neoplasia (HPV16 and 18 mainly) (Table 4).

The viruses associated with cutaneous lesions and *epidermodysplasia verruciformis* (EV) have been regrouped in the beta genera. Beta papillomavirus (e.g. HPV5) are usually associated with benign cutaneous infection but in immunocompromised individuals and in patients suffering from EV, they can be associated with the development of non-melanoma skin cancer (Table 4) [87, 88]. In immunosuppressed patient, HPV from beta type might have a role in the development of warts and squamous cell carcinomas [89]. The remaining HPV belong to the genera Gamma, Mu and Nu and generally cause papillomas and warts that do not progress to cancer.

The high-risk HPV have been associated with human cancer that will be described below. A group of 15 high risk HPV types seem to be involved in all cervical cancer cases worldwide. HPV 16 and 18 are the most prevalent high-risk type associated with 50 and 20% respectively of all cervical cancer cases (Table 4). The incidence rates of invasive cervical cancer tend to peak about 20-25 years after the peak of age for HPV infection prevalence. HPV16 has also been associated to anogenital and head and neck cancers. Therapy of cervical precancerous lesion consists in their surgical removal. Treatment of cervical cancer is mainly through radiotherapy in association with surgery in early stage disease. In advanced or recurrent diseases chemotherapy is used in combination with radiotherapy or surgery

The following parts of this report will be focused on the mucosal high type HPV 16 and the most prevalent HPV type in cancer.

b) Molecular aspects and viral cycle

(1) Molecular aspects

The HPV16 genome of 7905bp long (Figure 4) is composed of eight ORF mapped on three regions. The early region encodes six proteins: E1, E2, E4, E5, E6 and E7. The late region encodes the major capsid proteins L1 and the minor capsid protein L2. A non coding upstream regulating region or long coding region (URR/LCR) contains the transcription promoters and DNA replication elements. Most HPV genes are transcribed as polycistronic mRNAs from a single DNA strand and the precise identity of the mRNAs for each ORF has not been fully established. HPV uses alternate RNA splicing and alternative RNA polyadenylation to ensure the proper expression of all ORFs from a compact genome [90].

HPV double stranded circular DNA is encapsidated into an icosahedral capsid composed by the two capsid proteins L1 and L2. **L1** forms 72 star-shaped capsomers around HPV genome. **L2** less abundant in the capsid displays several important function beside its role in capsid stabilization [91]. L2 is involved in genome en-

capsidation [92] and endosomal escape of virion during infection [93]. L2 has been shown to recruit L1 to sites of the nuclear viral replication (ND10 domains) [94, 95]. Moreover HPV **E4** protein might be important in virions morphogenesis since it has been shown to induce the redistribution of the ND10 domains [96]. E4 is also involved in virion release. Indeed E4 compromise structural integrity of the corneocyte [97, 98] and induce apoptosis [99] to enable the release of the virions.

Prior to replication the viral DNA must be unwinded; this function is fulfilled by HPV **E1** protein which is the viral DNA helicase. Efficient recruitment of the E1 protein to the HPV Origin of replication is mandatory for viral replication and relies on interaction with HPV **E2** protein [100]. E2 is also responsible for viral persistence in dividing cells by associating with mitotic spindles to ensure correct genome segregation [101]. **E4** ensures an environment compatible with the replication of the virus in the differentiated layers of the mucosa. **E5** delays the process of endosomal acidification [102], leading to an increase in epidermal growth factor (EGF) signaling [103].

E6 and E7 are the two oncoproteins of HPV16. E6 expression is tightly regulated since one of its main functions is to induce the degradation of p53 [104]. E6 has the ability to bind PDZ (PSD95 Dlg1 zo-1) motifs that might contribute to the loss of cell polarity seen in HPV cancer. E6 PDZ binding is important for transformation since deletion of the PDZ motif of HPV16E6 prevented the immortalization of human epithelial cells in a model of Head and neck squamous cell carcinomas (HNSCC) [105]. E6 is also activating the human telomerase reverse transcriptase (hTERT) to probably bypass cellular senescence. As E6 in normal settings, **E7** expression is well regulated. One of its main functions is to activate the proliferation of the host infected cell mainly *via* pRb degradation [106]. Mechanisms of p53 and pRb deregulation will be detailed later on in the cell cycle regulation part.

The regulation of E6 and E7 is dependent on **E2** and E4. E2 acts as a transcriptional activator or inhibitor, high levels of E2 repress E6/E7 while low level of E2 activates E6/E7 expression.

(2) Viral cycle

HPV is sexually transmitted, reaches the epithelial basal layer and enters the dividing cells through micro wounds (Figure 5). HPV uses heparin sulfate proteoglycan (HSPGs) as primary attachment receptors [107-109]. Since Syndecan-1 is the predominant HSPG in epithelial tissue it was suggested to serve as the primary attachment receptor *in vivo*. This is further supported by its high level of expression in the appropriate target cell and up-regulation during wound healing [110, 111]. However, *in vivo* model suggests primary attachment to the basement membrane rather than cell surface indicating that a secreted HSPG must be involved [108]. *In vitro* studies have shown that PV can also bind to components of the extracellular matrix (ECM) secreted by keratinocytes and can be transferred from ECM to cells [112]. It has become clear in recent years that a secondary non-HSPG receptor is involved in infectious internalization of HPV particles [112, 113] but its identity is still unknown. Initial cell surface interactions are predominantly dependent on the major envelop protein L1. However, the minor envelop protein L2 may contribute to secondary interactions. The engagement with HSPG induces conformational changes which affect L1 and L2 that might help the handover from one receptor to another. The entry pathway of HPV16 has not been characterized further but may utilize tetraspanin-enriched micro-domains as entry platform [114]. Since successful infection requires acidification of endocytic vesicles, HPV must traffic through the endosomal compartment. Mechanisms of HPV internal-

ization are still under debate but might involved clathrin mediated endocytosis [115] or clathrin and caveole independent internalization of HPV16 [114]. Uncoating of HPV occurs in endocytic vesicles prior to transfer to the cytosol [116]. L2 mediates the escape of HPV DNA from endosomes and allow the transport to the nucleus with the microtubule network [93, 117]. The virus maintains its genome in the basal layer cells as low copy episomal DNA and replicates with the host cellular DNA during S-phase. Indeed, the virus is lacking a DNA polymerase and relies totally on the host cell proliferation. Since suprabasal cells are supposed to exit the cell cycle to enter in differentiation, the activities of the viral oncogenes E6 and E7 are required to keep a couple of cells proliferating and bypass the terminal differentiation [118].

The late events in HPV cycle include genome amplification and virus assembly and release. HPV DNA production seems to be tightly regulated by keratinocytes differentiation (Figure 5). The precise factor that triggers the late event in HPV16 life cycle is unknown but might depend on the changes of the cellular environment as the infected cell is progressing through the epithelium. For instance the CCAAT/enhancer binding protein beta (C/EBP β) a key transcription factor in the terminal differentiation of keratinocytes, induces the late promoter p670 of HPV16 [119]. The activation of this promoter increases the level of the proteins E1, E2, E4 and E5 that are necessary for viral replication.

The final step is the packaging of the genome. L1 and L2 accumulate after the genome amplification (Figure 5). E2 would improve the efficiency of genome encapsidation [120]. E2 mediates the association of the promyelocytic leukaemia (PML) bodies or ND10 with the viral genome, and then L2 binds the PML bodies in the nucleus. In the meantime L1 assembles in capsomers in the cytoplasm and is recruited by L2 to the PML bodies [94]. The assembly of the virus seems to be dependent on Hsp70 [121]. The virus maturation and stabilization is taking place as the cell approaches the epithelial surface and the virus is shed from the epithelial surface, which is facilitated by E4 disruption of the keratin network [122, 123].

c) HPV models of study

(1) Organotypic cultures

Organotypic raft cultures are a valuable tool for understanding the natural history of HPV. They consist in the *in vitro* generation of fully differentiated and permissive epithelium [124]. The raft culture mimics the *in vivo* three dimensional tissue morphology and differentiation of the epithelium therefore allowing the propagation of the virus. Organotypic cultures are constructed by placing epithelial cells on top of a dermal equivalent component (natural dermal equivalent or synthetic matrix collagen) and then raising the cells to the air-liquid interface. The epithelial cells can be primary keratinocytes for example that would differentiate into a healthy epithelium. When using cells derived from epithelial neoplasia it is possible to mimic tumoral architecture.

(2) Animal papillomavirus

Animal papillomavirus have been studied both as agents of disease in animals and as models of HPV infection. Bovine Papillomavirus (BPV) and cottontail rabbit papillomavirus (CRPV) have been for many years the model systems with which to study the biology of HPV [125]. Induction of papillomas and their neoplastic progression has been experimentally demonstrated and reproduced in cattle and rabbits, and virus-cofactor interactions have been elucidated in these systems.

(3) Transgenic Mouse models

Productive papillomavirus infection is species- and tissue-restricted. However it is possible to infect mice with HPV16 to study the establishment phase of HPV infection [108]. With this model it is not possible to study HPV oncogenesis. In order to study the role of HPV oncoproteins in cancer development or in immune response transgenic mice carrying the full or part of HPV genome under a promoter such as K14 specific of the basal epithelial cells have been engineered [126].

d) HPV therapeutic and vaccinal approaches

Prophylactic vaccine Gardasil® (Merck) and Cervarix® (GlaxoSmithKline) against HPV have been developed and validated. They are based on VLPs (virus-like particles) composed of the L1 major capsid protein coupled with adjuvants. They induced the production of neutralizing antibodies in vaccinated individuals [127]. Vaccines which are heavily adjuvanted might be an issue in some patients. They require two or three vaccinations to be efficient. Gardasil® is composed of VLPs from HPV low risk types 6 and 11 and from high risk types 16 and 18 with aluminum adjuvant. Cervarix® is made of VLPs from HPV high risk type 16 and 18 mixed with AS04 (aluminum hydroxide and MPL). These vaccines have been shown to be over 90% effective in preventing specific HPV type infection *in vivo* [128, 129]. While the results of the clinical trial and the first vaccinations look promising, it is important to stress that cervical cancer is a slowly progressive disease, so, the efficiency of the vaccines will be tested over 10 or 20 years.

As million women are infected by HPV therapeutic vaccine represent also an interesting prospect. Therapeutic vaccines targeting E6 and E7 activities are currently under development [130]. To enlarge the broad spectrum of the vaccines, different strategies to create multivalent vaccine are being developed. Sequences that would be immuno-stimulant, protective and common to a maximum of HPV type are searched to provide cross-protection between the HPV types. Mosaics VLPs made of L1 from different HPV types are being engineered.

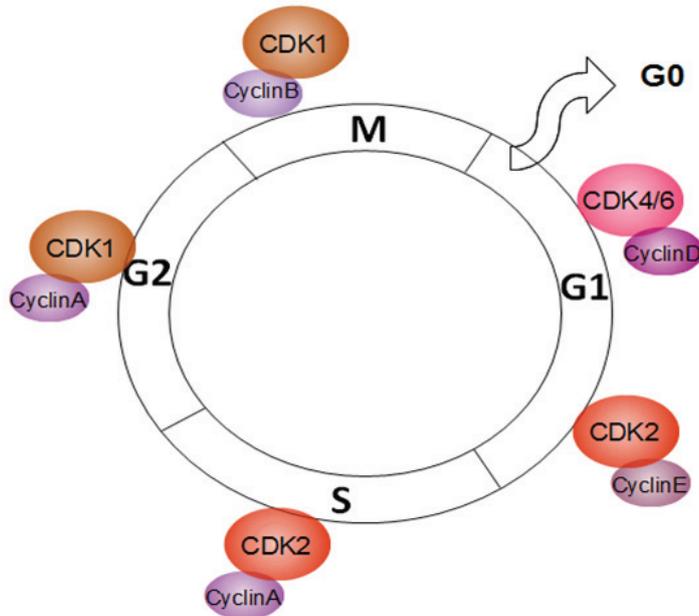


Figure 6: cell cycle progression through the different phases is controlled by cyclin/CDK complexes.

The path from viral infection to cancer is hopefully long and inefficient. Only few people infected will develop a cancer and it might take decades to arise. There are two prerequisite to the development of virally induced cancer: 1/ the virus must persist despite the host immune response and 2/ cell cycle alteration and cellular oncogene activation leading to genetic mutation must arise in infected cells.

The oncogenic potential of an oncovirus is either direct or indirect. Direct oncogenicity involves the activation of viral or human oncogenic genes (or both) in order to transform the cells. High risk HPV, EBV, HHV-8 and HTLV-1 belong to this category. Because the transformation is directly dependant on the persistence of the oncovirus, patient immuno-suppressed or immuno-compromised will be more at risk. Indirect oncogenicity involves chronic inflammation, tissue injury and repair like it is the case for the hepatitis B and C viruses. In that case, it's rather the inflammation resulting of the infection and the virus life cycle than the virus by itself, which lead to cancer. In this particular case the controversial theory of "hit and run" might be true; the virus would indirectly induce the tumor but would be lost from the cancer cells.

Some virus induced cancers arise when productive infection cannot be supported. Indeed when the host is inappropriate, the rate of cancer is higher than in natural host. This is for example true for CRPV in domestic rabbit, SV40 in hamster and adenovirus 5 in rat [131, 132]. Furthermore, this might also be true when the virus infects other sites in its natural host. Moreover, it has been shown that in immunosuppressed patients, the development of cancers due to oncoviruses is more frequent [133].

I will here detail the mechanisms of normal cell cycle and immune response as well as the mechanisms developed by HPV and EBV to deregulate those two keys events.

1. CELL CYCLE DeregULATION

As seen earlier, DNA oncoviruses such as EBV and HPV stay usually under an episomal form in the nucleus. In order to replicate their DNA, they need to push the cell to cycle to benefit from the host replicative machinery. Thus, they have developed several mechanisms to deregulate the cell cycle and promote cell growth. This deregulation is very important since one of the main engines that drive cellular transformation is the loss of proper control of the cell cycle [134].

a) Cell cycle

(1) Cell cycle phases

The cell cycle, or cell-division cycle, is the series of events that takes place in a cell leading to its division and duplication (replication). The cell cycle consists of three different phases: the resting phase (G₀) where the cells are quiescent, the interphase (G₁, S, G₂) where the cell are preparing for cell division, and the mitosis (M) where the cells are dividing: the chromosomes are segregated and the cell is diving into two (Figure 6).

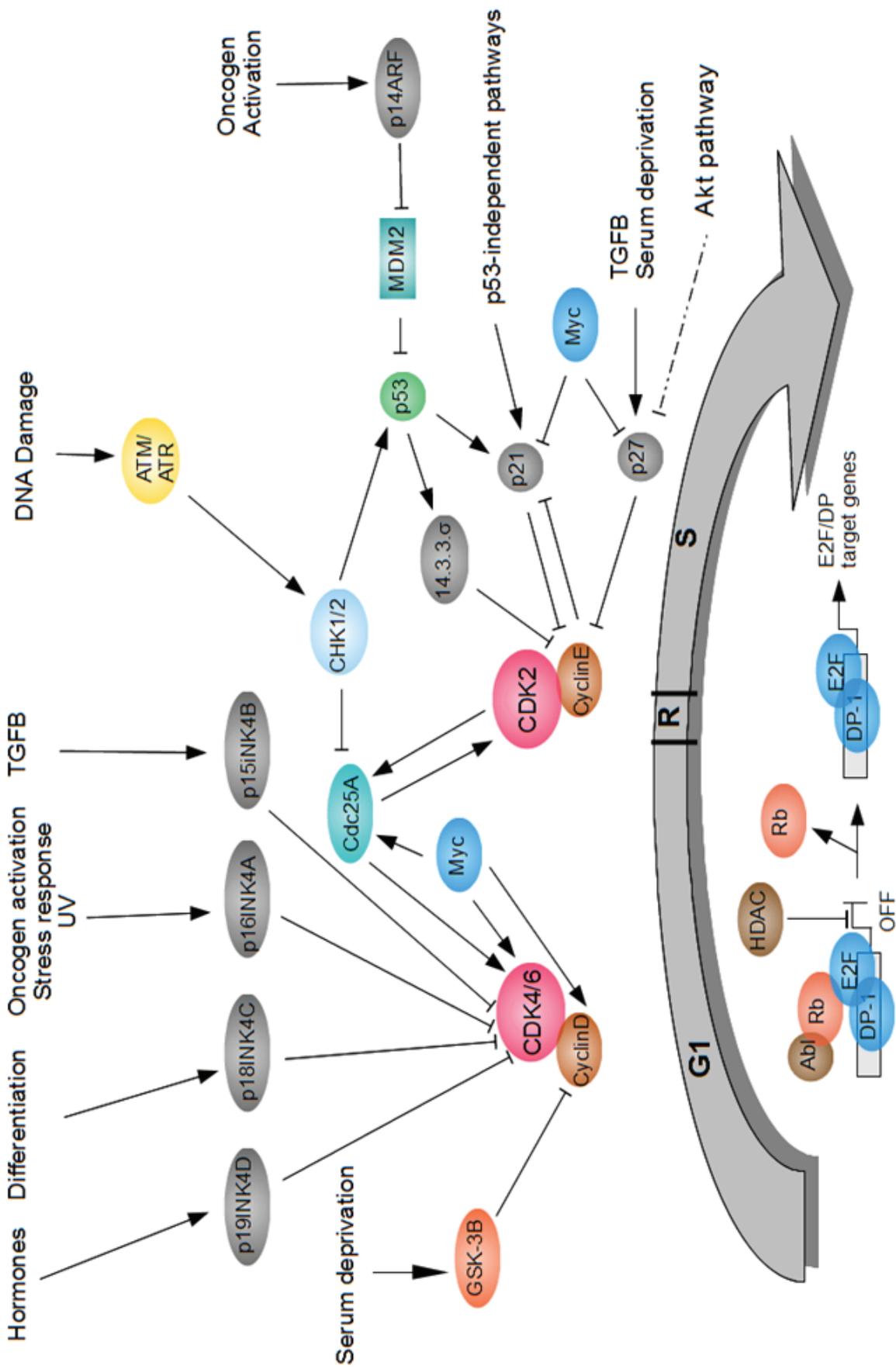


Figure 7 : Cell cycle transition from G1 to S. R: restriction point

The interphase is divided in three parts: the G1 phase (or growth phase) where the cells prepare for DNA synthesis *via* an increase in its biosynthesis activities; the S phase where the DNA replicates and the G2 phase where the cells check that the replication was correct and prepare for the mitosis (increase in microtubules formation for instance).

(2) Cell cycle regulation by cyclins and CDK

Each phase of the cycle is regulated (Figure 6). There are checkpoints that need to be passed before entering in the next phase. The cyclins and cyclin-dependent kinases (CDKs) are the master regulator of the cell cycle. They are only active as heterodimers. Cyclins and CDKs form respectively the regulatory and the catalytic subunits of an activated heterodimer. Cyclins have no catalytic activity and CDKs are serine threonin kinases inactive in the absence of a cyclin. When activated by cyclin binding, CDKs phosphorylate target proteins to orchestrate coordinated entry into the next phase of the cell cycle. Different cyclin-CDK combinations determine the downstream proteins targets. CDKs are constitutively expressed while cyclins are regulated by transcriptional and post-translational (ubiquitination) modifications at specific stages of the cell cycle in response to various signals. There are three cell cycle checkpoints, the first one between the phases G1/S, the second one between the phases G2 and M and the third one in M phase. The cell cannot proceed to the next phase until checkpoint requirements have been met.

The first control is taking place during the G1 phase. Prior to that, a cell exits the cell cycle and enters in G0 phase if specific mitogenic and growth signals are absent. After stimulation by mitogens, cyclin D is up-regulated by the Ras / MAPKinase pathway which activates the transcription factor Myc. Cyclin D associates with CDK4 or CDK6, depending on the cell type. Since cyclin D is constantly degraded by the S-phase promoting complex (SPC), the formation of the complex cyclin D / CDK is the result of equilibrium between cyclin D synthesis and degradation. CDK4 and CDK6 are then activated *via* dephosphorylation by CDC25 A (cell division cycle 25 A). The active cyclin D/CDK4/6 complexes phosphorylate the retinoblastoma protein; Rb (Figure 7). Rb is one of the pocket proteins (together with p107 and p130) and functions as a tumor suppressor. Rb in its hypophosphorylated form is active and represses cell cycle progression by inhibiting E2F transcription factors, which are necessary for S phase entry.

Later in the G1 phase, cyclin E is induced, which associates with CDK2. CDK2 is then activated *via* dephosphorylation by CDC25 A (Figure 7). The active complexes target Rb proteins for phosphorylation. Once Rb is phosphorylated over a certain threshold, it is deactivated and the bound transcription factors E2F are now released (Figure 7). E2F induces the transcription of genes such as cyclins, cyclin-dependent kinases, components of the pre-replication complex such as Mcms and Orc6, and DNA synthesis genes like DNA polymerase, topoisomerases, dihydrofolate reductase (DHFR) and thymidylate synthase (TS), that will allow the transition from G1 to S.

Two families of genes, the cip/kip family (CDK interacting protein/Kinase inhibitory protein) and the INK4a/ARF (Inhibitor of Kinase 4/Alternative Reading Frame) prevent the progression of the cell cycle *via* blockage in G1 phase. Because these genes are instrumental in prevention of tumor formation, they are classified as tumor suppressors. The INK4 family has four members (p16INK4a, p15INK4b, p18INK4c, p19INK4d) that

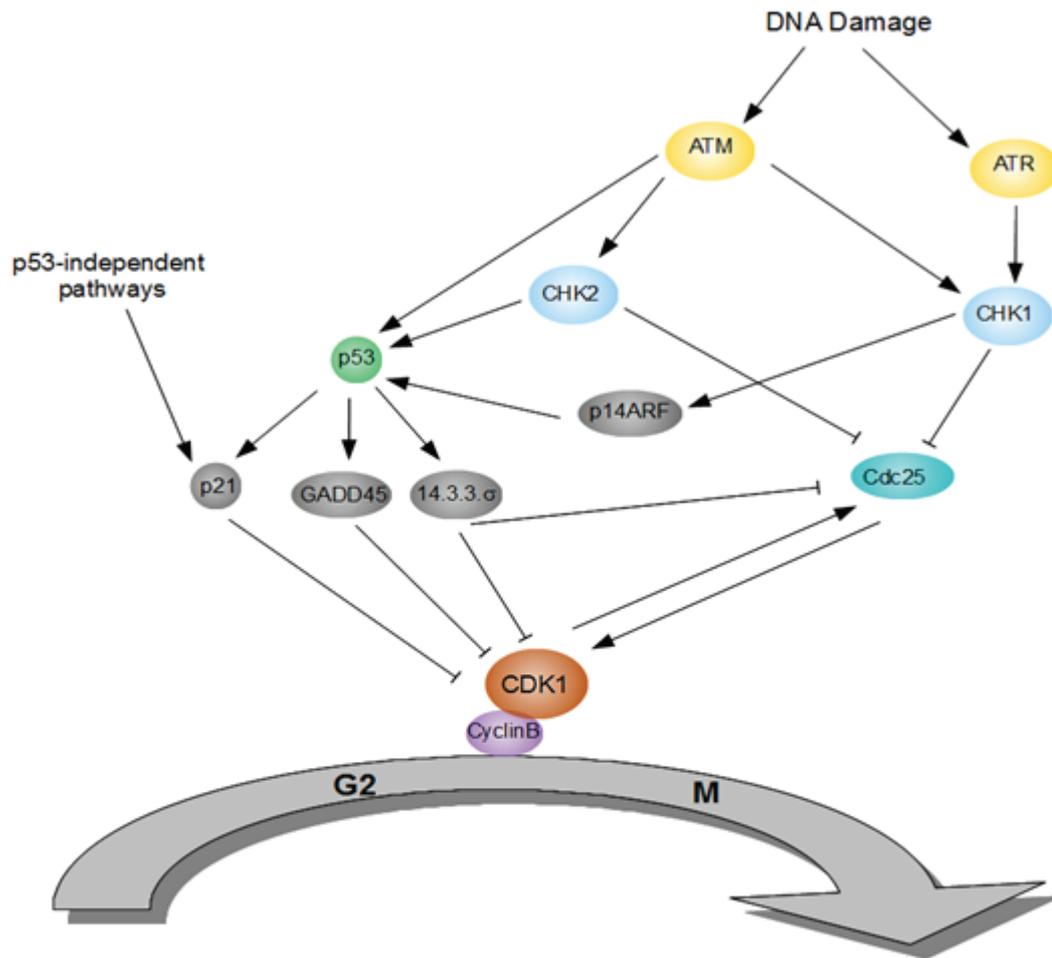


Figure 8 : Cell cycle transition from G2 to M

exclusively bind to and inhibit the D-type cyclin-dependent kinases (CDK4 and CDK6) (Figure 7). The Cip/Kip family has three members (p21^{CIP1/WAF1}, p27^{KIP1}, p57^{KIP2}) that inhibit all CDK activities during cell cycle.

During the S phase the complex Cyclin A/CDK2 activated *via* dephosphorylation by CDC25A phosphorylate proteins that maintain DNA replication, inactivate the transcription factors of the G1 phase and stop the degradation of the cyclinB.

Progression from G2 to M phase is regulated by the cyclinB/CDK1 complex (Figure 8). Inactive cyclinB/CDK1 complexes accumulate during the G2 phase. CDK1 is maintained in an inactive state by the tyrosine kinases Wee1 and Myt1. For the entry into mitosis CDK1 is activated by desphorylation by cdc25C (Figure 8).

The third and last checkpoint happens at the end of the mitosis *via* the anaphase-promoting complex (APC), an E3 ubiquitin ligase that marks target cell cycle proteins for degradation by the proteasome. APC induces ubiquitination and proteolytic degradation of cyclin B and inactivation of CDK1.

(3) DNA Damage Response

While the maintenance of genome integrity and fidelity is essential for the survival of all organisms, genotoxic agents, replicative mistakes and DNA instability are constantly modifying DNA [135]. Failure to repair DNA damage is involved among others in carcinogenesis [136, 137]. Eukaryotic cells have evolved DNA damage response (DDR) to counteract the effects of DNA damage. Upon sensing DNA damage or stalls in replication, cell cycle checkpoints are activated to arrest cell cycle progression to allow time for repair before the completion of cell cycle (Figure 9). In addition to checkpoint activation, the DNA damage response leads to induction of DNA repair pathways, and when the level of damage is severe, to initiation of apoptosis [138]. The DDR can regulate cell cycle checkpoints in response to damaged DNA. The DNA damage response pathway is a signal transduction pathway consisting of sensors, transducers and effectors (Figure 9). Once activated by DNA damage, the DDR sensors such as Rad family members lead to the activation of the upstream protein kinases ataxia telangiectasia mutated (ATM) and ATM and Rad-3 related (ATR). Then adaptor proteins, including MDC1, 53BP1, BRCA1 and claspin, are recruited and activate the downstream kinases checkpoint-1 (CHK1) and 2 (CHK2). CHK kinases propagate the damage signal to effector molecules such as CDC25 and p53. The effector molecules halt cell-cycle progression, either transiently or permanently (senescence), or trigger cell death (apoptosis) (Figure 9). Those mechanisms are dependent upon signaling pathways mostly involving p53 but also p53 independent p21 pathways.

(a) p53: guardian of the genome

P53 is known as the guardian of the genome. In cells, p53 levels are low due to interaction with MDM2, which targets p53 for nuclear export and proteasome-mediated degradation in the cytoplasm (Figure 10) [139]. Activation of p53 requires post-translational modifications. Phosphorylation of Serine 20 residue of p53 blocks p53/MDM2 interaction, resulting in p53 accumulation. While phosphorylation of Ser²⁰ is important to p53 stability, it is the phosphorylation of Ser¹⁵ that appears crucial in enhancing p53 transcriptional transactivation activity [140]. The transcriptional ability of p53 is further augmented through acetylation by p300/PCAF.

Two groups of protein kinases activate p53. First, protein kinases belonging to the MAPK family (JNK1-3, ERK1-2, p38 and MAPK) lead to p53 activation in response to several types of stress, such as membrane dam-

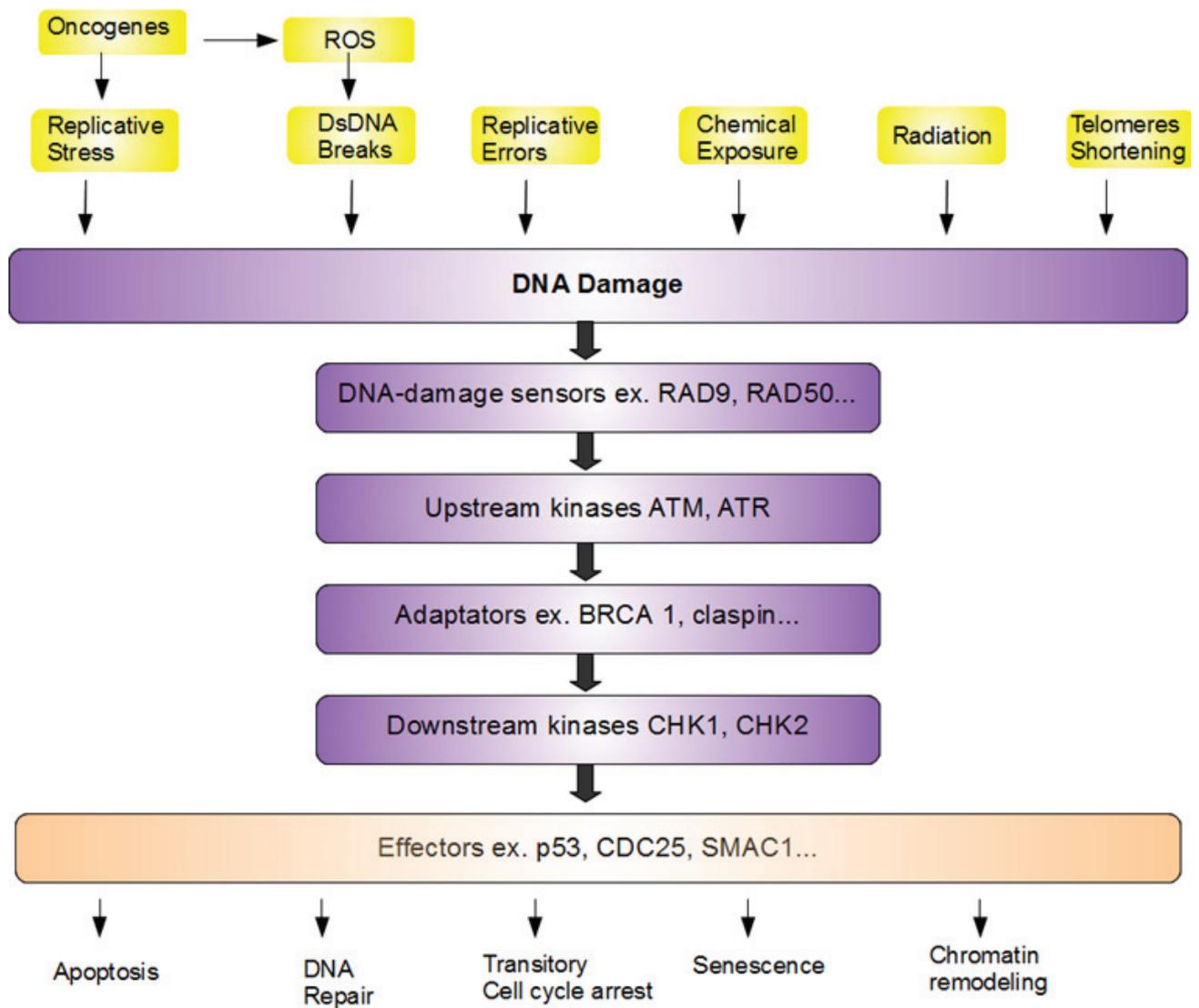


Figure 9 : The DNA damage response

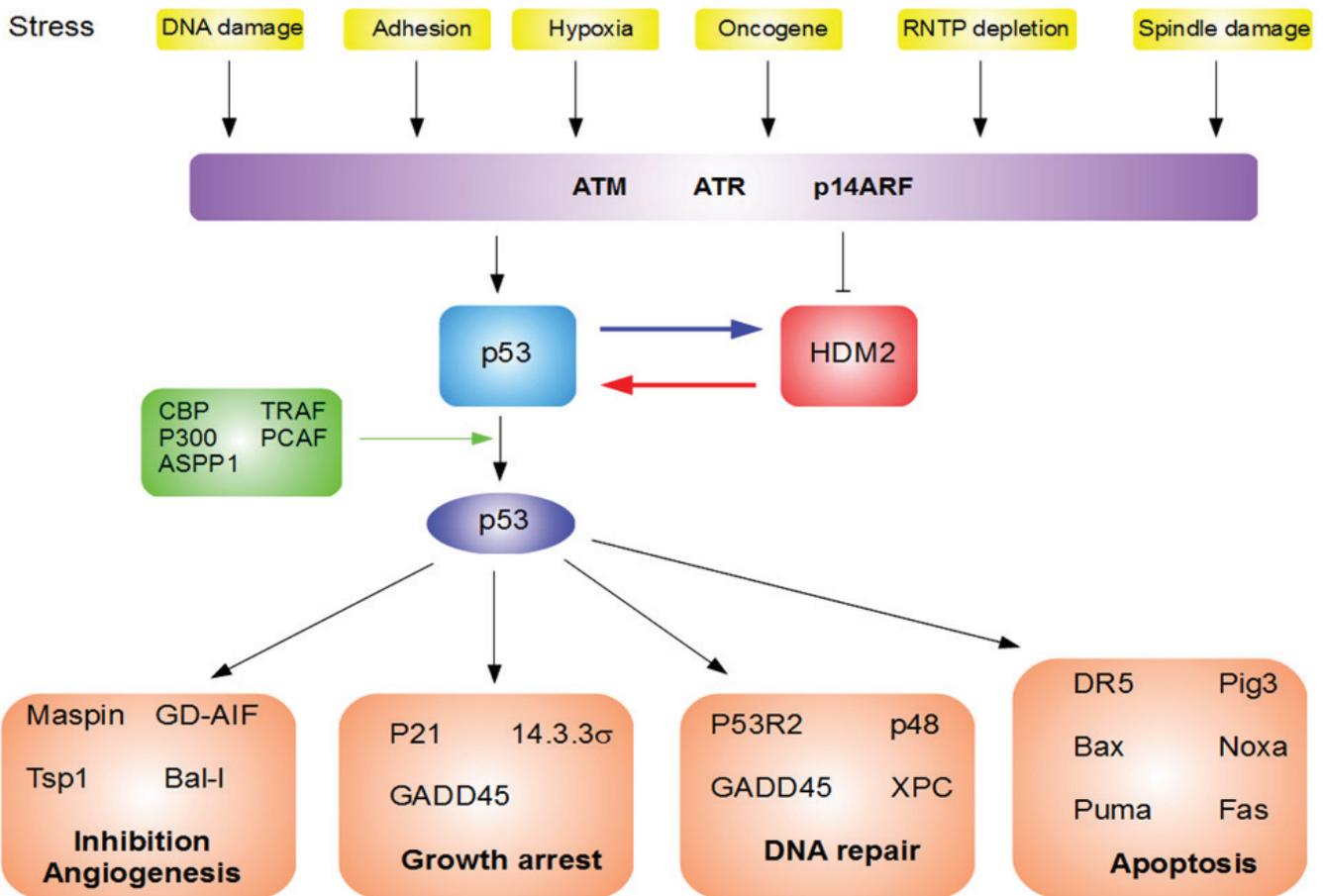


Figure 10 : p53 guardian of the genome

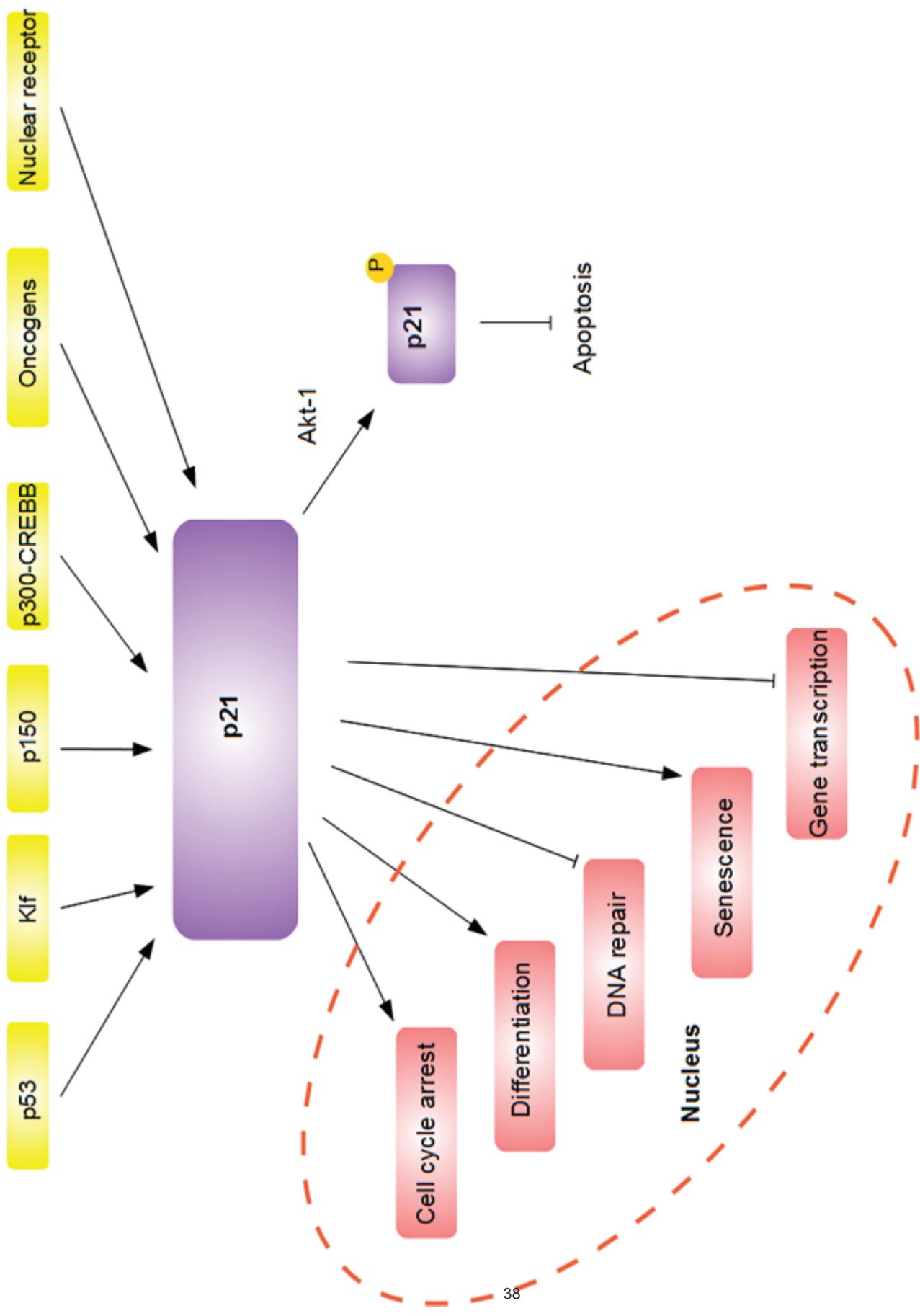


Figure 11 : Induction of p21 and effectors functions

age and oxidative stress. Second, the ATM/ATR protein kinases involved in the DDR pathway activate p53 in response to DNA damage and p14ARF mediates oncogene dependent p53 activation (Figure 10).

P53 is a transcription factor allowing the expression of several genes involved in cell cycle regulation (p21, GADD45..), angiogenesis (Tsp1), DNA repair (XPC, p48..) and apoptosis (Bax, Pig3..) (Figure 10). P53 dependent repression of genes regulating cell cycle progression including CDC25, CDC2, CHEK1, CCNB1 (encoding cyclin B1), TERT and BIRC5 (encoding survivin) are relying on p21 function [141, 142]. After a stress, p53 activation leads to a cell cycle block and induce DNA repair proteins. If the DNA lesions are too important, p53 induce pro-apoptotic genes.

(b) p21

As seen earlier, p21 belongs to the Cip/Kip family of CDK inhibitors. p21 binds and inhibits the kinase activity of the cyclin dependent kinases CDK1 and CDK2 leading to cell growth arrest in G2 and G1 respectively. p21 possesses several effectors functions (Figure 11). It mediates p53 dependent cell cycle arrest, stops the replication by inhibiting proliferating cell nuclear antigen (PCNA) [143], controls cellular growth in response to notch1 activation [144] and suppresses the transactivating activities of STAT3 [145] and MYC [146]. The p21 dependent inhibition of E2F1 activates gene transcription by de-repressing p300–CREBBP (CREB-binding protein) that activates *cdkn1a* gene expression [147]. The p21-dependent activation of p300–CREBBP-driven gene transcription induces the differentiation of ER α -positive cells [148]. This is important as p21 up-regulation is sufficient to prevent the growth of ER α -positive breast cancer cells [149] and may affect the efficacy of anti-estrogen treatments.

Upon phosphorylation by Akt, p21 accumulates in the cytoplasm. This form of p21 is found in human malignancies such as breast tumors and correlates positively with aggressive tumors and poor prognosis [150-152]. Cytoplasmic p21 represses apoptosis inhibiting directly pro-apoptotic proteins such as pro-caspase 3 [153-155] or by inhibition of apoptosis signal-regulating kinase 1 (ASK1) [156].

p21 is also regulated by many different pathways independently of p53 (Figure 11). Oncogenic Ras and Raf have been shown to activate *cdkn1a* transcription through p53 independent mechanisms and induce p21 dependent oncogene induced senescence (OIS) [157, 158]. *cdkn1a* transcription is also activated by several nuclear receptor including retinoic receptors, vitamin D receptors and androgen receptors [159] or several members of the Kruppel-like transcription factor (Klf) family, tumor suppressor key transcriptional regulators of proliferation and differentiation [160]. KLF family members have been shown down-regulated in several cancers [161-163]. p21 has been recently shown to be transactivated by p150^{sal2} [164]. p150^{sal2} belongs to the SALL family composed of SALL1, SALL2, SALL3 and SALL4 [165-167]. They are zinc fingers transcription factors. SALL1, SALL3 and SALL4 are involved in the embryonic development of limbs [167] and nerves [168] and stem cell differentiation [169]. SALL2 is not involved in embryonic development but seems to be a putative tumor suppressor. SALL2 was first identified as a binding partner of mouse polyoma virus large T antigen [170, 171] and later on has been shown to have a role in the establishment and maintenance of the quiescent state induced by serum deprivation [171].

(4) G1 and G2 checkpoints

As seen earlier the progression through G1 phase requires a phosphorylation of Rb dependent on cyclinD/CDK4-6 complexes (Figure 7). This complex is negatively regulated *via* phosphorylation by the INK4 family

members and Glycogen synthase kinase 3 β (GSK-3 β). Lack of growth factors, hormones, differentiation, TGF β , oncogen activation, stress or UV activates those inhibitors inducing an arrest of the cell cycle.

The main function of the restriction point is to prevent damaged DNA from being replicated. Central to the restriction point is the accumulation and activation of the p53 protein; two properties carefully controlled by the ATM and ATR kinases (Figure 7) [172, 173]. Following DNA damage, ATM activates downstream kinase CHK2 (by phosphorylation) [174], which in turn phosphorylates Ser²⁰ residue of p53. ATM exerts a second control measure on p53 stability by directly phosphorylating the p53 negative regulator MDM2 [175]. This modification allows MDM2/p53 interaction, but prevents p53 nuclear export to the cytoplasm where degradation would normally occur. The Ser¹⁵ residue of p53 can be phosphorylated directly by ATM or ATR in response DNA damage. p53 is also stabilized by p14ARF induced by oncogene activation or CHK1 (Figure 7). Activated p53 then up-regulates a number of target genes, several of which are also involved in the DNA damage response (14.3.3. σ , MDM2, GADD45a, and p21/Cip). The accumulation of p21, a cyclin-dependent kinase inhibitor, suppresses Cyclin E/Cdk2 kinase activity thereby resulting in G1 arrest. p21 can also be up-regulated by p53 independent pathways. The cyclin E / CDK2 complex is also inhibited by another member of the CIP/KIP family, p27Kip1, induced among others by TGF β and serum deprivation. In addition CHK1 activated by ATR phosphorylates CDC25A which induce its proteasomal degradation. Lack of CDC25A prevents CDK2 phosphorylation and subsequent activation.

The G2/M DNA damage checkpoint serves to prevent the cell from entering mitosis (M-phase) with genomic DNA damage. DNA damage activate the ATM/ATR kinases, which relay two parallel cascades that ultimately serve to inactivate the CDK1-Cyclin B complex (Figure 8). The first cascade rapidly inhibits progression into mitosis and relies on inhibition of CDC25C by CHK dependent phosphorylation, which creates a binding site for the 14-3-3 proteins. The 14-3-3/ CDC 25C protein complexes are sequestered in the cytoplasm, thereby preventing CDC25C from activating CDK1. The slower second parallel cascade involves p53 dependent pathway. The second cascade constitutes the p53 downstream regulated genes including: 14-3-3, which binds to the phosphorylated CDK1-Cyclin B complex and exports it from the nucleus; GADD45, which binds to and dissociates the CDK1-Cyclin B complex; and p21 [176, 177].

(5) Association of cell cycle actors to tumors development

In cancer, there are fundamental alterations in the genetic control of cell division, resulting in an unrestrained cell proliferation. Mutations mainly occur in two classes of genes: proto-oncogenes and tumor suppressor genes. Mutated versions of proto-oncogenes or oncogenes can promote tumor growth. Inactivation of tumor suppressor genes like pRb and p53 results in dysfunction of proteins that normally inhibit cell cycle progression. Cell cycle deregulation associated with cancer occurs through mutation of proteins important at different levels of the cell cycle. In cancer, mutations have been observed in genes encoding CDK, cyclins, CDK-activating enzymes, CDKi, CDK substrates, and checkpoint proteins [178].

CDK. Alterations of CDK in cancer have been reported, although with low frequency.

Cyclins. Aberrant cyclin D1 has been reported in many human cancers such as parathyroid adenomas [179], B-cell malignancies [180], breast, esophageal, bladder, lung and squamous cell carcinomas [181]. Cyclin D2 and cyclin D3 have also been reported to be over-expressed in some tumors and cyclin E has been found to be amplified, over-expressed or both in some cases of breast and colon cancer and in acute lymphoblastic

and acute myeloid leukaemias [182-185]. Both cyclin A and cyclin E are over-expressed in lung carcinoma and elevated expression of cyclin A but not cyclin E correlated with shorter survival [186].

CDK activating enzymes. Cdc25A and Cdc25B are potential human oncogenes [187]. CDC25B is over-expressed in 32% of primary breast cancers. Transcription of CDC25A and CDC25B genes is activated by c-myc, an oncogene found to be frequently mutated in human cancers [188]. Raf, a kinase downstream of the frequently mutated ras oncogene, is able to bind, activate and deregulate CDC25 protein [189].

CDKi. The p16 gene is altered in a high percentage of human tumors and can be inactivated by a variety of mechanisms including deletion, point mutations and hypermethylation [190]. Deletions of p16 have been reported in approximately 50% of gliomas and mesotheliomas, 40–60% of nasopharyngeal, pancreatic and biliary tract tumors and 20–30% of acute lymphoblastic leukaemias [181]. Large deletions of the ARF-INK4 locus can also affect the p19 gene, resulting in alteration of p14ARF and in deregulation of p53. The gene encoding p15 is located close to the p16 gene on chromosome 9 and is also often simultaneously deleted [191]. Loss of p27 expression has been reported for a number of human tumor types (lung, breast, and bladder) and has been correlated with poor prognosis and tumor aggressiveness. It has been shown in colorectal carcinomas that increased proteasome dependent proteolysis, rather than gene deletion, is responsible for p27 down-regulation [192, 193]. A few alterations have been found in p18 and p21 in breast tumor and leukaemia, respectively [194-196]. Although many human cancers such as colorectal, cervical, head and neck, and small-cell lung cancers are associated with reduced p21 expression [163, 197-201], the extreme rarity of loss-of-function mutations in *cdkn1a* in human cancer [202-204] argues that p21 may not be a classical tumor suppressor. *cdkn1a* *-/-* mice develop spontaneous tumors, but the loss of p21 by itself seems to be insufficient to promote malignancy [205]. However, *cdkn1a* deficiency accelerates the development of chemically induced tumors in mice [206-209]. *cdkn1a* deficiency also cooperates with the co-expression of HRAS and MYC [210] to promote transformation and proliferation of cells in culture. P21 promotes genomic stability, for example *cdkn1a* deficiency cooperated with a genomically unstable background i.e. loss of ATM in promoting aneuploidy that preceded tumor development [211].

pRB. pRb, the most important CDK substrate during G1 phase, is frequently mutated in human retinoblastoma and lung cancer [181, 212]. Approximately 90% of human cancers have abnormalities in some component of the pRb pathway [181].

DDR. p53 gene is known to be the most frequently mutated gene in human cancer [213, 214]. More than one half of human cancers expressed a mutated or deleted p53 [215]. This highlights the important role of p53 in preventing cancer development. A genetic disorder, Li-fraumeni syndrome due to an autosomal dominant mutation of p53 [216]. The cancers most often associated include breast cancer, osteosarcoma, soft tissue sarcomas, brain tumor and leukemia. Over-expression by gene amplification or other mechanisms of MDM2, the negative regulator of p53, has been reported in leukaemia and lymphoma, breast carcinoma, sarcoma and glioma and may represent an alternative mechanism to p53 mutation for escaping p53-mediated growth control [217-219].

A deletion-mutation of the CHEK2 gene is associated with an increased risk of breast cancer, particularly in the European population [220].

b) EBV cell cycle deregulation

The EBV proteins involved in the transformation of cells are EBNA1, EBNA2, EBNA3c, EBNA-LP, LMP1 and LMP2A. After infection EBNA2 induces cell proliferation and mediate the transcription of cellular genes which

contribute to the transformation [221-225]. *In vitro* it has been shown that EBNA2, EBNA3C and LMP1 can induce a LCL like phenotype when expressed individually in B cell lines [226].

More precisely, EBV is deregulating the cell cycle by affecting p53, cyclins, oncogenes, proteins of the DDR and miRNA (Table 5).

Effect	EBV protein	Cellular target	Refs
P53 inhibition	EBNA-1	p53 destabilization	[227]
	EBNA-3C	P53 inhibition	[228]
		HDM2 stabilization	[229]
		Inhibition of ING4 and ING5	[230]
	EBNA-LP	HMD2 stabilization	[231]
Cell cycle progression	EBNA2 and EBNA-LP	Cyclin D2	[39]
	EBNA-3C	Cyclin D1	[232]
	EBNA-3C	Increase cyclin A / Cdk2 activity	[233]
	LMP1	Up-regulation of cyclin D1	[234]
CDKi inhibition	LMP1	p16 reduction	[234]
	EBNA-3C	P27 inhibition	[235]
Cellular oncogene activation	EBNA-2	Increase c-myc transcription	[221]
	EBNA3-C	Increase c-myc stability	[236]
DDR inhibition	EBNA-3	Chk2 inhibition	[237]
pRb inhibition	EBNA-3C	MRS18-2 binding to pRb	[238, 239]

Table 5 : Alteration of cell cycle regulators by EBV proteins.

First, EBV interferes with p53. EBNA-1 has been show to bind USP7 an ubiquitin-specific protease [240]. Since USP7 was shown to bind and deubiquitinate p53, resulting in p53 stabilization [227], it is possible that the binding of USP7 by EBNA1 would induce p53 destabilization, therefore promoting cell cycle progression. EBNA-3C interacts also with p53 and modulates its transcriptional and apoptotic activities [228]. EBNA3C facilitates p53 degradation by stabilizing its negative regulator MDM2 [229]. EBNA-3C abrogates the effect on p53 of inhibitor of growth 4 and 5 (ING4 and ING5) proteins [230], which have been shown to enhance p53-dependent apoptosis in response to DNA damage [241]. EBNA-LP was found to bind MDM2 and forms a trimolecular complex EBNA-LP, p53 and MDM2 which might inactivate the transactivating function of p53 [231]. Altogether those data would explain why LCL can tolerate a high level of p53.

EBV acts also directly at the cyclin levels. EBNA2 and EBNA-LP cooperate to activate cyclin D2 therefore driving the B cells from G0 into G1 phase [39]. EBNA-3C has also been shown to stabilize and enhance the function of cyclin D1 thus facilitating the transition G1/S [232] and increase cyclin A dependent activity promoting S phase progression [233]. LMP1 up-regulates cyclin D1 expression and concomitantly reduces p16 expression [234], accelerating the G1/S phase transition and blocking the G2/M phase checkpoint.

EBV is also tempering with the proto-oncogene c-myc. EBNA-2 is driving its transcription thus inducing cell growth [221]. EBNA3-C interacts and increases the stability of c-myc [236].

The EBNA-3 proteins bind to and inhibit CHK2 and thereby may promote G1/S transition and disrupt the G2/M checkpoint [237].

EBV interferes with the pRb pathway. Indeed EBNA-3C decreases the level of phosphorylated pRb and p27 in rat fibroblasts [235]. However, it still remains controversial if EBNA-3C mediates the degradation of pRb and p27 *via* activation of the Skp, Cullin, F-box containing (SCF) complex leading to ubiquitylation and further degradation of the cell cycle regulators [242-244] and target MRS18-2, a pRb binding protein, to the nucleus increasing the amount of free E2F1 [238, 239].

The miRNA encoded by EBV are also playing a role in the alteration of the cell cycle. But their precise target are not very clear [72].

In Endemic Burkitt's lymphoma, the exact mechanistic of tumorigenesis is still under debate but two theories have emerged. The first one speculates that chronicles *Plasmodium falciparum* infection triggers B cells responses [245] and induce the expression of cytidine deaminase in germinal center which leads to c-myc translocation in EBV infected B cells [245, 246]. The translocation of c-myc induced an uncontrolled cellular proliferation (induction of cyclins and repression of p27) a reduced apoptosis threshold [247] and immune inhibitory activities. However the expression of c-myc alone would not be enough to induce a cancer since it will also lead the cells to apoptosis. Therefore these signals might be provided by other anti-apoptotic onco-genes such as EBNA1. The second based on immune escape will be described later.

c) HPV cell cycle deregulation

HPV is pushing the host cell to replicate in order to expand its genome. The mechanisms of cell cycle deregulation by HPV are well known and rely mainly on its oncoproteins E6 and E7 (Table 6).

HP16E7 associates with pRb and disrupt the binding between pRb and E2F [248]. Therefore E2F is free to transactivate cellular proteins required for the replication bypassing the need for cyclin D-CDK4 or -6. E7 interacts also with other cell cycle proteins. Furthermore, E7 can bind and activates directly the complexes cyclin E/CDK2 and cyclin A/CDK2 [249, 250], allowing CDK2 sustained activity. Moreover, HPV16E7 has been shown to increase indirectly CDC25A expression [251], thus reinforcing the activation of CDKs. E7 is also triggering cell proliferation by binding to HDAC [252] components of the AP1 transcription complex [253] and inhibiting p21 and p27 [254, 255].

	HPV protein	Cellular target	References
Effect			
pRb inhibition	E7	pRb binding	[248]
Cell cycle progression	E7	Activation of cyclin E/CDK2	[249, 250]
	E7	Activation of cyclin A/CDK2	[249, 250]
CDK activating enzymes	E7	increase CDC25A expression	[251]
AP1 transcription complex	E7	Inhibition of HDAC	[252]
	E6_PDZ	Degradation of DGL-1	[265]
CDKi	E7	Inhibition of p21	[254]
	E7	Inhibition of p27	[255]
p53	E6	E6AP mediated P53 degradation	[172, 173]
	E6	Direct inhibition of p53	[257]
	E6	Inhibition of Tip-60	[260]
	E6	Inhibition of p300-CBP	[259]
	E6	Inhibition of ADA3	[258]
Transformation	E6	Transformation / tumorigenesis	[263]
Apoptosis	E6	Inhibition of Bak	[264]
	E6_PDZ	Induction of cIAP	[266]
	E6	Inhibition of FADD and pro-caspase 8	[267]
	E6	Transactivation of Survivin	[268]

Table 6: Alteration of cell cycle regulators by HPV proteins.

The effect of E7 on the cell cycle depends on the amount of CDKI in the cell and also on the level of E7 itself. In fact to overcome the cell cycle arrest depending on p21 and p27, the level of E7 should be high enough. This might explain why not all the cells infected by HPV16 undergo malignant transformation [256]. The de-

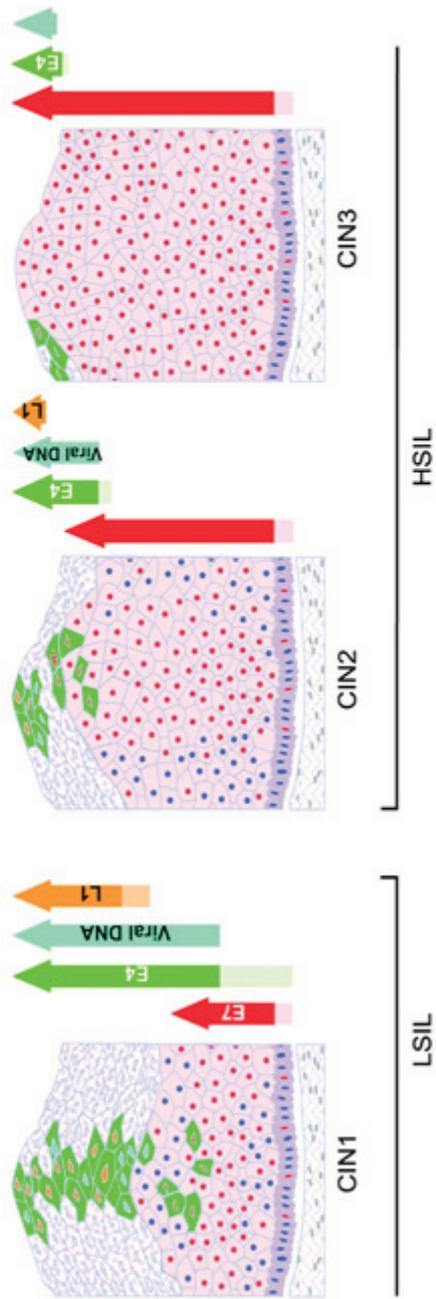


Figure 12: Changes in protein expression during neoplastic progression. LSIL: low grade squamous intraepithelial lesion, HSIL: high grade squamous intraepithelial lesion, CIN: cervical intraepithelial neoplasia. E7 expression is shown in red. (From Middleton, Peh et al. 2003.)

regulation of E7 expression is an important factor in the development of cancer.

In cells infected with HPV16 and other HPV high risk types, E6 interacts with several cellular proteins. In particular, it recruits the cellular E3 ubiquitin ligase E6-associated protein (E6AP) which leads to ubiquitylation and proteasomal degradation of p53 [172, 173]. This prevents growth arrest or apoptosis in response to E7 driven cell cycle entry. E2F increase and E2F/pRb complex decrease lead to the accumulation of p14ARF which inhibit MDM2 and induce an increase in p53 and growth arrest or apoptosis. To counteract this effect, E6 binds directly to p53 repressing its transactivational activities [257]. E6 reduces greatly p53 levels, but p53 is still able of being activated by DNA damage. Therefore, E6 by associating with the histone acetyltransferase Tip-60, p300–CBP and ADA3 prevents p53 acetylation therefore inhibiting the transcription of p53 dependent genes [258-260]. Since E6 mutants deficient for degradation of p53 can still immortalize cells, additional targets p53 independent must also play an important role in the development of cancers [261, 262]. Indeed, E6 plays also a role in mediating cell proliferation through its PDZ ligand domain mediating the degradation of its PDZ cellular partners since transgenic mice encoding E6 proteins defective for binding for PDZ proteins do not develop hyperplasia or tumors [263]. Moreover E6 inhibits the pro-apoptotic pathways by associating with other proteins such as Bak [264].

Although the main actions of HPV on the cell cycle are through its oncoproteins, some others proteins such as E5 may play a role in this phenomenon. Indeed it has been shown that E5 synergized with EGF-receptor and the Akt pathway to enhance cell cycle progression *via* p27 down-regulation [269]. This protein seems also to cooperate with E6 in inhibiting pro-apoptotic pathways. It has been shown that E5 is stimulating the degradation of bax by the proteasome [270].

The cervical neoplasia arises usually in an anatomical region called the cervical transformation zone. It is thought that in this region high risk HPV type cannot properly regulate their viral cycle leading to a deregulate expression of viral proteins. The timing of viral genes product expression is then disturbed and leads to progression (Figure 12). The changes in viral protein expression might reflect the changes in the levels of E6 and E7 expression following the integration of the viral DNA into the host genome. Furthermore, integration might be an early event in cancer progression since integrated DNA is found in invasive cancer and CIN3 (Cervical intraepithelial neoplasia) but also in some CIN1 lesions [271]. The integration in the genome has two main consequences. The first one is the loss of E2 and E4 that lead to the deregulation of the expression of E6/E7 and a suppression of the G2 arrest. The second one is the deregulation of cellular genes at the site of integration that might participate in cancer development [272]. Other environmental factors influence the development of cancer such as glucocorticoids and progesterone that affect the expression of viral genes [273, 274].

The combined action of E7 and E6 induce cell cycle growth *via* E2F activation and impairment of the DNA damage response *via* E6 degradation of p53. Cell cycle checkpoints are compromised by the action of these oncoproteins. Indeed, in case of persistent active infection the constant proliferation induced by E7 and the loss of p53-mediated DNA damage response due to E6 induce genome instability increasing the chance of mutation for the host cell [275]. As a matter of fact, the accumulation of somatic mutations in the infected cell is a prerequisite to cancer development.

2. IMMUNE RESPONSE TO ONCOVIRUSES AND ESCAPE

As the persistence of oncogenic virus is required to cancer development, the quick clearance of those oncoviruses by the host immune system is crucial. Indeed, immunosuppressed patient are more prone to develop oncovirus associated cancer. First, I will introduce the immune responses involved in recognition and further clearance of oncoviruses. Then I will develop the mechanisms of immune evasion developed by oncoviruses. The immune response relies on two steps: the innate and adaptive immune responses. The innate immune response is “non-specific” of the pathogen and provides immediate response, and then the adaptive immune response specific of the antigen is then activated. Both innate and adaptive immune responses will allow the clearance of the infection.

a) Immune response to oncoviruses

(1) Innate response

The innate immune system is the first line of defense against invading pathogens and depends on immune and non immune cells. It includes physical barriers, humoral barriers and cellular components. The epithelium forms a physical barrier impermeable to most infectious agents. Furthermore epithelium secretes chemical factors such as lysozyme and phospholipase (found in tears) to limit infection. The humoral components of the innate immunity include the complement system, the coagulation system, antimicrobial peptides and chemokines and cytokines. In response to an invading pathogen, epithelial cells will release cytokines and chemokines that will activate immune cells present in the epithelium such as Langerhans cells (LC) in the skin

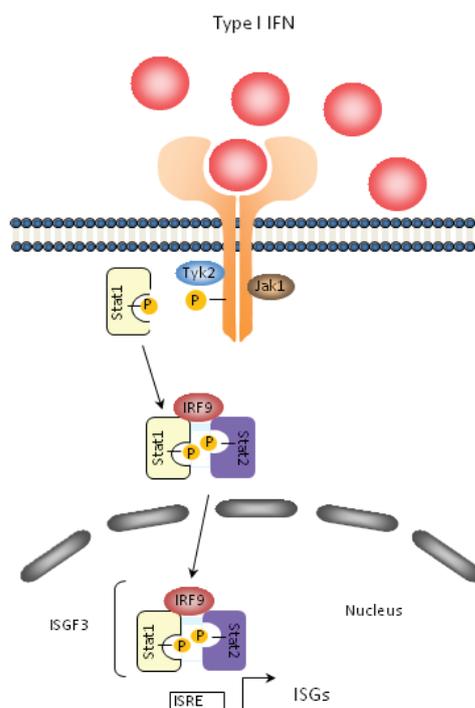


Figure 13: Type I interferon signal transduction

and recruit immune cells to clear the infection. Innate immune responses rely on Pattern Recognition Receptor (PRR) able to recognize Pathogen Associated Molecular Patterns (PAMPs) of all class of microorganisms. There are four families of receptors including toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) and the Pyhin family. PRRs were recently involved in the sensing of endogenous ligands, referred as Danger Associated Molecular Patterns (DAMP), released by cells in non infectious conditions such as stress, injury or cell death [276]. Those receptors are localized at the membranes (TLR) or in the cytosol (RLR, NLR and pythin sensors). Their localization is closely linked to the nature of the DAMP/PAMP they sense. For instance, TLRs (1/2/4/5/6) involved in bacterial and fungi recognition are expressed at the cell surface, with TLRs specialized in virus recognition through sensing of nucleic acids (TLR3/7/8/9) are located within the endosomes. In addition to TLRs, two recently described families of cytosolic innate sensors are involved in virus recognition; (i) the RNA helicases and (ii) the cytosolic DNA receptors (DAI, AIM2, and IFI16/p204). The cytoplasmic RNA helicases, RIG-I and melanoma differentiation associated gene 5 (MDA5) and DDX1/DDX21/DHX36 complex, sense RNA or DNA viruses that replicate in the cytosol *via* an RNA intermediate. Pyhin family of DNA sensors would be involved in the detection of DNA from viruses and intracellular bacteria. Finally, NLRs includes NOD1/2 involved in bacterial recognition as well as the NLRP family members that sense various PAMP and DAMP. The binding of those receptors with their cognate ligands lead to recruit members of the interleukin-1 receptor-associated kinase (IRAK) and tumor necrosis factor receptor-associated factor (TRAF) families leading to the activation of transcription factors such as Nuclear factor kappa b (NFκB), the interferon regulatory factors (IRFs) and the mitogen activated proteins kinases (MAP kinases). The activation of these pathways relies on various adaptors downstream of the different PRR: myeloid differentiation factor 88 (MYD88) and TIR-domain containing adaptor inducing interferon β (TRIF) for TLR, mitochondrial antiviral signaling (MAVS) (also known as IPS1/CARDIF/VISA) for RNA helicases, stimulator of interferon genes (STING) for most DNA sensors and apoptosis-associated speck-like protein (ASC) for NLRP. Triggering of those PRRs results in the induction of pro-inflammatory cytokines, type I IFN and chemokines that altogether participate in the establishment of an adjusted innate and adaptive immune response.

One of the most important group of cytokines in the response against viruses is the type I IFN [277]. Type I IFN comprises 13 subtypes of IFN α , IFN β , IFN ω and IFN κ specific of keratinocytes [278, 279]. Type I IFNs bind to alpha interferon receptors 1 and 2 (IFNAR1 and -2), resulting in receptor dimerization. In the classical pathway, the receptor-bound Janus kinases (Jaks) and the non receptor tyrosine kinase 2 (Tyk2) are activated and cross-phosphorylate each other (Figure 13). Activated Jaks phosphorylate IFNAR1 and -2, which then serve as Src homology 2 domain docking sites for signal transducers of activated transcription 1 and 2 (STAT1 and -2). STAT1 and -2 are subsequently phosphorylated by activated Jak1 and Tyk2. Phosphorylated STATs heterodimerize and interact with interferon regulatory factor 9 (IRF9) to form the active transcriptional factor complex interferon-stimulated gene factor 3 (ISGF3), which regulates the expression of interferon-stimulated genes (ISGs) [280]. Majority of human cells are able to produce type I IFN during viral infection. However the pDCs can induce up to one thousand time more IFN α than other cell types in response to enveloped viruses especially *via* TLR7 and TLR9. Beside their direct anti-viral activities, type I IFN display anti-proliferative, anti-angiogenic and immunostimulatory properties. Actually, type I IFN can modulate the innate immune response by contributing to NK cell homeostasis and activation [281]. They also modulate adaptive immune response by inducing phenotypical and functional maturation of immature DCs such as up-regulation of CD83, MHC Class I and II, CD40, CD80 and CD86 and increase ability to stimulate T cell proliferation [282-284]. Type I IFNs license DCs to present exogenous antigens to CD8⁺ T cells, then allowing the cross-priming of CD8⁺ [285].

This cross-priming has been shown to be important in clearing viral infection and in cancer treatment [286]. Moreover, they also drive the T cell response toward a Th1 type [287] and enhance CD8+ T cells responses during cross-priming [288]. In addition, they enhance the antigen presentation of infected cells by increasing the level of MHC Class I molecules [289]. The earliest immune response to EBV infection is the production of type I IFNs. EBV recognition by the innate immune system and trigger this cytokine production. In vitro, IFN α production peaks 24 hours after infection and is mainly produced by T cells and NK cells [290, 291]. This production of type I IFN is important within the first 24h to inhibit EBV infected B cell proliferation because afterwards the cells become resistant to type I IFN [292].

Direct antiviral activities rely on three major interferon responsive genes induced by type I IFN; the protein kinase K (PKR), the 2'5'oligoadenylate synthetase (2'5'OAS) and the Mx proteins. Mx proteins are GTPases which bind to nucleocapsids of some viruses altering their intracellular transport [293] and interfere with the viral polymerase activity during the viral transcription [294]. The 2'5' OAS activates the RNaseL in response to dsRNA to degrade viral and cellular RNAs thereby blocking viral infections [295]. PKR is activated by dsRNA, polyanionic molecules or caspases 8, 3 or 7. Upon activation the main function of PKR is to phosphorylate eIF-2 α therefore blocking protein translation [296]. PKR regulates also phosphatase 2A (PP2A)-mediated dephosphorylation of BCL2 *via* B56 α phosphorylation to induce apoptosis [297]. In addition, PKR enhance the induction of IFN β and apoptosis mediated by RLR in response to measles virus infection [298] and is required for type I IFN production in response to encephalomyocarditis, Theiler's Murine encephalomyelitis, Semliki Forest virus and west Nile virus [299, 300].

Additional cytokines play also a key role in innate immune response, IL1 β and IL18 that belong to the IL1 family. Those cytokines are produced in a pro-form that must be cleaved into an active form by caspase-1. Activation of NLRP-1 and -3, as well as the DNA sensors AIM2, triggers the formation of a multiprotein complex termed inflammasome, which leads to caspase 1 activation and proteolytic cleavage of pro-interleukin (IL)-1/IL-18 after microbial, DAMPs or genomic DNA sensing. IL-1 β is a pro-inflammatory cytokine involved in several inflammatory events, such as fever [301], hyperalgesia [302], and hepatic acute-phase protein stimulation [303], bone marrow cell number increases [304], neutrophil progenitors proliferation [305], activation of mast cells [306] and induction of neutrophil migration and survival [307, 308]. IL18 plays an important role in the priming of NK cells which are important in the killing of cancer cells lacking MHC Class I molecules [309]. Furthermore, IL1 β and IL18 stimulate innate immunity by activating neutrophils and macrophages to engulf pathogens and to release reactive oxygen species (ROS) and nitrogen radicals [310, 311]. IL-1 β and IL-18 play prominent roles in polarizing T helper responses. IL-18 is important for the induction of the Th1 response [312]. By inhibiting IFN γ and Th1 immunity, IL-1 β contributes to polarization towards a Th17 response. In addition, IL1 β /IL18 responses are very important in host defense against virus [313] or in the induction of innate immunity against tumor [314]

(a) Toll like receptors

(i) Introduction

The Toll protein was first described in drosophila where it mediates dorso ventral pattern during development [322] as well as immunity to pathogens [323]. The Toll protein is a receptor that mediates the production of anti-fungal peptides. Similar genes have been found in vertebrates [324]. So far, 13 TLRs have been identified in mammals, TLRs 1-9 are conserved between humans and mice, TLR10 is expressed only in humans whereas

TLRs 11-13 are present in mice but either absent or non functional in humans [325].

(ii) *Structure and signaling.*

The TLR are type I transmembrane glycoprotein. They are characterized by three domains, an ectodomain constituted of 16 to 28 extracellular leucine rich repeat (LRR) domains [315], a transmembrane α -helix domain and an intracellular domain Toll IL1 receptor (TIR) domain homologous to the intracellular domain of the IL1 and IL18 receptor [316]. The LRR domains form a horseshoe-like structure involved directly in the binding of PAMPs and DAMPs. The study of the crystal structure of TLR3 bound to dsRNA has suggested that the ligand bridge two TLR molecules forming a dimer between the ectodomains to dimerize the cytoplasmic TIR domain [317, 318]. Most TLRs are homodimeric but TLR1/2 and TLR2/6 function as heterodimers. The conformational change of the receptors triggers the recruitment of specific adaptors to the intracellular TIR domains [319]. To date four positive adaptors MyD88, TIR domain-containing adaptor protein (TIRAP or Mal), TIR domain-containing adaptor inducing IFN β (TRIF or TICAM-1) and TRIF-related adaptor molecule (TRAM or TICAM2) and one negative adaptor sterile alpha and TIR motif containing (SARM), which block TRIF dependent signaling have been described [319, 320]. The adaptor MyD88 is used by all TLRs but TLR3. However TLR1, TLR2, TLR4 and TLR6 require Mal as a bridge between their TIR domain and MyD88.

(iii) *Ligand and expression.*

TLRs can be broadly divided in two groups. The cell surface expressed TLRs (TLR1, TLR2, TLR4, TLR5 and TLR6 in humans) that will respond mainly to bacterial and viral surface associated PAMPs and the endosomal TLRs (TLR3, TLR7, TLR8 and TLR9 in human) that will mainly respond to nucleic acid. As specified in table 7, TLRs are also cell type specific and thereby regulate innate immune responses by different cell type. There is increasing evidence that TLRs beside their role in recognizing PAMPs are also able to recognize DAMPs released by injured tissue or dying cells (Table 7). One can question some of the endogenous ligands since some demonstrations have used ligands purified from bacterial systems or that display high affinity for bacterial products [321]. However the work from Zitvogel and collaborators showed *in vivo* that endogenous ligands (such as HMGB1) were generated during chemotherapy-induced cell death and triggered TLR4 [276].

TLR2 recognizes PAMPs derived from viruses such as hemagglutinin from measles virus, bacteria such as triacyl lipopeptides, peptidoglycan (PGN) and lipoteichoic acid (LTA) from gram positive bacteria, porin from *Neisseria*, fungi such as zymosan and parasites such as GPI from *T. gondii* [322]. TLR2 heterodimerize with TLR1, TLR6 or non TLR molecules such as CD36, CD14 or dectin-1 depending on the structure of the ligands. For the recognition of MALP2 and PGN it needs to associate with TLR6. TLR2 is also activated by high mobility group box 1 protein (HMGB1)/nucleosome complexes released by apoptotic cells involved in systemic lupus erythematosus (SLE) pathology [323]. *Hmgb1*, *Hmgb2* and *Hmgb3* code for proteins highly similar containing DNA-binding domains. HMGB1, the most studied is ubiquitously expressed nuclear protein that modulates chromatin accessibility and transcription [324, 325]. Active HMGB1 is released from cell following necrosis, immune cell activation, TLR stimulation, autophagy or secondary necrosis due to apoptosis [326-330]. Post-translational modifications including phosphorylation, acetylation and oxidation influence the localization and the function of HMGB1 [331-333]. HMGB1 has a pleiotropic chemotactic activity and is involved in tissue regeneration [324, 334, 335].

TLR4 was first recognized to be the receptor for LPS of gram-negative bacteria [336]. It is involved in the recognition of bacteria through LPS, viruses through their structural proteins, parasite through GPI and fungi

through mannan. In order to recognize LPS TLR4 needs a co-receptor MD2 that is triggering TLR4 clustering [337]. The TLR4 response to LPS is enhanced by seric or cell bound CD14 and HMGB1 that bind specifically to LPS [338].

The engagement of TLR4 with its ligand recruits the TIR domain-containing adaptor TIRAP and Myd88 which leads to early NF κ B activation. The complex is then internalized in the endosome where it recruits TRAM and TRIF which leads to IRF3 and late NF κ B activation and production of inflammatory cytokines (early and late NF κ B activation are required) and type I IFN [339] (Figure 14). TLR4 is the only TLR that can recruit the four adapters.

TLR5 has been shown to recognize only the flagelin of bacteria. It is expressed mainly on monocytes and epithelial cells where it allows the recognition of bacteria that have invaded the intestinal epithelium [340]]. TLR5 is also important in the defense of the urinary tract against bacterial infections [341].

In order to detect virus infection, the cells display PRRs that are able to recognize nucleic acid in the endosomes. After binding to the cell membrane, viruses may enter the cells by endocytosis and reach the endosomes or directly into the cytosol after membrane fusion. TLR3, TLR7, TLR8 and TLR9 are expressed in the endosomes and can sense viral genome. TLR3, TLR9 and TLR7 are sequestered in the ER of unstimulated cells and translocate to the endosome *via* UNC93B binding [342] after stimulation. Deficiency in UNC93B abrogates the signaling of the endosomal TLRs but also affects antigens presentation [343]. PRAT4A associates with TLR9 and is required for its trafficking to the endolysosome [344] and HMGB1 seems to accelerate the redistribution of TLR9 to the endosomes [345]. TLR9, and more recently TLR3 and TLR7, were shown to require proteolytic cleavage by intracellular protease in endolysosomes to become a functional receptor [346-350].

TLR3 was originally identified as the receptor of polyinosinic-polycytidylic acid (poly (I:C)), synthetic analog of dsRNA which mimics viral infection. TLR3 uses TRIF to activate a pathway leading to an antiviral response *via* activation of NF- κ B and IRF3 and the induction of type I IFN and inflammatory cytokine. The structure of TLR3 bound to its ligand was solved by crystallography and gave precious insight on the recognition mechanism between TLRs and their ligand [386]. TLR3 recognizes dsRNA viruses such as reoviruses and dsRNA produced during the course of the replication of ssRNA viruses such as West Nile virus [387]. TLR3 deficiency in humans is associated with susceptibility to herpes simplex virus type 1 (HSV-1) [388].

Single-stranded (ss) RNA from viruses are the natural ligands for **TLR7 and TLR8**. Stimulation of TLR7 and TLR8 is sequence dependent. AU-rich sequences only stimulate hTLR8 response while GU-rich sequences can trigger activation of human and mouse TLR7/8 with the exception of mTLR8. The first ligand identified for TLR7 and TLR8 were synthetic small molecules such as imidazoquinolines and nucleoside analogues, such as Resiquimod (R848). Human TLR7 is expressed in B cells, pDC and eosinophils and hTLR8 in monocytes and mDC. TLR7 was shown to be central for *in vivo* IFN α induction, mediated by pDCs, in response to ssRNA viruses (VSV, influenza). TLR7 signaling has been shown to be important for B cell and CD4+ T cell responses [389], but also for the differentiation of CD8+ T cells [390]. TLR7 was shown to be central *in vivo* for the development of antibodies against both MMTV and MuLV [391]. Recently, TLR7 has been also shown to recognize RNA from Group B streptococci in lysosomes of cDCs [392].

	Cellular expression			Ligands		Ref
	Immune cells	Non immune cells	PAMP	DAMP		
PRR						
TLR1/2	Monocyte, mDC, B cell, NK, neutrophil, basophil	ubiquitous	LAM, PGN, GPI (<i>Toxoplasma gondii</i>), LTA, triacyl lipopeptide, zymosan			[351-354]
TLR2/6	Monocyte, mastocytes, mDC	epithelial cell, neural cell, MSC, endothelial cell, keratinocytes	LTA, diacyl lipopeptide			[351, 355-357]
TLR3	mDC	epithelial cell, neural cell, MSC, keratinocytes	RNA virus (WNV, RSV, MCMV), synthetic dsRNA (Poly[I:C], poly [A:U])	Self dsRNA		[354, 358, 359]
TLR4	Monocytes, macrophages, mDC, mastocytes, basophil	renal cell, hepatic cell, keratinocytes, MSC, endothelial cell	LPS, viral proteins (HIV, VSV, RSV, retroviruses)	HSPs, fibronectin, hyaluronic acid, fibrinogen, HMGB1		[355-357, 360-362]
TLR5	mDC, monocyte, NK, T cell	gastric epithelial cell, keratinocytes, MSC	flagellin			[356, 361, 362]
TLR7	pDC, B cell, eosinophil		RNA virus (influenza), synthetic ssRNA, imidazoquinoline (R848/CL097/imiquimod)	Self ssRNA		[352, 363-366]
TLR8	mDC, T and B cell, monocyte		synthetic ssRNA (AU-rich) imidazoquinoline (R848/CL075)	Self ssRNA		[352, 363, 364, 366, 367]
TLR9	pDC, B cell, basophil, eosinophil, T cells, B cells, NK	epithelial cell, keratinocytes, MSC, endothelial cell	unmethylated CpG dsDNA, dsDNA virus (HSV, MCMV)	DNA/LL-37, chromatin DNA immune complexes, mitochondrial DNA		[354, 368-383]
TLR10	pDC, neutrophil, B cell, basophil		unknown			[352, 384, 385]

Table 7: Cellular expression and ligands for TLRs. MDCs, myeloid dc; NK, natural killer cell; pDC, plasmacytoid DC; MSC, mesenchymal stem cell; LTA: lipotechoic acid; LAM: lipoarabinomannan; PGN: peptidoglycan.

Historically **TLR9** was thought to recognize unmethylated CpG motifs commonly present in the genome of bacteria and viruses [376, 393]. Therefore the majority of the studies on TLR9 function were done with synthetic oligonucleotides (ODNs) termed CpGs. There are four classes of CpGs that differs by their backbone modification, secondary structure, number and positioning of CpG motifs (Table 8).

Class	Backbone	PolyG tail	Palindrome	Structure	Example	Ref
A	PS and PD	+ (PS)	Internal containing CpG motif(s) with PD linkage	G-Tetrads	2216	[394]
B	PS	-	-	Linear	2006	[395]
C	PS	-	3' GC rich	Duplex	2395	[396]
P	PS	-	2 sequences	Multimeric	23617	[397]

Table 8: Structural properties of CpGs ODN. PS: phosphorothioate PD: phosphodiester.

The sequence composition, sugar, base, or backbone modifications, as well as secondary and tertiary structures affect the immune-modulatory effects of CpGs. Secondary and tertiary structures formed by CpG ODNs appear to be crucial for strongest IFN-inducing activities [398, 399]. Class A CpGs are the stronger inducer of type I IFN by human pDCs but are weak inducer of NFκB activation (Table 9). On the contrary class B CpGs are weak inducer of type I IFN but potent human B cell activators resulting in increased Major Histocompatibility

Class II complex (MHCII) expression, secretion of immunoglobins and B cell proliferation [400, 401]. C class CpGs induce both type I IFN and B cell stimulation [396, 402]. P class CpGs are an improved version of C class CpG since they induce more type I IFN but are still able to activate NFκB [397]. TLR9 dependent secretion of type I IFN in pDCs is dependent on the ability of the CpGs to form higher order secondary structures through the poly-G tail or the palindromic sequences [403].

Class	Type I IFN	B cell activation	NK and APCs activation	pDCs activation	Ref
A	+++	+/-	+++	+	[394, 400, 404, 405]
B	+/-	+++	+/-	+++	[376, 400, 405-407]
C	+	++	+	++	[400-402]
P	++	+	++	++	[397]

Table 9: Activities of the different classes of CPG-ODN.

Synthetic ODN have been shown to elicit a TH1 like immune response, which is important in the context of immunity against cancer and adjuvant effect in vaccination [408, 409]. CpGs used in clinical trial are usually from the B or the C class.

To achieve greater biological stability through nuclease resistance and cellular uptake, the CpGs ODN are phosphorothioate-modified (PS): one of the non-bridging backbone oxygen atoms of the phosphodiester linkage is replaced with sulfur [410, 411]. However this modification is not neutral, in particular regarding the activation of TLR9. Experiments made with PS ODNs showed that the recognition of the DNA by TLR9 was dependent on the presence non methylated CpG motifs in genomes of virus and bacteria [395]. CpG dinucleotides are methylated and scarce in vertebrate, which was thought to allow the discrimination between self and non self and limit activation of TLR9 by self DNA [412]. However, recent experiment showed that non CpGs PD ODNs and self DNA “forced” to the endosomes activate TLR9 [413]. While TLR9 activation by synthetic PS ODNs is sequence specific (CpG motifs), natural DNA with PD linkage is not. Furthermore, base free PD 2’deoxyribose 20-mers “forced” into the endosomes can activate TLR9 while base free PS 2’deoxyribose is an antagonist of TLR9 activation [414]. The discrimination between self and non self is a matter of compartmentalization. TLR9 can signal only in the endosome where it can be activated by pathogens invading the cells by endocytosis. In homeostatic condition self DNA cannot access spontaneously the endosome and is rapidly degraded by the extracellular environment. However in some pathological condition such as SLE or psoriasis, self genomic DNA can reach the endosome, activate TLR9 and participate to autoimmunity and inflammation [415, 416]. Host factors such as the antimicrobial peptide LL37 [415] and DNA specific autoantibodies [417] bind to self DNA and may facilitate its endosomal translocation. Other factors such as extracellular HMGB1 binds to self DNA and through the receptor for advanced glycation end products (RAGE) potentialize TLR9 activation [345, 418, 419].

TLR9 is signaling through MyD88 to induce pro-inflammatory cytokines and type I IFN. Adaptor protein 3 (AP-3) complexes mediate the trafficking of UNC93B and TLR9 to a specialized lysosome-related organelle in pDCs allowing the production of type I IFN through IRF7 [378]. TLR9 is critical in the control of bacterial infections by *Brucella abortus*, *Streptococcus pneumonia*, *Mycobacterium tuberculosis* and helicobacter [420]. It also detects many DNA viruses including HSV type 1 and type 2, murine cytomegalovirus, adenovirus, EBV and recently, adenovirus-associated virus (AAV).

TLR triggering leads to the activation of IRF, NFkB, p38, ERKs and MAPKs pathways [421]. Besides inducing cytokines, TLR ligands trigger innate immune cells activation. They also activate DC maturation and NK cells to increase their lytic capacity. They increase the phagocytic ability of neutrophils [353] and macrophages [422] and the production of reactive oxygen species [423] nitrogen intermediates [424] and provoke neutrophils oxidative burst [425]. They also play a role in adaptive immune response. TLRs induce B-cell proliferation, immunoglobulin isotype switching and somatic hypermutation [426]. Moreover, they promote the differentiation and proliferation of TH17 cells [427] and the inhibition of the regulatory T cell activity [428]. While TLR are primarily expressed on immune cells, they have been described in human keratinocytes [371], epithelial cells from the intestinal, urogenital and respiratory tracts [362], endothelial cells [429], mesenchymal stem cells [430] and various neural cells [357]. In these tissues they are likely to provide a first line of innate anti-microbial defense. Indeed, TLRs stimulation of epithelial cells or endothelial cells has been shown to attract immune cells *via* chemokines secretion and cell surface adhesion molecule induction [431, 432].

(b) Toll-like recognition of oncoviruses

(i) *Cell surface recognition of oncoviruses*

TLR1, TLR2, TLR4 and TLR6 are localized at the cell surface and are involved in the recognition of oncoviruses; however TLR5 has not been associated with the recognition of viruses. TLR4 is the only TLR that can recruit the four adapters TIRAP, MyD88, TRAM and TRIF (figure 14).

TLR2 homodimerizes or dimerizes with TLR1 or TLR6 and induce NFkB trough the recruitment of TIRAP and MyD88 which lead to proinflammatory cytokines production. It has been shown that intact EBV virions can be recognized by TLR2 on epithelial cells and monocytes. This recognition is likely mediated by GP350 (encoded by BLLF1), the major envelope glycoprotein, which might be a ligand for TLR2 and is responsible for MCP-1 production [433]. In addition, the non structural protein dUTPase (encoded by BLLF3) might also be recognized by TLR2 and induces the expression of proinflammatory cytokines by macrophages [434]. The complexes TLR2/1 and TLR2/6 were also involved in recognition of HCV PAMPs, such as the non-structural NS3 and core proteins [435](Table 10).

Concerning EBV it is not known whether TLR2 is associated with TLR1 or TLR6. A recent study found a mechanism of induction of type I IFN *via* TLR2 [436]. TLR2 would follow vaccinia virus in the endosomal compartment and activates IRF3 and IRF7 to induce type I IFN production. This new mechanism has only been shown in mouse inflammatory macrophages. However, similar mechanism possibly exists in human and would be involved in the recognition of oncoviruses that trigger TLR2 (Figure 14). It might also be the case for EBV.

Oncovirus	PRR	Ligand	Ref
EBV	TLR2	dUTPase	[434]
		GP350	[433]
HCV	TLR2	Core protein	[435, 437]
	TLR2	NS3	[435]
KSHV	TLR4	unknown	[438]
HPV	TLR4	HPV16 L1 VLP	[439, 440]

Table 10 : Recognition of oncoviruses by membrane bound TLRs.

TLR4 is also involved in the recognition of HCV. Indeed *in vitro* infection of human B cell line lead to IFNβ

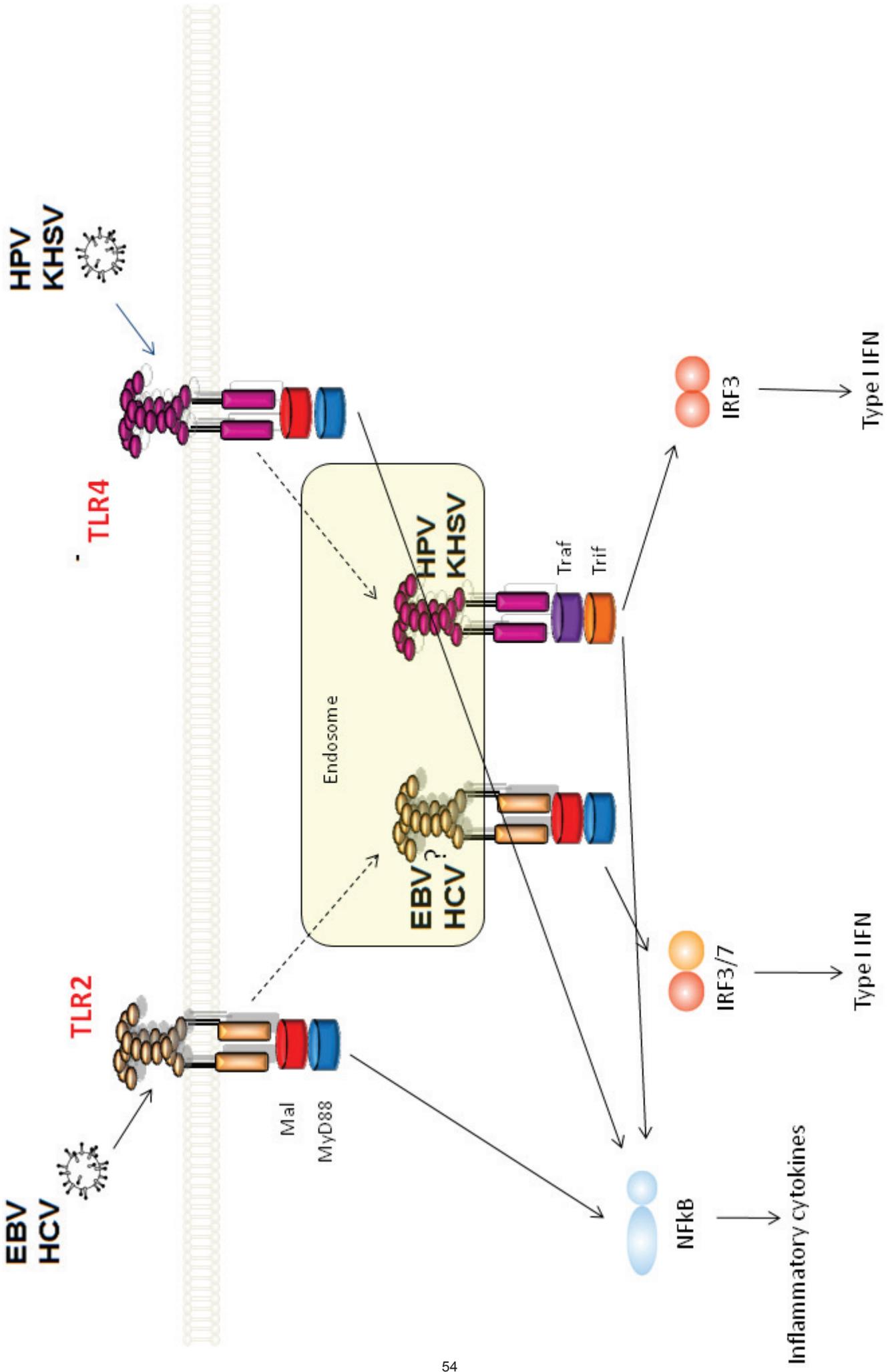


Figure 14 : Cell surface recognition of oncoviruses.

an IL6 secretion in a TLR4 mediated way [441]. However, TLR4 is also induced upon virus triggering, so it is unclear whether the virus is triggering directly TLR4 or if the increase of this PRR is leading to a higher basal activation without ligand involved (or at least not HCV). As for HPV, VLPs constituted of L1 are recognized *in vitro* by TLR4 (Table 10). It might not be as relevant *in vivo* since the keratinocytes of the mucosa at the site of infection do not respond to TLR4 ligand, but might be important to activate immune cells following HPV vaccination. KSHV has been found to induce TLR4 signaling but to date the specific viral molecule involved is still unknown (Table 10).

(ii) Endosomal sensing of oncoviruses

Infection of hepatoma cell line with HCV leads to a TLR3, TLR7 and RIG-I dependent production of type I IFN [442, 443]. In addition it seems that the RNA dependent RNA polymerase activity of NS5B would be recognized by TLR3 and trigger IFN β promoter activity when transfected in HepG2 cells (Figure 15) [444]. EBV EBERs associated with La protein are released *in vitro* by EBV infected B cells but are also found in the sera of patients with chronic active EBV. Those complexes induce TLR3 dependent type I IFN and inflammatory cytokines productions as well as TLR3 dependent monocytes derived DCs maturation, activation and increase of antigen presentation ability [445].

HTLV-1 viral particles trigger IFN α production in murine pDC *via* TLR7 [446]. In addition it has been shown that IFN α produced by murine FLT3-derived DCs were critical to control HTLV1 infection [447]. As mentioned earlier, HCV infection of hepatoma cell line induce type I IFN secretion partly dependent on TLR7 [442, 443]. DNA oncoviruses have not been shown to trigger this receptor.

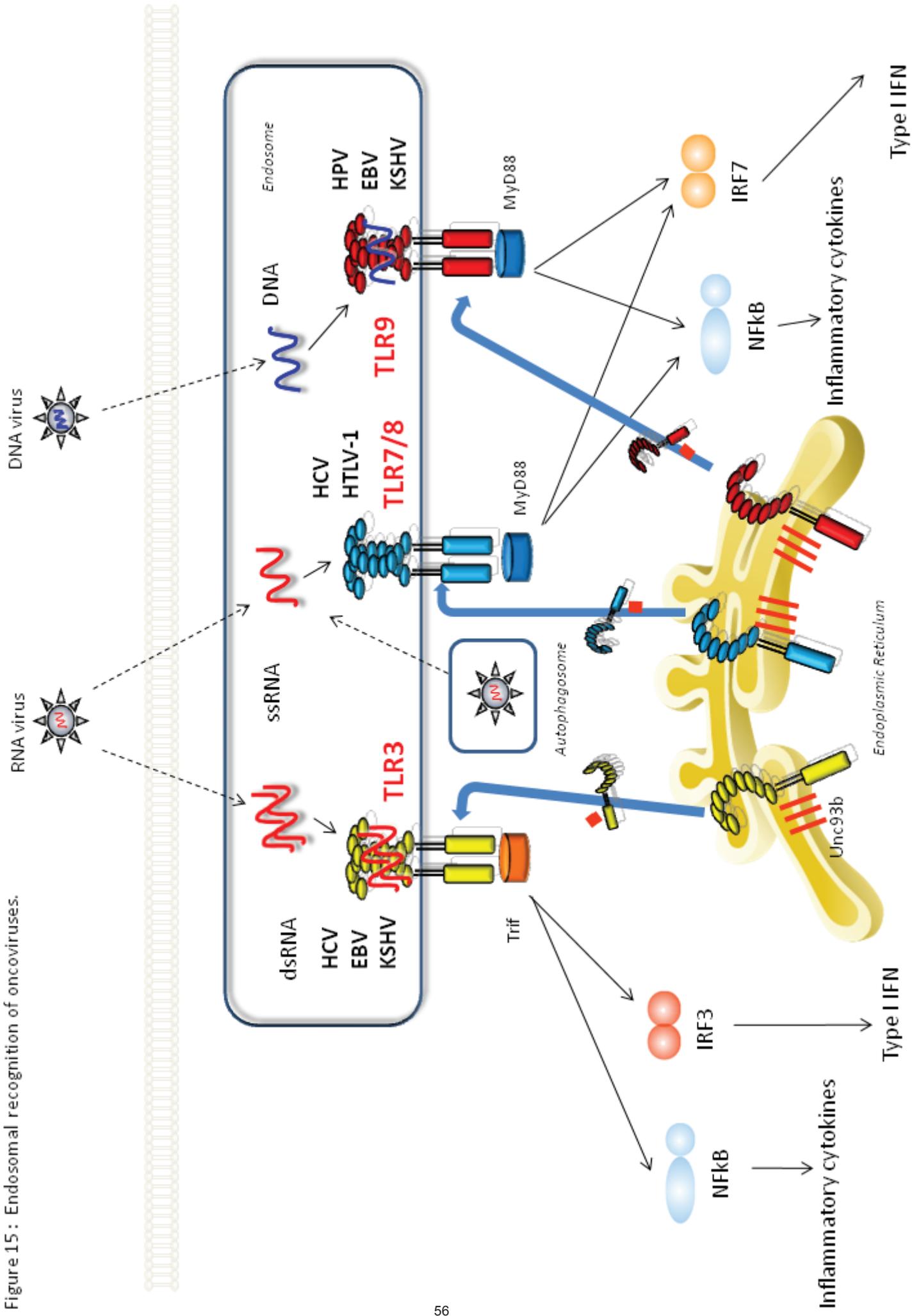
TLR9 is the primary sensing mechanism of DNA viruses in the endosome and thereby is as well able to recognize oncoviruses. HPV16 viruses have been shown to induce NF κ B activation *via* TLR9 (Hasan, U. unpublished data). TLR9 seems also to contribute to antiviral immunity during gammaherpesvirus infection [448]. The authors used murine gammaherpesvirus 68 as a model to study gammaherpesvirus pathogenesis and showed that *in vivo* TLR9 dependent signaling is important to control viral load to induce organ-specific immunity during latency and lytic cycle. Furthermore, *in vitro* KSHV and EBV have been shown to trigger type I IFN in human pDCs *via* TLR9 [449, 450]. *In vitro* EBV infection activates TLR9 signaling pathway leading to IFN α production in pDCs and IFN β in monocytes, and promotes activation of NK cells and IFN γ production by CD3+ T cells [451] [450]. However, the responsiveness of pure monocytes to TLR9 is controversial [352]. As for B cells EBV induces an increase in activation markers in B cells [452].

(iii) Cytosolic virus sensing

During their life cycle most viruses will reach the cytosolic compartment. DNA viruses such as EBV and HPV are usually transported from the endosome *via* cellular transport mechanisms to the nucleus where they either stay as episomes or are integrated in the host DNA.

HTLV-1 is first reverse transcribed in the cytosol, and then the DNA negative strand is replicated and shuttled to the nucleus. As for HCV, the replicon complex is found in the cytoplasm of most of the cells. The cytosolic sensing of virus can lead to the production of type I IFN and inflammatory cytokines such as IL1 β and IL18. Cytosolic sensing of viruses relies on various redundant PRR. RNA viruses may be sensed by RLR, PKR and NLR while DNA viruses are sensed by DNA sensors (including ZBP1, LRRFIP1 and the pyhin family), NLR or

Figure 15: Endosomal recognition of oncoviruses.



RLR (Table 11 and 12). HMGB1, HMGB2 and HMGB3 proteins bind nucleic acids and are required for the full-blown activation of innate immune cells by nucleic acids, VSV and HSV-1 through TLR, RLR and cytosolic DNA sensors [453].

Sting/MITA was recently identified as a molecule able to activate type I IFNs. It is a transmembrane protein localized in the ER of macrophages, DC, endothelial and epithelial cells [454, 455]. Over-expression of Sting leads to the production of type I IFN *via* NF κ B and IRF3 activation (Figure 16). In mice model sting has been found to be essential to the type I IFN production in response to negative stranded RNA viruses such as VSV or SV. Therefore one role of sting might be to facilitate RIG-I signaling (Figure 15-16). But it has been shown that sting display also a central role in the type I interferon production in response to ISD (interferon stimulatory DNA) and to infection with HSV-1 and HIV-1 [454-456]. Sting seems to be an essential component of both the RLR-mediated and DNA sensing pathways that induce type I IFN (Figure 15-16). ZBP1 a Z-DNA binding protein was the first molecule to be identified as DNA sensor in the cytoplasm. ZBP1 induces type I IFN production in response to synthetic DNA and HSV-1 infection through TBK1/IRF3 signaling [457]. It has been recently shown that the ZBP1 pathway was redundant and probably cell type or species specific [458]. ZBP1 signals through Sting in order to induce type I IFN production (Figure 9). LRRFIP1 bound exogenous DNA and associate with β -catenin to increase type I IFN expression IRF3 mediated [459]. This molecule is essential for maximal type I IFN production in response to VSV for example.

DEXD/H BOX RNA HELICASES:

The RLRs receptors are a family of DExD/H box RNA helicases that are cytoplasmic sensors of RNA (Figure 16 and Table 11).

	Cellular expression		Ligands		Ref
	immune cells	non immune cells	PAMP	DAMP	
PRR					
MDA5	ubiquitous	ubiquitous	long dsRNA [poly(I:C), EMCV		[460]
RIG-I	ubiquitous	ubiquitous	5'tri-phosphate dsRNA, short Poly(I:C), RNA viruses (NDV, VSV, SeV, flu Δ NS1, HCV, JEV)		[461, 462]

Table 11: Cellular expression and ligands for RLRs. EMCV: encephalomyocarditis virus, NDV: Newcastle disease, VSV: Vesicular stomatitis virus, SeV: Sendai virus, flu: Influenza, JEV: Japanese encephalitis virus.

To date, three RLR members have been identified: RIG-I, MDA5 and LGP2. RIG-I and MDA5 are mainly responsible for the production of type IFN in response to RNA viruses in most cell types but pDCs. They are able to bind and unwind ssRNA through their helicase domain and signal *via* their caspase recruitment domains (CARD). Comparison of RIG-I and MDA-5 interaction with synthetic dsRNA poly (I:C) suggest that while MDA-5 preferentially recognizes high molecular weight branched RNA [463], RIG-I interacts preferentially with shorter blunt-ended 5' triphosphate (5'-PPP) dsRNA [464]. Interaction between the helicase and the RNA recruits the mitochondrial protein MAVS and through FADD, RIP1 and TBK1 leads to the production of inflammatory cytokines and type I IFN (Figure 10). The third member of the family, laboratory of genetics and physiology 2 (LGP2), lacks CARD domains and has two ambivalent actions; it may function as a co-factor of RLR signaling [465] and as an inhibitor of RIG-I [466]. MDA5 has been involved in the recognition of DNA viruses such as HSV [467]. RIG-I has been involved in the recognition of AT-rich dsDNA such as poly(dA-dT) that can be transcribed by RNA pol III into 5'-triphosphate, the real ligands for RIG-I in human fibroblasts [468]. Therefore

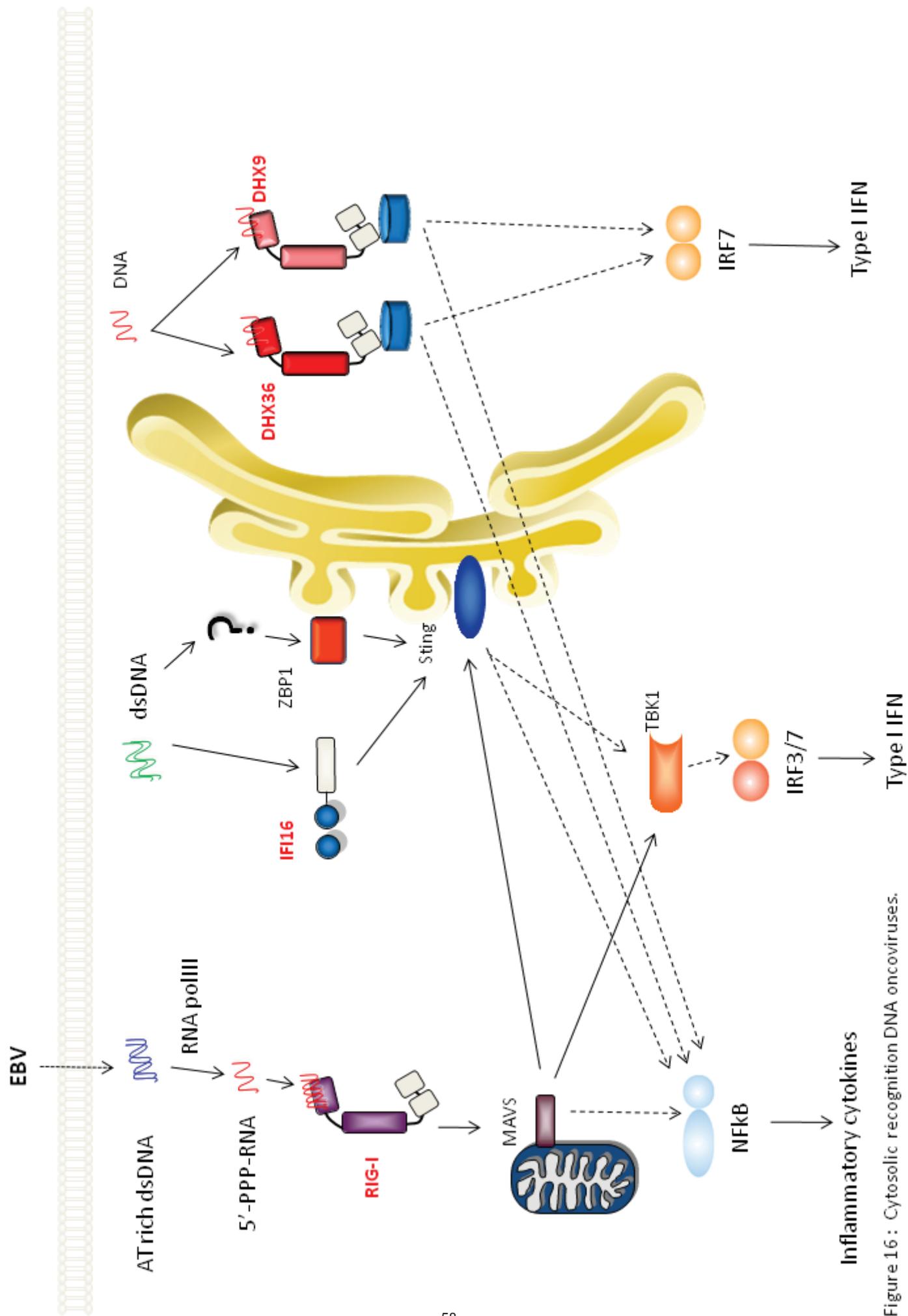


Figure 16 : Cytosolic recognition DNA oncoviruses.

RIG-I is a RNA receptor that can sense cytosolic DNA, so RIG-I is predicted to be able to respond to dsDNA from intracellular pathogens. RIG-I has been shown to be able to trigger both NF- κ B-dependant production of pro-IL1 β and in inflammasome activation in response to VSV (Figure 17-18). In this context, RIG-I engages the CARD9–Bcl-10 module for NF- κ B activation and triggers ASC for inflammasome activation [469]. RIG-I has been shown to sense HCV genome (uncapped RNA with 5'-PPP) [470] but also HCV viral particles [442] and EBERs from EBV (dsRNA with a 5'-PPP ends) [471] (Figure 16). In addition, HCV unraveled the existence of MAVS [472, 473]. Indeed HCV protease NS3/4A cleaves MAVS off the mitochondria abolishing RIG-I signaling [472]. EBERs recognition by RIG-I after RNA pol III processing induces type I IFNs and IL10 secretion [471, 474, 475]. Since the EBERs are released by infected cells, they also stimulate RIG-I pathway in non infected cells. This systemic activation of RIG-I might be partly responsible for the symptoms of acute IM seen during EBV primo infection. By stimulating RIG-I, EBERs might engage NK cells for the production of IFN γ [476]. Actually, NK cells are very important in EBV immune responses as patients suffering from NK disabilities such as X-linked lymphoproliferative diseases are unable to control EBV infections [477]. The control of EBV dependent B cells transformation by NKs in the tonsil, site of the infection, is mainly *via* IFN γ secretion. Indeed *in vitro* studies shown that DCs matured with polyI:C were activating allogenic tonsillar NK cells which were able to restrict B cell transformation *via* IFN γ secretion [478]. The IFN γ is central in anti EBV immune response, it has been shown it was 7 to 10 times more efficient than type I IFN to limit EBV infected B cells outgrowth and it was also efficient even if administrated three to four days after EBV infection [292]

Other RNA helicases were recently described. DHX36 and DHX9 have recently been involved in the detection of CpGA and B respectively in the cytosol of human pDCs [479]. Upon stimulation, these helicases binds the TIR-domain of MyD88 to induce the activation of IRF7 and NF κ B (Figure 16). In experiments using Gen2.2 pDC cell line, the authors showed that the response to CpG A and B was significantly reduced when using a siRNA for DHX36 and DHX9 respectively. The remaining TNF α and IFN α production accounted for TLR9 signaling. Furthermore, those receptors seem also important in the innate immune response to HSV. Their putative role in DNA oncoviruses immunity has not been assessed yet. RNA helicases DDX1, DDX2 and DHX36 have been also identified as cytosolic dsRNA sensors in bone marrow GM-CSF derived mDCs [480]. Upon poly I:C triggering they activate type I IFN response *via* the TRIF pathway. The helicase DDX41 has been described as a cytosolic sensor of intracellular B and Z form DNA and HSV-1 in murine mDCs and human myeloid cell line THP1 that depends on STING signaling [481].

NLR

Nucleotide-binding oligomerization domain-like receptor (NLR) proteins are a family of proteins with diverse functions in the immune system, characterized by a shared domain architecture that includes a nucleotide-binding domain (NBD) and a leucine rich repeat (LRR) domain. The NBD can bind nucleotides and is possibly involved in the induction of conformational changes and self-oligomerization that are necessary for NLR function. On the basis of the presence of additional domains, NLRs were grouped into five subfamilies: the NLRA (CIITA), the NLRB (NAIP), the NLRC (NOD1, NOD2, NLRC3, NLRC4 and NLRC5), the NLRP (NLRP1 to 14) and the NRLX1 (NLRX1). Typical domains present in NLRs are CARD and pyrin domains (PYDs). The PYD domain is a death domain protein fold that forms homotypic interactions with other PYD-containing proteins such as ASC to form multiproteic complexes involved in inflammation, apoptosis and cell cycle. Some NLR proteins, including NLRP1, NLRP3 and NLRC4, are involved in anti-microbial responses *via* inflammasome formation.

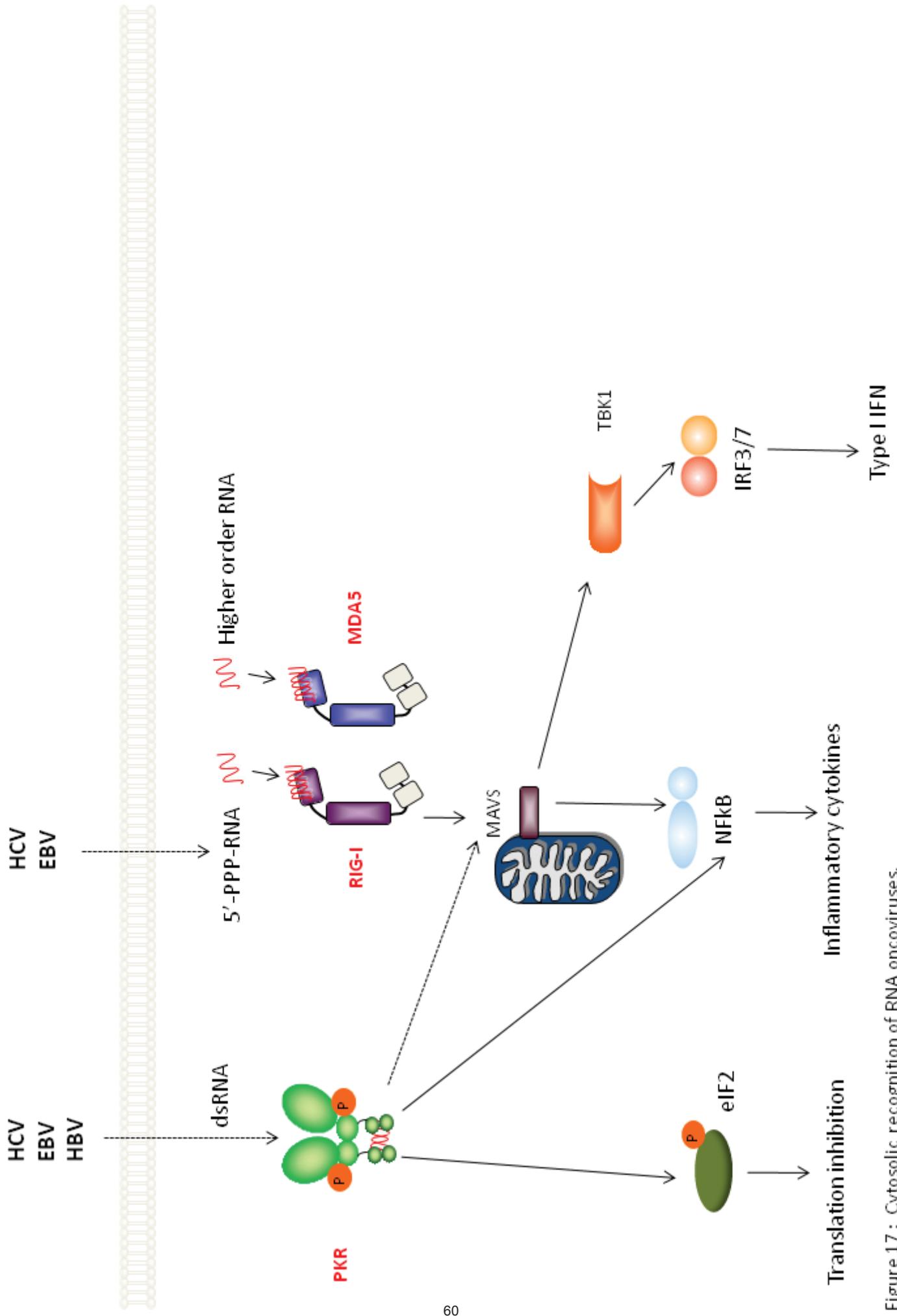


Figure 17: Cytosolic recognition of RNA oncoviruses.

Table 12 depicts the NLRs involved in virus recognition. NLRP3 has been shown to recognize several PAMPs such as bacterial toxins and viral DNA such as influenza A and PAMPs such as crystal and ROS [313]. As for the other ligands, the precise mechanism by which NLRP3 senses viral DNA is unknown. However, it was shown to either sense ROS and potassium efflux, lysosomal or endosomal rupture due to crystals such as silica or cholesterol crystal [482, 483]. NLRP3 associates with the adaptor protein ASC to activate the caspase 1 which cleaves pro-IL1 β in active IL1 β (Figure 18). In that respect it would be interesting to assess whether the viruses such as HPV, that escape endosomal compartment, might damage it and trigger this pathway.

PRR	Cellular expression		Ligands		Ref
	Immune cells	Non immune cells	PAMP	DAMP	
NOD2	Monocytes, macrophages, DCs, T cells, peripheral B cells	Paneth cells, stromal cells	MDP, viral ssRNA (RSV, influenza...), bacteria (Mycobacterium tuberculosis...)		[485-487]
NLRP1	Granulocytes, monocytes, DCs, T and B cells	Neurons	MDP, KHSV?		[484, 488]
NLRP3	Monocytes, granulocytes, T cells	Epithelial cells, keratinocytes, osteoblast, microglia	ssRNA and dsRNA Virus (SeV, Flu, ECMV..), bacteria (Listeria monocytogenes..), bacterial pore forming toxins, MDP, fungus (candida albicans...)	Crystals (MSU, alum, cholesterol...), ATP, β -amyloid, hyaluronan, glucose, imidazoquinoline compounds, ROS	[313, 489-493] [482, 483, 494-500]
IFI16	CD34+, monocytes, lymphocytes, DCs	Fibroblast, epithelial cells, endothelial cells	dsDNA viruses (KHSV, HSV1, VV), cytosolic dsDNA		[501, 502]
Aim2	Monocytes, macrophages, DCs	keratinocytes	dsDNA viruses (VV, mCMV), cytosolic bacteria (FT), cytosolic dsDNA	Self dsDNA	[503, 504]

Table 12: Cellular expression and ligands for NLRs and pythrin family members involved in virus recognition. MSU: monosodium urate, CPPD: calcium pyrophosphate dihydrate, Ad: adenovirus, MDP: muramyl-dipeptide, VV: vaccinia virus, mCMV: mouse cytomegalovirus.

Recently, Orf63 a KHSV protein has been described to be a viral NLR homolog that inhibits the inflammasome [484]. This protein has been shown to interact with NLRP1, NLRP3 and NOD2 to block their functions which might indicate an important role for NOD receptors in the immune response to KHSV. Inhibition of NLRP1 by Orf63 was shown to be critical for KHSV viral gene expression and viral genome replication during KSHV primary infection as well as KSHV reactivation from latency. It is then possible that KHSV would trigger NLRP1 signaling.

NOD2 has been shown to recognize viral ssRNA and some bacterial components such as MDP. Upon triggering it activates IRF3 leading to IFN β production and proinflammatory cytokine through NF κ B activation. It has been shown essential in the response to RSV in mice [485] (Table 12).

PYTHIN FAMILY MEMBERS

The pythrin protein family consists of a PYD domain and one or more HIN 200 domains. Four members constitute the human pythrin family: IFI16, IFIX (Pythrin1), MNDA and AIM2. The HIN 200 domain is a DNA binding domain that is involved for AIM2 and IFI16 in the detection of viral DNA. IFI16, IFIX and MNDA bear a nuclear localization sequence while AIM2 does not and is exclusively cytoplasmic (Figure 18).

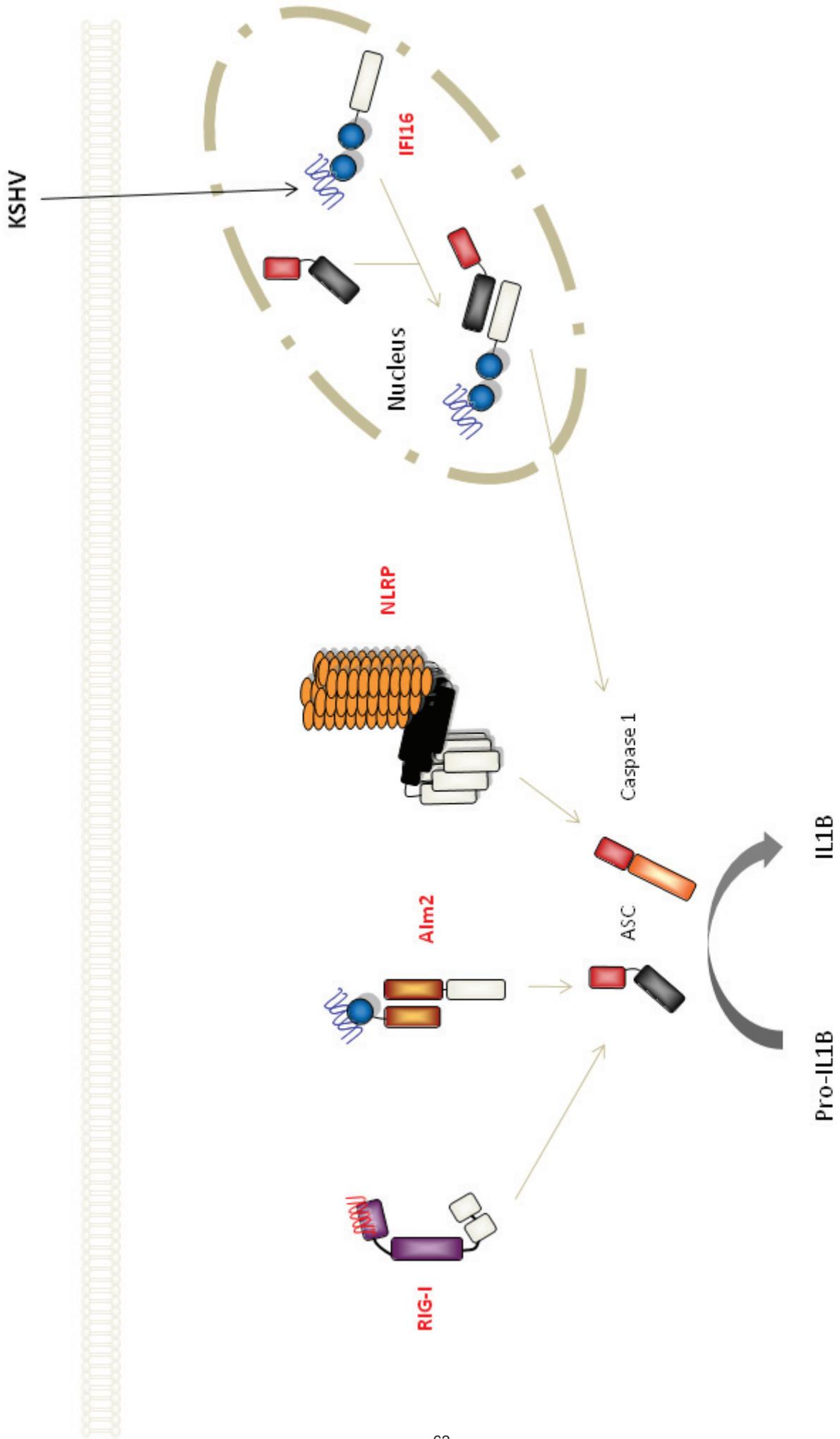


Figure 18: Induction of IL1B by cytosolic sensors

Aim2 is a crucial component involved in dsDNA-induced production of IL1 β *via* ASC/Caspase 1 but is dispensable for type I IFN production (largely dependent on sting) [503]. Upon DNA ligation in the cytoplasm, AIM2 associates with ASC and the caspase 1 which is then activated and can trigger the maturation of IL1 β (Figure 17). It has also been shown as an essential component of the IL1 β response to the cytosolic DNA viruses such as MCMV and vaccinia virus and the cytosolic bacteria *Franciscella tularensis* [504] (Table 12).

IFI16 is the only PRR that has been shown to be involved in type I IFN production and in IL1 β maturation and secretion in response to DNA viruses [501, 502] (Figure 16 and Table 12). IFI16 is localized predominantly in the nucleus; therefore it could possibly sense nuclear replication of viral DNA. Indeed, IFI16 associates with ASC in endothelial cells infected with KSHV and triggers inflammasome caspase 1 dependent formation and NF κ B activation (Figure 18 and Table 12) [502]. It will be interesting to look if the other DNA oncoviruses that replicates in nucleus would be able to trigger an IFI16 dependent response.

PKR

During viral infection, dsRNA activates PKR and lead to the phosphorylation of eIF2a and the inhibition of the protein translation (Figure 16). HCV is using PKR to inhibit RIG-I dependent type I IFN [505] and antiviral interferon-stimulated gene [506] protein translation while HCV IRES-dependent translation remains unaffected. As for HCV, PKR might have a deleterious role in EBV infection. LMP1 induces the production of IL6 and IL10 through PKR activation [507]. IL-6 and IL-8 are known to have autocrine growth factors that play a role for uncontrolled growth of LCLs [508, 509]. Additionally, IL-10 is known to suppress T cell proliferation through the inhibition of IFN- γ secretion by macrophages. Taken together, cytokine dysregulation induce by PKR helps the virus to evade the host immune system during EBV infection.

Beside its deleterious role in HBV and EBV infection, PKR is involved in the inhibition of HBV replication mediated by IFN α [510]. The mechanism underlying PKR activation by HBV is unknown.

(2) Adaptive immune response:

(a) Introduction

Adaptive immunity refers to antigen-specific immune response. The antigen first must be processed by professional antigen presenting cells (APC) and presented as antigen/MHC complexes to lymphocytes together costimulatory signals. The adaptive immunity indeed relies on B lymphocytes via the production of immunoglobulin and on the activation of CD4 and CD8+ T lymphocytes. Exogenous antigen are classically recognized by CD4 T cells as conjugate with MHC class II molecules while endogenous antigen will be loaded on MHC class I molecules to trigger CD8+ T cells. It has been shown that for an efficient anti-tumor immunity both specific CD8+ and CD4+ T cells are required [511, 512].

The HLA Class II molecules are constitutively expressed by APC, such as macrophages, DCs and B cells [513]. However, DCs are the most potent CD4 helper T cell activators because they express at steady state basal levels of co-stimulatory molecules such as CD80/86. Macrophages and B cells can also present MHC class II antigen after activation. HLA class II molecules present exogenous antigens such as viral and tumor antigens that enter the cells by endocytosis. HLA class II molecules are composed of α and β chains that are assembled in the ER and associated with the invariant chain (Ii). Ii aids in transporting class II molecules to the endolysosomal compartments, where Ii is sequentially degraded by cathepsins, leaving class II associated invariant-chain peptide (CLIP) on the class II binding groove. Ags/Peptides are also processed in the endolysosomal

compartments by acidic cathepsins for class II loading and presentation to T cells [514]. Removal of CLIP and formation of stable class II-peptide complexes is mediated by a non classical class II protein, HLA-DM. Once peptide is bound to a class II protein, the HLA class II-peptide complex is transported to the cell surface for presentation to CD4+ T cells. Human CD4+ effector T cells can differentiate into different subsets (Th1, Th2, Th9 and Th17 cells) producing different cytokines, depending on the antigen and the cytokine microenvironment encountered during activation. They will then direct T CD8 and B lymphocytes activation and differentiation. B cells are activated by cross-linking of their BCR in an antigen dependent manner. In order to differentiate into plasma cells and produce large amount of antibodies, they need co-stimulation signals from CD4+ Th2 cells one of which is IL4.

The activation of CD8+ cytotoxic cells (CTL) is very important in the elimination of virus infected cells and cancer cells. CD8+ T cells *via* their TCR recognize peptides-bound MHC class I, to be fully activated they need a second signal delivered by APCs and/or IFN γ released by CD4+ Th1 cells. Nucleated non immune cells when infected may present cytosolic peptides on HLA Class I molecules to cytotoxic CD8+ cells [515]. As proteins are produced in the cytosol, they may become ubiquitinated, marking them for degradation by the proteasome. Peptides resulting from proteasomal degradation are transported into the lumen of the endoplasmic reticulum (ER) via the transporter associated with Ag presentation (TAP). HLA class I molecules bind peptides in the ER lumen. Peptide-HLA class I complexes are then transported to the cell membrane for presentation to CTL. If the peptide-MHCI complex is recognized by a CTL as being non-self, the CTL may induce apoptosis in the target cell through the perforin/granzyme pathway. Viral proteins are synthesized in the cytosol and are subjected to the same proteasomal degradation and HLA class I presentation than cellular proteins. This process is essential for the immune system ability to monitor viral infections and to mount an appropriate response. Indeed, the importance of this pathway is revealed by the strategy of HLA class I down-regulation employed by many viruses and transformed cells to reduce their immunogenicity. In the case of cancer cells sensing and elimination, CTL response are also crucial. However, such antigens are exogenous. In that case, additional mechanisms exist for HLA class I presentation called cross presentation, they allow presentation by HLA class I molecules of exogenous peptides [516]. Cross presentation capacity seems to be restricted to DC and more recently $\gamma\delta$ T cells. The antigen engulfed by endocytosis reached the cytosol by debated mechanisms where it then follows the regular pathway for MHC class I presentation. Priming of naïve CD8+ T cell by cross-presentation of extracellular viral antigens by non infected DCs is also mandatory for immunity against viruses that do not infect DCs [517]. As for HLA class I presentation an alternate way exists for endogenous peptides to be presented to CD4 T cells. Endogenous peptides could be presented on HLA Class II molecules after degradation by macroautophagy [518].

(b) Adaptive Immune response to HPV

In general, the development of cervical cancer seen with mucosal high risk HPV (including HPV16) arises in women who were not able to control the infection. However, the immune response following HPV infection is not fully unraveled. A successful immune response against HPV is characterized by a strong local cell mediated response and the generation of neutralizing antibodies.

Sero-conversion in natural infection is slow and does not occur for all the infected patients. Fifty to seventy percent of women infected with HPV16 develop antibodies against L1 after 8-9 months [519]. In addition, these antibodies are protecting against infection (they are at the basis of the prophylactic vaccines) [127] and they might persist over 10 years [520]. Some neutralizing antibodies against L2 might also be found but they

are less protective than the anti-L1 [521].

The CD4+ T cell response is central for the clearance of HPV infection since AIDS patients and allograft recipients showed multiple recurrences of cervical HPV infections [133]. In healthy individuals HPV16E6 and HPV16E2 specific T CD4+ cells were shown to proliferate and secrete IFN γ upon *ex-vivo* stimulation with E6 or E2 antigenic peptides [522]. It is thus possible that those T CD4+ responses have protected those healthy individuals from a prior HPV16 infection.

Cell mediated cytotoxicity has been shown to be important in the control of viral infection and in the elimination of cancer cells. HPV specific CD4+ and CD8+ CTL can be detected in patients with previous or ongoing HPV infection [523-525]. Lack of CTL responses to E6 correlates with HPV persistence [526]. The NK immune response seems also an important factor in the clearance of HPV infection. Indeed, patients suffering from NK deficiencies were more prone to symptomatic HPV infection [527].

It is important to note that the immune response is affected by genetic factors. Since different allele of MHC molecules will present different peptides to the immune system, the HLA haplotype might play a role in the clearance or persistence of the HPV virus. Indeed, a correlation has been found between some HLA haplotypes and cervical cancer [528].

(c) Adaptive Immune response to EBV

Because of the lack of good animal model, IM patients and healthy EBV carriers are investigated for acute and chronic EBV infection respectively. At the early stage of the infection EBV triggers innate immune responses in the host.

Infection by EBV is followed by T cell responses that control the infection. It has been shown *in vitro* that EBV transformed B cells are unable to initiate the immune responses and it is indeed the DCs that prime naive T cells to recognize EBV latent antigen [529]. After primo-infection a cytotoxic CD8+ T cell response predominantly aimed at controlling naïve B cells expressing the latency III program takes place [530]. The recognition of EBNA3, the immunodominant protein, allows the elimination of infected cells by specific T cells. These cells might mediate the resolution of primary infection. In addition, a CD8+ T cell response against lytic antigens is also seen in acute infection [531]. Since the lytic cycle of EBV down-regulates HLA class I molecules, the CD8+ T cells might not target infected B cells undergoing lytic EBV replication. Indeed CD8+ T cell EBV specific have very low cytolytic potential against LCL [532, 533]. *In vitro* CD8+ T cell dependent IFN γ secretion is able to control of LCL outgrowth [534].

CD4+ T cells are crucial for the priming and the maintenance of CD8+ T cells [535-538]. With the exception of IL10 producing LMP1 specific CD4+ T cells [539], EBV specific CD4+ T cell seem to display a Th1-like phenotype [540]. EBNA1 specific CD4+ T cells responses are believed to be also very important in the control of EBV infection. *In vitro* CD4+ T cells specific of EBNA1 were shown to lyse EBV transformed LCL *via* a Fas/Fas ligand mechanism [541]. It has been also shown in a murine Burkitt's lymphoma tumor model that EBNA1-specific CD4+ T cells recognized peptide-pulsed targets as well as EBNA1-expressing tumor cells and were necessary and sufficient for suppressing tumor growth *in vivo* [542]. In human the nuclear location of EBNA1 limits its accessibility to the macroautophagy pathway, therefore limiting the delivery of antigenic peptides derived from EBNA1 to MHC class II molecules [543].

EBV triggers also the production of specific IgM, IgA and IgG antibodies against the nucleocapsid, the immediate early and the early lytic antigens by EBV negative plasma cells. It seems that only the IgG antibodies against gp350 are neutralizing [544, 545]. Several vaccine candidates based on gp350/220 have been

developed in order to prevent PTLD for example. Live recombinant vaccinia virus vectors have been used to express the gp350/220 antigen and were found to confer protection in primates and elicit antibodies in EBV-negative Chinese child [546]. Soluble recombinant gp350/220 produced in CHO cells was found to be safe in humans but needed strong adjuvants to elicit acceptable immunogenicity (co-development by MedImmune, GSK and Henogen). Phase II clinical trials of this candidate vaccine started in the USA in 2008.

b) Escape to immune responses

It is important that an efficient immune response would be mounted following the recognition of oncoviruses to eliminate them and avoid the development of cancer. Some viruses have developed escape mechanisms to persist. The mechanism developed by oncoviruses to avoid immune recognition will be described in this part with a special focus towards EBV and HPV.

(1) EBV

(a) Impairment of innate immunity

As seen earlier, IFN α is one of the most important cytokine secreted in response to viral infection. The majority of viruses have developed strategies to impede type I IFN production or to limit its effect. EBV is no exception and a lot of its proteins are targeting the IFN pathway (Table 13).

In order to dampen type I IFN production, EBV uses two principal mechanisms. The inhibition of the transcription factors involved in type I IFN production and the activation of inhibitor of type I IFN. BRLF1 limits type I IFN production by inhibiting the transcription of IRF3 and IRF7 inducing a decrease in type I IFN transcription as well as other interferon-induced genes [547, 548]. EBV LF2 tegument protein specifically interacts with the central inhibitory association domain of IRF7, and this interaction leads to inhibition of IRF7 dimerization, which suppresses IFN- production and IFN-mediated immunity [549]. BGL4 binds and phosphorylates IRF3 which lead to an inhibition of its transactivation activities and a subsequent impairment of type I IFN production [443]. BZLF1 induces suppressor of cytokine signaling 3 (SOCS3) in monocytes which impede type I IFN production [550]. EBV is also mediating resistance to type I IFN anti-proliferative and pro-apoptotic activities. LMPA2A and 2B accelerate the turnover of IFNAR1 limiting the effect of type I IFNs [551]. LMP-1 prevents Tyk2 phosphorylation and inhibits IFN-stimulated STAT2 nuclear translocation and interferon-stimulated response element transcriptional activity [552]. EBERs protect from apoptosis induced by type I IFNs, however the mechanism is controversial [553, 554]. Furthermore EBNA2 mediates resistance to type I IFN anti-proliferative effect but the precise mechanism is unknown [555, 556]. The apoptosis is also inhibited by up regulation of Bcl2 by LMP1 [557].

EBV inhibits also the TLR9 pathway by down-regulating this PRR. Indeed *tlr9* is down regulated during latencies II and III by LMP1 [381] but also during lytic phase by BGL5 [452] (Table 13). *tlr6* and *tlr7* are also down-regulated during lytic cycle [452], however less drastically than *tlr9*. TLR7 and TLR8 proliferative responses are dampened after infection of B cells with EBV [558].

EBV acts also on immune cells by modulating the cytokines environment. Those cytokines as well as growth factors produced by infected B cells [52, 559], T cells [560] and epithelial cells [561] sustain the cellular proliferation of infected cells.

Effect	Protein	Immunomodulatory activity	Ref
Impediment of type I IFN production	BGLF4	Suppression of IRF3 signaling	[548]
	LF2	Suppression of IRF7 signaling	
	BRLF1	Inhibition of IRF3 and IRF7 transcription	[547]
	BZLF1	Activation of SOCS3	[550]
Resistance to type I IFNs	EBNA-2	Resistance to anti-proliferative activities of type I IFN	[555, 556]
	LMP2A-2B	Increase of IFNAR1 turn-over	[551]
	LMP1	Binding to Tyk preventing his phosphorylation	[552]
	LF2	Antagonist of type I IFN	[549]
	EBERs	Resistance to type I IFN mediated apoptosis	[568]
Resistance to type II IFN	BZLF1	Blocks STAT nuclear translocation and IRF1 activation decrease IFN γ α chain receptor	[569]
	LMP2A-2B	Increase of IFN γ R1 turn-over	[551]
Apoptosis resistance	LMP1	Bcl-2 upregulation	[557]
	EBERs	Resistance to Fas mediated apoptosis	[553]
Induction of immunomodulatory molecules	EBERs	Induction of IL10 IN B cells	[559]
	LMP1	Enhancing of IL10	[52, 539]
	BZLF1	Induction of IL10 production by b cells	[570]
		Induction of GM-CSF & cox-2/PGE2 from NPC cells	[571]
	BCRF1	Homolog of IL10	[572, 573]
	Soluble form of BARF1	Decoy CSF receptor	[567]
Autocrine growth factors	EBERs	Induction of IL9 in T lymphocytes	[560]
		Induction of IGF-I in epithelial cells	[561]
	LMP1	sCD23 & IL6 production	[52]
Inhibition of TLR	BGL5	Down-regulation of TLR9	[452]
	LMP1	Down-regulation of TLR9	[381]
Inhibition of T cells activities	LMP1	Secretion of immunosuppressive domain LALLFWL	[58]
		EBI3 mediated th2 polarization	[574, 575]
Deregulation of MHC class I and II	BNLF2A	Inhibits MHC class I antigen presentation	[576]
	BILF1	Degradation of MHC class I molecules	[577]
	BZLF1	Down-regulation of MHC class I and II	[578]
	BGLF5	Down-regulation of MHC class I and II	[579]

Table 13: Immunomodulatory activities of EBV latent and lytic proteins.

EBV has been shown to replicate in primary human monocytes [562] where it can alter a number of cellular defense mechanisms. EBV negatively regulates monocytes secretion of TNF- α [563], MIP1- α [564], PGE2 [565] and reduce monocytes phagocytic activities [566]. In addition the soluble form of BARF3 is a decoy receptor for CSF-1 which inhibits IFN α production by monocytes and inhibits macrophage proliferation [567] (Table 13).

(b) Adaptive Immune escape

During latency, EBV is trying to stay invisible by reducing its antigenicity. Indeed none of the EBV proteins are expressed during latency 0 in memory B cells [33].

During latencies I, II and III EBNA1 is expressed but is poorly antigenic. The glycine-alanine encoding repeats

(GA domain) of EBNA-1 minimize translation, bind to the proteasome thus inhibiting proteasomal degradation [580, 581]. As a consequence EBNA-1 peptides are poorly presented in the context of MHC class I molecules. Similarly, EBNA3 expressed in latency III limits its protein expression to one copy per cell [582] (Table 13).

Another mechanism is the down-regulation of MHC class I molecules on infected cells (Table 13). In endemic Burkitt's lymphoma the number of HLA Class I molecule per cell is greatly reduced [583], therefore the detection of infected B cells by CTL is greatly limited. Several proteins of the lytic cycle inhibit MHC Class I presentation. BILF1 associates with MHC class I molecules, induces an increased turn over from the cell surface and an enhanced degradation *via* lysosomal proteases [577]. BNLF2A co-localizes with TAP and blocks TAP-mediated peptide transport leading to a defect in MHC Class I presentation [576]. Host shutoff is a well described feature of α - but not β -herpesviruses and lead to a global mRNA degradation that can result in down-regulation of surface expression of MHC class I and II molecules, which normally display peptide fragments of viral antigens for activation of specific T cells [584-586]. BGLF5 induces host shutoff in infected B cells leading to a defect in MHC Class I and II presentation [579].

EBV lytic phase proteins are also impeding MHC Class II presentation (Table 13). Ectopic expression of BZLF1 strongly inhibits the constitutive expression of MHC class II and the major histocompatibility complex transactivator in B cell line [578]. Finally, it has been shown that EBV positive cell lines express HLA-DO that correlates with the inhibition of HLA-DM allowing the accumulation of CLIP at the cell surface which may interfere with peptide binding to class II molecules [587].

IL-10 is a potent immunosuppressive cytokine. It plays an essential role in dampening down overt immune responses, especially the pro-inflammatory Th1 type of response by directly inhibiting the proliferation and IL-2 production of Th1 cells [588-594]. The cytokine is known to inhibit DC maturation and functions, including their ability to produce IL-12 essential for driving Th1-cell differentiation [595], and to suppress antiviral activities of TH1 cells, NK and macrophages [596]. Several EBV proteins and RNA induce IL10 (Table 13) either directly or indirectly during latency or lytic cycle. EBERs, LMP1 and BZLF1 induce directly IL10 production by B cells. BZLF1 induces GM-CSF and Cox-2/PGE2 from Nasopharyngeal carcinoma cells which increase IL-10 production by monocytes [571]. EBV secretes also a homolog of IL-10 that down-regulates MHC molecules, costimulatory molecules and inflammatory cytokines of monocytes and macrophages [572, 573].

T cells which are central in immune responses against virally infected cells and cancer cells are also targeted by EBV. LMP1 is secreted from infected cells and LMP1 derived LALLFWL peptides show strong inhibition of T cell proliferation and NK cytotoxicity and Ag-specific IFN- γ release [59]. In addition LMP1 induces Epstein-Barr virus-induced gene 3 (EBI3), an IL-12p40-related protein [574]. EBI3 associates as a heterodimer with either IL-12p35 or an IL-12p35 homologue, p28, to create IL-27. EBI3 plays a critical regulatory role in the induction of Th2-type immune responses and the development of Th2-mediated tissue inflammation *in vivo* [575]. By inducing this cytokine EBV might drive the immune response toward a Th2 phenotype.

Despite all the immune escape mechanisms displayed by EBV, the host immune system is able to keep the EBV infection under control in a normal setting. Nevertheless, in some circumstances like co-infection, immunosuppression or action of environmental or genetic factors, the balance might be unsettled and oncogenic potential of EBV might rise. In case of HIV coinfection, tumors develop as a consequence of the immunosuppression induced by the virus. It has been shown that CD4+ specific for EBNA1 are lost during early HIV infection despite a CD4+ count normal [597]. One hypothesis might be that during latency the CD4+ EBV specific are constantly activated, which renders them vulnerable to HIV infection. In endemic Burkitt's lymphoma, it

is speculated that the malaria infection impairs the immunity anti-EBV and allows the escape of cells carrying a c-myc mutation. Indeed, T cell mediated immune control of EBV was found to be impaired in malaria infected individuals [598]. In nasopharyngeal carcinoma, the local immunosuppression might facilitate the growth of the tumor and impairs EBV-specific immune response locally. While LMP1 and LMP2 specific CD8+ cells are present within the tumor infiltrating lymphocytes, their functions are impaired, possibly because of Treg [599, 600]. In Hodgkin's lymphomas, only a small proportion of cells the Hodgkin-Reed-Sternberg (HRS) cell are EBV positive. The tumor is infiltrated with lymphocytes, but the microenvironment is suppressing the immune cell function. HRS cells were shown to produce immunosuppressive cytokines [601], furthermore regulatory T cells are found at the site of the tumor [602, 603]. The tumor area displays elevated galactin-1 level that inhibits EBV specific T cell proliferation [604]. In addition systemic selective impairment of EBV specific T cell responses have been shown [605].

(2) HPV

(a) General mechanisms of immune escape

(i) Low profile

The failure of the immune system to recognize incoming or progeny virus may also be explained by the fact that the life cycle of HPV is non-lytic and therefore does not elicit any pro-inflammatory signals that activate DCs and induce their migration into the local environment. The essential signals required for initiation of immune responses in squamous epithelia are absent [606]. The non-lytic nature of HPV infection limits the production of antigens that are processed and presented to the adaptive immune system. The majority of these, the early proteins, are expressed at low levels and primarily in the nucleus of infected cells [607-609]. Furthermore, the production of the highly immunogenic capsid proteins is limited to the terminally differentiated outer layer, which is shed from the epithelium [610, 611]. Since there is no blood-borne phase of the HPV life cycle and only minimal amounts of replicating virus are exposed to the immune system, the virus is essentially invisible to the host. Thus, the first strategy that HPV has evolved to avoid detection is to maintain a very low profile.

(ii) Codon usage

The genes of papillomaviruses use codons that are not commonly used by mammalian cells. Substituting them with codons preferentially used by the mammalian genome results in increased translation of the genes, as described for L1 [612], and their immunogenicity, as described for E7 [613]. These findings imply that HPV has evolved to exploit the redundancy in genetic code to control the expression levels of its gene products. This, combined with a variety of other transcriptional and translational control mechanisms that prevent expression of the L genes in the undifferentiated layers of the epithelium [614], allows escape from detection by the immune system.

(iii) Molecular mimicry

Molecular mimicry is defined as the process in which structural properties of an introduced molecule imitate or simulate molecules of the host. Either the linear amino acid sequences or the conformational fits of the molecules may be shared, even though their origins are as separate as, for example, a virus and a normal host self determinant [615]. To maintain tissue and organ integrity, the host immune system must be able to distinguish between self and non-self molecules and be tolerant to self-molecules. Mimicking host-proteins and thereby taking advantage of the host's selftolerance toward important cellular proteins may be a mechanism by which HPV escapes functional antigenspecific immune recognition. Evidence for this comes from an analysis of the HPV16 E7 protein that has wide spread similarity to several human proteins for instance xeroderma pigmentosum group G complementing protein (XPGC) and the retinoblastoma binding protein 1 (RBP-1) involved in critical regulatory processes [616]. Thus, a cell-mediated response that targets the common motifs of these proteins would result in inhibition of excision repair [617, 618] or derangement of cell cycle regulation [619]. Since there is no evidence that human T-cells target the shared epitopes from these proteins, it may be that molecular mimicry is one of the mechanisms that HPV has evolved to escape immune recognition.

(b) Modulation of innate immunity by HPV

(i) Interference with type I IFN

Like the majority of viruses, papillomaviruses have developed strategies to inhibit type I IFN production, signaling and effect (Table 14).

High-Risk HPV viruses down-regulate IFN α inducible gene expression [620]. IFN α does not effectively inhibit transcription of E6/E7 immortalized by recombinant HPV-16. In several human cervical epithelial cell lines, E6 and E7 were shown to inhibit the interferon receptor signaling pathways and the activation of the interferon response genes. HPV+ condylomas biopsies from patients were analyzed for type I IFN responsiveness and HPV gene expression. A correlation has been found between E7 expression and type I IFN resistance suggesting that E7 is mediating this resistance [621]. E7 binds to p48/IRF9 and sequester the complex in the cytoplasm inhibiting the formation of the Interferon-stimulated gene factor 3 (ISGF-3) that binds the interferon-stimulated response elements (ISRE) mandatory for the transcription of ISGs [622, 623]. HPV18E7 binds and inhibits the transactivation functions of IRF-1 leading to a reduced expression of IRF-1 target genes such as TAP-1, IFN β and MCP-1 [624, 625]. HPV E6 modulates also type I IFN production and resistance in infected cells. Analysis of gene expression by cDNA micro-array showed that HPV16 E6 inhibits expression of type I IFNs genes and ISGs [620]. Indeed E6 binds and inhibits the transactivation activities of IRF3 therefore preventing the transcription of IFN β [626]. E6 is also down-regulating nuclear STAT-1 protein thereby preventing its binding to the ISRE and the transcription of ISGs [620]. HPV18E6 binds also to Tyk2 preventing Tyk2 binding to IFNAR1 thus inhibiting type I IFN mediated signaling [627].

(ii) Modulation of PRRs

TLR9 is able to recognize HPV viral DNA *in vitro*. HPV16-positive cancer-derived cell lines and primary cervical cancers show a down-regulation of TLR9 [371]. The oncoproteins E6 and E7 are responsible for this deregulation since infection of human primary keratinocytes with HPV16 E6 and E7 recombinant retroviruses inhibits

tlr9 transcription and hence functional loss of TLR9-regulated pathways [371]. It has to be noted that HPV low risk type E6 (HPV 6) is unable to down-regulate TLR9. It is then possible that abolishing TLR9 function would be important either in viral persistence or in transformation induced by HPV high risk type. The study of TLR expression and viral persistence or clearance upon infection with HPV16 revealed that the clearance of the infection was associated with an increase in expression of the four viral nucleic acid-sensing TLRs (TLR3, TLR7, TLR8 and TLR9) as well as TLR2 upon viral acquisition [628].

Effect	Protein	Immunomodulatory activity	Ref
Interference with type I IFN	HPV16E7	Inhibition of ISGF-3	[622, 623]
	HPV18E7	Inhibition of IRF1	[624, 625]
	HPV16E6	Inhibition of IRF3	[626]
	HPV16E6	Down-regulation of STAT-1	[620]
	HPV18E6	Inhibition of Tyk2	[627]
Modulation of PRR	HPV16 E6 E7	Deregulation of TLR9	[371]
Modulation of antigen presentation	HPV16 E7	Inhibition of MHC Class I	[629]
	HPV16 E5	Down-regulation of MHC Class I	[630]
Modulation of chemokines and cytokines	HPV16 E6 and E7	Decrease of MCP-1	[631] [632]
	HPV16 E6 and E7	Down-regulation of IL-8	[633]
	HPV16 E6	Down-regulation of IL-18	[634]
	HPV16 E6 and E7	Inhibition of IL-18 mediated IFN γ production	[635]
	Unknown	Increase in IL-10 and TGF β	[636, 637]
	Unknown	Shift from Th1 to Th2 cytokines profile	[638, 639]
	Unknown	IL6 expression	[640]
	HPV16 E6	Induction of VEGF	[641]
Modulation of APCs	HPV16 E6	Decrease of E-cadherin	[642]
	Unknown	Decrease of MIP-3 α	[636]
	HPV16 L2	Inhibition of LCs	[643]

Table 14: Immunomodulatory activities of HPV proteins.

(c) Modulation of adaptive immunity

(i) Modulation of Antigen presentation

There are evidences that HPV infection might interfere with antigen processing and presentation (Table 14). Indeed, the oncoproteins E7 has been shown *in vitro* to repress MHC Class I heavy chain promoter activity [629]. Furthermore, it has been shown recently that HPV-16 E5 down-regulates expression of surface HLA class I molecules and reduces recognition by CD8⁺ T cells [630].

(ii) Modulation of chemokines and cytokines profiles

Identified as one of the first chemokines, MCP-1 attracts a variety of cell types including monocytes, memory T cells and NK cells [644], and is therefore particularly relevant in the clearance of viral infections. In addition, MCP-1 produced by keratinocytes has been shown to induce recruitment of DCs and LCs to the skin [645]. MCP-1 expression in CIN 3 was shown to be strongly decreased *via* a mechanism mediated by E6 and E7 [631]. MCP1 loss might diminish antigen uptake by APCs and subsequent adaptative immune response against HPV.

Initially identified as a chemo-attractant for neutrophils, IL-8 is now known to function as a potent activator and chemo-attractant for neutrophils, basophils and T cells [646, 647]. In the setting of HPV infections, ex-

pression of *il-8* is down-regulated when E6 and E7 are expressed. Together, E6 and E7 inhibited transcription of the IL-8 promoter [633].

Transfection experiment showed that HPV16 E6 down-modulates also IL18 expression [634]. Moreover HPV16 E6 and E7 inhibit the IL18 dependent IFN γ production by PBMCs and NK. Indeed, HPV16 E6 and E7 bind the IL18 receptor alpha chain and compete for the binding for IL18 [635]. Since HPV16 E6 and E7 can be found in the extracellular fluids of HPV-containing cervical cancer cell lines, one can imagine they would be also secreted by tumor cells *in vivo* and could inhibit IL18 mediated IFN γ production at the tumor site.

HPV may modulate the environment of the transformation zone to be immunosuppressive, which in turn is affecting the immune response (Table 14). IL10 and TGF β have been found expressed at higher level in the transformation zone than in normal cervix [636, 637]. In addition, analysis of cytokine profiles in cervical secretions of HPV DNA positive and negative cervix showed an increase in IL10 in HPV DNA positive samples [648]. The CD1A+ stromal DC shown to display immunotolerant activities are increased in high grade squamous intraepithelial lesion (HSIL) [649]. They are expressing IL10 and TGF β and might therefore induce an immunosuppressive environment in HSIL. Using a HPV16 associated tumor model in mice deficient or not for IL-10, it was shown that IL-10 produced by tumor macrophages induces T regulatory phenotype on T cells, an immune escape mechanism that facilitates tumor growth [650].

Concerning TGF β , some studies have shown that *in vivo* the expression of this cytokine is reduced concomitant to CIN aggravation [651]. This is a paradox since TGF β is known to be a potent immunosuppressor that may favor cancer development. In fact, TGF β is known to be anti-proliferative and stop the cells in G1 phase [652]. Since HPV depends on cell proliferation for its own replication, TGF β might be deleterious to HPV infected cells. Furthermore, it was demonstrated that TGF β might inhibit the expression of E6 and E7 [653, 654]. HPV16E7 *via* its action on pRb might render the infected cells more resistant to TGF β anti-proliferative effect, however the complete resistance to TGF β might be achieved in the later stages during malignant transformation.

A shift from Th1 to Th2 cytokines profile is observed when the SIL is progressing from low-grade to high grade. It is unclear if this shift is predisposing to HPV persistence or is a result of HPV infection. But it has been suggested that the Th2 phenotype is associated with the persistence of the viral infection and the dysplasia [638, 639] and the draining lymph nodes appear to have an increased proportion of T regulatory cells [655]. At later stage of malignancy, carcinoma cells produce high level of IL6, but since they show a limited expression of the IL6 binding subunit gp80 the silencing of the autocrine IL6 response prevents MCP-1 production [640]. Increasing evidences showed that IL6 has a role in cancer. It can lead to the differentiation of DC into macrophage like cells [656, 657], affects the differentiation of DC via STAT3 activation [658] and impairs the function of DC [659]. *il-6* gene expression in cervical cancer is associated with a negative disease prognosis. Indeed, vascular endothelial growth factor (VEGF) is produced *via* a STAT3 pathway depending of IL6 and is correlated with cervical tumor growth [660]. VEGF is an important mediator of angiogenesis but also exerts immunosuppressive activities on DC [661]. Moreover VEGF has also been shown *in vivo* to increase with disease progression [662, 663]. *In vitro* this VEGF production seems to be E6 dependent since HPV16E6 can induce the VEGF promoter [641].

(iii) Modulation of APCs

As seen earlier, DCs are very important in immune responses. The confinement of HPV infection to the epi-

thelia assigns the epithelial dendritic cells, the Langerhans cells (LC), in charge of the induction of T cell-dependent immunity. As HPV-infected keratinocytes cannot reach the regional lymphoid organs, priming of antiviral T-cells depends on LC, which continuously monitor the epidermal microenvironment for infection and damage. HPV infected keratinocytes seem to alter LC biology. These alterations reduce the HPV antigen presentation relying on LCs in the mucosa (Table 14). Adhesion molecules, such as E-cadherin are necessary to mediate contact between LCs and keratinocytes [664]. *In vitro*, E6 decreases E-cadherin expression on keratinocytes [642]. While DCs and LCs generated *in vitro* were randomly distributed throughout the full thickness of organotypic cultures of E-cadherin- HPV-transformed cells, they rapidly adhered to the keratinocyte cell layers when HPV-transformed cells transfected with E-cadherin were used [665]. E6 by modulating E-cadherin expression in keratinocytes may indirectly limit presentation of viral antigen by LCs. Although LCs are maintained in the epidermal microenvironment, the precursors are recruited under inflammatory conditions [666]. This recruitment is dependent upon cytokines and chemokines secreted by epidermal cells, among which macrophage inflammatory protein-3alpha (MIP-3alpha) is the most potent chemotactic agent for LC precursors [667]. Normal keratinocytes express constitutively MIP-3a. Cells expressing the receptor for this chemokines such as memory T cells [668] and LCs precursor [669], migrate in response to this chemokine. The attraction of LCs to the transformation zone is reduced as a consequence of the diminished expression of MIP-3 α [636]. In addition, HPV16 L2 VLPs have been shown to alter the phenotype and the maturation of LCs by activation of the Phosphoinositide-3-Kinase and down-regulation of Akt [643].

It is to note that in most of the case, the immune system will be able to clear the infection; only 1% of CIN1 will progress to invasive cancer [1, 670].

c) TLR modulation and cancer association

The major role of TLRs is to defend host against infection. As seen earlier, the TLRs recognize PAMPs and trigger innate immune response. They also play a role in tissue repair, cell proliferation, apoptosis and angiogenesis [6, 671-673]. Those later activities link TLRs signaling to cancer. Moreover, TLRs are expressed by cancer cells [674] and their precise role carcinogenesis is quite controversial. In some studies TLRs activation is inhibiting cancer progression but in others it is promoting carcinogenesis and metastasis. I am going to describe how the TLR modulations in cancers modify tumoral development with a special focus on TLR9. TLRs modulation has an impact on the cancer cells and cells from the tumor micro-environment (immune cells, endothelial cells and fibroblasts) modulating tumor growth.

(1) Association of TLR SNPs to cancer

Single nucleotide polymorphisms (SNP) in TLRs have been linked to cancer. The more striking example is the SNPs linking TLR4 to *H. pylori* infection and gastric cancer induction. Patients suffering from the SNP Asp299Gly in TLR4 show an exaggerated inflammatory reaction with tissue destruction when infected by *H. pylori* which increase their cancer risk [9, 675]. So far, the association between TLR9 SNPs and cancer has not been well documented. The TLR9-1237T/C polymorphism, enhancing TLR9 transcriptional activity, has been shown to be a risk factor in the development of *H. pylori* induced premalignant gastric lesions [676]. A study of association between TLRs SNPs and lymphomas showed a 20% decreased risk of all lymphoma associated with the TLR9_1237 C-allele [677].

(2) TLRs modulation and cancer

(a) Direct effect on cancer cells

(i) Expression on cancer cells

TLR9 is differentially expressed on tumor and healthy tissue. In some tumors TLR9 has been found up-regulated when compared to healthy tissue. In prostate or lung cancer, expressions of TLR9 and ER α are simultaneously increased especially in poorly differentiated tumors [678]. However in breast cancer, TLR9 up-regulation has been associated with a lower probability of metastasis. A study on the clinical relevance of TLRs in breast cancer showed that tumors with high TLR9 expression by fibroblast like cells were associated with low probability of metastasis [679]. On the contrary high TLR3 and TLR4 expression respectively on breast tumor cells or mononuclear inflammatory cell were significantly associated with higher probability of metastasis [679]. However treatment of these cancer cells with TLR3 agonist lead to apoptosis and significant tumor regression [680, 681].

In contrast, TLR9 levels are decreased during the transformation of bone marrow cells of myelodysplastic syndromes (MDS) to overt leukemia (OL) [682]. TLR9 was mainly localized in neutrophils in healthy and refractory anemia bone marrow; strongly expressed in the immature myeloid cells in refractory anemia with excess blasts bone marrow, but not expressed anymore in OL. TLR9 expression and function has been found to be abolished in cervical cancer cell lines and biopsies associated with HPV high risk type but not with HPV low risk type [371]. A study by Daud et al analyzed the changes in TLRs expression in women that either cleared or not HPV 16 infection [628]. The clearance of the virus was associated with an increase in TLR2, TLR3, TLR7, TLR8 and TLR9, while the viral persistence was associated with a decrease in TLR3, TLR7, TLR8 and TLR9 expression [628]. Furthermore, TLR9 expression and responsiveness was abolished upon expression of HPV16 oncoproteins but not upon expression of protein from low risk type [371].

TLRs and especially TLR9 are differentially regulated depending on the type of cancer. Their dysregulation might impact on the ability of TLR ligands to drive immune responses or apoptosis

(ii) Apoptosis

Apoptosis pathways and regulation:

Apoptosis or programmed cell death results from the sequential activation of caspases (cysteine proteases). The process of apoptosis is induced by two main pathways the extrinsic and the intrinsic pathways.

Extrinsic apoptosis signaling is mediated by the activation of “death receptors” which are cell surface receptors that transmit apoptotic signals after ligation with their cognate ligands. Death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily, including TNFR-1, Fas/CD95, and the TRAIL receptors DR-4 and DR-5. Adapter molecules like FADD or TRADD are recruited through their death domain (DD) to the DD of the receptor thereby forming the death inducing signaling complex (DISC). Pro-caspase 8 is sequestered to the DISC. In type I cells, the local concentration of several procaspase-8 molecules at the DISC leads to their autocatalytic activation and release of active caspase-8. Active caspase-8 then processes downstream effector caspases such as caspase-3 that subsequently cleave specific substrates resulting in cell death. **Intrinsic apoptosis pathway** relies on mitochondria that plays a central role in the integration and propagation of death signals originating from inside the cell such as DNA damage, oxidative stress, starvation, as well as those induced by chemotherapeutic drugs. Activation of the intrinsic apoptotic pathway

leads to the rupture of the outer mitochondrial membrane, resulting in the release of proapoptotic proteins from the mitochondrial intermembrane space into the cytoplasm. Released proteins include cytochrome c, which activates the apoptosome and therefore the caspase cascade, but also other factors such as the apoptosis-inducing factor (AIF). Cytosolic cytochrome c is binding to monomeric apoptotic protease activation factor 1 (Apaf-1) which then, in a dATP-dependent conformational change, oligomerizes to assemble the apoptosome, a complex that triggers the activation of the initiator procaspase-9. Activated caspase-9 subsequently initiates a caspase cascade involving downstream effector caspases such as caspase-3, caspase-7, and caspase-6, ultimately resulting in cell death. The changes in mitochondrial membrane also cause a loss of the biochemical homeostasis of the cell: ATP synthesis is stopped, redox molecules such as NADH, NADPH, and glutathione are oxidized, and reactive oxygen species (ROS) are increasingly generated. Increased levels of ROS directly cause the oxidation of lipids, proteins, and nucleic acids, thereby enhancing the apoptosis as part of a positive feedback.

The apoptosis pathways are tightly regulated. Bcl2 family members are either pro-survival or pro-apoptotic. In addition to Bcl-2 itself, there are a number of other pro-survival proteins (Bcl-XL, Bcl-w, A1, and Mcl-1) that are induced by NF κ B. The pro-apoptotic group of Bcl-2 members can be divided into two subgroups: the Bax-subfamily consists (Bax, Bak, and Bok) and the Bcl-2 homology domain 3 (BH3)-only proteins (Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma, Blk, BNIP3, and Spike). Bax and Bak are the central core of the proapoptotic Bcl-2 death machinery. They are held in check by the pro-survival members of Bcl-2 family Bcl-2 and Bcl-XL. Other regulators of apoptosis exist such as Inhibitors of apoptosis proteins (IAPs). IAPs are a family of anti-apoptotic proteins transactivated by NF κ B. The PI3K/Akt pathway interferes with apoptosis regulation. Upon activation Akt negatively regulates the function or expression of BH3-only proteins such as BAD, which exert their pro-apoptotic effects by binding to and inactivating pro-survival Bcl-2 family members. Akt also phosphorylates and displaces FOXO transcription factors from target genes and triggers their export from the nucleus. Through this mechanism, Akt blocks FOXO-mediated transcription of target genes that promote apoptosis, cell-cycle arrest, and metabolic processes. In addition, Akt phosphorylates MDM2 which promotes its translocation to the nucleus, where it negatively regulates p53 function. Two transcriptional targets of p53 are the BH3-only proteins Puma and Noxa, which appear to be the essential targets in p53-induced apoptosis.

TLR9 and apoptosis

Stimulation of TLRs has been shown to be pro- or anti- apoptotic depending on the TLR, the cell type and the metabolic condition of the cell. In some conditions TLRs engagement lead to recruitment of pro-apoptotic proteins such as caspase 8 *via* MyD88 and FADD or TRIF, RIP1 and FADD [683].

A couples of reports showed that CpG stimulation of TLR9 induced apoptosis via caspase 3 cleavage in fibroblast and human glioma cell lines [684, 685]. However the exact mechanism remains unknown. It was also shown that treatment with TLR9 ligand increases the survival of nude mice with experimentally induced brain tumors *via* caspase 3 dependent apoptosis [685]. Opioids induced microglia apoptosis has been shown to be mediated *via* TLR9 dependent induction of Bax and decrease of Bcl-2 [686, 687]. However the precise mechanism remains unknown. In addition, TLR9 triggering may also induce extrinsic apoptosis *via* cytokine secretion. TLR9 ligand stimulation of B-CLL induced NF κ B dependent IL10 secretion activating STAT1 phosphorylation and leading to apoptosis *in vitro* in 78 % of the patients (n=23) and in a xenograft model in NOD-scid mice where the B-CLL were pretreated with CpG prior to injection [688].

On the contrary, it has been shown that TLR9 ligands can inhibit spontaneous apoptosis. *Ex vivo* TLR9 triggering by CpG ODN (phosphothiorate backbone) can inhibit the spontaneous apoptosis of human neutrophils *via* PI3K / AKT signaling pathway [689].

Several studies analyzed the potential link between TLR9 engagement and induction of apoptosis by extracellular factors. It was shown that triggering of TLR9 inhibit apoptosis induced by serum starvation *via* HSP70 up-regulation or Akt activation [690], dexamethasone [691] or staurosporine *via* up-regulation of HSP70 [690]. However, the protection induced by TLR9 ligands against TRAIL-induced apoptosis in MM was not dependent on TLR9 by itself but on the binding of the phosphorothioate backbone of CpG to TRAIL and DR4 and therefore inhibition the apoptosis [692].

In conclusion, TLR9 engagement depending on the cell type, the ligand used and the experimental conditions displays pro- or anti-apoptotic activities that dependent mainly on STAT-1 and Akt signaling pathways respectively.

(iii) *TLR9 pathway and tumorigenesis*

The TLRs agonists have been used in a plethora of xenograft models or *in vitro* studies to determine their pro or anti-tumor role on tumor growth and cellular proliferation.

CpG dependent TLR9 activation has been shown to have anti-proliferative action on different cancer cells. In xenograft model of ovarian carcinoma, human small cell lung cancer, human colon cancer and human lung adenocarcinoma cell lines, treatment of the mice with CpG-B led to a decrease in tumor size when compared to controls [693, 694]. Furthermore, *in vitro*, TLR9 agonists are able to decrease human colon cancer cells proliferation and survival independently of p53 [695]. TLR9 agonists have been shown to inhibit the EGFR/ Akt pathway leading to a decrease in proliferation of cancer cells [696].

In contrast in other studies, TLR9 engagement has been shown to induce proliferation and invasiveness of cancer cells. *In vitro*, CpG treatment has been found to induce matrix metalloproteinase-13 (MMP13), which enhanced the invasiveness of prostate cancer cell line [697]. MMP13 secretion by cancer cells was induced *via* TRAF6 signaling regulated by estrogen independently of MyD88 [698, 699]. The same group showed the same effect of CpG stimulation in human breast cancer (MDA-MB-231), astrocytoma (U373) and glioblastoma (D54MG) cell lines [700]. The results obtained *in vitro* or in xenograft models have to be taken with caution. The cell lines have accumulated a lot of mutations in culture that are probably not found *in vivo* in tumors. Moreover the xenograft model is very artificial. The mice are immunodeficient to be permissive to allogenic tumor growth so all the interactions between immune system and tumors are lost.

The link between TLRs and chemically induced skin carcinogenesis in mice has been analyzed. It was shown that the induction of tumors was dependent on TLR4 and MyD88 but not on TLR2 and TLR9 [701].

Since MyD88 is the adaptor involved in TLR9 signaling, modulation of tumorigenesis by this molecule might be relevant to TLR9. MyD88 has been shown to promote tumorigenesis in a mouse model of spontaneous intestinal tumorigenesis and in azoxymethane-induced colon carcinogenesis in IL10 deficient mice, *via* the IL6-STAT3 pathway [7, 702]. In addition, MyD88 has also been involved in oncogene-induced (Ras) carcinogenesis in mice [703]. These findings have been confirmed in humans where a mutation leading to a hyperactivation of Myd88 induced a tumor cell survival [704]. Conversely, MyD88 as adaptor of the IL18 receptor has been shown to be protective in the azoxymethane/ DSS injury-induced experimental model of colitis-associated colorectal cancer. Innate receptor recognition of commensal flora through MyD88 signaling is important to maintain mucosal homeostasis. The inability of MyD88-deficient mice to heal ulcers generated by injury with DSS may create an altered inflammatory environment that exacerbates the mutation rate in mucosal epithe-

lial cells following exposure to the mutagen azoxymethane and results in augmented adenoma formation and cancer progression [705].

(b) Indirect effect of tumor micro-environment

The tumor microenvironment, which includes for instance immune cells, fibroblasts and endothelial cells, is also affected by TLRs modulation and has been implicated as a major factor for progression and metastasis of cancer [706]. When considering mice model limitation to assess the role of the microenvironment, use of TLR ligands *in vivo* in immunocompetent mice is thus crucial.

(i) Angiogenesis

Wound healing and cancer progression have striking similarities, including the angiogenesis and the rearrangement of the molecular matrix around the cells. Cancer progression can be seen as an uncontrolled wound repair. Vascular endothelial growth factor (VEGF) is the main factor involved in tumor angiogenesis [668] and in wound healing [669]. TLR9 agonist can modulate VEGF production in macrophage *in vitro* or *in vivo* [670]. During wound healing, TLR9 activation has been shown to induce VEGF in different murine macrophages [671, 672]. In addition, TLR9 stimulation enhanced VEGF release in *H. pylori* infected gastric cells and lung tumor cells *via* Cyclooxygenase-2 Induction (Cox-2), which was associated with increased tumor lesions [673, 674]. On the contrary TLR9 ligand stimulation of colon cancer cells has been shown to reduce VEGF production by inhibiting the EGFR-Akt pathway [657]. The tissue repair and regeneration process has been reported to depend on MyD88 signaling since wound healing was impaired in MyD88- deficient mice [675]

All together those data show that TLR9 stimulation has a dual role in angiogenesis depending on the cell type and the experimental condition it might induce or inhibit VEGF production.

(ii) TLRs modulation on Immune cells

TLR modulation on cell of the micro-environment affects immune recognition thereby modulating tumor development. Cancer progression and metastasis are thought to be due to a down-regulation of the anti-tumor activity of infiltrating immune cells (macrophages, DCs and T cells) [706-708].

Alteration of TLR9 signaling in immune cells has been found altered in numerous cancers such as HBV and HCV related cancers, HNSCC or ovarian cancer as a consequence of soluble factors released by tumor cells such as TGF β or IL10 or binding of viral antigen to the immune cells [396, 709-711]. Beside soluble factors, ligands expressed on tumor cells were shown to inhibit the ability of pDCs to produce IFN α in response to TLR-L. ILT7, a receptor expressed by pDCs specifically whose ligand is BST2, has been shown upon cross-linking or BST2 ligation to diminish TLR7 and TLR9 dependent IFN α production [712, 713]. Since BST2 has been found expressed on brain, breast cancer, multiple myeloma cells and melanoma cell lines and its expression could therefore modulates TLRs responses of neighboring pDCs [712, 714-717]. Because IFN α is required for immunoediting process to inhibit primary tumor growth [718, 719] interaction between BST2 expressed on cancer cells and ILT7 that suppress pDCs IFN α responses may contribute to immune tolerance.

Different immune cells expressed different TLRs and their activation induces an immune response that may inhibit tumor development notably IFN α or may favor tumor development. Indeed it has been shown that

TLRs stimulation was affecting directly regulatory T cell (T reg) biology. While TLR8 stimulation has been shown to revert the suppressive activity of human Treg, TLR5 triggering was found to enhance the suppressive activities of human T reg [720, 721]. In addition, it was shown that murine DCs activated by TLR4 and TLR9 ligands overcame Treg-mediated suppression and thereby restored proliferation of responder T cells [428].

(iii) Use of TLRs agonist as anti-cancer therapeutic

The first use of TLRs ligand to treat cancer goes back 30 years ago, with Mycobacterium BCG (bacillus Calmette Guerin) a potent activator of TLR2 and TLR4 used to treat bladder cancer [722, 723]. Several TLR agonists have been demonstrated to produce antitumor effects and nowadays are used in clinic to treat cancer [724, 725]. R837 (imiquimod), a TLR7-L, is used to treat human papilloma virus infection induce genital wart [726] and basal cell carcinoma of the skin [726, 727]. CpG-ODN were used in numerous clinical trial, in non Hodgkin's lymphoma with rituximab (phase I) [728], in refractory chronic lymphocytic leukemia (phase I) [676, 729], in refractory cutaneous T cell lymphoma (phase I) [730], in basal cell carcinoma or metastatic melanoma (phase I) [731, 732]. TLR9 agonists are also used to treat chronic lymphocytic leukemia [733], brain and renal cancer. While phase I and II clinical trial of CpG used in combinatory approach with radiotherapy or chemotherapy were promising, a lot of trial did not proceed to phase III [734].

Agonists of TLR9 have shown antitumor activity, alone and in combination with chemotherapy and radiotherapy. They were shown able to activate natural killer, dendritic and cytotoxic T cells and enhance the antibody-dependent cell-mediated cytotoxicity (ADCC) of monoclonal antibodies such as anti-EGFR or anti-CD20 antibody *in vitro* and *in vivo* [735-737]. However, the molecular mechanisms by which they affect tumor growth and angiogenesis have not been fully elucidated.

As seen earlier the role of TLRs in cancers remains highly controversial. One of the problems that researchers are facing is the non specificity of TLRs tools which renders the research quite difficult. Furthermore, differences might also appear depending of experimental settings, for example CpG stimulation has different effect depending on the backbone of the CpG [692]. The purely *in vitro* data and the xenograft models in immunodeficient mice are good models to start with, but they should be confirmed by more physiological models. We have also to consider interspecies differences between human and mice. In human immune cells TLR9 expression is restricted to B cells, pDCs and polymorphonuclear leukocytes while in mouse B cells, macrophages and neutrophils, and all DC subpopulations do express TLR9 [354, 370, 372].

II.PRESENTATION OF THE THESIS PROJECT

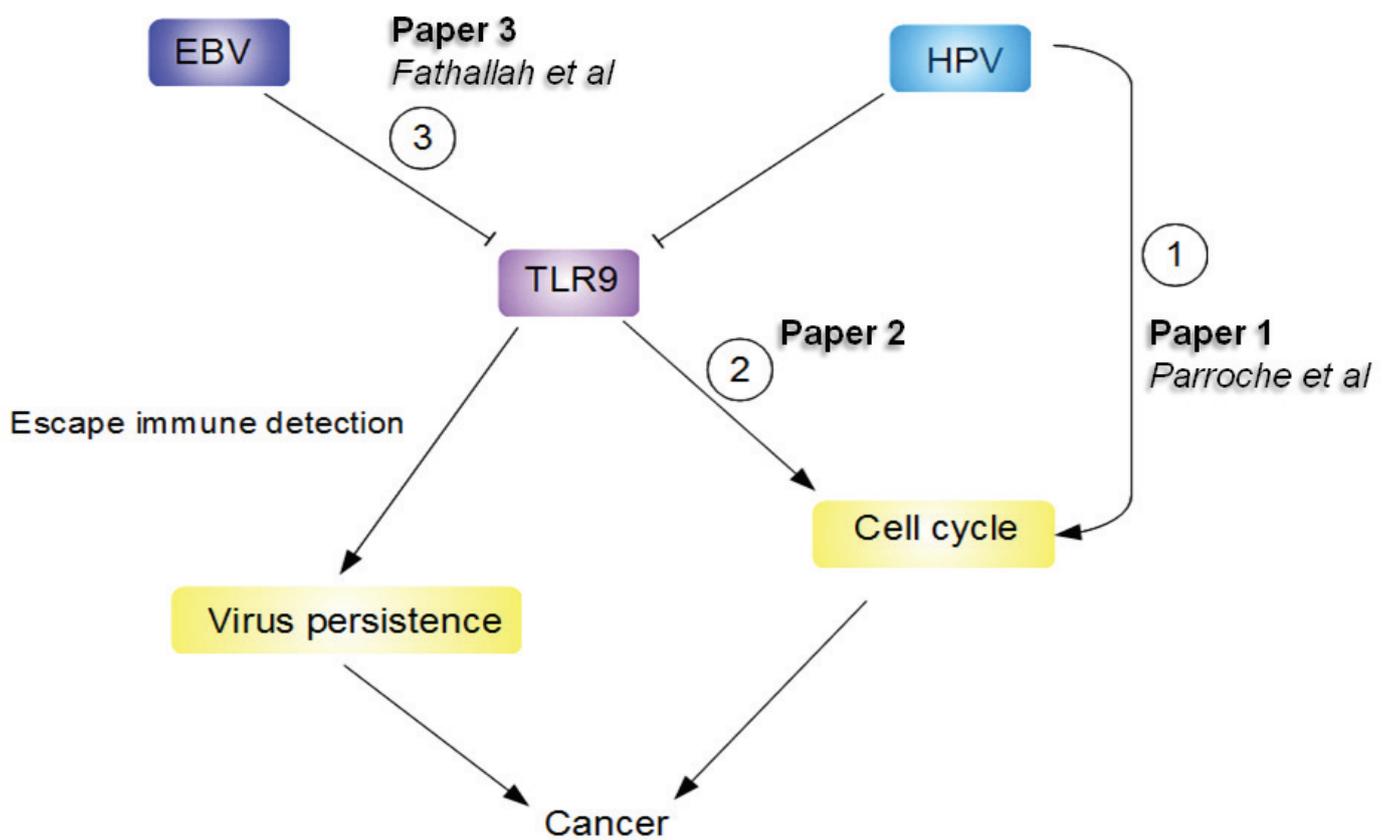


Figure 20: Thesis projects.

1-Inhibition of p21 by HPV16E6 by inactivation of p150.

2-Role of TLR9 in proliferation and cell cycle.

3-Modulation of TLR9 by EBV to induce viral persistence.

My thesis project focused on the relationship between the oncoviruses EBV and HPV and the ways they deregulate innate immune responses or cell cycle to persist, replicate and at last induce cancer. Cancer represents the second most common cause of death in industrialized countries. Epidemiological and biological studies have now conclusively proved that a variety of infectious agents constitute one of the main causes of cancer worldwide. It has been pointed out that more than 20% of cancers are from infectious origin [1]. EBV and HPV are respectively associated with 16% and 44% of virus induced cancers. In both case it is now widely accepted that persistent infection is mandatory for the development of cancer.

Key features for oncoviruses to induce cancer are viral persistence and induction of genomic alteration within the host cell. In order to persist, HPV and EBV need to escape innate and adaptive immune response and deregulate host cell cycle. Altogether these escape mechanisms are required to carry on their life cycle inducing therefore genomic instabilities that might lead to cancer.

The purpose of this thesis was to find new mechanisms by which EBV and HPV can promote carcinogenesis through three independent but interconnected projects (Figure 19).

(1) The first aspect of my project dealt with the mechanism of cell cycle deregulation by the oncoprotein HPV16E6. The ability of HPV16E6 to deregulate the G1/S phase of the cell cycle through p53 degradation preventing transcription of the CDK inhibitor p21 was already identified [172, 173]. However, additional mechanisms independent of p53 were previously described [738, 739]. Here, we reported that HPV16 E6 targets the cellular factor p150^{Sal2}, which positively regulates *cdkn1a* transcription. HPV16 E6 associates with p150^{Sal2}, inducing its functional inhibition by preventing its binding to cis elements on the p21WAF1 promoter. These data described a novel mechanism by which HPV16E6 induces cell cycle deregulation in a p53-independent pathway. The viral oncoprotein targets p150^{Sal2}, a positive transcription regulator of p21WAF1 gene, preventing G1/S arrest and allowing cellular proliferation and efficient viral DNA replication.

(2) As TLR9 has been shown to be deregulated in several viral and non-viral induced cancer, we secondly analyzed whether TLR9 may also have a direct role in the process of cell cycle control and that loss of its expression may lead to transformation of the cell. Our overall objective here was to study the role of TLR9 in suppressing the events that initiate transformation of epithelial cells in the setting of cervical cancer (virus-associated) and in head and neck cancer (non-virus-associated). Therefore, we tried to determine if TLR9 is able to control cell cycle progression in the context of viral or non-viral induced carcinogenesis. In particular, we tested the hypothesis that TLR9 controls cell cycle regulation and that suppressing its expression as seen in several cancers leads to cell cycle entry and unrestrained cellular proliferation.

(3) Based on previous observation in the group [371] showing HPV-mediated TLR9 dysregulation, we at last aimed at assessing whether another oncovirus would be able to deregulate TLR9, a key effector molecule of the viral innate immune response. This is of interest especially as human B cells strongly express TLR9 which is involved in their physiology such as antibody class switch [740], proliferation and enhance antigen presentation abilities [741]. We have shown that EBV infection of human primary B cells can alter the regulation and expression of *tlr9*. This resulted in the inhibition of its functionality. We demonstrated that this mechanism was through NF- κ B activation via the latent LMP1 protein.

III. RESULTS

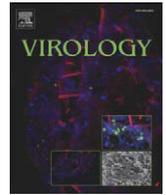
A. PAPER 1: HUMAN PAPILLOMAVIRUS TYPE 16 E6 INHIBITS P21WAF1 TRANSCRIPTION INDEPENDENTLY OF P53 BY INACTIVATING P150SAL2

HPV16 E6 deregulates G1/S cell cycle progression through p53 degradation preventing transcription of the CDK inhibitor p21WAF1. However, additional mechanisms independent of p53 inactivation appear to exist. Here, we report that HPV16 E6 targets the cellular factor p150Sal2, which positively regulates p21WAF1 transcription. HPV16 E6 associates with p150Sal2, inducing its functional inhibition by preventing its binding to cis elements on the p21WAF1 promoter. A HPV16 E6 mutant, L110Q, which was unable to bind p150_{Sal2}, did not affect the ability of the cellular protein to bind p21_{WAF1} promoter, underlining the linkage between these events. These data describe a novel mechanism by which HPV16 E6 induces cell cycle deregulation with a p53-independent pathway. The viral oncoprotein targets p150Sal2, a positive transcription regulator of p21WAF1 gene, preventing G1/S arrest and allowing cellular proliferation and efficient viral DNA replication.



Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Human papillomavirus type 16 E6 inhibits p21^{WAF1} transcription independently of p53 by inactivating p150^{Sal2}

Peggy Parroche^{a,b,1}, Majid Touka^{a,1,2}, Mariam Mansour^{a,3}, Véronique Bouvard^a, Amélie Thépot^{a,4}, Rosita Accardi^a, Christine Carreira^a, Guillaume G. Roblot^b, Bakary S. Sylla^a, Uzma Hasan^{a,b}, Massimo Tommasino^{a,*}

^a International Agency for Research on Cancer, Lyon, France

^b Institut Fédératif de Recherche 128 BioSciences Gerland-Lyon Sud, France

ARTICLE INFO

Article history:

Received 4 March 2011

Accepted 27 May 2011

Available online xxxx

Keywords:

HPV16 E6

p150^{Sal2}

p21^{WAF1} down-regulation

p53

ABSTRACT

HPV16 E6 deregulates G1/S cell cycle progression through p53 degradation preventing transcription of the CDK inhibitor p21^{WAF1}. However, additional mechanisms independent of p53 inactivation appear to exist. Here, we report that HPV16 E6 targets the cellular factor p150^{Sal2}, which positively regulates p21^{WAF1} transcription. HPV16 E6 associates with p150^{Sal2}, inducing its functional inhibition by preventing its binding to cis elements on the p21^{WAF1} promoter. A HPV16 E6 mutant, L110Q, which was unable to bind p150^{Sal2}, did not affect the ability of the cellular protein to bind p21^{WAF1} promoter, underlining the linkage between these events. These data describe a novel mechanism by which HPV16 E6 induces cell cycle deregulation with a p53-independent pathway. The viral oncoprotein targets p150^{Sal2}, a positive transcription regulator of a p21^{WAF1} gene, preventing G1/S arrest and allowing cellular proliferation and efficient viral DNA replication.

© 2011 Elsevier Inc. All rights reserved.

Introduction

Epidemiological and biological studies have clearly demonstrated that approximately 15 mucosal human papillomaviruses (HPVs), referred to as high-risk HPV types, are the etiological agents of cervical cancer (zur Hausen, 2000). Among these, HPV16 is the most frequent type associated to cervical cancer worldwide (Clifford et al., 2003). The products of two early genes, E6 and E7, act as major oncoproteins in HPV-induced carcinogenesis (Munger et al., 2004). Since the viral DNA replication is totally dependent on host cellular machinery, these oncoproteins have developed a number of mechanisms to drive cells into S phase (Munger et al., 2004). In a normal situation, the exposure of quiescent cells to mitogens gives rise to activation of cyclin/cyclin dependent kinase (CDK) complexes, with concomitant dissociation and/or degradation of CDK inhibitors, such as p21^{WAF1}, a key regulator of the G1/S transition (Ekholm and Reed, 2000; Kaldis and Aleem, 2005). The transcription of p21^{WAF1} gene is known to be regulated by several cellular factors, including the tumor suppressor protein p53

(el-Deiry et al., 1993). Upon DNA damage, accumulation of p53 leads to increased p21^{WAF1} levels, inducing a G1 arrest and allowing DNA repair before replication (Sherr and McCormick, 2002). Furthermore, it has been shown that p21^{WAF1} can bind to PCNA (proliferating-cell nuclear antigen), an essential DNA replication factor, and this binding is sufficient for the inhibition of DNA replication based on simian virus 40 (Chen et al., 1995).

HPV16 E6 mediates the interaction between the ubiquitin protein ligase E6AP and p53, promoting degradation of the latter via the proteasome pathway (Kao et al., 2000). Thus, HPV16 E6 expressing cells in presence of DNA damages do not accumulate p21^{WAF1} and continue to proliferate (Mantovani and Banks, 2001). We have previously presented several lines of evidence that E6 protein has an additional p53-independent mechanism to down-regulate p21^{WAF1} expression and promote cell cycle progression. In fact, E6 from the benign cutaneous HPV1 and a HPV16 E6 mutant that are unable to target p53 for degradation, were still competent to inhibit p21^{WAF1} transcription and induce cellular proliferation (Malanchi et al., 2004, 2002). However, this E6 p53-independent mechanism still remains to be elucidated.

Few reports describe that the product of Sal2 gene, p150^{Sal2}, acts in part as a p53-independent positive regulator of p21^{WAF1} transcription (Li et al., 2004). Interestingly, p150^{Sal2} (Li et al., 2001) has been found to be targeted by the oncoprotein large T antigen from the mouse polyomavirus.

Here, we show that HPV16 E6 is able to bind to and inactivate the transcriptional functions of p150^{Sal2} in several experimental models.

* Corresponding author at: Infections and Cancer Biology Group, International Agency for Research on Cancer, 150 cours Albert-Thomas, 69372 Lyon, France. Fax: +33 4 72738442.

E-mail address: tommasino@iarc.fr (M. Tommasino).

¹ The first two authors equally contributed to the study.

² Current address: Invitrogen & Applied Biosystems, Paris, France.

³ Current address: Monash University, Department of Biochemistry and Molecular Biology, Faculty of Medicine, Nursing and Health Sciences, Victoria, Australia.

⁴ Current address: CellSeed Europe, Lyon, France.

Results

HPV16 E6 inhibits p21^{WAF1} transcription in the absence of p53

We have previously observed that HPV16 is able to alter the regulation of the restriction point in the G1 phase of the cell cycle by down-regulation of p21^{WAF1} expression by a mechanism that is independent of p53 inactivation (Malanchi et al., 2004, 2002). To investigate whether this E6 p53-independent mechanism affects p21^{WAF1} mRNA transcription, the activity of the p21^{WAF1} promoter linked to a luciferase reporter gene was evaluated in primary human fibroblast (POF) in the presence of a siRNA targeting p53 or a scramble control. We observed that the activity of the p21^{WAF1} promoter declined with increasing concentrations of HPV16 E6 wild type in presence and absence of p53 (Figs. 1A and B). In addition, HPV16 E6 was able to inhibit p21^{WAF1} promoter in a human osteosarcoma cell line (SaOS-2) that is p53 null (Fig. 1C).

We next analyzed the ability of two HPV16 E6 mutants, L110Q and G130V, to modulate p21. It has been previously reported that both mutants are unable to bind E6AP and induce p53 degradation (Liu et al., 1999). The mutation G130V was still able to inhibit the p21 promoter in a dose dependent-manner, whereas the mutation L110Q totally abolished the suppression of the p21^{WAF1} promoter by HPV16 E6 (Fig. 1B).

Besides p53, another cellular protein, p150^{Sal2}, positively regulates p21^{WAF1} expression (Li et al., 2004). Therefore, we next determined whether p150^{Sal2} could also be involved in the HPV16 E6-mediated inhibition of p21^{WAF1} transcription. To do this, several different deletion constructs of the p21^{WAF1} promoter were analyzed in transient transfection experiments in human osteosarcoma cell line (SaOS-2) (Fig. 1C). These data showed that after complete deletion of the two p150^{Sal2} cis elements, p21^{WAF1} transcription is no longer down-regulated by HPV16 E6, suggesting the possibility that the viral oncoprotein targets p150^{Sal2}.

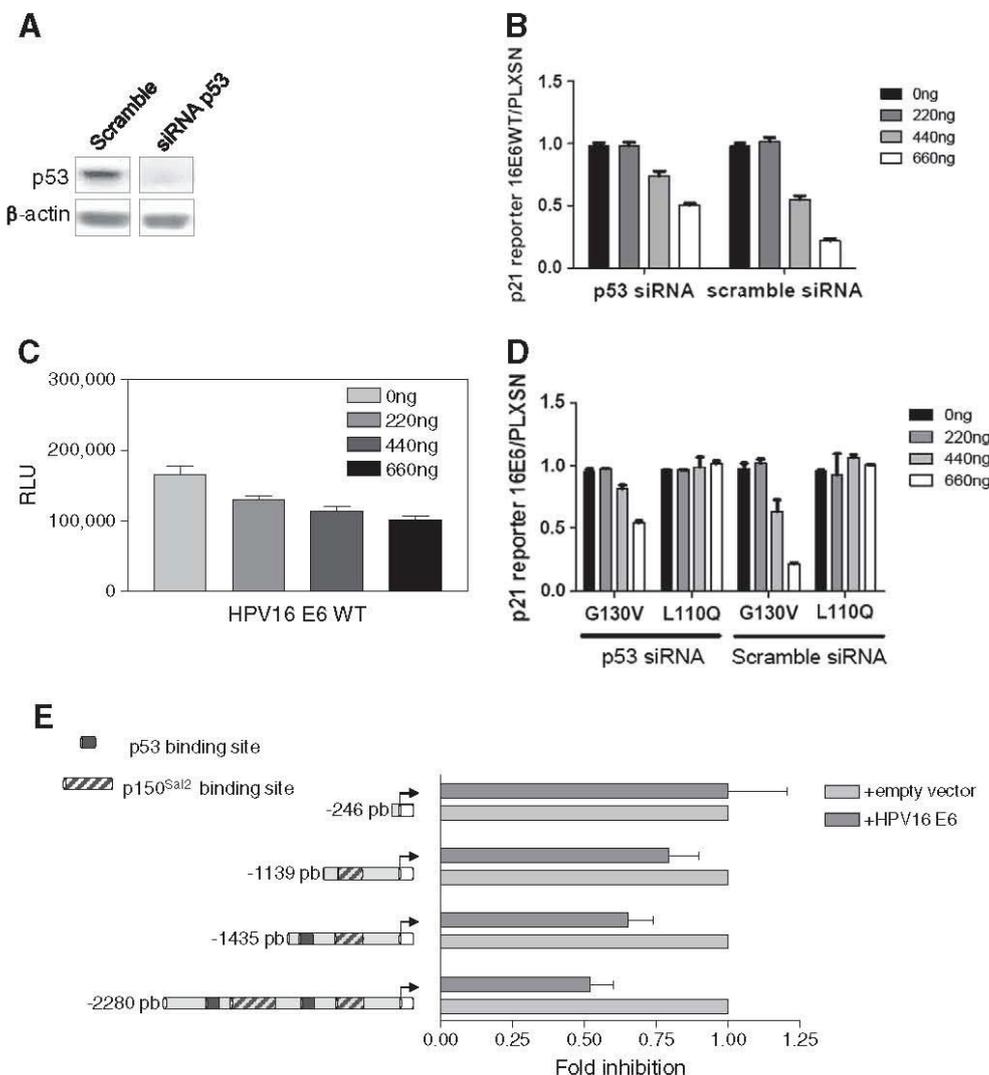


Fig. 1. HPV16 E6 inhibits p21^{WAF1} transcription in the absence of p53. (A) Determination of p53 levels in POFs stably expressing scramble shRNA or shRNAp53 by immunoblotting. (B) Luciferase assays were performed using increasing concentrations of pLXSN-HPV16 E6 wild type co-transfected with 1 µg of p21^{WAF1} promoter-luciferase construct in POFs stably expressing scramble shRNA or p53 shRNA. Luciferase activities were expressed as fold induction compared to empty pLXSN vector. Results are from at least three independent experiments; SEM is indicated by the error bars. (C) Luciferase assays were performed using increasing concentrations of HPV16 E6 co-transfected with 1 µg of p21^{WAF1} promoter-luciferase construct in SaOS-2 cells (p53-null). Luciferase activities are expressed as relative luciferase unit (RLU) measuring p21^{WAF1} promoter activity. (D) Luciferase assays were performed as described in the legend of B. Results are from at least three independent experiments; SEM is indicated by the error bars. (E) Schematic representation of full length and 5' end deletions of p21^{WAF1} promoter with the two p53 and p150^{Sal2} binding sites (left panel). CAT assays were performed with extracts from SaOS-2 cells transfected with the empty vector or HPV16 E6 wild type and the p21^{WAF1} promoter-reporter constructs. The data are the means of three independent experiments (right panel).

The ability of HPV16 E6 to inhibit p21^{WAF1} expression correlates with its affinity for p150^{Sal2}

We next determined whether the down-regulation of p21^{WAF1} promoter activity in absence of p53 by HPV16 E6 could be due to its ability to associate with p150^{Sal2}. We first performed immunoprecipitation experiments. HPV16 E6 fused with hemagglutinin (HA) at the C terminal (HPV16 E6HA) was stably expressed in SaOS-2 cells, which were subsequently transfected with a plasmid expressing Flag-p150^{Sal2} fusion protein. As shown in Fig. 2A, an anti HA antibody efficiently immunoprecipitated p150^{Sal2} protein in HPV16 E6-HA SaOS-2 cells transfected with p150-Flag, but not in the mock cells. Also an anti-Flag antibody efficiently immunoprecipitated HPV16 E6 protein (Fig. 2B), providing an additional evidence that the two proteins can interact. Based on the fact that HPV16 E6 G130V mutant is still able to inhibit p21^{WAF1} expression in a p53-independent mechanism, we hypothesized that this mutant has also retained the ability to interact with p150^{Sal2}, while this property should not be shared with the HPV16 E6 L110Q mutant. To confirm this hypothesis, we next analyzed the ability of the two HPV16 E6 mutants, L110Q and G130V, to bind p150^{Sal2} in a GST-pull down assay. The L110Q mutation completely abolished the binding to p150^{Sal2} (Fig. 2C), while G130V was still able to interact with p150^{Sal2}, although with reduced efficiency in respect to the HPV16 E6 wild-type protein (Fig. 2C).

Next we compared the ability of HPV16 E6 wild type and mutants (L110Q and G130V) to down-regulate the expression of the endogenous p21^{WAF1} gene. POFs were transduced with recombinant retroviruses expressing wild type, L110Q or G130V HPV16 E6 (Fig. 3A) and the endogenous mRNA levels of p21^{WAF1} were determined in all cell lines by quantitative RT-PCR (Fig. 3B). The wild type HPV16 E6 protein, as expected, strongly repressed p21^{WAF1} transcription. HPV16 E6 G130V was still able to down-regulate p21^{WAF1} mRNA, although with less efficiency than the wild type protein. L110Q mutant was even less efficient than G130V in blocking p21^{WAF1} transcription, although it appeared to retain a

weak inhibitory activity (Fig. 3B). Accordingly, the proliferative status of the cell lines expressing the different HPV16 E6 proteins correlated with their efficiency in binding p150^{Sal2} and down-regulating p21^{WAF1} expression (Figs. 3C and D).

HPV16 E6 induces the accumulation of a transcriptional inactive p150^{Sal2} form

Several independent studies reported that the interaction of E6 protein with cellular factors often results in their rapid degradation (Mantovani and Banks, 2001). Therefore, we next determined whether HPV16 E6 decreases the intracellular levels of p150^{Sal2} in POFs. Unexpectedly, p150^{Sal2} levels were clearly elevated in POFs transduced with the HPV16 E6 wild-type retrovirus (Fig. 4A). This accumulation was less evident or completely undetectable in cells expressing the HPV16 E6 G130V or L110Q mutant, respectively (Fig. 4A). HPV16 E6 induced p150^{Sal2} accumulation also in the p53-null SaOS-2 cells (Fig. 4B), demonstrating that the stabilization of p150^{Sal2} is not a consequence of the E6-mediated p53 inactivation. In addition, the HPV16 E6-mediated p150^{Sal2} accumulation was detected in HPV-positive cervical cancer-derived cell lines SiHa (HPV16), CaSki (HPV16) and HeLa (HPV18), but not in the cervical cancer line C33A that is HPV-negative (Fig. 4C). Gene silencing of HPV16 E6 and E7 expression in CaSki cells resulted in a strong reduction of p150^{Sal2} levels (Fig. 4D). Importantly, we observed that p150^{Sal2} accumulation always correlated with the down-regulation of p21^{WAF1} levels in POFs, SaOS-2 and cervical-cancer derived cells (SiHa, CaSki and HeLa) (Figs. 4B–D), suggesting that the stabilized form of p150^{Sal2} is inactive. To confirm this hypothesis, we investigated whether the oncoprotein could affect the ability of p150^{Sal2} to bind the p21^{WAF1} promoter by chromatin immunoprecipitation (ChIP). Based on the predicted p150^{Sal2} binding sites on the p21^{WAF1} promoter (Li et al., 2001) and Fig. 1E, primers were designed outside this region. Cellular extracts of SaOS-2 cells transduced with empty (pLXSN) or HPV16 E6 wild type or mutant retrovirus were immunoprecipitated using an anti-p150^{Sal2} antibody. As shown in

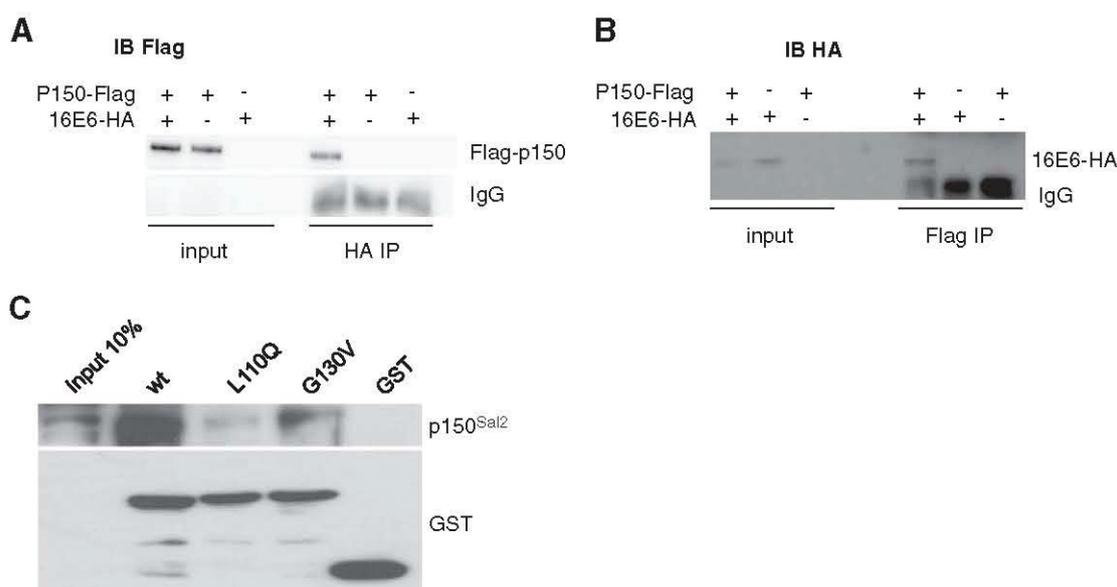


Fig. 2. HPV16 E6 targets and inhibits p150^{Sal2}. (A–B) SaOS-2 cells were transduced with retroviral empty vector (pLXSN) or recombinant retrovirus expressing HPV16 E6-HA fusion protein (pLXSN-HPV16 HA-E6) and then transfected with plasmids expressing pcDNA-p150-Flag or pcDNA-Flag. The cell lysates were immuno-precipitated (IP) using an anti-HA antibody (A) or an anti Flag antibody (B). The input represents 10% of the protein extract used for incubation. HA and Flag-tagged proteins were detected by immunoblotting (IB). (C) HPV16 E6 wild type, G130V and L110Q proteins were expressed as a GST fusion in BL21 *E. coli* strain purified and immobilized on Glutathione-sepharose beads. Purified recombinant proteins were incubated with POF extracts. Beads were washed and captured proteins were analyzed by SDS-PAGE (12% gel) and immunoblotting with anti-p150^{Sal2} or anti-GST antibodies. The input represents 10% of the protein extract used for incubation.

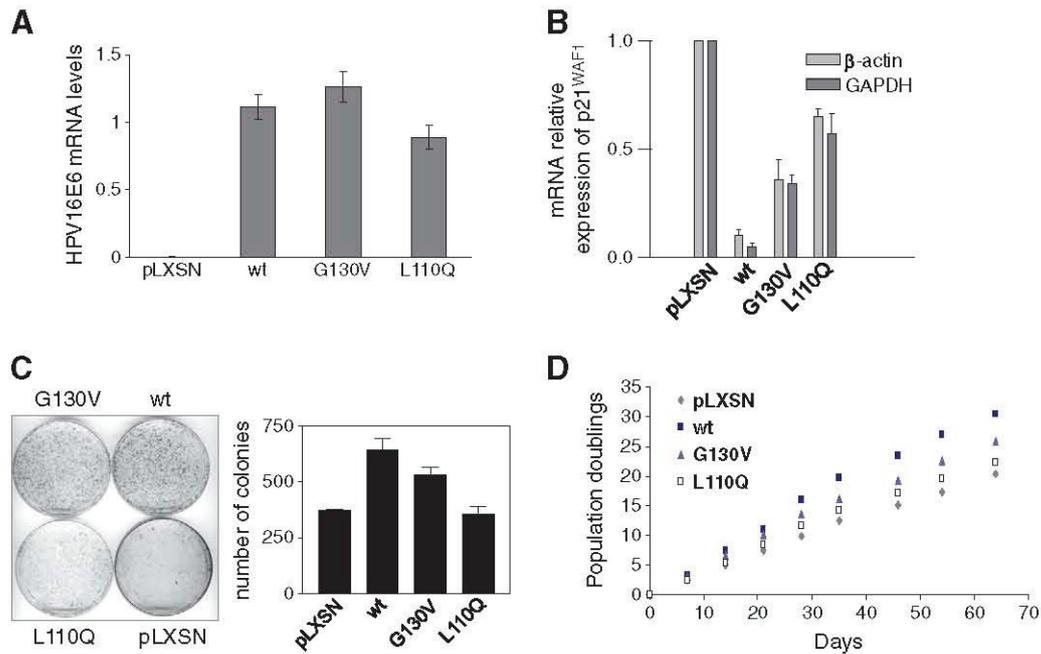


Fig. 3. Abilities of the HPV16 E6 mutants to down-regulate p21^{WAF1} and to induce cellular proliferation. (A) The mRNA levels of infected HPV16 E6 wild-type, HPV16 E6 G130V and HPV16 E6 L110Q were controlled in POF infected cells by quantitative RT-PCR. (B) p21^{WAF1} mRNA levels were quantified by real-time RT-PCR in POF expressing HPV16 E6 wild-type or mutants. Quantification was normalized with respect to two different control mRNAs, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The data are the means of three experiments performed with three independent RNA extractions and RT-PCR. (C) POF cells were transduced with HPV16 E6 wild-type, HPV16 E6 G130V, HPV16 E6 L110Q and pLXSN retroviruses. At 24 h post-transduction, G418 (0.5 mg/ml) was added to the cells for 2 weeks. The plated clones were stained with crystal violet. A representative colony formation assay is shown (left panel). Histograms represent the number of colonies quantified in each plate. Results are from three independent experiments (lower panel). (D) Growth profiles of POF cells expressing the HPV16 E6 viral proteins and respective mutants. After selection, the growth of the different cell populations was monitored for more than 60 days.

Fig. 4E, p150^{Sal2} was found bound to the p21^{WAF1} promoter in pLXSN cells, but not in HPV16 E6 expressing cells. As for the E6 mutants, their abilities to prevent the binding of p150^{Sal2} on the p21^{WAF1} promoter correlate with their efficiency in binding p150^{Sal2}.

Together these data show that HPV16 E6 is able to prevent the binding of p150^{Sal2} on the p21^{WAF1} promoter.

Discussion

We have previously observed that HPV16 is able to alter the regulation of the restriction point in the G1 phase of the cell cycle by down-regulation of p21^{WAF1} transcript by a mechanism that is independent of p53 inactivation (Malanchi et al., 2004, 2002). Here, we show that HPV16 E6 binds, stabilizes and inactivates the transcription factor p150^{Sal2}, which is implicated in the positive regulation of the p21^{WAF1} promoter (Li et al., 2004). Mutation of HPV16 E6 L110 greatly diminished p150^{Sal2} binding, stabilization and inactivation. Importantly, the oncoprotein is able to prevent p150^{Sal2} association with p21^{WAF1} promoter, therefore inhibiting its transcriptional functions.

It has been previously shown that p150^{Sal2} associates with another protein from a DNA tumor virus, the large T antigen of mouse polyomavirus (Li et al., 2001). This interaction was characterized in a cellular assay, which allowed the identification of p150^{Sal2} as a negative regulator of viral replication (Li et al., 2001). Since viral replication is dependent on the host DNA replication machinery and G₁-to-S progression, the inhibition of p150^{Sal2}, and consequently down-regulation of p21^{WAF1}, represent a key event in the life cycle of the virus. Therefore, based on the polyomavirus model, a similar scenario could be envisaged for HPV16. The importance of the neutralization of p21^{WAF1} for HPV16 life cycle is underlined by the existence of several virus-mediated mechanisms to block the functions of the CDK inhibitor. In fact, in addition to the ability of E6 to

repress p21^{WAF1} transcription with p53-dependent and -independent mechanisms, it has been shown that the other major HPV16 oncoprotein, E7, binds p21^{WAF1}, preventing its interaction with CDK2 and PCNA (Funk et al., 1997; Jones et al., 1997).

The existence of two distinct mechanisms of HPV16 E6 to repress p21^{WAF1} transcription is not surprising. In fact, it is very likely that p150^{Sal2} and p53 activate p21^{WAF1} transcription in different circumstances. In this scenario, E6 would be able to inhibit p21^{WAF1} transcription independently of the situation of the infected cell and guaranteeing a constant and efficient viral DNA replication. In addition, it is very likely that p150^{Sal2} regulates the transcription of additional cellular genes encoding proteins, which may play a negative role in the HPV life cycle, and therefore needs to be neutralized.

Our data indicate that HPV16 E6 induces the accumulation of an inactive form of p150^{Sal2}. Interestingly, p150^{Sal2} levels were found to be elevated in synovial sarcomas (Nielsen et al., 2003). In light of our findings, we could speculate that the accumulation of p150^{Sal2} in this pathological setting reflects the loss of its function.

In summary, we describe here a new mechanism for HPV16 E6 down-regulation of p21^{WAF1} transcription, independently of p53 in which inactivation of p150^{Sal2} is required. It is likely that a similar mechanism of inactivation of p21^{WAF1} expression occurs in other cancers non- and virally induced.

Materials and methods

Cell culture

SaOS-2, SiHa, CaSki, HeLa, C33A, Phoenix and primary human oral fibroblast (POF) were grown in DMEM supplemented with 10% fetal calf serum. Recombinant retroviruses, infections, doubling population counting and colony assays were performed as previously described (Caldeira et al., 2003; Mansour et al., 2007). Gene silencing of HPV16

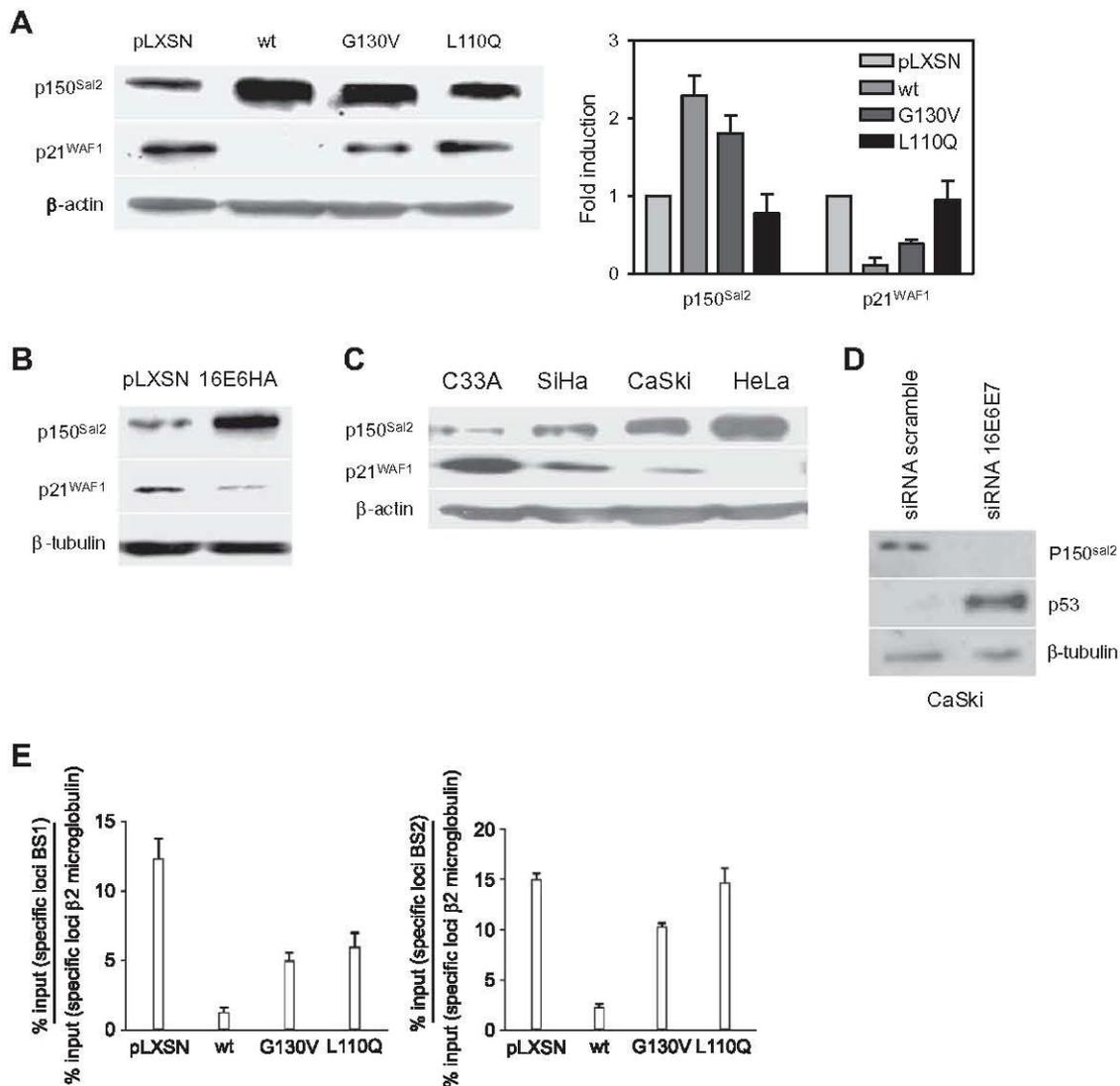


Fig. 4. HPV16 E6 induces accumulation of an inactive form of p150^{Sal2}. (A) Immunoblotting of p21^{WAF1} and p150^{Sal2} levels in POFs transduced with empty retroviruses (pLXSN) or wild type or mutated HPV16 E6 (left panel). Quantification of p150^{Sal2} signal in indicated cells in three independent immunoblottings (right panel). (B) Immunoblotting of p21^{WAF1} and p150^{Sal2} levels in SaOS-2 cells transduced with empty retroviruses (pLXSN) or expressing HPV16 HA-E6. (C) Immunoblotting of p21^{WAF1} and p150^{Sal2} levels in C33A (HPV-negative cervical cells line), CaSki, SiHa (HPV16-positive cell lines) and HeLa (HPV18-positives cells line). (D) p150^{Sal2} levels in CaSki cells transfected with HPV16 E6/E7 small interfering RNAs. After preparation of total protein extracts p150^{Sal2} and β-tubulin protein levels were determined by immunoblotting using specific antibodies. (E) ChIP assay determining the in vivo binding of p150^{Sal2} to the p21 promoter in SaOS-2 cells transduced with empty retroviruses (pLXSN) or expressing wild type or mutated HPV16 E6. Chromatin was immunoprecipitated with the p150^{Sal2} antibody. A rabbit IgG isotype control was used as negative control. The sites BS1 and BS2 on the p21 promoter were amplified by qPCR from transcription factor bound to DNA and the values obtained are referred as relative to the initial amount of DNA (input).

E6 and E7 in CaSki was achieved by transient transfection using the following siRNA 5' UUAAAUGACAGCUCAGAGG 3'.

Plasmid constructs

The retroviral vectors pBabe-puro and pBabe-neo were described previously (Morgenstern and Land, 1990), while the pLXSN construct was obtained from Clontech (Palo Alto, CA, USA). The open reading frames of HPV16 E6 wild-type and the two mutants G130V and L110Q (kindly provided by Elliot Androphy, Indiana University, Indianapolis, IN, USA) were amplified by PCR and cloned in pBabe-neo, pLXSN or pGEX-4T1 expression vector (Amersham Biosciences, Piscataway, NJ, USA) with or without the hemagglutinin (HA) tag sequence at the 5' end. Full-length and serial deletions of p21^{WAF1} promoter in pJFACT1 were kindly provided by Bert Vogelstein (el-Deiry et al., 1993) and Xiao-Fan Wang (Duke University Medical Center, Durham, NC, USA). The p53 pRetroSuper construct was used as previously described

(Accardi et al., 2006). The p150^{Sal2} pcDNA was kindly provided by Benjamin Thomas (Harvard Medical School, Boston, MA, USA). The p150sal2-Flag was cloned in pcDNA-Flag between HpaI and BamH1.

Determination of p21^{WAF1} promoter activity

POF were transfected with 1 μg or 3.5 μg of the p21^{WAF1} luciferase or chloramphenicol acetyl transferase (CAT) reporter plasmids respectively, HPV16 E6 plasmid at different concentrations. Luciferase activity was measured with a dual luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions using the luminometer (Optocomp I; MGM instrument, Hamden, CT, USA). Renilla luciferase activity or beta-galactosidase activity was used to normalize transfection efficiency. CAT assays were performed with 40 μg of protein incubated with 2.5 μl acetyl-CoA and 1.5 μl [¹⁴C] chloramphenicol (Amersham, Piscataway, NJ, USA). Following extraction with ethyl acetate, samples were analyzed by thin layer

chromatography and visualized by autoradiography. Quantification was performed by excising the products and liquid scintillation counting.

Biochemical analyses

Preparation of total protein cellular extracts and immunoblotting were performed as previously described (Accardi et al., 2006). Immunoblottings were performed with following antibodies: anti-p150^{Sal2} antibody (Benjamin Thomas, Harvard Medical School, Boston, MA, USA and Santa Cruz Biotechnology, Santa Cruz, USA), anti- β -tubulin (Sigma-Aldrich, St Louis, MI, USA), anti-p21^{WAF1} (Cell Signaling, Danvers, MA, USA), anti- β -actin (Sigma-Aldrich, St Louis, MI, USA), anti-GST (generous gift from Georges Mosialos, Institute of Immunology, Biomedical Sciences Research Center Al. Fleming, Vari, Greece), anti-Flag M2 (Sigma-Aldrich, St Louis, MI, USA), and anti-HA (Roche, Mannheim, Germany). All densitometry analyses were performed using the BIORAD phosphor-imager.

For the immunoprecipitation, cleared lysates were incubated overnight at 4°C with anti-Flag or anti-HA. After addition of protein A/G plus agarose (Santa Cruz Biotechnology), immunoprecipitates were collected and subjected to a 15% or a 10% SDS-PAGE and transferred to a membrane.

The GST pull down assays were performed as previously described (Caldeira et al., 2003).

ChIP assay was performed using previously described protocol (Zannetti et al., 2010) using the p150^{Sal2} provided by Santa Cruz Biotechnology. The qPCR was carried out with a set of two primers amplifying the two predicted regions of the p150^{Sal2} binding site (p150^{Sal2} BS) on the human p21^{WAF1} promoter (Table 1, Supplementary material).

Quantitative RT-PCR

Total RNA extraction and cDNA synthesis were performed as previously described (Hasan et al., 2007). Real-time PCR was performed by using the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kit (Roche, Mannheim, Germany) in a LightCycler fluorescence temperature cyler according to the manufacturer's instructions. Primers used for RT-PCR and real-time PCR are listed in Table 1 (Supplementary material).

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2011.05.016.

Acknowledgments

This work was supported by grants from La Ligue Contre le Cancer (Comités du Rhône, Drôme and Savoie), the Association pour la Recherche sur le Cancer, European Union (LSHC-2005-018704), the Association for International Cancer Research and the "Applied Tumor Virology" German-French cooperation, DKFZ-Cancéropôle du Grand-Est. Peggy Parroche was supported by L'ARC and Lyon Biopole. M. Touka was supported by a doctoral fellowship from the Egide Foundation and by the ICB group. We are grateful to Drs E. Androphy, D. Galloway, G. Mosialos, B. Thomas, B. Vogelstein and XF Wang for the reagents.

References

- Accardi, R., Dong, W., Smet, A., Cui, R., Hautefeuille, A., Gabet, A.S., Sylla, B.S., Gissmann, L., Hainaut, P., Tommasino, M., 2006. Skin human papillomavirus type 38 alters p53 functions by accumulation of deltaNp73. *EMBO Rep.* 7 (3), 334–340.
- Caldeira, S., Zehbe, I., Accardi, R., Malanchi, I., Dong, W., Giarre, M., de Villiers, E.M., Filotico, R., Boukamp, P., Tommasino, M., 2003. The E6 and E7 proteins of the cutaneous human papillomavirus type 38 display transforming properties. *J. Virol.* 77 (3), 2195–2206.
- Chen, J., Jackson, P.K., Kirschner, M.W., Dutta, A., 1995. Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature* 374 (6520), 386–388.
- Clifford, G.M., Smith, J.S., Plummer, M., Munoz, N., Franceschi, S., 2003. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *Br. J. Cancer* 88 (1), 63–73.
- Ekhholm, S.V., Reed, S.L., 2000. Regulation of G(1) cyclin-dependent kinases in the mammalian cell cycle. *Curr. Opin. Cell Biol.* 12 (6), 676–684.
- el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., Vogelstein, B., 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* 75 (4), 817–825.
- Funk, J.O., Waga, S., Harry, J.B., Espling, E., Stillman, B., Galloway, D.A., 1997. Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. *Genes Dev.* 11 (16), 2090–2100.
- Hasan, U.A., Caux, C., Perrot, I., Doffin, A.C., Menetrier-Caux, C., Trinchieri, G., Tommasino, M., Vlach, J., 2007. Cell proliferation and survival induced by Toll-like receptors is antagonized by type I IFNs. *Proc. Natl. Acad. Sci. U. S. A.* 104 (19), 8047–8052.
- Jones, D.L., Alani, R.M., Munger, K., 1997. The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. *Genes Dev.* 11 (16), 2101–2111.
- Kaldis, P., Aleem, E., 2005. Cell cycle sibling rivalry: Cdc2 vs. Cdk2. *Cell Cycle* 4 (11), 1491–1494.
- Kao, W.H., Beaudenon, S.L., Talis, A.L., Huibregtse, J.M., Howley, P.M., 2000. Human papillomavirus type 16 E6 induces self-ubiquitination of the E6AP ubiquitin-protein ligase. *J. Virol.* 74 (14), 6408–6417.
- Li, D., Dower, K., Ma, Y., Tian, Y., Benjamin, T.L., 2001. A tumor host range selection procedure identifies p150(sal2) as a target of polyoma virus large T antigen. *Proc. Natl. Acad. Sci. U. S. A.* 98 (25), 14619–14624.
- Li, D., Tian, Y., Ma, Y., Benjamin, T., 2004. p150(Sal2) is a p53-independent regulator of p21(WAF1/CIP). *Mol. Cell. Biol.* 24 (9), 3885–3893.
- Liu, Y., Chen, J.J., Gao, Q., Dalal, S., Hong, Y., Mansur, C.P., Band, V., Androphy, E.J., 1999. Multiple functions of human papillomavirus type 16 E6 contribute to the immortalization of mammary epithelial cells. *J. Virol.* 73 (9), 7297–7307.
- Malanchi, I., Accardi, R., Diehl, F., Smet, A., Androphy, E., Hoheisel, J., Tommasino, M., 2004. Human papillomavirus type 16 E6 promotes retinoblastoma protein phosphorylation and cell cycle progression. *J. Virol.* 78 (24), 13769–13778.
- Malanchi, I., Caldeira, S., Krutzfeldt, M., Giarre, M., Alunni-Fabbroni, M., Tommasino, M., 2002. Identification of a novel activity of human papillomavirus type 16 E6 protein in deregulating the G1/S transition. *Oncogene* 21 (37), 5665–5672.
- Mansour, M., Touka, M., Hasan, U., Bellopede, A., Smet, A., Accardi, R., Gabet, A.S., Sylla, B.S., Tommasino, M., 2007. E7 properties of mucosal human papillomavirus types 26, 53 and 66 correlate with their intermediate risk for cervical cancer development. *Virology* 367 (1), 1–9.
- Mantovani, F., Banks, L., 2001. The human papillomavirus E6 protein and its contribution to malignant progression. *Oncogene* 20 (54), 7874–7887.
- Morgenstern, J.P., Land, H., 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* 18 (12), 3587–3596.
- Munger, K., Baldwin, A., Edwards, K.M., Hayakawa, H., Nguyen, C.L., Owens, M., Grace, M., Huh, K., 2004. Mechanisms of human papillomavirus-induced oncogenesis. *J. Virol.* 78 (21), 11451–11460.
- Nielsen, T.O., Hsu, F.D., O'Connell, J.X., Gilks, C.B., Sorensen, P.H., Linn, S., West, R.B., Liu, C.L., Botstein, D., Brown, P.O., van de Rijn, M., 2003. Tissue microarray validation of epidermal growth factor receptor and SALL2 in synovial sarcoma with comparison to tumors of similar histology. *Am. J. Pathol.* 163 (4), 1449–1456.
- Sherr, C.J., McCormick, F., 2002. The RB and p53 pathways in cancer. *Cancer Cell* 2 (2), 103–112.
- Zannetti, C., Bonnay, F., Takeshita, F., Parroche, P., Menetrier-Caux, C., Tommasino, M., Hasan, U.A., 2010. C/EBP(delta) and STAT-1 are required for TLR8 transcriptional activity. *J. Biol. Chem.* 285 (45), 34773–34780.
- zur Hausen, H., 2000. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *J. Natl. Cancer Inst.* 92 (9), 690–698.

B. PAPER 2: RESTORATION OF TOLL-LIKE RECEPTOR 9 INDUCES A SLOWDOWN IN CELL PROLIFERATION: INVOLVEMENT OF THE DOWN-REGULATION OF TLR9 IN CARCINOGENESIS.

Oncoviruses such as EBV (Epstein Barr Virus), HPV (Human Papilloma Virus) and HBV/HCV (Human Hepatitis B/C Virus) involved respectively in Non Hodgkin's lymphoma, cervical cancer and liver cancer contribute to 12.1% of all cancer. As the immune response is a key component for the development of these cancers, de-regulating the innate immune response would be imperative to promote viral cellular transformation. The innate immune system senses pathogen components via many innate sensors of which the predominant members are the Toll Like Receptor (TLR) family. TLR9 recognizes unmethylated dsDNA sequences from bacteria or viruses in the form of CpG motifs. Therefore, we studied the role of TLR9 in suppressing the events that initiate transformation of epithelial cells in the setting of cervical cancer (virus-associated) and in head and neck cancer (non-virus-associated). TLR9 expression lead to a transient slow-down in cell proliferation associated with an increase of the cell cycle regulators p53, p21 and p27 and a longer S-phase. Our study shows that besides its function in innate immune response TLR9 might also play a role in inhibiting cellular transformation.

Restoration of Toll-Like Receptor 9 expression in epithelial tumor cells induces a slowdown in cell proliferation

Parroche P^(1,2), Goutagny N⁽³⁾, Malfroy M⁽³⁾, Zannetti C⁽¹⁾, Roblot G⁽¹⁾, Varisio D⁽⁴⁾, Gissman L⁽⁴⁾, Chopin S⁽²⁾, Le Calvez-Kelm F⁽⁵⁾, Mckay J⁽⁵⁾, Tommasino M⁽²⁾, Hasan UA⁽¹⁾.

(1) U851 oncoviruses and innate immunity, Lyon Sud, France (2) Infection and cancer biology group, IARC, Lyon, (3) U1052, Centre de recherche en Cancérologie de Lyon Lyon (4) Genome modification and carcinogenesis, DKFZ germany, (5) Genetic Cancer Susceptibility Group, IARC, Lyon.

INTRODUCTION

The innate immune system senses pathogen components *via* many innate sensors of which the predominant members are the Toll Like Receptor (TLR) family. The TLRs are Pathogen Recognition Receptors (PRRs) expressed on immune cells as well as on non immune cells such as epithelial or endothelial cells. TLRs can be broadly divided in two groups; the cell surface expressed TLRs (TLR1, TLR2, TLR4, TLR5 and TLR6) that are activated mainly by bacterial and viral surface associated PAMPs; and the endosomal TLRs (TLR3, TLR7, TLR8 and TLR9) that respond to nucleic acid of virus and bacteria. TLR9 is involved in the recognition of unmethylated CpG motifs 2-deoxyribose sugar backbone. [376, 393]. A number of viruses have been shown to trigger TLR9 responses, such as herpesviruses (HSV-1) [377], KHSV [449] and EBV [450]. There is increasing evidence that TLRs beside of their role in recognizing pathogen products are also able to recognize danger products released by injured tissue or dying cells such as HMGB1 or host DNA. TLRs seem to have a dual role in cancers. Indeed, as TLRs are expressed on tumor cells but also on stromal cells including endothelial cells, immune cells and fibroblasts, they may influence tumor growth *via* many ways [742]. TLR9 is differen-

tially expressed on tumor and healthy tissue. In some tumors, such as prostate cancer, TLR9 has been found up-regulated when compared to healthy tissue [678]. TLR9 is down-regulated in several cancers including breast cancer, myelodysplastic syndrome to overt leukemia transformation and HPV high risk type associated cancers [371, 381, 628, 679, 682]. Furthermore, loss of TLR9 function in pDCs has been associated with several cancers such as ovarian cancer [710], head and neck squamous cell carcinomas [396], hepatitis B and C virus-associated cancer [709, 711]. Therefore, we studied the role of TLR9 in suppressing the events that initiate transformation of epithelial cells in the setting of cervical cancer (virus-associated) and in head and neck cancer (non-virus-associated). We assessed whether TLR9 is able to control cell cycle progression in the context of viral or non-viral induced carcinogenesis. In particular, we tested the hypothesis that TLR9 controls cell cycle regulation and that suppressing its expression as seen in several cancers leads to cell cycle entry and unrestrained cellular proliferation. We have shown that TLR9 expression induced a cell proliferation slow-down and a longer S-phase due to p21/p53 activation.

RESULTS

Characterization of TLR9 constructs and cell lines expressing TLR9

To investigate the role of TLR9 in cell cycle regulation we used several over-expression strategies; (i) a transient transfection using a pcDNA vector encoding TLR9; (ii) cell lines stably transduced with a retroviral vector (pbabe) and (iii) cell lines stably expressing TLR9 under an inducible promoter (PLVUT''). We first assessed that the non inducible vectors encoding TLR9 were functional. We transiently transfected Hek 293 cells with increasing concentration of TLR9 expressed in pcDNA or in pbabe (Figure 1A) and measured the NFκB activity after overnight stimulation by 3μM of CpG-B. The expression of both plasmids was sufficient to elicit a TLR9 response (Figure 1A). However it has to be noted that the pcDNA-TLR9 plasmid induced a 40 times higher NFκB response when the cells were stimulated with CpG as compared to the unstimulated control (Figure 1A) whereas the pbabe-TLR9 induced only a response four times higher. The difference might be due to the fact that pbabe has a retrovirus backbone and might be transfected at a lesser efficiency than pcDNA derived plasmids. We next generated and tested a Caski cell line derived from HPV16 + cervix cancer that would stably express TLR9 under control of a tetracycline inducible promoter: PLVUT''-TtKRAB. In this cell line TLR9 could be expressed upon doxycyclin treatment. We next checked the inducibility of the cell line. We first analyzed TLR9 mRNA expression by quantitative PCR after induction with doxycyclin (Figure 1B). The mRNA is induced almost fifteen fold in the Caski PLVUT''-TLR9 upon doxycyclin treatment. However in the condition doxycyclin/Tetracycline free, the mRNA for TLR9 is fourteen fold more elevated in the Caski PLVUT'-TLR9 than in the Caski PLVUT'-GFP (Figure 1B). We next analyzed the TLR9 protein expression by western blotting of the two Caski cell lines. As shown in Figure 1C, the TLR9 protein is not expressed in tetracycline / doxycyclin free medium but is well induced upon doxycyclin treatment. While there was some TLR9 mRNA expression in absence of doxycyclin, the TLR9 protein is not detected without induction (Figure 1C). We also analyzed TLR9 expression by flow cytometry and seen that 100% of the Caski PLVUT'-TLR9 and PLVUT'-GFP were expressing TLR9 and GFP respectively upon doxycyclin treatment (Data not shown). We next investigated if the PLVUT'-TLR9 construct was functional. PLVUT'-GFP and PLVUT'-TLR9 were transiently transfected in Hek 293 in presence of doxycyclin and NFκB activation was measured after stimulation with various ligands (Figure 1D). The cells transfected with PLVUT'-GFP showed an increase of NFκB activation after TNFα stimulation but not upon CpG and R848 treatment. The cells transfected with PLVUT'-TLR9 responded as well to TNFα with a similar NFκB activation than the GFP cells, showing that both plasmids did not alter NFκB activation. The cells transfected with TLR9 acquired the ability to respond CpG an agonist of TLR9 whereas a TLR7 ligand R848 was

unable to induce NF κ B activation by those cells (Figure 1D). The PLVUT'-TLR9 construct allowed an inducible expression of TLR9 which is functional since it can bind TLR9 ligand and signal. We next decided to investigate the effect of TLR9 expression on cell proliferation.

Re-expression of TLR9 inhibits cell proliferation

We investigated the effect of TLR9 expression in different cell types. TLR9 was introduced in Hek 293 cells and the cell growth was assessed by MTT assay after 48 hours (Figure 2A). The quantity of TLR9 correlated with a decrease in cell proliferation in a dose dependent manner in these heavily transformed cell lines. The effect of the expression of TLR9 was assessed in non transformed immortal Near Diploid Immortalized Keratinocytes (NiKs) and in HPV transformed NiKs expressing the oncoproteins of HPV E6 and E7 (NiKs 16E6E7). Similarly to the previous data, TLR9 was able to decrease cellular proliferation in those cell lines (Figure 2 B and C). NiKs and NiKs16E6E7 were stably transduced with pbabe or pbabe_TLR9 (Figure 2B), selected and tested in a doubling population assay. TLR9 expression slowed down cell proliferation as assessed by the doubling population counting. TLR9 and GFP were stably expressed in a cervical cancer cell line HPV+ (SiHa) and a HNSCC transformed cell line (124). Accordingly, TLR9 was also able to slow down cell proliferation (Figure 2C). Cells used in a MTT or doubling population assay after ten fifteen days in culture were not showing any sign of cell growth inhibition (Data not shown). The cells that were able to survive the crisis induced by TLR9 would recover and not show any sign of inhibition of cell proliferation despite the expression of TLR9. Upon expression and selection, TLR9 was able to diminish colony formation in NiKs, NiKs16E6E7, Caski and SiHa (Figure 2D). In conclusion TLR9 expression induced a slowdown in cellular proliferation of both immortal and heavily transformed cell lines. We next determined the mechanism involved in TLR9 inhibition of cell proliferation.

TLR9 mediated effects on cell cycle

Since TLR9 has been linked to apoptosis, we first checked whether TLR9 re-expression in cervical Caski and HSCNN 136 lines was increasing cell death by necrosis or apoptosis. We analyzed by flow cytometry the Caski cell lines positive and negative (Figure 3A) for TLR9 after annexin V and propidium iodide (PI) staining. As shown in Figure 3A, both cell lines exhibited similar percentage of cells in necrosis (AnnV+/PI+) or apoptosis (AnnV+/PI-). TLR9 induced slow-down of cell proliferation did not involved cell death induction. We next try to determine if the cellular replication was altered by TLR9 expression. First we analyzed the DNA content by PI staining and flow cytometric analysis. There was no difference between the cells expressing or not TLR9 (Figure 3B). The percentage of cells in G1, S and G2 phases of the cell cycle was roughly the same. To investigate the apparent poor efficiency of TLR9 expression on cell cycle inhibition, we performed bromodeoxyuridine (BrdU) uptake experiments where BrdU+ cells indicate the percentage of cells in S-phase. The PLVUT'-GFP and TLR9 cell lines were induced for five days and pulsed thirty minutes with BrdU fixed and analyzed for BrdU content by flow cytometry. As seen in Figure 3 C, when cells are in cycling condition (cycling), the percentage of cells that replicated within 30 minutes is the same independently of TLR9 expression. To study the impact of TLR9 in the progression through the cell cycle, we synchronized both cell lines with a thymidine double block then added BrdU, released the cells and harvested the cells at different time points (Figure 3C Time after sync). After synchronization, TLR9 expression leads to a decrease number of BrdU positive cells. However this effect seems to be rather short lived since after 8 hours the percentage of BrdU positive cells in both conditions was the same. To further define the mechanism of TLR9-mediated growth inhibition we analyzed the cell cycle distribution. HNSCC 136 expressing empty pbabe (Figure 3D left panel) or pbabe-TLR9 (Figure 3D right panel) or pbabe-GFP (Figure 3D middle panel) were pulsed for twenty minutes with BrdU

and cell cycle phase were analyzed by flow cytometry. There is no significant difference between the cells expressing the empty vector or the GFP but the cells expressing TLR9 showed a delayed S-phase, marked by the arrow on Figure 3D right panel. TLR9 expression is able to delay the S-phase inducing a transient slow-down of cell proliferation.

TLR9 re-expression effect on cell cycle regulators

Based on the observation that TLR9 restoration could promote cell cycle arrest we tried to identify which cell cycle regulators could mediate these effects. The effect of TLR9 on the p53 dependent *cdkn1a* and *pig3* expression was analyzed. The p21 (Figure 4A) and *pig3* (Figure 4B) promoters cloned upstream of the luciferase were co-transfected into cells with either empty or TLR9 or p53 encoding pCDNA plasmid and luciferase activity was measured. TLR9 expression was able to drive *pig3* promoter (Figure 4B) and to a less extent p21 promoter (Figure 4A). We then examined the effect of TLR9 over-expression on p21, p27 and p53 by western blotting in stable Caski, Hela and SiHa cell lines. As seen in Figure 4C, the expression of TLR9 induced the protein expression of p21, p27 and p53. TLR9 expression induced an increase in p53, p21 and p27 that may delay the replication of the cells by blocking transiently cells in S phase.

Cell death and TLR9

As previously mentioned, late passage TLR9 expressing cells lost the TLR9 dependent inhibition of proliferation (data not shown). We hypothesized that the anti-proliferative response induced by TLR9 effect was ligand mediated and that a danger associated molecular pattern (DAMP) could be released. We tried to determine whether cell death inducer could induce the release of such DAMPs and then decrease the proliferation of late passage TLR9 expressing cells. We treated stable TLR9 or GFP expressing SiHa cells with increasing concentrations of cisplatin (CPPD) for four days. As shown in Figure 5A, TLR9 expression did not affect the survival as measured by MTT of cells treated with various concentration of cisplatin. Likewise, when we used Caski, HNSCC 124 or HNSCC 136 we did not see any effect of the TLR9 expression on cell death. In addition similar results were obtained when cells were treated with other death inducers such as H₂O₂ and doxorubicin (data not shown). We next tried to determine if a soluble factor released during puromycin induced cell death could account for the differences seen between TLR9 or GFP expressing cells. We added supernatant from cells (puromycin sensitive) treated with puromycin for three days to cells stably expressing TLR9 or GFP that are resistant to puromycin and assessed the proliferation by MTT after four days. The supernatant from dying cells affected the proliferation of both TLR9 and GFP expressing cells in a similar way as shown in Figure 5B for the HNSCC 136. Such experiment was also performed with SiHa, Caski and HNSCC 124 cell lines and displayed similar results (data not shown). Cell proliferation in TLR9 or GFP expressing cells was similar in all the cell lines. We next co-cultured mock and GFP or TLR9 HNSCC 136 expressing cells with cells treated with puromycin, cisplatin or H₂O₂ (killed cells) and analyzed cell proliferation after four days. In order to determine cell proliferation and analyze for GFP or TLR9 expression specifically on HNSCC cells, killed cells were previously loaded with fluorescent PKH26 dye. Furthermore, cell proliferation of HNSCC cells was monitored using violet cell trace reagent dye. Violet cell trace reagent has the ability to stably label molecules within cells, with each cell division resulting in a successive decrease of its fluorescence intensity; thereby the more the cells will proliferate the less bright they will be. After four days of co-culture the mock and TLR9 expressing 136 cells showed identical proliferation (Figure 5C). Similarly the proliferation of the GFP and the mock expressing cells was the same (data not shown). 136 cells pretreated with CPPD were added to a co-culture of mock and TLR9 136 expressing cells for four days (Figure 5D). The addition of killed cells had the same effect

on TLR9 and mock expressing cells. Likewise the proliferation of the GFP and the mock expressing cells was the same when co-cultured with killed cells. The supernatant of the killed cells was added to the co-culture of mock and TLR9 or GFP expressing cells. As shown in Figure 5E the proliferation of the cells was identical between the TLR9 and mock expressing cells. In the same way GFP and mock expressing cells showed an identical proliferation. Altogether those data suggest that short-lived effect of TLR9 on cell proliferation as observed early after cell transduction is not mediated by the released of DAMP generated upon cell death.

Search for pathway-TLR9 inducible genes

In order the pathway involved in anti-proliferative activity of TLR9, we performed transcriptome analysis in TLR9 expressing cells. Hek 293 cells were transiently transfected either with mock or TLR9 pcDNA. After 48 hours cells were harvested, and counted before RNA isolation. As expected we found fewer cells in TLR9 expressing conditions. RNA was analyzed with illumina bead array in triplicate. Raw data were analyzed with the genespring software (agilent) and are summarized in Table 1. One gene was significantly up-regulated in response to TLR9 expression colony stimulating factor 3 (G-CSF) when compared to mock or TLR7 expressing conditions. The transcript for G-CSF was increased 6.9 and 14.9 times in TLR9 transfected cells as compared to the control. One transcript Heat Shock Protein 70 (HSP70) was significantly down-regulated after the expression of TLR9.

DISCUSSION

The major role of TLRs is to defend host against pathogens via PAMP recognition and triggering of innate immune response. They also play a role in tissue repair, cell proliferation, apoptosis and angiogenesis [6, 632-634]. Those later activities link TLRs signaling to cancer. Moreover, TLRs are expressed by cancer cells [635] and their role carcinogenesis is still quite controversial.

Our results point out the association of TLR9 expression with cell cycle control. We have shown that re-expression of TLR9 in cervical and head and neck cancer cells was able to inhibit transiently cell proliferation. However this effect was short lived; when the cells were let to rest long enough after the expression of TLR9 there was no difference between TLR9 and mock expressing cells. One explanation might be that the transfection or the transduction of the cells might transiently release DAMPs that will trigger TLR9 and induce cell growth inhibition. Indeed it has been shown that TLR9 would respond to histone during hepatic ischemia/reperfusion injury and induce sterile inflammatory liver injury [713]. Furthermore another report showed that injury would induce the release of mitochondrial DNA that would activate cells in a TLR9 dependent manner [714]. Using various experimental conditions, we were unfortunately unable to show any effect of cell death induced DAMPS on proliferation of TLR9 expressing cells.

The DNA content as shown by PI staining was not affected by TLR9 expression. In contrast, the percentage of cells positive for BrdU after synchronization was significantly decreased in TLR9 positive cells when compared to mock expressing cells for few hours. It was previously published that when two cell populations differ by the duration of cell cycle phases without variations in the relative proportions of cells in each phase, flow cytometry by PI analysis shows similar DNA content profiles while the percentage of cells in S-phase uptaking 3H-Thymidine during the same incubation period will vary [715]. This is consistent with our data and might suggest that TLR9 expression would rather affect length of the S phase. In order to really quantify the length of the S-phase in TLR9 positive versus negative cells, pulse chase experiment consisting of a successive labeling by 5-Iodo-2'-deoxyuridine (IdU) for 2 hours and BrdU for 15 min should be carried out.

We have shown that TLR9 was inducing a transient increase of some inhibitors controlling the cyclin / cyclin dependent kinase complexes. Indeed, in TLR9 positive cells the protein levels of p53, p21 and p27 were transiently increased. The cell cycle is regulated by the activity of different cyclin/CDK complexes. CyclinD/CDK4-6 and cyclin E/CDK2 promote G1 phase progression into S-phase while Cyclin A/CDK2 and cyclin A-B/CDK1 ensures S-phase progression from G2 to M phases [716]. The CDKs are regulated by CDK inhibitors belonging either to the INK4 family or to the Cip/Kip family. The INK4 family has four members p16, p15, p18 and p19 that may inhibit CDK4 and CDK6 activities during the G1 phase. The Cip/Kip family has three members p21, p27 and p57, shown to inhibit CDK activities during all phases of the cell cycle. P53 is a central tumor suppressor protein that in response to DNA damage or cell cycle abnormalities is inducing cell cycle arrest and repair or apoptosis. Among others p53 is transactivating p21 that would in return inhibit CDK activities. In order to know exactly at what point the cell cycle is affected in TLR9 positive cells it would be interesting to compare the status of these CDK/cyclin complexes in TLR9 or mock expressing cells. However the gene transcription analysis did not show any down-regulation of cyclins or up-regulation of cell cycle regulator. One explanation might be that the cells were not synchronized which made the differences in cell cycle regulators impossible to detect. Hsp70 was down-regulated in TLR9 expressing cells. Hsp70 over-expression is typical of several types of tumors, and strong evidence suggests that it plays a role in the control of the cell cycle and growth. Under unstressed conditions, Hsp70 is expressed in some cases in proliferating cells during the G1/S- and S-phases of the cell cycle [717, 718]. Its role in proliferation has been demonstrated by over-expression and antisense administration assays [719, 720]. It is thus possible that TLR9 would be involved in the down-regulation of HSP70, which might inhibit cell proliferation.

In conclusion, in addition of escaping immune recognition, the deregulation of TLR9 induces by oncoviruses such as HPV and EBV might also favor the carcinogenesis.

MATERIAL AND METHODS

Cell lines and expression plasmids

Human TLR9 encoding pcDNA construct was previously described [721]. Human TLR9 and GFP were cloned in the retroviral vector pBabe-puro [722] using BamH1 and EcoRI. Human TLR9 cDNA was cloned using BamH1 and EcoRI in lentiviral pLVUT' vector. pLVUT is a lentiviral vector expressing GFP downstream the ubiquitin promoter in a doxycyclin inducible fashion [743]. PLVUT' was created from pLVUT to generate unique EcoRI site downstream GFP. EcoRI site at position 6235 was removed by digestion with BstB1 (present on either sides of EcoRI site) and re-ligation of the vector. PLVUT' vector and TLR9 encoding pLVUT' were generated by the ISP platform (<http://canceropole-clara.com/page.asp?page=463>). The pLXSN_HPV16E6E7, pGL3-NFkB luciferase and pGL4-TK constructs were already described [355]. 293 and cervical cancer-derived cell lines, SiHa and Caski were obtained from American Type Culture Collection. HNSCC 124 and 136 cell lines were described elsewhere [723]. Cells were maintained in DMEM supplemented with 10% FBS and 10ug/mL ciprofloxacin. NiKs were maintained as previously [744]. The Caski PLVUT'-GFP and TLR9 were made after lentiviral transduction of Caski cells and cloning by dilution limit. Ten clones for each cell line positive for WPRE were picked and mixed together to work with polyclonal cell lines.

Lentiviral and retroviral infections

Retroviral infections were described elsewhere [355]. After infection, the cells were selected with puromycin (1ug/mL) for 3 days (corresponding to 100% of killing of uninfected cells), let to recover 24 hours and plated

for a day and used for experiments. Lentiviral particles were produced by the “plateau technique analyse génétique et vectorologie” (IFR128, Lyon). Lentiviral infections were done accordingly to the protocol of the ISP platform (<http://canceropole-clara.com/page.asp?page=463>).

RNA extraction, RT-PCR and Quantitative PCR

RNA was extracted using Nucleospin RNA/protein kit following the manufacturer protocol (Macherey-Nagel, Germany). Reverse transcriptase (RT) reaction was performed using 500 to 1000ng of RNA. For qPCR, cDNA were diluted 1/20 using Mesa green qPCR Master Mix (Eurogentec, Angers, France). PCR was conducted in a 40 3 3000P real-time PCR system (Stratagene, La Jolla, CA). Two sets of PCR assays were conducted for each sample, the TLR9 and β 2-microglobulin primers were already used elsewhere [365, 725]. Amplification specificity was assessed for each sample by melting curve analysis, and the size of the amplicon checked by electrophoresis (data not shown). Relative quantification was performed using standard curve analysis. TLR9 mRNA levels were normalized to β 2-microglobulin mRNA levels and are presented as a ratio of gene copy number per 100 copies of β 2-microglobulin in arbitrary units (A.U).

Luciferase assay

Transient transfection of the reporter plasmid TLR9 luciferase, NF- κ B, p21 or pig3 luciferase was performed as previously described [355, 726].

Immunoblot analysis

Briefly, harvested cells were lysed in mild lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 0.5 mM and complete protease inhibitor (Roche, Meylan, France). Cellular protein content was determined by the Bradford assay (Bio-Rad, Marnes-la-Coquette, France); used for SDS-PAGE and immunoblotting onto a PVDF membrane. After incubation with primary antibodies, proteins were detected with secondary peroxidase-conjugated antibodies (promega, Madison, USA) and ECL. All the primary antibodies for western-blotting were from cell signaling but the β -actin (MP biomedical, Santa Ana, USA).

Proliferation assay

Doubling population assay and clonogenicity assay were previously described [726]. For MTT assays, cells were plated at 50 000, 100 000 and 150 000 cell/mL in 96 wells plate in quadruplicate. After 48 hours ten percent of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (5mg/mL) (sigma-aldrich, St Louis, USA) was added for four hours then the supernatants were removed and the cells resuspended in DMSO. The 550nm absorbance was read on a plate reader. For the flow cytometry proliferation analysis, the cells were either stain with PHK 26 fluorescent dye (sigma-aldrich) or CellTrace violet Cell proliferation kit (invitrogen, Villebon sur Yvette) following manufacturer recommendations.

Flow cytometry analysis

For synchronization experiments, 200 000 cells per mL were plated in a six wells dish. The next day thymidine (2mM final) was added to the cells, 18 hours later the cells were washed three times with PBS and complete medium was added for 9 hours. Then thymidine (2mM final) was added for 18 hours. The cells were then washed three times in PBS and complete medium containing BrdU was added. For BrdU cell cycle analysis of non synchronized cells, 100 000 cells per well were plated. The next day BrdU was added to the plate for 20 to 30 minutes. The cells were harvested and washed in PBS then fixed in 70% ethanol overnight at 4C. The

cells were treated with 3N HCl that was neutralized with 0.1M Na₂B₄O₇ pH8.5. The cells were then blocked and stained with an anti-BrdU (Biolegend, SanDiego, USA) and 7-AAD (Invitrogen, Villebon sur Yvette). For apoptosis experiment, Apoptosis detection kit I (Becton Dickinson, Le Pont de Claix, France) was used following manufacturer protocol. For TLR9 intracellular staining, one million cells were fixed with 2% paraformaldehyde and permeabilized in PBS containing 0.25% saponin. After blocking the cells were stained with a rat anti-human TLR9 (eBioscience) and with a secondary goat anti-rat alexa 633. The staining was analyzed on a BD LSRII using the software Diva and FlowJo (treestar, Ashland, USA).

Statistical analysis

All the statistical analysis have been performed using a two-tailed non paired t-test.

FIGURES LEGEND:

Figure 1: Characterization of TLR9 constructs and inducible cell line expressing TLR9. (A) Hek 293 were co-transfected with TLR9 encoded by pcDNA (Left panel) or pbabe (Right panel), the NFκB minimal consensus site promoter linked to the luciferase gene and the TK-Renilla reporter. Eighteen hours after transfection cells were stimulated with 3μM of CpG-B. Cells were harvested and luciferase activity measured. (B) (C) (D) Caski were stably transduced with PLVUT'-GFP and PLVUT'-TLR9 (tet-ON system). The cells were treated for 5 days with doxycyclin or left in tetracyclin free medium. (B) The mRNA was extracted and analyzed for TLR9 by qPCR and the level of expression was normalized to the β2-microglobulin. (C) TLR9 protein levels of the Caski cell lines were determined by western blotting. (D) Caski PLVUT'-GFP and PLVUT'-TLR9 were co-transfected with the NFκB luciferase and TK-renilla reporter. Eighteen hours after transfection cells were stimulated with 3μM of CpG-B. Cells were harvested and luciferase activity measured.

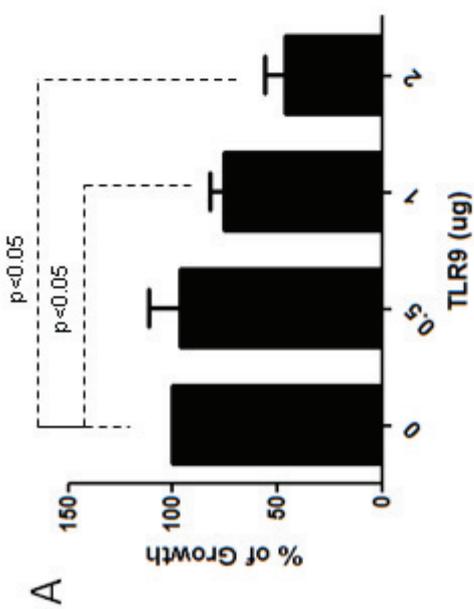
Figure 2: TLR9 expression slows down cell proliferation. (A) Hek 293 cells were transfected with increasing concentrations (0-2μg) of pcDNA-TLR9. Proliferation was assessed after forty eight hours by MTT assay. NiKs and NiKs HPV16E6E7 (B), or cervical cancer cell line SiHa and the HNSCC 124 cell line (C) were stably transduced with pbabe or pbabe_TLR9 and plated for a doubling population assay. The cells were harvested and reseeded every 5 days and the doubling populations were counted. The asterisk represent a condition where the pvalue was inferior at 0.05. (D) NiKs, NiKs stably expressing HPV16E6E7, CasKi and SiHa were stably transduced with an empty or TLR9 coding pbabe. After selection, cells were plated in a 6 wells plate. After four weeks the plated clones were stained with crystal violet.

Figure 3: TLR9 expression did not affect apoptosis but delayed S-phase. (A) Caski cells stably transduced with empty pbabe (left panel) or TLR9 encoded pbabe (right panel) were stained with Annexin V and propidium iodide to determine percentage of apoptotic and necrotic cells. (B) Caski-pbabe and Caski-TLR9 were ethanol fixed and DNA content was analyzed by propidium iodide staining. (C) The Caski PLVUT'-GFP and PLVUT'-TLR9 cell lines were induced with doxycyclin. After five days the cells were left untreated (cycling condition) or synchronized with a thymidine double block (numbered condition). BrdU was pulsed for thirty minutes in the cycling condition and added at the time of the release for cells the synchronized cells. The cells were harvested and fixed with ethanol at different time points indicated on the chart. BrdU content was analyzed by flow cytometry. (D) HNSCC 136 cells were stably transduced with pbabe (left panel), pbabe_GFP (middle

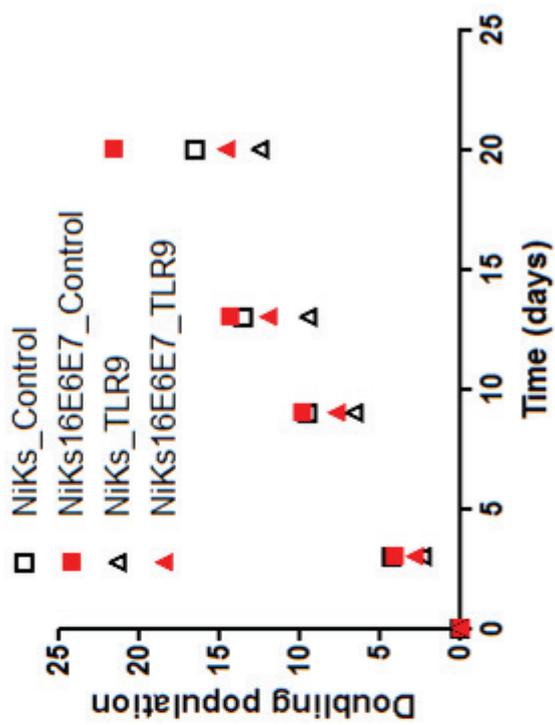
panel) or pbabe_TLR9 (right panel). Cells were pulsed with BrdU for twenty minutes and cell cycle analysis was performed after BrdU and 7-AAD staining by flow cytometry.

Figure 4: TLR9 expression induces cell cycle inhibitors expression. Hek 293 cells were transiently co-transfected with pcDNA_TLR9 or pcDNA_p53, and the p21 (A) or pig3 (B) promoter luciferase constructs and the TK-Renilla reporter. Forty eight hours after transfection cells were harvested and luciferase activity measured. (C) Caski, Hela and SiHa were stably transduced with pbabe or pbabe TLR9. Cell lysates were analyzed for TLR9, p21, p53, p27 and β -actin protein content by Western Blotting.

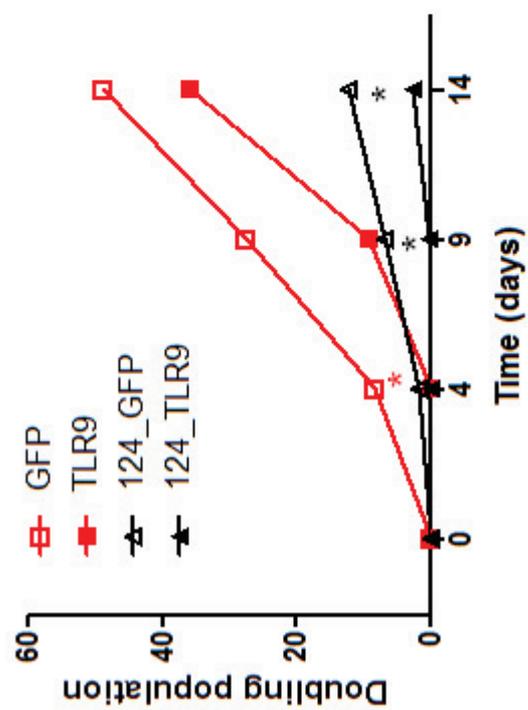
Figure 5: Cell death associated DAMP do not trigger TLR9 mediated inhibition of proliferation. SiHa, Caski, HNSCC 124 and 136 were transduced with pbabe or pbabe TLR9; the cells were selected with puromycin and kept in culture for two months. (A) SiHa expressing pbabe or pbabe TLR9 were treated with increasing concentrations of cisplatin (5 to 75 μ M), and proliferation was measured by MTT assay after four days. The bars indicate the percentage of surviving cells as compared to the non treated condition. (B) Puromycin sensitive cells were treated with puromycin for three days and the supernatant was transferred onto HNSCC 136 expressing TLR9 or not. The cellular proliferation was measured after four days by MTT assay. The bars indicate the percentage of survival as compared to the non treated condition (NT). (C-E) HNSCC 136 and TLR9 expressing cells were stained with a violet cell tracing reagent. The proliferation was analyzed by flow cytometry after intracellular staining for TLR9. The pbabe empty or TLR9 cells were co-cultured for four in regular medium (C), in presence of cells pretreated with cisplatin (D) or in presence of the supernatant of cells treated with puromycin (E).



B



C



D

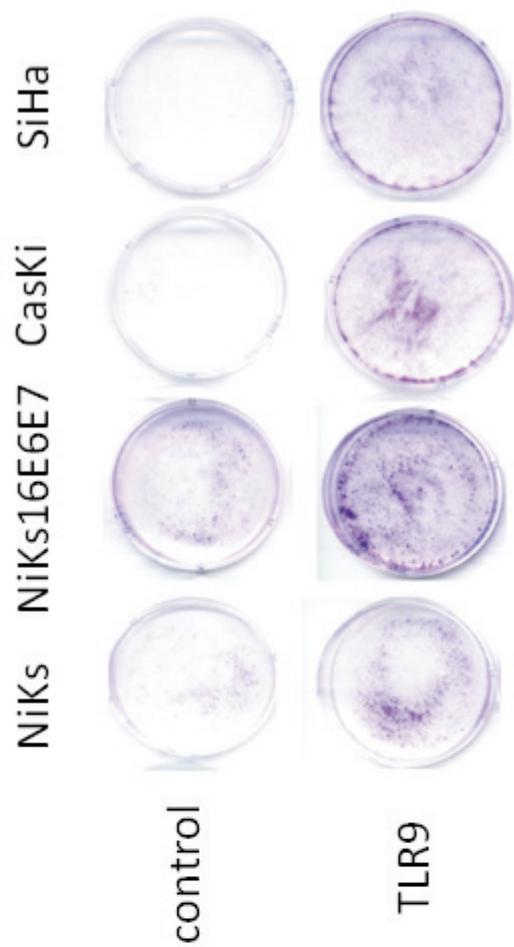


Figure 2

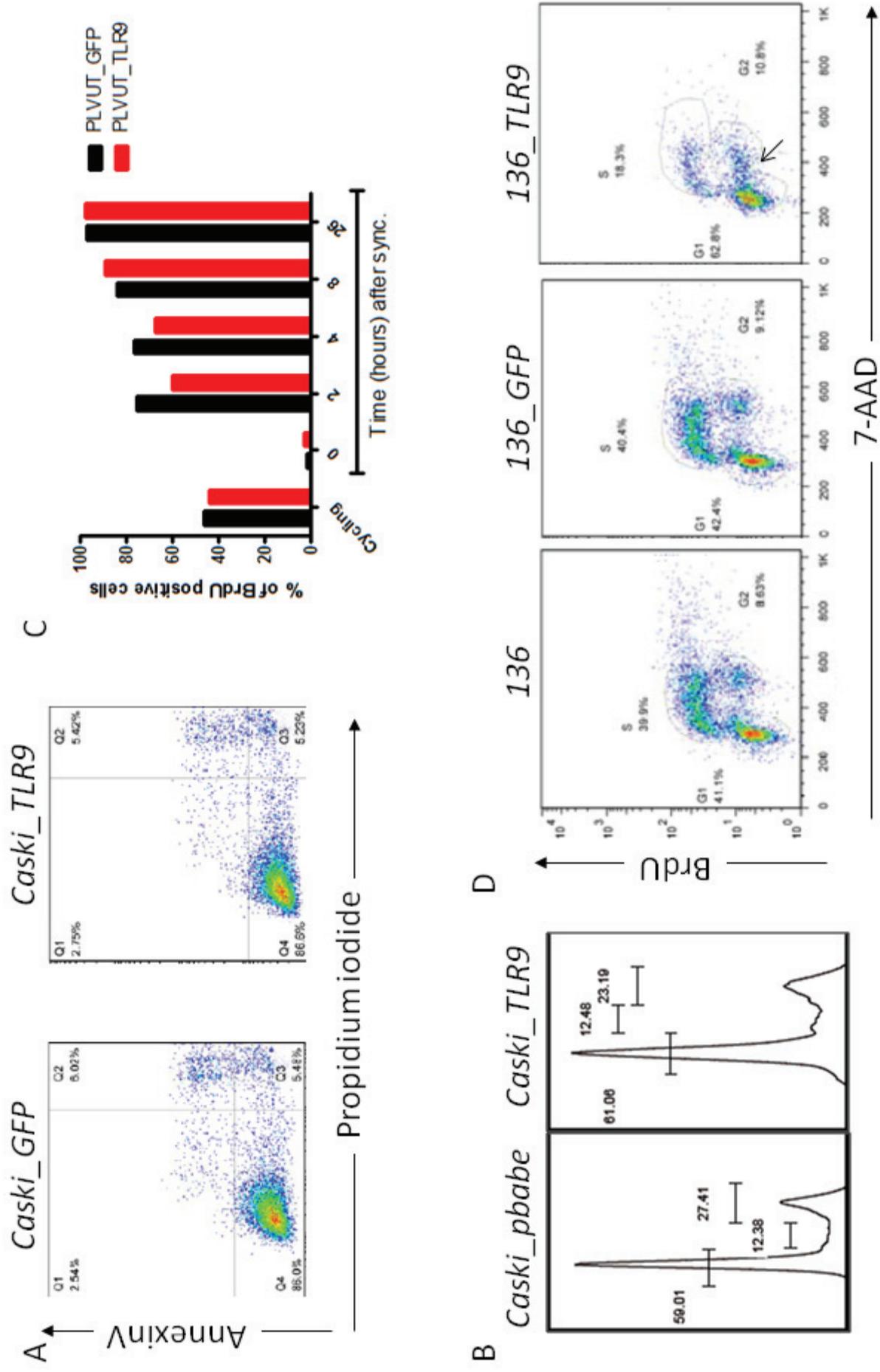
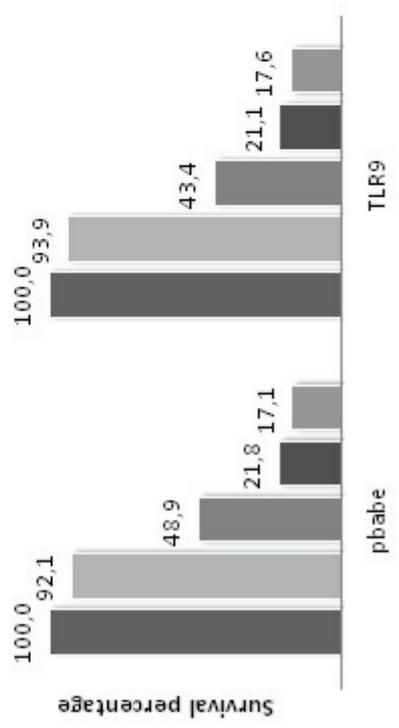


Figure 3

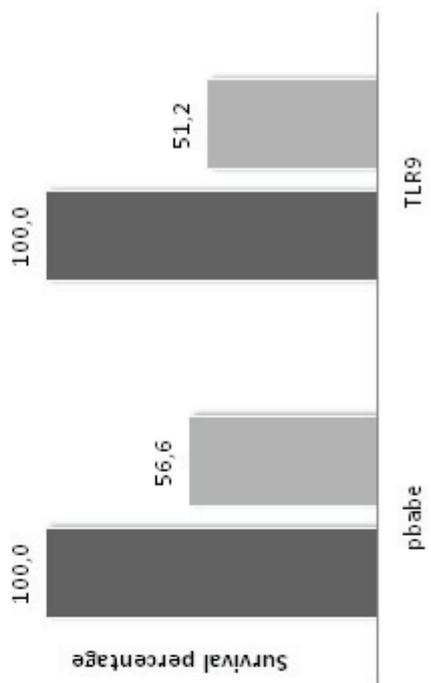
A

■ NT ■ CDDP_5uM ■ CDDP_25uM
 ■ CDDP_50uM ■ CDDP_75uM

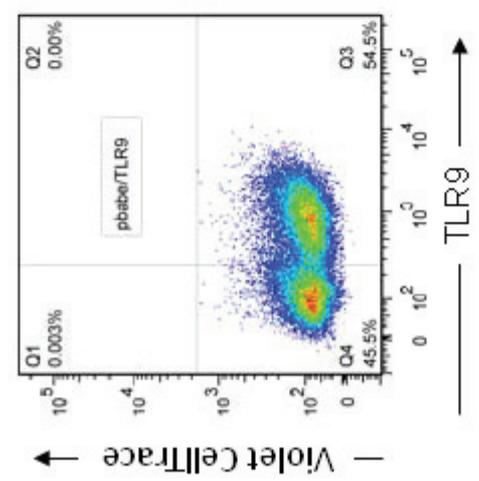


B

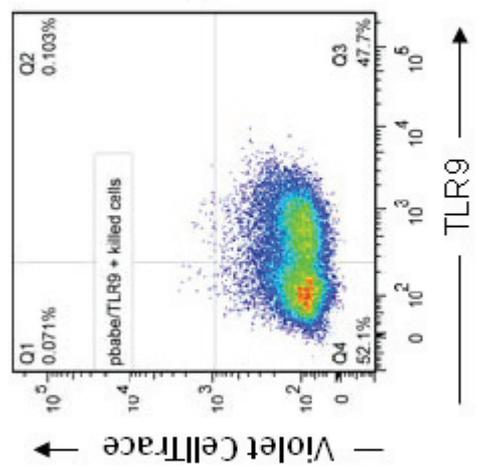
■ NT ■ Sup



C



D



E

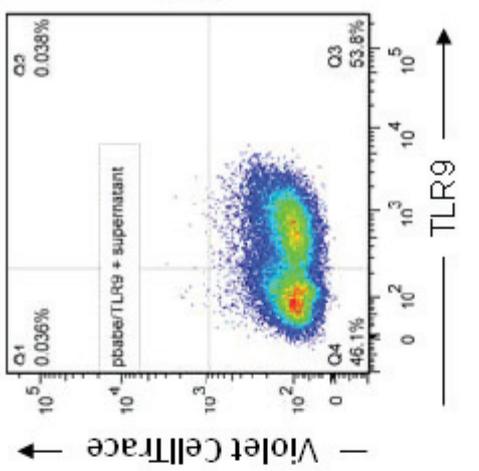
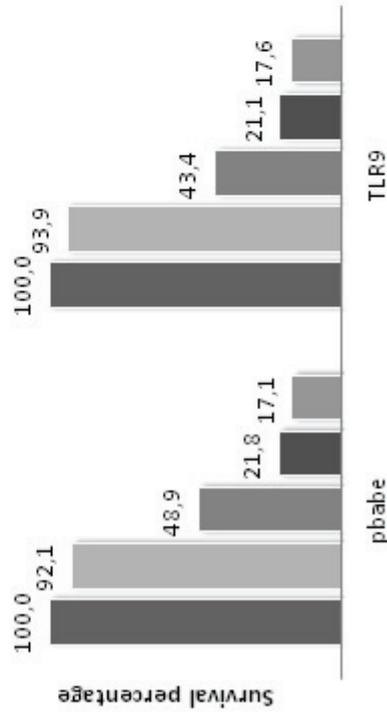


Figure 5

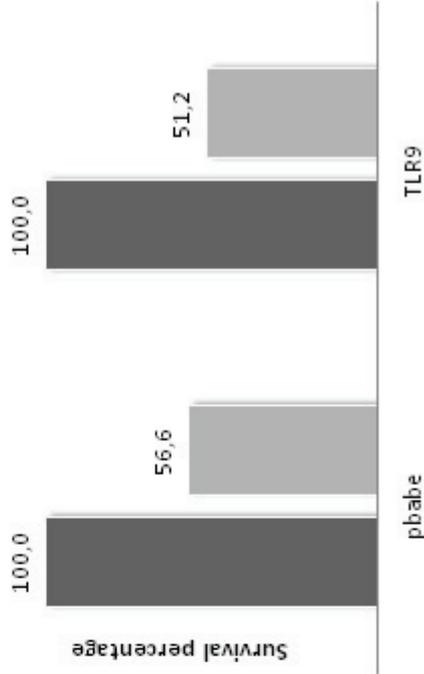
A

■ NT ■ CDDP_5uM ■ CDDP_25uM
 ■ CDDP_50uM ■ CDDP_75uM

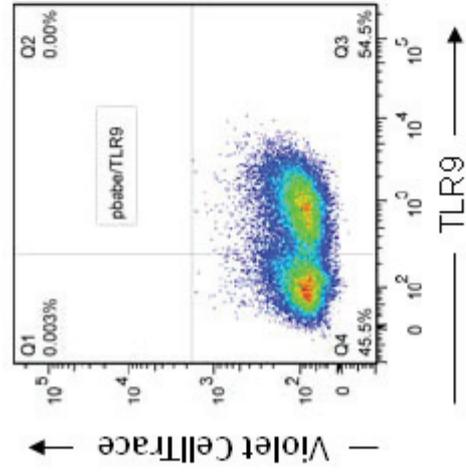


B

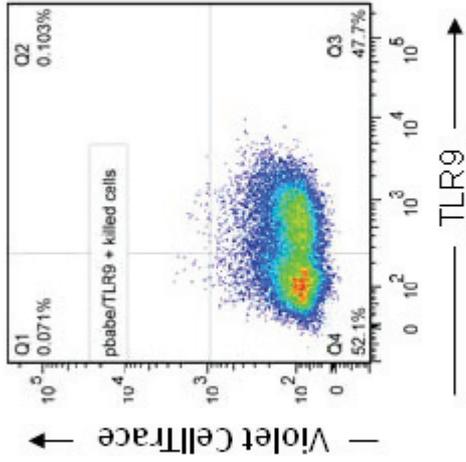
■ NT ■ Sup



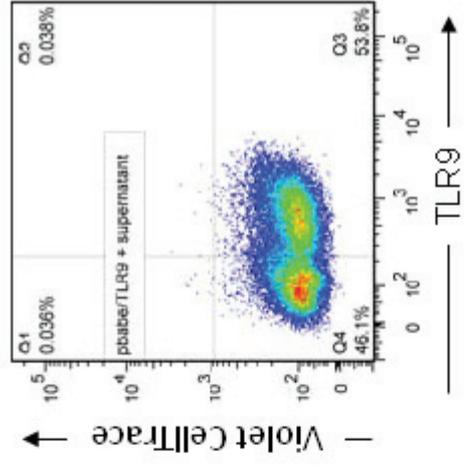
C



D



E



[TLR9]vs[Mock]

Fold change	Regulation	Definition
20.149519	up	Homo sapiens toll-like receptor 9 (TLR9), transcript variant A, mRNA.
6.898789	up	Homo sapiens colony stimulating factor 3 (granulocyte) (CSF3), transcript variant 1, mRNA.
14.917988	up	Homo sapiens colony stimulating factor 3 (granulocyte) (CSF3), transcript variant 1, mRNA.
-1.4177902	down	Homo sapiens heat shock 70kDa protein 6 (HSP70B') (HSPA6), mRNA.
-3.0508826	down	Homo sapiens heat shock 70kDa protein 6 (HSP70B') (HSPA6), mRNA.

Table 1: Microarray analysis of hek 293 cells transiently expressing TLR9 Hek 293 cells were transiently transfected with pcDNA or pCDNA_TLR9. After 48h the cells were harvested and the RNA extracted. Quality control and quantification of extracted RNAs will be performed using Agilent 2100 bioanalyzer. The genes expression profiling was examined in quadruplicate using an Illumina HT 12v4 array. The raw data were analyzed using genespring. The data are summarized in the table 1. The first column indicate the fold change in expression between the cells expressing TLR9 and the control, the second column the sense of the regulation and the third column the name of the gene.

EBV infects most of the human population and is associated with a number of human diseases including cancers. Moreover, evasion of the immune system and chronic infection is an essential step for EBV-associated diseases. In this paper, we show that EBV can alter the regulation and expression of TLRs, the key effector molecules of the innate immune response. EBV infection of human primary B cells resulted in the inhibition of TLR9 functionality. Stimulation of TLR9 on primary B cells led to the production of IL-6, TNF- α , and IgG, which was inhibited in cells infected with EBV. The virus exerts its inhibitory function by decreasing TLR9 mRNA and protein levels. This event was observed at early time points after EBV infection of primary cells, as well as in an immortalized lymphoblastoid cell line. We determined that the EBV oncoprotein latent membrane protein 1 (LMP1) is a strong inhibitor of TLR9 transcription. Over-expression of LMP1 in B cells reduced TLR9 promoter activity, mRNA, and protein levels. LMP1 mutants altered in activating the NF- κ B pathway prevented TLR9 promoter deregulation. Blocking the NF- κ B pathway recovered TLR9 promoter activity. Mutating the NF- κ B cis element on the TLR9 promoter restored luciferase transcription in the presence of LMP1. Finally, deletion of the LMP1 gene in the EBV genome abolished the ability of the virus to induce TLR9 down-regulation. Our study describes a mechanism used by EBV to suppress the host immune response by deregulating the TLR9 transcript through LMP1-mediated NF- κ B activation.



EBV Latent Membrane Protein 1 Is a Negative Regulator of TLR9

This information is current as of August 3, 2011

Ikbal Fathallah, Peggy Parroche, Henri Gruffat, Claudia Zannetti, Hanna Johansson, Jiping Yue, Evelyn Manet, Massimo Tommasino, Bakary S. Sylla and Uzma A. Hasan

J Immunol 2010;185:6439-6447; Prepublished online 27 October 2010;
doi:10.4049/jimmunol.0903459
<http://www.jimmunol.org/content/185/11/6439>

-
- Supplementary Data** <http://www.jimmunol.org/content/suppl/2010/10/28/jimmunol.0903459.DC1.html>
- References** This article **cites 39 articles**, 23 of which can be accessed free at: <http://www.jimmunol.org/content/185/11/6439.full.html#ref-list-1>
- Article cited in:
<http://www.jimmunol.org/content/185/11/6439.full.html#related-urls>
- Subscriptions** Information about subscribing to *The Journal of Immunology* is online at <http://www.jimmunol.org/subscriptions>
- Permissions** Submit copyright permission requests at <http://www.aai.org/ji/copyright.html>
- Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at <http://www.jimmunol.org/etoc/subscriptions.shtml/>

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994. Copyright ©2010 by The American Association of Immunologists, Inc. All rights reserved. Print ISSN: 0022-1767 Online ISSN: 1550-6606.



EBV Latent Membrane Protein 1 Is a Negative Regulator of TLR9

Ikbal Fathallah,^{*,1} Peggy Parroche,^{*,†,‡,1} Henri Gruffat,^{‡,§} Claudia Zannetti,^{*,†,‡} Hanna Johansson,^{*} Jiping Yue,^{*} Evelyn Manet,^{‡,§} Massimo Tommasino,^{*} Bakary S. Sylla,^{*} and Uzma A. Hasan^{*,†,‡}

EBV infects most of the human population and is associated with a number of human diseases including cancers. Moreover, evasion of the immune system and chronic infection is an essential step for EBV-associated diseases. In this paper, we show that EBV can alter the regulation and expression of TLRs, the key effector molecules of the innate immune response. EBV infection of human primary B cells resulted in the inhibition of TLR9 functionality. Stimulation of TLR9 on primary B cells led to the production of IL-6, TNF- α , and IgG, which was inhibited in cells infected with EBV. The virus exerts its inhibitory function by decreasing TLR9 mRNA and protein levels. This event was observed at early time points after EBV infection of primary cells, as well as in an immortalized lymphoblastoid cell line. We determined that the EBV oncoprotein latent membrane protein 1 (LMP1) is a strong inhibitor of TLR9 transcription. Overexpression of LMP1 in B cells reduced TLR9 promoter activity, mRNA, and protein levels. LMP1 mutants altered in activating the NF- κ B pathway prevented TLR9 promoter deregulation. Blocking the NF- κ B pathway recovered TLR9 promoter activity. Mutating the NF- κ B *cis* element on the TLR9 promoter restored luciferase transcription in the presence of LMP1. Finally, deletion of the *LMP1* gene in the EBV genome abolished the ability of the virus to induce TLR9 downregulation. Our study describes a mechanism used by EBV to suppress the host immune response by deregulating the TLR9 transcript through LMP1-mediated NF- κ B activation. *The Journal of Immunology*, 2010, 185: 6439–6447.

Epstein-Barr virus belongs to the human γ -herpesvirus type 4 family (HHV4) and infects >95% of the adult population with a specific tropism for B and epithelial cells (1). When primary infections occur at young ages, EBV may cause a benign lymphoproliferative disease known as infectious mononucleosis. Chronic EBV infections are associated with a set of severe human diseases including Burkitt's lymphoma, Hodgkin's disease, posttransplant lymphoma, nasopharyngeal, and some forms of gastric carcinomas (2). EBV also induces lymphoma development in genetically predisposed immune deficiency families affected by the X-linked lymphoproliferative syndrome (3–5). In vitro, EBV can efficiently infect human resting

B lymphocytes and transform them into indefinitely proliferating lymphoblastoid cell lines (LCLs). In these cells, several latent viral genes are expressed including the latent membrane protein 1 (LMP1) (2). LMP1 is essential for EBV-induced B cell immortalization and long-term proliferation, and it is the only latent protein that displays in vitro transforming properties (6). LMP1 is a plasma membrane aggregating protein that mimics the activated form of TNFR, CD40, or CD30 and exerts its transforming functions via a long C-terminal cytosolic domain (7–9). Accordingly, LMP1 can activate several signal transduction pathways, such as MAPK, PI3K and JAK3/STAT, and NF- κ B (8, 10–13). EBV LMP1 C-terminal activation region 1 (CTAR1) and CTAR2 both activate NF- κ B (14). NF- κ B is a transcription factor with the prototype being p65/Rel A, which is kept inactive in the cytoplasm of quiescent cells by its inhibitor I κ B. NF- κ B-inducing agents, including proinflammatory cytokines, lead to activation of I κ B kinase complex (IKK α / β / γ), which, in turn, phosphorylates and promotes degradation of I κ B. This event results in NF- κ B nuclear translocation and expression of genes encoding key players of cell survival, cellular proliferation, and immune response. NF- κ B is constitutively activated in many types of viral and nonviral-associated human cancers.

The normal life cycle of EBV infection and the function of the LMP1 in relation to the adaptive immune response are subtle and foremost, very complex. The virus must be able to persist for life in the host without doing much harm to ensure its survival (6, 15, 16). For instance, EBV targets preferentially circulating memory B cells expressing low levels of MHC class I and costimulatory molecules to avoid presentation to the immune cells (17). In addition, EBV also has the ability to downregulate the expression of MHC molecules in proliferating B cells (15). During latent infection, a small number of EBV Ags are expressed at a low copy number, which leads to low T cell CD8⁺ epitope presentation (15, 18). Thus, escaping adaptive immune responses play a key role in

*International Agency for Research on Cancer, 69008 Lyon; [†]Innate Immunity and Cancer Team, INSERM U851, Faculté de Médecine Lyon Sud, 69495 Pierre Benite; [‡]Institut Fédératif de Recherche 128 BioSciences Gerland-Lyon Sud, 69366 Lyon Cedex 7; and [§]Laboratoire de Biologie Moléculaire des γ -Herpesvirus INSERM U758, F-69007 Lyon, France

[†]I.F. and P.P. contributed equally to this work.

Received for publication October 23, 2009. Accepted for publication September 16, 2010.

This work was supported by grants from La Ligue contre le Cancer (Comité de Savoie to I.F. and P.P.; Comité du Rhône et de la Drome to B.S.S.); Association pour la Recherche contre le Cancer (to P.P. and U.H.; Grants 4914 and 3420); Government Français and DEMINAP Région Rhône-Alpes (to M.T. and U.A.H.); International Agency for Research on Cancer postdoctoral fellowship program (to J.Y.); Agence Nationale pour la Recherche (Programme MIB 2009); and Réseau INSERM Herpesvirus et Cancer.

Address correspondence and reprint requests to Dr. Uzma A. Hasan, Faculty of Medicine, Lyon Sud, 165 Chemin du Grand Revoyet, 69495 Pierre Benite, France. E-mail address: uzma.hasan@inserm.fr

The online version of this article contains supplemental material.

Abbreviations used in this paper: CTAR, C-terminal activation region; IKK, I κ B kinase; LCL, lymphoblastoid cell line; LMP1, latent membrane protein 1; qPCR, quantitative PCR; RT, reverse transcriptase; WT, wild-type.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/\$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0903459

EBV survival, although how EBV escapes from the innate immune response is still to be studied (15).

The innate immune response is the first line of defense against infection. Innate immunity uses several pattern recognition receptors to sense pathogen-associated molecular patterns. The TLRs are the most studied pattern recognition receptors, and activation by pathogen components leads to robust immune response (19). TLR2 and TLR4 recognize Gram-positive and -negative bacteria cell wall products, respectively. TLR5 recognizes a structural epitope of bacterial flagellin, whereas TLR3, TLR7, and TLR8 have been demonstrated to recognize different forms of nucleic acid derived from viruses. TLR9 recognizes dsDNA sequences from bacteria or viruses in the form of nonmethylated CpG oligonucleotides. TLR activity has been intensely investigated in the B cells, the host cell of EBV infection. B cells express a variety of TLRs such as 1, 2, 6, 7, 9, and 10 (20). Furthermore, TLR7 and TLR9 expression and functionality were historically characterized in B cells (21, 22). Classically, B cell activation was shown to require BCR cross-linking and CD40 signaling. Yet, a third signal needs to be delivered coupled by BCR-TLR7 or -TLR9 in response to R848 or CpG, respectively, to produce optimal Ab responses to T cell-dependent Ags (23). In terms of viral recognition, it has been shown that TLR9 is required for the innate immune response to human DNA viruses including HSV-2 (21, 24) and EBV (25), and the involvement of TLR9 in the antiviral immune response to the murine γ -herpesvirus MHV-68 (a murine model of EBV infection) was demonstrated in a recent study (26). However, the direct effect of EBV infection in B cells on the TLR9-mediated innate immune response has not been elucidated.

We show that EBV escapes the innate immune response by downregulating TLR9 transcription. Most importantly, the virus exerts this activity by using its major oncoprotein LMP1 via activation of NF- κ B signaling.

Materials and Methods

Cell culture and EBV infection

RPMI 8226 cells were a kind gift from the laboratory of Christophe Caux (Centre Leon Berard, Lyon, France). PBMCs were freshly isolated by density-gradient centrifugation using Ficoll Hypaque (σ). B cells were purified from the PBMCs using anti-CD19-coated nanoparticle (EasySep; StemCell Technologies, Vancouver, British Columbia, Canada) or using the B cell isolation kit II from Miltenyi Biotec (Auburn, CA). The purity of the cells was assessed by flow cytometry (FACSCalibur, BD Biosciences Pharmingen, San Diego, CA, and FACLSRII) using an anti-CD19 (BD Pharmingen). In total, 10 PBMC donors were used for this study in which primary B cells and corresponding LCLs were generated. B cells and RPMI 8226 cells were infected with recombinant EBV expressing GFP, as described previously (27). In addition, purified B cells were also infected for 5 d with a recombinant EBV deleted of the LMP1 gene (EBV_{ΔLMP1}) also expressing the GFP, as described previously (28). Primary B cells, infected B cells, and RPMI 8226 cells were maintained in culture in RPMI 1640 medium (GIBCO; Invitrogen life Technologies, Cergy-Pontoise, France) supplemented with 10% FBS, 100 U/ml penicillin G, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate (PAA, Pasching, Austria). HEK293 T cells were obtained from American Type Culture Collection and maintained in DMEM supplemented as described earlier.

TLR ligands

CpG2006-G and GpC2006 were purchased from Sigma-Aldrich (St. Louis, MO). Pam2CysKKKK was purchased from EMC (Tubingen, Germany) microcollection and used at 4 nM. Poly(I:C) and R848 were purchased from Invivogen and used at 10 μ M and 10 μ g/ml, respectively. Repurified LPS was used at 500 ng/ml (Invivogen); Flagellin (*Salmonella muenchen*) was from Calbiochem (San Diego, CA) and used at 50 ng/ml.

Retroviral infections

RPMI 8226 cells were transduced using retroviral supernatant generated by transfection of pLNSX and pLNSX LMP1 (gift from L. Young, University of Birmingham, Birmingham, U.K.) into phoenix cells using the method previously described (29).

RNA extraction and reverse transcription

Total RNA was extracted from cells (RNeasy; Qiagen [Courtabouef Cedex, France] or Macherey Nagel [Hoerd, France]). One microgram of RNA was reverse transcribed using the first strand cDNA synthesis kit according to the manufacturer's protocol (Fermentas, St. Leon-Rot, Germany).

Quantitative PCR

The reverse transcriptase (RT) reaction was diluted according to detection sensitivity, and 5 μ l of the diluted sample was added to a 15- μ l PCR mixture containing 4 μ M of each primer and 8.75 μ l Mesa green quantitative PCR (qPCR) Master Mix (Eurogentec, Angers, France). PCR was conducted in a 40 \times 3000P real-time PCR system (Stratagene, La Jolla, CA). Two sets of PCR assays were conducted for each sample using the following primers specific for TLR9: forward: 5'-CGT CTT GAA GGC CTG GTG TTG A-3'; reverse: 5'-CTG GAA GGC CTT GGT TTT AGT GA-3'. Housekeeping genes β_2 -microglobulin or β -actin were used. The sequence of the LMP1 primers was: forward: 5'-CCC CCT CTC CTC TTC CATAG-3'; reverse: 5'-GCC AAA GAT GAA CAG CAC AA-3'. Amplification specificity was assessed for each sample by melting curve analysis, and the size of the amplicon checked by electrophoresis (data not shown). Relative quantification was performed using standard curve analysis. TLR9 expression data were normalized with β_2 -microglobulin or β -actin and are presented as a ratio of gene copy number per 100 copies of β_2 -microglobulin or β -actin \pm SD.

Western blotting

Whole-cell lysates were prepared using lysis buffer (20 mM Tris/HCl, pH 8, 200 mM NaCl, 0.5% Nonidet P40, 1 mM EDTA, 10 mM NaF, 1 mM PMSF, 1 μ g/ml leupeptin, and 1 mM Na₃VO₃). The protein concentration was measured using BCA (BC Assay reagent; Uptima Interchim, Montluçon, France). Adequate quantity of proteins (between 15 and 25 μ g) was used for SDS-PAGE analysis and immunoblotting.

Luciferase assay

Transient transfection of the reporter plasmid TLR9 luciferase or NF- κ B luciferase was performed as previously described (30, 31).

ELISA

Cells were seeded at 2×10^5 /200 μ l/well and stimulated for 24 h. Supernatant was harvested, and IL-6 (R&D Systems, Minneapolis, MN) and TNF- α (Bender MedSystems, Vienna, Austria) were measured following the manufacturer's protocol. Protein concentration of three wells per cell type was measured to normalize cytokine secretion. The IgG secretion was evaluated in supernatants harvested 120 h after CpG and GpC stimulation using the Human IgG ELISA Quantitation kit (Bethyl Laboratories, Montgomery, TX).

Confocal staining

Primary B cells and LCL from the same donor were fixed using cold acetone/methanol (v/v) and then saturated using 5% BSA (PAA) in 0.05% TBS-Tween-20 at room temperature for 1 h. Slides were mounted using Vectra shield mounting media with DAPI. Analysis of the slides was performed using a Leica confocal TCS SP5 System (Leica, Wetzlar, Germany).

Plasmid constructs

The NF- κ B luciferase and Renilla reporter gene constructs were purchased from BD Clontech (Palo Alto, CA). The pGL3-TLR9 promoter was obtained from Fumihiko Takeshita (31). The NF- κ B site (⁻⁴¹³GGG-GAGTGCCC⁻⁴⁰³) was mutated to (⁻⁴¹³CTCGAGTGCCC⁻⁴⁰³) using the QuikChange site-directed mutagenesis kit (Stratagene) according to manufacturer's instructions. pcDNA3-LMP1 (15) was obtained from E. Kieff (Harvard Medical School, Boston, MA). The expression vectors for LMP1 and LMP1 mutants: LMP1AxAxA (Mut 1), LMP1 378 STOP (Mut 2), and LMP1AxAxA/378STOP (Mut 3) were a kind gift from L. Young (University of Birmingham) and described by Eliopoulos and colleagues (32). NF- κ B superrepressor Δ N-I κ B α , which lacks the sequence that codes for the first N-terminal 36 aas, was obtained from E. Kieff and cloned into pcDNA3. The dominant negative constructs IKK α -DN (pcRK5-Flag-IKK α -KA) and IKK β -DN (pcRK5-Flag-IKK β -KA) were obtained from D. Goeddel (Tulirak, San Francisco, CA).

Abs

The following Abs were used: anti-TLR9 (2254; Cell Signaling Technology, Beverly, MA), anti-LMP1 Ab (S12; a gift from Georges Mosialos, Alexandra Fleming Institute, Varkiza, Greece), anti-rabbit/mouse IgG-conjugated to HRP (W401B and W40213; Promega, Madison, WI),

Alexa Fluor 488 mouse anti-human CD19 (BD Pharmingen), Alexa Fluor 488 mouse IgG1 κ isotype control (BD Pharmingen), and anti-GFP (Roche, Basel, Switzerland).

Results

EBV infection hampers TLR9 pathway in B cells

To address whether EBV affects TLR-regulated pathways, primary human B cells (Fig. 1A) were first infected with recombinant EBV virus GFP gene (27). The efficiency of EBV infection was evaluated by monitoring GFP expression using confocal microscopy (Supplemental Fig 1). Additional evidence of viral infection was seen in viral gene expression, unscheduled proliferation, and B cell immortalization into LCL (data not shown).

We next determined the functionality of TLR2-9 pathways in noninfected primary B cells and in LCL derived from the same batch of primary B cells by adding their specific ligands and monitoring the secretion of IL-6. We identified the optimal TLR ligand concentrations for maximal IL-6 secretion poststimulation in primary B cells (data not shown). No significant difference in R848-induced TLR7 activation was detected in primary B cells or LCL, indicating that EBV has no effect on the TLR7 activity

(Fig. 1B). Primary B cells, as well as LCL, were refractory to LPS-induced TLR4 stimulation. Poly(I:C) marginally activated the TLR3 pathway in primary cells; however, this activity was inhibited in LCL. In contrast, the levels of secreted IL-6 were strongly decreased in LCL in comparison with primary cells on engagement of TLR2 (pam2Cys4K), TLR5 (Flagellin), and TLR9 (CpG; * $p < 0.006$, Student nonpaired t test; Fig. 1B).

Previous work has highlighted the importance of TLR9 in controlling EBV or murine γ -herpesvirus in animal experimental models (26); thus, we focused our study on the characterization of the events involved in the alteration of the TLR9 pathway. The EBV-mediated inhibition of TLR9 pathway was further confirmed using serial dilutions of CpG, whereas no significant changes in IL-6 levels were observed in primary cells and LCL on exposure to GpC control oligos (Fig. 1C).

To further demonstrate the impairment of TLR9 functionality after EBV infection, we monitored TNF- α and IgG secretion on TLR9 stimulation. As shown in Fig. 1D, TNF- α secretion was severely altered in EBV-infected LCL compared with primary B cells. Similarly, a significant reduction of IgG secretion was also observed in LCL after stimulation with TLR9 ligand (Fig. 1E).

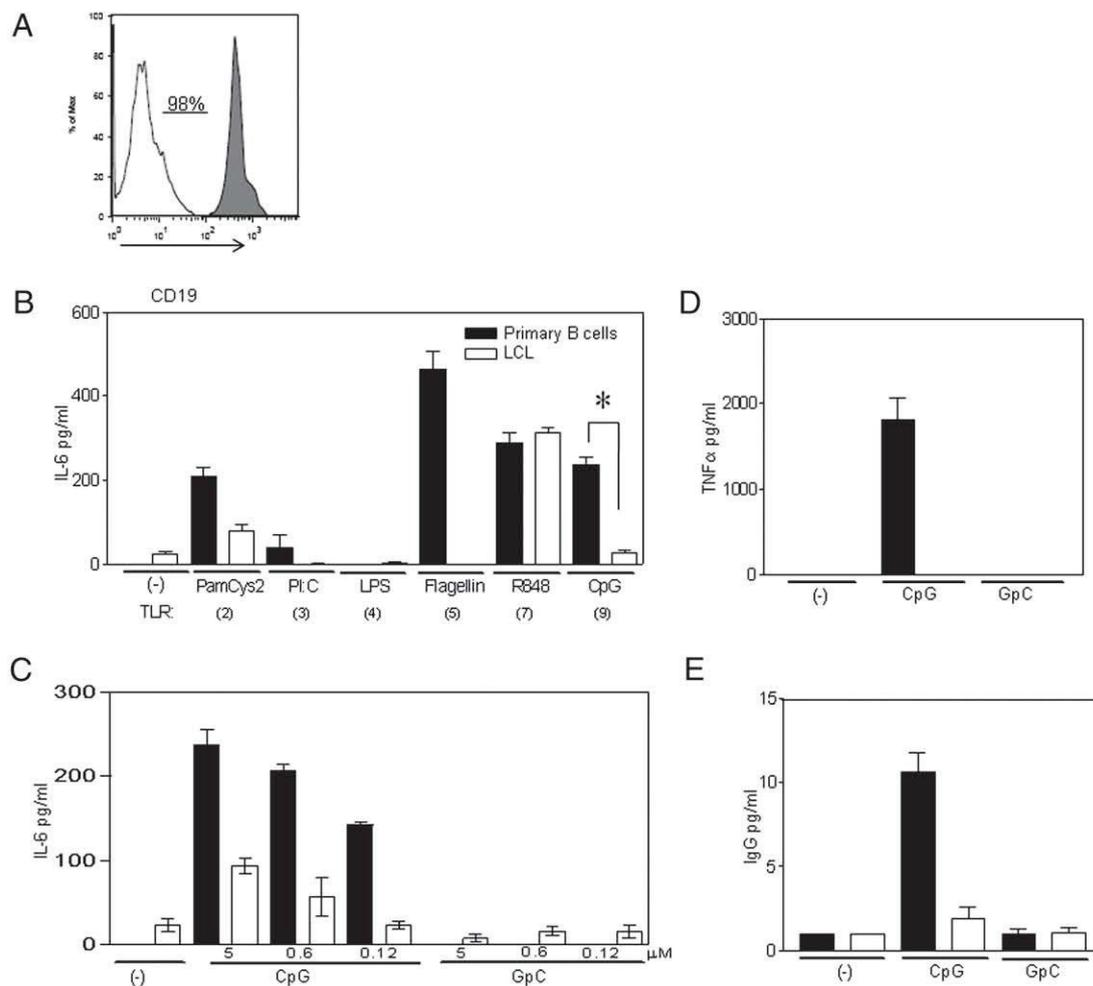


FIGURE 1. EBV affects TLR-mediated cytokine production in B cells. *A*, Purified primary B cells from PBMCs were extracted from several donors. B cell purity was between 96 and 98% as measured by flow cytometry. *B*, TLR-induced secretion of IL-6 in primary B cells and in EBV-immortalized LCLs. Levels of secreted IL-6 were measured by ELISA from primary B cells and LCLs (>5 wk infection), 24 h poststimulation with different TLR ligands. *C*, Secretion of IL-6 in primary B cells and LCLs stimulated with increasing concentrations of TLR9 ligand CpG 2006. GpC was used as a control for CpG stimulation. *D*, TLR9-induced secretion of TNF- α in B cells and LCLs. Secretion of TNF- α was measured by ELISA from primary B cells and LCLs, 24 h poststimulation with CpG. *E*, Production of IgG from primary B cells and LCLs was measured by ELISA, 120 h poststimulation with CpG. Data for IgG levels were normalized by dividing values of stimulated cells by nonstimulated cells. Results shown represent the SD of the mean of four experiments performed in B cells purified by CD19⁺ and three experiments performed with negative selection of B cells.

Taken together, our data demonstrated that EBV inhibits TLR2, TLR5, and TLR9 signaling pathway in B cells.

EBV downregulates TLR9 expression

To elucidate the mechanisms underlying the EBV-induced TLR9 inhibition, we measured the TLR9 expression levels in LCL compared with primary B cells by RT-qPCR. As shown in Fig. 2A, a significant reduction of TLR9 mRNA was observed in LCLs in comparison with primary B cells. This inhibition of TLR9 mRNA expression correlated with a lower level of TLR9 protein in LCLs (Fig. 2B). In these cells, infection with EBV was monitored by GFP expression (Supplemental Fig. 1).

TLR9 downregulation observed in LCLs might be a consequence because of the immortalization of B cells induced by EBV or, alternatively, associated with a specific EBV function. To evaluate whether EBV has a direct role in TLR9 downregulation, we infected the immortalized EBV-negative RPMI 8226 B cell line, which expressed high levels of TLR9 (30, 31), with EBV. We observed that EBV infection led to downregulation of TLR9 mRNA and protein levels in RPMI 8226 cells (Figs. 2C, 2D), providing evidence for a direct involvement of EBV in the event. In addition, the EBV-induced TLR9 protein downregulation was observed at early stages of infection in B cells, before immortalization (Fig. 2E). Together, these data show that EBV plays a direct role in downregulating TLR9 expression.

LMP1 negatively regulates TLR9 expression in B cells

We have previously reported that the main oncoproteins of HPV16, namely, E6 and E7, suppress TLR9 levels in primary keratinocytes and B cells (30). Therefore, we hypothesized that EBV may use its main oncoprotein LMP1 to suppress the TLR9 transcript in B cells. RT-qPCR analysis showed that LMP1 expression correlates with a decrease in expression of TLR9 mRNA (Fig. 3A). Indeed, the appearance of a few copies of LMP1 at 24 h is associated with a significant decrease of TLR9 mRNA. A further decrease of TLR9 mRNA occurred when a high expression of LMP1 is observed at 48 h and 5 d postinfection (Figs. 2E, 3A). LMP1 protein expression was confirmed by Western blotting (data not shown). To determine the direct role of LMP1 in TLR9 downregulation, we transduced the RPMI 8226 B cell line with recombinant retrovirus expressing LMP1 and, as a control, with an empty retrovirus (pLNSX). LMP1 expression resulted in a decrease of TLR9 mRNA (Fig. 3B). In addition, infection with a recombinant EBV lacking LMP1 (EBV $_{\Delta}$ LMP1) in primary B cells had no effect on TLR9 protein expression (Fig. 3C). Thus, LMP1 plays a central role in EBV-mediated downregulation of TLR9.

LMP1 inhibits TLR9 promoter activity

LMP1 may decrease TLR9 by affecting the stability of TLR9 transcript or directly inhibiting TLR9 transcription. To discriminate between these two possibilities, we determined whether LMP1

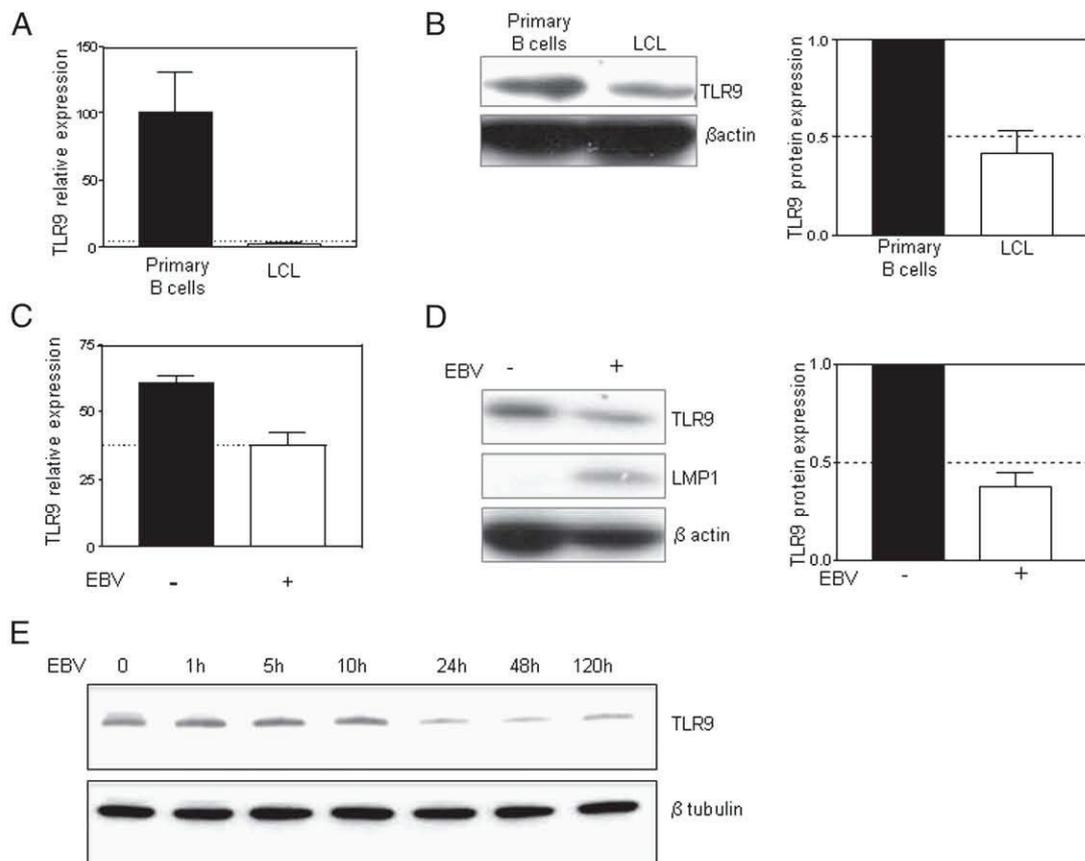
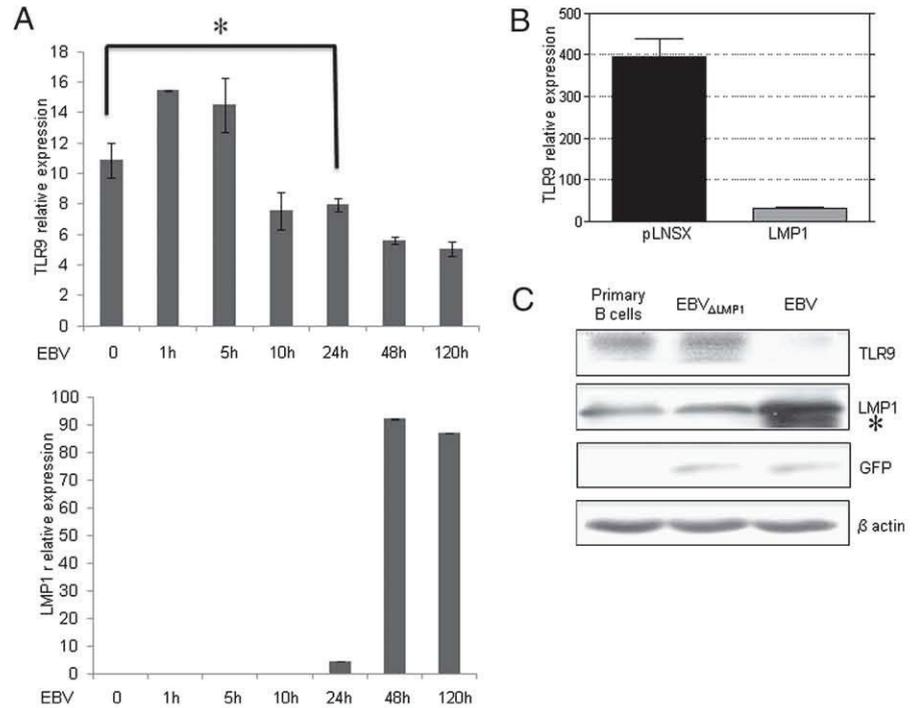


FIGURE 2. EBV downregulates TLR9 mRNA and protein expression in B cells. *A*, Total RNA was extracted from primary B cells and LCL. After reverse transcription, qPCR was performed using TLR9-specific primers, and the level of TLR9 mRNA was normalized to β_2 -microglobulin levels. *B*, The effect of EBV infection on TLR9 protein expression in primary B cells was monitored by immunoblotting (*left*) and normalized to β -actin levels (*right*). *C*, mRNA expression of TLR9 in an immortalized B cell line (RPMI 8226) not infected or infected with EBV. The level of TLR9 mRNA was determined by qPCR in RPMI 8226 cells infected with EBV >2 mo postinfection compared with noninfected cells. *D*, Protein expression of TLR9 in RPMI 8226 cells infected with EBV. Immunoblotting (*left panel*) and quantification of TLR9 protein expression relative to β -actin (*right panel*) are presented. *E*, Early kinetics of EBV infection to measure the effects on TLR9 protein expression. TLR9 protein in EBV-infected primary B cells was monitored by immunoblotting at different time points postinfection.

FIGURE 3. LMP1 downregulates TLR9 expression. *A*, Expression of TLR9 and LMP1 during viral infection. Expression of TLR9 (*top panel*) and LMP1 (*bottom panel*) mRNAs was monitored by qPCR at different time points postinfection with EBV in primary B cells. The levels of TLR9 and LMP1 expression were normalized to housekeeping genes mentioned in *Materials and Methods*. *B*, Expression of TLR9 mRNA in RPMI 8226 cells transduced with LMP1. Expression of TLR9 mRNA in RPMI 8226 transduced with retrovirus expressing pLNSX-LMP1 (LMP1) or the vector alone was analyzed by qPCR, and the level of expression was normalized to the β_2 -microglobulin. *C*, TLR9 protein levels in not infected human primary B cells, infected with EBV defective for LMP1 (Δ EBV), or WT EBV (EBV). Protein expression was determined by immunoblotting for TLR9, LMP1 (asterisk indicates background band in the WB, which comigrates with LMP1), GFP (marker of viral infection), and β -actin. Results shown here represent the SD of the mean of four experiments performed in B cells purified by CD19⁺ and three experiments performed with negative selection of B cells.



protein influences the activity of the TLR9 promoter. The TLR9 promoter cloned in front of luciferase reporter gene was introduced in RPMI 8226 B cells together with increasing amounts of LMP1 expression plasmid. High basal luciferase activity was detected in these cells in absence of LMP1. (However, the expression of the oncoprotein LMP1 correlated with a strong inhibition of TLR9 promoter activity in a dose-dependent manner [Fig. 4A], indicating that LMP1 can alter the transcription of the TLR9 gene.) Similarly, transient transfection of the TLR9 reporter vector in LMP1 transduced RPMI 8226 cells led to a significant decrease of the promoter activity compared with the control cells (Fig. 4B). Similar results were obtained in a different cell system using HEK293 T cells (Fig. 4C). These data correlated with LMP1 ability to increase the NF- κ B luciferase activity in the same dose-dependent manner (Fig. 4C, *bottom panel*). The relative low level of NF- κ B activation observed with high level of LMP1 (0.5 and 1.0 μ g) could result from a saturation effect because of a large amount LMP1 protein in the cells on NF- κ B signaling. Indeed, no further significant decrease of TLR9 promoter activity was observed between 0.2 or 0.5 and 1.0 μ g ($p = 0.12$, NS, Student nonpaired t test). In summary, these data show that the oncoprotein LMP1 downregulates TLR9 promoter activity in B cells and nonhematopoietic cells.

LMP1-mediated NF- κ B activation is required for the downregulation of TLR9 promoter activity

Many *cis*-acting elements that regulate TLR9 transcription have been characterized along the -3277 bp region of the TLR9 promoter (30, 31). In particular, Takeshita et al. (31) first identified NF- κ B as being one of the suppressive transcription factors potentially associated with TLR9 downregulation. Therefore, we next determined whether the LMP1-induced TLR9 downregulation is mediated by its ability to activate NF- κ B signaling. We used LMP1 mutants in CTAR1 and/or CTAR2 (Fig. 5A) that are respectively responsible for 30 and 70% of NF- κ B activity induced by wild-type (WT) LMP1 (12). Mut 1 and Mut 2 LMP1 mutants retain part of the WT ability to activate NF- κ B pro-

motor, whereas Mut 3 was completely inactive (Fig. 5B). The impairment of the three LMP1 mutants to activate NF- κ B promoter tightly correlated with their inability in TLR9 downregulation (Fig. 5C). NF- κ B activation appears to be important in inhibiting TLR9 expression. To corroborate this observation, we determined whether inhibition of NF- κ B signaling results in increase of TLR9 promoter activity in LCL. Indeed, overexpression of dominant-negative mutants of IKK α or IKK β resulted in high level of TLR9 promoter activity (Fig. 5D, *top panel*) coinciding with a successful inhibition in the NF- κ B pathway (Fig. 5D, *bottom panel*). Similar data were obtained when the NF- κ B pathway was blocked by expressing the I κ B superrepressor Δ N-I κ B α , an I κ B α deletion mutant that is not phosphorylated by the IKK complex, thereby promoting the sequestering of NF- κ B p65 in the cytoplasm (Fig. 5E) (33). Thus, LMP1-induced TLR9 downregulation occurred via the activation of NF- κ B signaling pathway.

We speculated that LMP1 exerts its inhibitory effect on the TLR9 promoter by activating NF- κ B, which would, in turn, suppress TLR9 transcription. The promoter of TLR9 gene contains a NF- κ B binding site at the position -413 to -403 within the -3327 bp sequence, and overexpression of the NF- κ B p65 resulted in the inhibition of TLR9 promoter activity (31). We performed three point mutations on the NF- κ B site within the full-length TLR9 promoter. RPMI B cells or LCLs were cotransfected with either the WT or the mutated TLR9 luciferase reporter plasmid together or not with an LMP1 expression plasmid (Fig. 5F, 5G, respectively). The effect of LMP1 on the mutated TLR9 promoter was drastically reduced. Furthermore, transfection of the mutated construct in LCLs from three donors revealed once again that NF- κ B activity by LMP1 is required to suppress TLR9 promoter activity of the luciferase reporter gene. In all, these data strongly show the involvement of NF- κ B as a negative regulator in the LMP1-mediated inhibition of TLR9.

Discussion

Understanding the importance of host innate immune responses in the context of EBV infection will allow us to determine how EBV

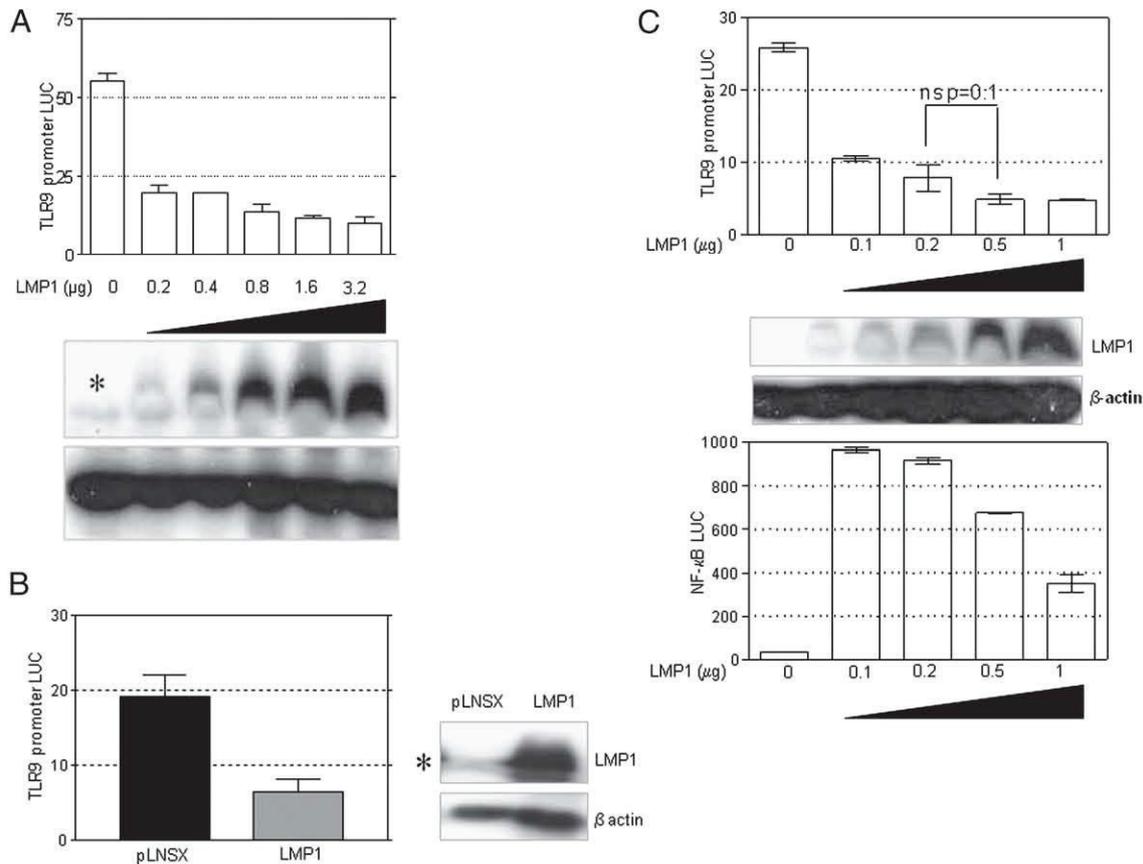


FIGURE 4. LMP1 negatively regulates TLR9 promoter activity. *A*, RPMI 8226 cells were transiently transfected with TLR9 promoter coupled to the luciferase gene with increasing concentrations of the LMP1 expression vector. After 48 h, cells were harvested, and luciferase activity was measured. The data are the mean of three independent experiments performed in triplicate (*top panel*). LMP1 protein expression was monitored by immunoblotting as shown (*bottom panel*). *B*, LMP1 downregulates TLR9 promoter in RPMI 8226 cells stably expressing LMP1. RPMI 8226 cells were transfected with LMP1 and transfected with TLR9 luciferase promoter. TLR9 promoter activity was monitored as described earlier (*left panel*), and the expression of LMP1 was controlled by immunoblotting (*right panel*). Asterisk indicates that the band seen in the pLNSX vector control is a nonspecific band that comigrates with LMP1, observed when using the S12 Ab. *C*, LMP1-induced downregulation of TLR9 promoter activity correlates with increased LMP1-mediated NF- κ B activation. HEK293 T cells were cotransfected with increasing concentrations of LMP1 and the TLR9-luciferase reporter plasmid (*top panel*). To control for LMP1 ability to activate the NF- κ B pathway, HEK293 T cells were transiently transfected with NF- κ B luciferase reporter plasmid with increasing concentrations of LMP1 (*bottom panel*). The luciferase activity was measured 48 h posttransfection. LMP1 expression was evaluated by immunoblotting in both cases but represented as a control for TLR9 promoter versus LMP1 (*middle panel*). Results shown here represent the SD of the mean of six experiments performed.

can persist to promote cellular transformation. Whereas viral escape during the adaptive immune response toward EBV infection has been well studied, the direct effect on the innate immune response has not been elucidated (15). Our study reveals that EBV is able to efficiently target TLR9-regulated pathways. Primary B cell infection with EBV resulted in lower levels of IL-6, TNF- α , and IgG secretion on TLR9 engagement with CpG. This impairment of TLR9 pathway functionality in EBV-infected cells correlated with a decrease of TLR9 mRNA and protein levels in comparison with noninfected primary B cells. Our observations in terms of TLR9 mRNA levels confirmed the findings of Martin and colleagues (34), who observed that both UV-inactivated and untreated EBV downregulated the expression of TLR9 in B cells. They also showed an increase in TLR7 functionality in EBV-infected B cells. However, we did not detect any alteration in TLR7 function (Fig. 1*B*), although mRNA levels were reduced (Supplemental Fig. 2). The discrepancies between our findings and those of Martin and colleagues could be because of the time course of assessing TLR7 functionality and expression, in which Martin et al. looked at proliferation alteration by TLR7, not cytokine secretion. In addition, most of their studies were performed

at earlier time points after EBV infection with either UV-inactivated or live virus.

Several studies highlight the importance of the role played by TLR9 in anti-EBV immunity (25, 26). We demonstrated that the inhibition of TLR9 expression by EBV is mainly mediated by its major oncoprotein LMP1, as EBV mutant lacking LMP1 is impaired in its ability to downregulate TLR9. In the 95% of the human population asymptomatic for EBV infection, the virus has evolved to evade the immune system to persist “normally” within the host, and LMP1 expression is tightly controlled and regulated. LMP1 can contribute to the immune suppression at earlier stage of infection by blocking IFN- α secretion and increasing the transcription of the anti-inflammatory cytokine IL-10 (16). Yet, deciphering the precise role of LMP1 in EBV-associated carcinogenesis has proved a wide task because it can activate many pathways. What can be noted is that signaling pathways associated to B cell proliferation and LMP1-induced NF- κ B signaling has a significant role in EBV latency and persistence. Our data show that LMP1 is able to inhibit TLR9 transcription in primary and immortalized B cells by activating NF- κ B pathways. In fact, inhibition of the NF- κ B in EBV-infected cells by different means

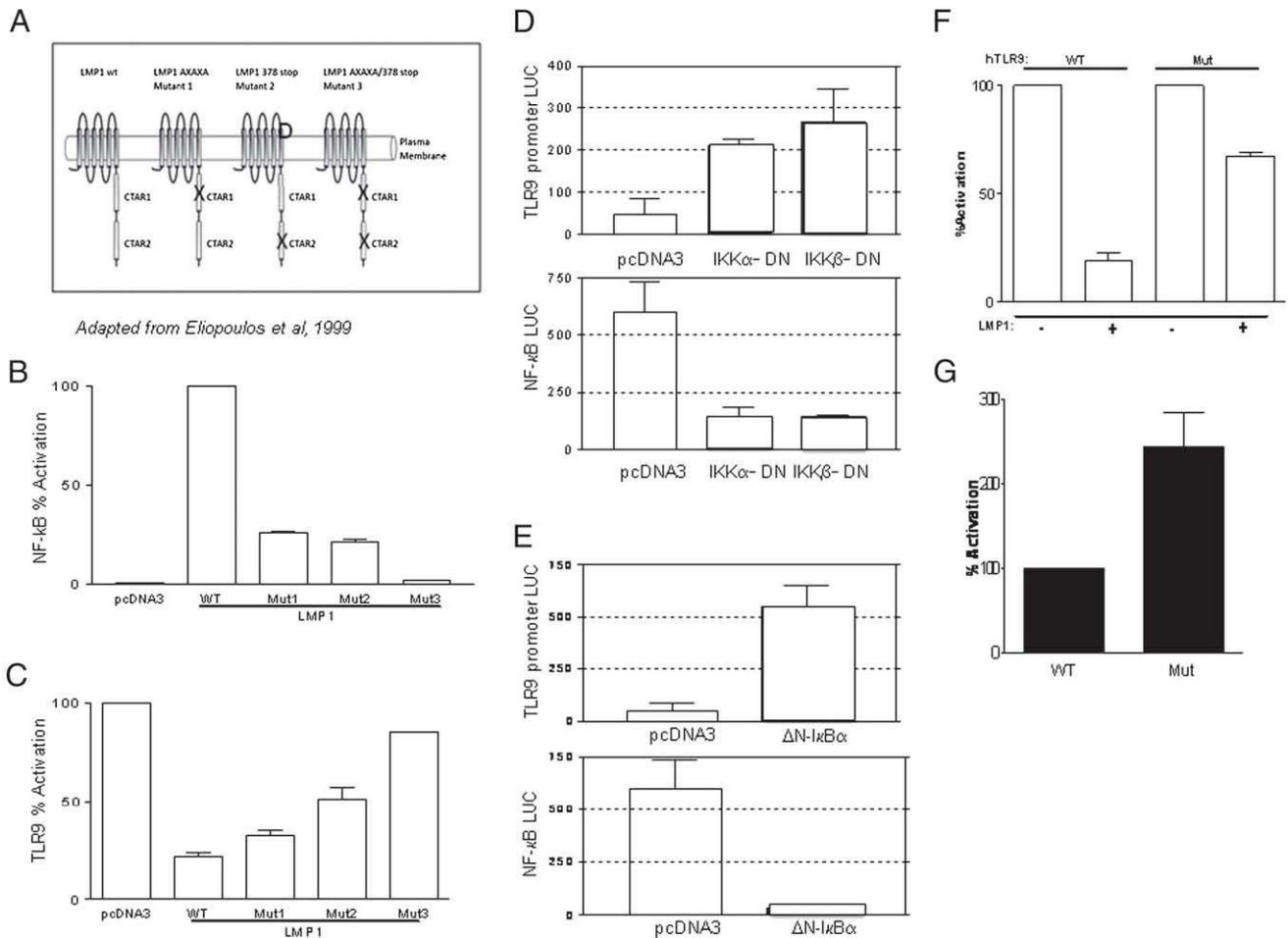


FIGURE 5. TLR9 downregulation is mediated by NF- κ B signaling induced by LMP1. *A*, Schematic representation of LMP1 WT and mutations made in the NF- κ B activator domains of CTAR1 and CTAR2. Mut 1 is within the CTAR1 domain (aas 196–231), has a triple PxQxT3AxAxA point mutation, which abrogates TNFR-associated factor binding. Mut 2 in the CTAR2 domain of LMP1 contains a stop codon at aa 378 that abolishes the interaction of TRADD and RIP. Mut 3 contains both. This research was originally published in *The Journal of Biological Chemistry*. Aristides G. Eliopoulos, Neil J. Gallagher, Sarah M. S. Blake, Christopher W. Dawson, and Lawrence S. Young. Activation of the p38 mitogen-activated protein kinase pathway by Epstein-Barr virus-encoded latent membrane protein 1 coregulates interleukin-6 and interleukin-8 production. *J. Biol. Chem.* 1999; 274: 16085–16096. © The American Society for Biochemistry and Molecular Biology. *B*, HEK293 T cells were transfected with expression plasmids for WT LMP1, and its mutants, together with NF- κ B promoter luciferase reporter plasmid and the relative activation, are presented. *C*, LMP1 mutants less potent for NF- κ B activity are partially defective in inhibiting TLR9 promoter activity. LMP1 (WT) and the three LMP1 mutants were transfected in RPMI8226 cells together with TLR9 promoter luciferase reporter plasmid, and luciferase activity was monitored. Data for *B* and *C* are reported as percentage of luciferase activity normalized by dividing relative luciferase units obtained for LMP1 WT for *C* and empty vector for *D*. LMP1 and mutants were monitored for expression by Western blotting using the S12 Ab (data not shown). *D*, Blocking NF- κ B activation increases TLR9 promoter activity. Plasmids expressing dominant-negative forms of IKK α or IKK β and vector control were cotransfected with either the TLR9 (*top panel*) or NF- κ B promoter (*bottom panel*) in LCLs, and luciferase activity was measured 48 h post-transfection. *E*, NF- κ B superrepressor Δ N-I κ B α and vector control were cotransfected with either the TLR9 (*upper panel*) or NF- κ B promoters (*lower panel*) in LCL, and luciferase activity was measured 48 h posttransfection. Results shown here represent the SD of one of three experiments performed. RPMI 8226 B cells (*F*) or LCLs (*G*) were cotransfected with the TLR9 WT or with mutated at the NF- κ B promoters \pm LMP1; luciferase activity was measured 48 h posttransfection.

resulted in an increase of TLR9 expression. In addition, LMP1 deletion mutants lacking the well-characterized domains CTAR1 and CTAR2 important for the activation of NF- κ B pathways (12) showed a reduced efficiency in downregulating TLR9. Together, these data showed the implication of NF- κ B in the mechanism used by LMP1 to downregulate TLR9 expression. However, the precise contribution of NF- κ B pathways in this event remains to be elucidated. The characterization of the TLR9 promoter revealed the presence of several transcription factors sites including four NF- κ B sites (30). It has been previously reported that the NF- κ B *cis* element at position -413 bp plays a negative role in regulating TLR9 levels (31). Our data suggest that LMP1 may use the same NF- κ B *cis* element to inhibit TLR9 transcription. In fact,

a deletion mutant of TLR9 promoter containing only the first 700 bp upstream of the ATG and including the NF- κ B *cis* element still resulted in TLR9 repressed by EBV (unpublished data).

Data from our group have showed that the major oncoproteins, E6 and E7, from carcinogenic HPV type 16 inhibit the expression of TLR9. Thus, the ability to downregulate TLR9 expression appeared to be a shared property between DNA tumor viruses. Interestingly, the low-risk HPV types that are normally associated with benign cervical lesions are not able to alter TLR9 expression, suggesting that TLR9 downregulation is an event exclusively associated with carcinogenic viruses (30). Whether EBV promotes TLR9 downregulation, as a means to exist within the host or promote carcinogenesis, needs to be addressed. We observed that

initial infection up to 120 h with EBV did decrease TLR9 mRNA levels compared with primary B cells (Fig. 2E). However, in LCLs (minimum 5 wk in culture), we noted a further decrease in TLR9 expression (data not shown) in which decreasing the dose of the TLR9 ligand conferred a wider decline in IL-6 levels between primary B cells and LCLs (Fig. 1C). This subtle difference observed might indicate the difference between the virus escaping TLR9 recognition to coexist at the initial stages of EBV infection versus type III latency (LCL) in which TLR9 expression is further reduced. Iskra et al.'s recent study (35) highlights the role of TLR9 agonist CpG2006 in synergistically increasing proliferation and activation of B cells by EBV at an early time point postinfection. Thus, EBV uses TLR9 to infect and induce proliferation; yet, our study demonstrates that later it downregulates TLR9 expression to escape the immune response.

In addition to TLR9, we have observed that EBV infection of primary B cells led to inhibition of TLR2 transcription and functionality (Fig. 1B, Supplemental Fig. 2). Interestingly, it has recently been shown that the nonstructural EBV protein, DUTPase, is recognized by TLR2, leading to NF- κ B activation (36). It is thus not surprising that we have observed that the virus efficiently downregulates both TLR2 and TLR9 mRNA, as both receptors are activated by two distinct EBV components, similarly to HSV-2 (37). Furthermore, EBV-encoded small nonpolyadenylated RNAs are recognized by RIG-I and TLR3, enhancing the induction of type I IFN (38). We observed that TLR3 responses for IL-6 secretion were low in primary B cells and abolished in LCLs. Discrepancies between our findings and those reported in terms of TLR3 activity could deviate from the cellular models used, and the time points postinfection chosen for expression and functionality studies (39, 40). Most strikingly, TLR5 functionality was halted in our EBV-infected cells; although there are no reports correlating TLR5 to EBV, this observation merits further investigation.

In summary, our data showed that LMP1, in addition to its key role in cellular transformation, is able to efficiently target innate sensors, although the possibility that additional viral protein may synergize with LMP1 remains to be investigated. This study reveals a mechanism used by EBV to escape innate immune TLR9 recognition, and it highlights the importance of immune deregulation mediated by tumor-inducing viruses.

Acknowledgments

We thank Annick Rivoire for her administrative aid and E. Kieff, T. Gilmore, G. Mosialos, and L. Young for contributing reagents. We thank Dr. W. Hammerschmidt for the gift of the HEK293_{EBV} and HEK293_{EBV/LMP1} cells. We also thank the team of Christine Delprat at Lyon Sud for providing PBMCs for B cell purification and Nadege Goutagny at the Centre Leon Berard, Lyon, France, for continual availability and advice.

Disclosures

The authors have no financial conflicts of interest.

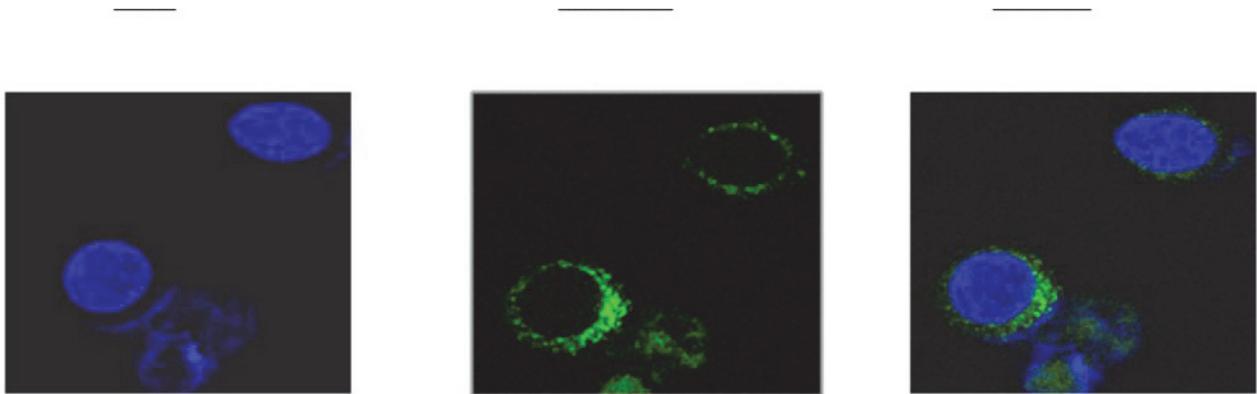
References

1. Parkin, D. M., F. Bray, J. Ferlay, and P. Pisani. 2005. Global cancer statistics, 2002. *CA Cancer J. Clin.* 55: 74–108.
2. Kieff, E. D., and A. B. Rickinson. 2007. Epstein-Barr virus and its replication. In *Fields Virology*, 5th Ed. D. M. Knipe, and P. M. Howley, eds. Philadelphia: Lippincott Williams & Wilkins, p. 2603–2655.
3. Coffey, A. J., R. A. Brooksbank, O. Brandau, T. Ohashi, G. R. Howell, J. M. Bye, A. P. Cahn, J. Durham, P. Heath, P. Wray, et al. 1998. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat. Genet.* 20: 129–135.
4. Lamartine, J., K. E. Nichols, L. Yin, M. Krainer, F. Heitzmann, A. Bernard, S. Gaudi, G. M. Lenoir, J. L. Sullivan, J. E. Ikeda, et al. 1996. Physical map and

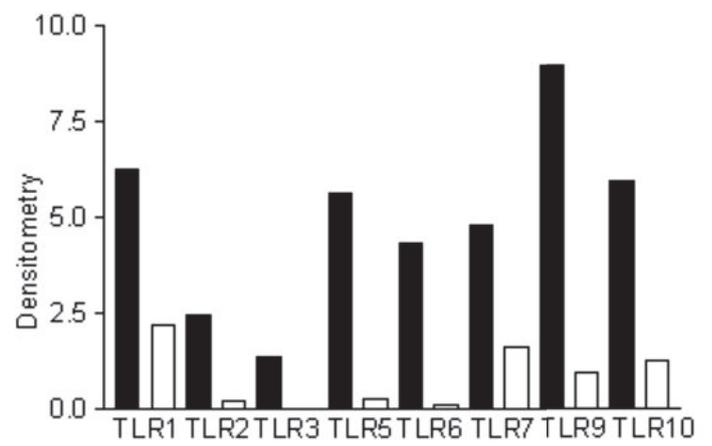
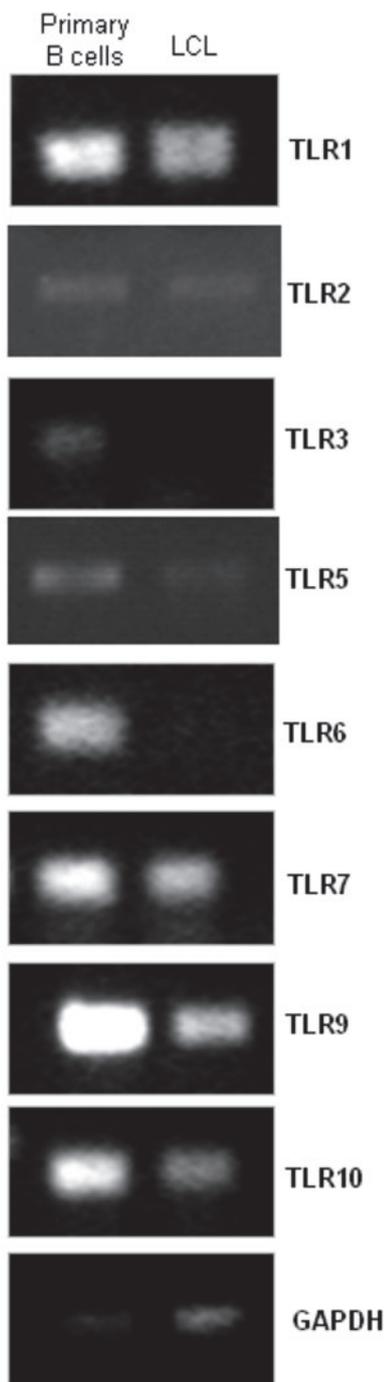
- cosmid contig encompassing a new interstitial deletion of the X-linked lymphoproliferative syndrome region. *Eur. J. Hum. Genet.* 4: 342–351.
5. Purtilo, D. T., R. S. Strobach, M. Okano, and J. R. Davis. 1992. Epstein-Barr virus-associated lymphoproliferative disorders. *Lab. Invest.* 67: 5–23.
6. Kaye, K. M., K. M. Izumi, and E. Kieff. 1993. Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. *Proc. Natl. Acad. Sci. USA* 90: 9150–9154.
7. Cahir McFarland, E. D., K. M. Izumi, and G. Mosialos. 1999. Epstein-Barr virus transformation: involvement of latent membrane protein 1-mediated activation of NF- κ B. *Oncogene* 18: 6959–6964.
8. Eliopoulos, A. G., and L. S. Young. 2001. LMP1 structure and signal transduction. *Semin. Cancer Biol.* 11: 435–444.
9. Mosialos, G., M. Birkenbach, R. Yalamanchili, T. VanArsdale, C. Ware, and E. Kieff. 1995. The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell* 80: 389–399.
10. Dawson, C. W., G. Tramontanis, A. G. Eliopoulos, and L. S. Young. 2003. Epstein-Barr virus latent membrane protein 1 (LMP1) activates the phosphatidylinositol 3-kinase/Akt pathway to promote cell survival and induce actin filament remodeling. *J. Biol. Chem.* 278: 3694–3704.
11. Gires, O., F. Kohlhuber, E. Kilger, M. Baumann, A. Kieser, C. Kaiser, R. Zeidler, B. Scheffer, M. Ueffing, and W. Hammerschmidt. 1999. Latent membrane protein 1 of Epstein-Barr virus interacts with JAK3 and activates STAT proteins. *EMBO J.* 18: 3064–3073.
12. Huen, D. S., S. A. Henderson, D. Croom-Carter, and M. Rowe. 1995. The Epstein-Barr virus latent membrane protein-1 (LMP1) mediates activation of NF- κ B and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain. *Oncogene* 10: 549–560.
13. Izumi, K. M., and E. D. Kieff. 1997. The Epstein-Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-associated death domain protein to mediate B lymphocyte growth transformation and activate NF- κ B. *Proc. Natl. Acad. Sci. USA* 94: 12592–12597.
14. Sylla, B. S., S. C. Hung, D. M. Davidson, E. Hatzivassiliou, N. L. Malinin, D. Wallach, T. D. Gilmore, E. Kieff, and G. Mosialos. 1998. Epstein-Barr virus-transforming protein latent infection membrane protein 1 activates transcription factor NF- κ B through a pathway that includes the NF- κ B-inducing kinase and the I κ B kinase kinases IKK α and IKK β . *Proc. Natl. Acad. Sci. USA* 95: 10106–10111.
15. Levitsky, V., and M. G. Masucci. 2002. Manipulation of immune responses by Epstein-Barr virus. *Virus Res.* 88: 71–86.
16. Middeldorp, J. M., and D. M. Pegtel. 2008. Multiple roles of LMP1 in Epstein-Barr virus induced immune escape. *Semin. Cancer Biol.* 18: 388–396.
17. Miyashita, E. M., B. Yang, G. J. Babcock, and D. A. Thorley-Lawson. 1997. Identification of the site of Epstein-Barr virus persistence in vivo as a resting B cell. *J. Virol.* 71: 4882–4891.
18. Babcock, G. J., L. L. Decker, M. Volk, and D. A. Thorley-Lawson. 1998. EBV persistence in memory B cells in vivo. *Immunity* 9: 395–404.
19. Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. *Int. Immunol.* 17: 1–14.
20. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdörfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* 168: 4531–4537.
21. Bowie, A. G., and I. R. Haga. 2005. The role of Toll-like receptors in the host response to viruses. *Mol. Immunol.* 42: 859–867.
22. Finberg, R. W., J. P. Wang, and E. A. Kurt-Jones. 2007. Toll like receptors and viruses. *Rev. Med. Virol.* 17: 35–43.
23. Pasare, C., and R. Medzhitov. 2005. Control of B-cell responses by Toll-like receptors. *Nature* 438: 364–368.
24. Lund, J., A. Sato, S. Akira, R. Medzhitov, and A. Iwasaki. 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *J. Exp. Med.* 198: 513–520.
25. Lim, W. H., S. Kireta, G. R. Russ, and P. T. Coates. 2007. Human plasmacytoid dendritic cells regulate immune responses to Epstein-Barr virus (EBV) infection and delay EBV-related mortality in humanized NOD-SCID mice. *Blood* 109: 1043–1050.
26. Guggemoos, S., D. Hangel, S. Hamm, A. Heit, S. Bauer, and H. Adler. 2008. TLR9 contributes to antiviral immunity during gammaherpesvirus infection. *J. Immunol.* 180: 438–443.
27. Delecluse, H. J., D. Pich, T. Hilsendegen, C. Baum, and W. Hammerschmidt. 1999. A first-generation packaging cell line for Epstein-Barr virus-derived vectors. *Proc. Natl. Acad. Sci. USA* 96: 5188–5193.
28. Dirmeier, U., B. Neuhierl, E. Kilger, G. Reisbach, M. L. Sandberg, and W. Hammerschmidt. 2003. Latent membrane protein 1 is critical for efficient growth transformation of human B cells by Epstein-Barr virus. *Cancer Res.* 63: 2982–2989.
29. Caldeira, S., I. Zehbe, R. Accardi, I. Malanchi, W. Dong, M. Giarrè, E. M. de Villiers, R. Filotico, P. Boukamp, and M. Tommasino. 2003. The E6 and E7 proteins of the cutaneous human papillomavirus type 38 display transforming properties. *J. Virol.* 77: 2195–2206.
30. Hasan, U. A., E. Bates, F. Takeshita, A. Biliato, R. Accardi, V. Bouvard, M. Mansour, I. Vincent, L. Gissmann, T. Ifitner, et al. 2007. TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16. *J. Immunol.* 178: 3186–3197.
31. Takeshita, F., K. Suzuki, S. Sasaki, N. Ishii, D. M. Klinman, and K. J. Ishii. 2004. Transcriptional regulation of the human TLR9 gene. *J. Immunol.* 173: 2552–2561.

S1. *Confocal monitoring of EBV infection.* **A**, For confocal staining, LCL were directly fixed on coverslides coverslides and mounted using a mounting reagent containing DAPI. **B**, LMP1 staining was performed by using anti LMP1, and a secondary PE-conjugated anti-mouse was used. As negative control, LCL were incubated with secondary antibody only (data not shown).

S2. *TLR gene expression in primary B cells and LCL.* Primary B-cells and EBV-infected LCLs were stimulated with ligands for TLR family members (TLR1 to TLR10, with exception of TLR4 and 8) and TLR mRNA expression was determined by RT-PCR (left panel) with the quantification normalized to GAPDH (right panel).



S1



S2

IV. CONCLUSION AND PERSPECTIVES

As detailed in the introduction, two key events are required for an oncovirus to induce cancer. First the virus needs to persist and second it must induce genetic alterations by deregulating the cell cycle for example. While the host immune response tends to clear the infection, viruses have developed mechanisms of immune escape to persist. This persistence leads to chronicity that might result in the long run into cancer development.

Through the three articles presented, we participated to unravel the mechanisms by which EBV and HPV promote carcinogenesis. First we showed that EBV deregulates TLR9, a key actor of DNA virus recognition by the innate immune system, *via* its LMP1 protein [381]. This probably favors EBV escape of immune recognition by B cells. Besides its involvement in immune responses, we demonstrated a direct role for TLR9 in inducing cell cycle modulation. TLR9 re-expression in cancer cell lines favored cell cycle arrest, by inhibiting transiently cell proliferation *via* a short lived increase of the S-phase (Paper 2 in preparation). Altogether those data tend to suggest that TLR9 down-regulation observed in many cancers may promote both immune escape and cell cycle deregulation. This new finding demonstrated that innate receptors can have two functions; first to sense viral infection and thus induce an effective immune response and second to control cell cycle regulation. Finally, we found a new role for HPV16E6 protein in cell cycle deregulation. E6 is able to inhibit p21 transactivation in a p53 independent way by inhibiting the transcription factor p150^{sal2}.

A. NEW MECHANISM E6 DEPENDENT P21 DEREGULATION

HPV promotes cell cycle deregulation mainly by acting on E2F and p53. E7 is activating E2F dependent genes transcription by inhibiting pRb and E6 prevents p53 acetylation and is inducing its proteasome mediated degradation. This later effect is inhibiting p53 pathways including p53 dependent transcription of p21, a key mediator of p53-dependent induction of cell cycle arrest and apoptosis for instance. However it was previously proposed that E6 might also alter p21 independently of p53 by unknown mechanisms, since E6 mutants unable to induce p53 degradation were still able to down-regulate p21 [738]. In *Paper A* [745], we have indeed shown that HPV16E6 is able to inhibit *cdkn1a* expression independently of p53. HPV16E6 bound to the transcription factor p150^{sal2} (Figure 2 Paper 1). This association abolished the transactivation function of p150^{sal2} towards *cdkn1a* and the transcription of this later one was inhibited.

1. ACTIVATION OF P150^{SAL2}

123

Little is known about the regulation of p150^{sal2}. It has been shown to play a key role into cellular quiescence induced by serum deprivation and in the NGF dependent growth arrest of neuronal cells [746]. Upon serum deprivation or NGF treatment, p150^{sal2} expression increased and the protein translocates to the nucleus. Very little is known regarding the signals that could trigger p150^{sal2} activation beside serum deprivation and NGF. It will thus be interesting to dissect the precise mechanisms and extracellular signals leading to p150^{sal2} accumulation and activation upon E6 expression. We will check which of the classical inducers of p21 such as DNA damage (by chemical exposure), radiation or oncogene expression (such as c-myc or h-ras) will activate the transcription factor p150^{sal2} in primary cells such as primary oral human fibroblast POF. p150^{sal2} increase and nuclear translocation will be assessed by immunofluorescence staining for example. Furthermore, we have seen that tumors biopsies HPV16⁺ showed an increased in p150^{sal2} immunostaining as compared with

healthy tissues (data not shown). Similarly, p150^{sal2} expression was high in HPV16⁺ cancer cell lines. Thus p150^{sal2} accumulates upon HPV16 infection. We have shown that E6 could participate to this increase of p150^{sal2} however we will investigate which other viral proteins are also required. We will first perform *in silico* studies of the p150^{sal2} promoter in order to determine which transcription factors could be involved in its regulation. Then we will look at the effect of the expression of HPV viral proteins on a promoter luciferase assay in POF (p150^{sal2} luciferase reporter plasmid will be constructed). Modulation of p150^{sal2} mRNA and protein expression (qPCR and western blot) will be followed upon over expression of HPV16 proteins into POF cells in order to confirm the luciferase data. We will narrow down the protein domains involved in this regulation by biochemical approaches using mutant constructs deleted for various domains of these proteins.

p150^{sal2} accumulates in several cancers such as oral tongue cancer, testicular germ cell tumors and synovial sarcoma as well as in HPV16 associated cancers [728-730]. While oral tongue squamous carcinoma have been linked to HPV infection, the etiology of testicular germ cell tumors and synovial sarcoma appear to be HPV independent [731, 732]. Theoretically, the accumulation of p150^{sal2} should lead to an activation of p21, thus arresting cell proliferation. However such effect was not observed in those cancers. Thus a similar mechanism of p150^{sal2} inactivation as the one we observed can be also potentially found in HPV independent tumors and to a large extent in non virus induced cancers. To find other potential p150^{sal2} inhibitors, we could perform a pull-down of p150^{sal2} protein and analyze bound partners by mass spectrometry in cancer cells accumulating p150^{sal2}.

2. TARGET GENES OF P150^{SAL2}

Little is known on the target genes of p150^{sal2} beside p21. The E6-dependent deregulation of the transcriptional activity of p150^{sal2} might also affect other genes that might be important in HPV induced malignancies. Understanding how the viral oncoproteins are affecting the host cell is of major importance. In order to find genes regulated by p150^{sal2}, we could perform bioinformatics analyses searching for genes that express the putative p150^{sal2} binding site in their promoter. We could then assess whether p150 accumulation does indeed modulate the expression of those genes (qPCR) and in the long run analyze the potential involvement of those genes in cancer development.

3. P150^{SAL2} INVOLVEMENT IN OTHER VIRUS INDUCED CANCER

Binding of Large T antigen from polyomavirus to p150^{sal2} was found to be essential to overcome p150^{sal2} dependent viral replication inhibition [170]. We have shown that HPV16E6 is also deregulating p150^{sal2}. We could speculate that the deregulation of this transcription factor is a common feature of oncoviruses. For example, it is already known that EBV can deregulate p21 expression during its life cycle. While during lytic cycle EBV induces senescence by Rta-mediated transactivation of p21 [747, 748], during EBV latency EBER1 mediated p21 suppression is linked to an increase resistance to drug induced apoptosis in Hodgkin's lymphoma [749]. We could investigate if the effect of EBER1 on p21 might be partially p150^{sal2} mediated. For that purpose we would express EBER1 in primary cells such as POF and see if the regulation of *cdkn1a* is dependent on p150^{sal2}. We could analyze if p150^{sal2} would still be able to bind *cdkn1a* promoter by Chip when EBER1 is expressed.

TLR9 is down-regulated in several cancers including breast cancer, myelodysplastic syndrome to overt leukemia transformation and HPV high risk type associated cancers [371, 679, 682]. Our group indeed demonstrated that TLR9 expression and responsiveness is abolished upon expression of HPV16 oncoproteins but not upon expression of protein from low risk type [371] thus raising the question of the role of TLR9 in the carcinogenesis. As detailed in the introduction, TLR9 stimulation induces or inhibits cell proliferation and/or apoptosis, depending on the cellular model and the ligand. As a today there was no report on the link between TLR9 expression and cell cycle regulation. In an ongoing study (Paper 2), we demonstrated that besides its role in innate immunity, TLR9 has a role on the cell cycle by inhibiting cell proliferation without inducing apoptosis. Short and long term perspectives will be presented hereafter

1. SHORT TERM: TLR9 AND CELL PROLIFERATION

Indeed, we have shown that re-expression of TLR9 in HNSCC and HPV⁺ cell lines slowed-down cell proliferation by MTT and double population assays (Paper 2). To further extend these observations and demonstrate the role of TLR9 on cell proliferation, we will in the near future perform the opposite experiment. For that matter, we will silence TLR9 in cells expressing TLR9 and determine whether this increases cell proliferation or deregulates the normally occurring senescence. We will perform those experiments in primary keratinocytes using validated lentiviral vector encoding shRNA directed against TLR9 and check the impact on cell proliferation by BrdU incorporation and MTT assays and senescence by β -galactosidase staining and chromatin H2AX analysis.

While TLR9 re-expression does not affect the percentage of cells in each phase of the cell cycle (PI staining, Figure 3 Paper 2), we showed that it delayed the S-phase of the cell cycle (BrdU/7AAD staining, Figure 3 Paper 2). In order to further demonstrate these observations and really quantify the duration of the S-phase, we will perform, in TLR9 versus negative HNSCC and Caski cell lines, pulse chase experiment consisting of a successive labeling by IdU and BrdU. S phase duration will then be calculated using the formula defined by Shibui *et al* [750].

We showed as well that TLR9 was inducing an increase of some inhibitors controlling the CDK/cyclin complexes (Figure 4 Paper 2). We indeed observed in preliminary experiment that the protein levels of p53, p21 and p27 were transiently increased in TLR9 positive cells (Figure 4 Paper 2). We will first confirm those data after cell synchronization in both Caski and Siha cell lines. To further investigate the cell cycle proteins that are directly targeted by TLR9, we will look, after cell synchronization, for the expression of the cyclins and the activation of the CDKs in TLR9 as compared to mock expressing cells. A special focus should be put on the cyclin A / CDK2 complex that ensures the S-phase progression and might be differentially regulated in TLR9 and mock expressing cell.

The transcriptome analysis of TLR9 versus mock expressing cells revealed some interesting findings. We found that *hsp70* was down-regulated in TLR9 expressing cells. As discussed in the Paper 2 discussion, HSP-70 over-expression is typical of several types of tumors, and strong evidences suggest that it plays a role in the control of the cell cycle and growth [717-720]. While CpG-B ODN has been shown to up-regulate *hsp70* via the *TLR9/MyD88/PI3K pathway* [690], no correlation has been found as of today between TLR9 expression and *hsp70*. After confirmation of the microarray data by qPCR, we will assess whether the down-regulation of *hsp70* by TLR9 is involved in the inhibition of the proliferation. HEK 293 (cell line used for the micro-array)

cells will be transfected with TLR9 and *hsp70* mRNA expression will be assessed by qPCR. To demonstrate a role for HSP70 in TLR9 induced cell proliferation inhibition, we will assess whether *hsp70* silencing affects TLR9 mediated effect as compared to mock transfected cells. If the down-regulation of HSP70 does specifically affect TLR9 mediated effect, we will analyze the precise role of HSP70 in cell proliferation. Knock-down or over-expression approaches will be performed to depict the precise effect of HSP70 in cell cycle using western blotting approaches as previously done.

2. SHORT TERM: IS TLR9 ACTIVITY ON PROLIFERATION A CONSEQUENCE OF ITS ACTIVATION?

In our model the effect of TLR9 on cell proliferation was short lived. After few passages TLR9 expressing cells proliferated at the same rate than the mock expressing cells, unless cells were synchronized and then the effect of TLR9 was again visualized.

Besides a possible adjustment of the cells to TLR9 over-expression *via* compensatory mechanisms, our main hypothesis is that TLR9 modulates cell proliferation under stress conditions. This would explain why such effect is observed shortly after puromycin selection or after thymidine double block for cell synchronization. Experimental setting used in both approaches might induce transitory DAMPs that would specifically trigger a TLR9 dependent cell growth inhibition. Indeed it has been suggested that TLR9 might be stimulated by histone preparation during hepatic ischemia/reperfusion injury and induce sterile inflammatory liver injury [713]. Furthermore another report showed that injury would induce the release of mitochondrial DNA that would activate cells in a TLR9 dependent manner [714]. We thus propose that DNA or histone released during cell death, induced by puromycin selection or thymidine double block, might be responsible for TLR9 triggering and further effect on cell proliferation. We indeed investigated whether DAMPs could induce the TLR9 dependent inhibition of cell proliferation (Figure 5 Paper 2). We tried to induce DAMP release by killing cells with puromycin, cisplatin, H2O2 or doxorubicin, incubated them with TLR9 or mock expressing cells and assessed the effect on cell proliferation. In those conditions, we did not observe any proliferation inhibition of TLR9 versus mock expressing cells. It is possible that either the ligand was not released (in cisplatin, H2O2 or doxorubicin treated conditions) or that the ratio dead/live cells (in puromycin condition) was not appropriate to visualize such effect. We will further investigate this hypothesis using another approach with purified TLR9 ligand such as mitochondrial DNA (as described in [714]), or CpG DNA and look for their effect on cell proliferation. Preliminary experiments using CpG-B DNA in MTT assay showed contradictory results and shall be repeated using QC controlled batch of CpG ODN from different classes

3. LONG TERM: ROLE OF TLR9 IN LIMITING ONCOVIRUS INDUCED CARCINOGENESIS

In order to really demonstrate the role of TLR9 in carcinogenesis, we will follow two long term approaches, one using *in vitro* cellular models and the other *in vivo* in xenograft models.

Addressing this question *in vitro* is quite difficult. In the case of HPV, quasivirions can only infect and replicate in raft 3D culture of primary keratinocytes. HPV dependent transformation being a long process, it is thus impossible to monitor transformation this *in vitro* model. It would however be possible to address this question in EBV context. We could knock-down TLR9 in primary B cells before infection with EBV, follow-up the appearance of immortalized and transformed cells and see whether TLR9 decrease favors transformation. In

order to assess the immortalization of the cells we could analyze the telomerase activity (Millipore TRAPeze kit) of the B cells silenced or not for TLR9. The transformation could be tested by soft agar cell transformation assay. We already validated TLR9 knock-down approach in primary B cells using lentiviral vectors. We could also assess the role of TLR9 expression in non-viral transformation models. The standardized model of *in vitro* oncogenic transformation of primary human mammary epithelial cells (HMEC) might be valuable to test our hypothesis [737]. There are three steps in the model of normal HMEC transformation. In this experimental system, HMEC undergo sequential retroviral transduction to express -the catalytic subunit of the human telomerase (HMEC/hTERT cells), then the SV40 large T and small t antigens (HMLE cells) and finally the constitutively active form of Ras, H-Ras^{V12} (HMLER cells) [738]. We will like first to analyze the expression of TLR9 both RNA and protein in these cells and then determine the effect of a modulation of TLR9 (using approaches previously described) on the events leading to the oncogenic transformation assessed by the growth of the cells in soft agar.

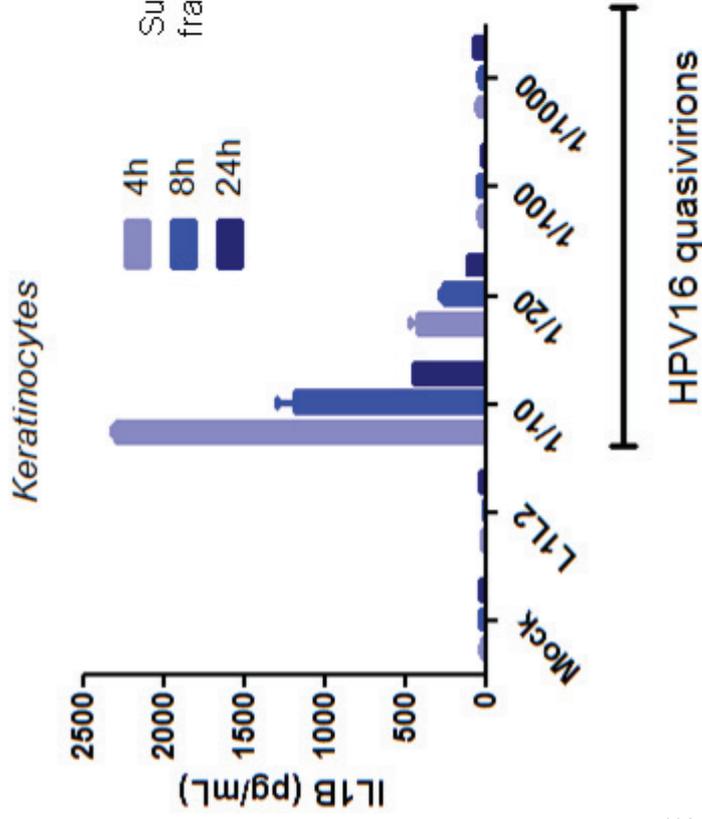
In a second step, we will assess *in vivo* whether the loss of TLR9 is involved in the first set of events leading to the transformation. For that purpose we will use the human keratinocyte cell line HACAT that is not transformed and expresses functional TLR9. This cell line has already been used in xenograft model [739] and does not induce tumor unless it expresses exogenously an oncogene such as h-Ras. We will first create stable keratinocyte cell lines expressing either (i) h-Ras alone, (ii) a shRNA targeting TLR9 or (iii) both h-Ras and sh-TLR9. These cell lines will be injected subcutaneously in nude mice, which lack T and B cells, and tumor growth monitored in the three groups. We have preliminary results in nude mice that were injected with a stable Caski cell line expressing an inducible TLR9 construct. Half of the mice were fed with doxycyclin and tumor growth was monitored. As tumor sizes were not homogenous within each group; it is quite difficult to conclude. This experiment has to be repeated with larger number of mice in each group.

We will then determine whether the loss of TLR9 increases the tumorigenicity of already transformed cells, using RPMI8226 cells. RPMI8226 is a multiple myeloma derived B cell line expressing TLR9. This cell line is transformed since it induces tumors when injected subcutaneously in nude mice [751]. RPMI8226 knocked-down for TLR9 were previously established. We will then inject RPMI8226 expressing sh-targeted TLR9 or mock in NOD/SCID mice and compare the tumour growth over time. We here hypothesize that down-regulation of TLR9 might not be *per se* sufficient to promote immortalization and transformation but will impact oncogene-induced transformation.

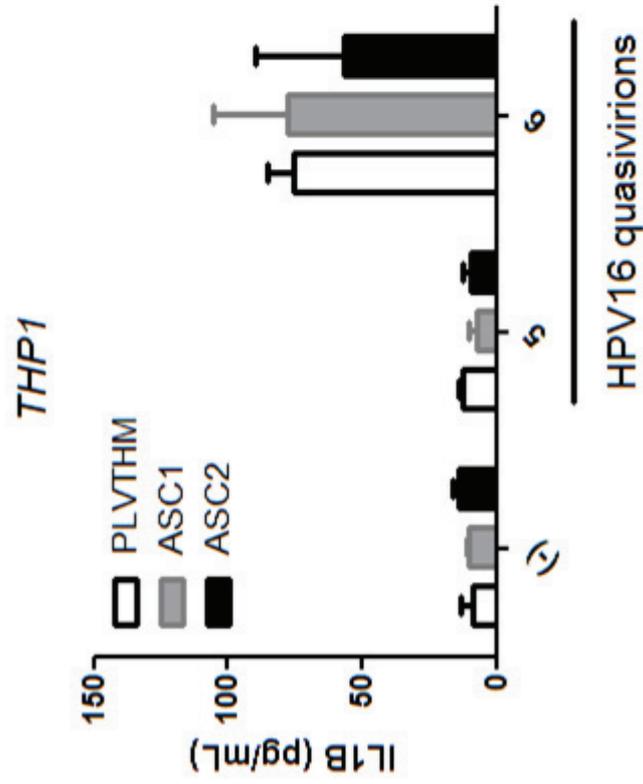
While those xenograft models are necessary to really demonstrate the tumorigenicity of cell lines, they are partially deficient in their immune response and do not allow to assess in parallel the role of TLR9 in immune escape. Syngenic mouse tumour models would be required for a global role of TLR9 in limiting tumour development.

C. INNATE IMMUNE RESPONSE AND ONCOVIRUSES

Studies on TLR9 expression showed that this PRR is repressed during breast cancer, overt leukemia and HPV16 malignant transformation. In the case of EBV, we and others [752] have observed that TLR9 transcription is suppressed from 24 hours after infection without restoration over 5 weeks later. We showed that LMP1 via activation of NFκB pathway was inhibiting *tlr9* transcription and functional response. It is of particular interest since EBV is able *in vitro* to activate TLR9 in B cell lines, primary monocytes and pDCs [450, 452, 753].



Supplementary Figure 1: IL1 β production by HPV16 virions or control L1L2 fraction in keratinocytes



Supplementary Figure 2: IL1 β production by HPV16 virions in stable THP1 expressing a shRNA for ASC (ASC1 and ASC2) or a control plasmid (PLVTHM) after 6 hours of stimulation.

1. EXPRESSION OF TLRs IN PRIMARY AND EBV INFECTED B CELLS

We and others have seen that human primary B cells express low TLR2, -3, -5 and high TLR1, -6, -7, -9 and -10 levels [352, 754]. Upon differentiation into memory B cells, the expression of TLR6, -7, -9 and 10 is increased [352, 755]. EBV infection drastically changes TLRs expression in B cells. We indeed observed that all TLRs were down-regulated in LCL 5 weeks after infection when compared to corresponding primary B cells. In contrast, another study has reported an increase in *tlr7* expression after EBV infection of primary B cells [752]. In our hands, *in vitro* infection of primary B cells slightly decreased *tlr7* expression but did not affect their responsiveness in term of cytokine and IgG secretion of LCL to TLR7 ligands. The discrepancies between those two studies could be due to the time point post infection chosen for the analysis of *tlr* expression (5 weeks for our study and 72 hours for Martin *et al.*).

2. RELATIONSHIP BETWEEN TLRs, B CELL BIOLOGY AND TRANSFORMATION

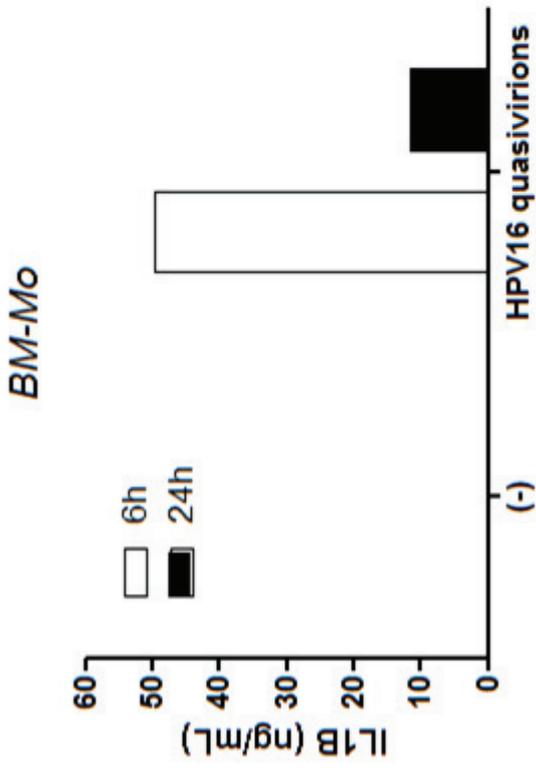
Full activation of naïve B cells require three signals: BCR triggering, T cell help *via* CD40 ligation and PRR stimulation [756]. As described earlier, the activation of naïve B cells is crucial in EBV life cycle. Therefore this virus closely mimics or constitutively activates the pathways leading to naïve B cell activation. Indeed, signal 1 and 2 are provided respectively by LMP2A and LMP1 [64, 757]. While some mechanisms are proposed, the precise nature of the third signal is still unknown. PRR stimulation might come from non EBV-PAMPs present in the B cell environment or EBV-associated PAMPs as EBERs shown to trigger RIG-I. Based on the observation that *tlr7* and *myD88* are increased upon EBV infection, intrinsic TLR7 pathway might as well be involved in this last signal [752].

Because CpG-B have been shown to activate primary B cells and induce their proliferation [758], their effects on B cells upon EBV infection were studied. When administrated simultaneously, CpGs and EBV synergize to increase activation and proliferation of B cells compared to EBV alone [759]. CpGs were also able to increase outgrowth of LCLs [759, 760].

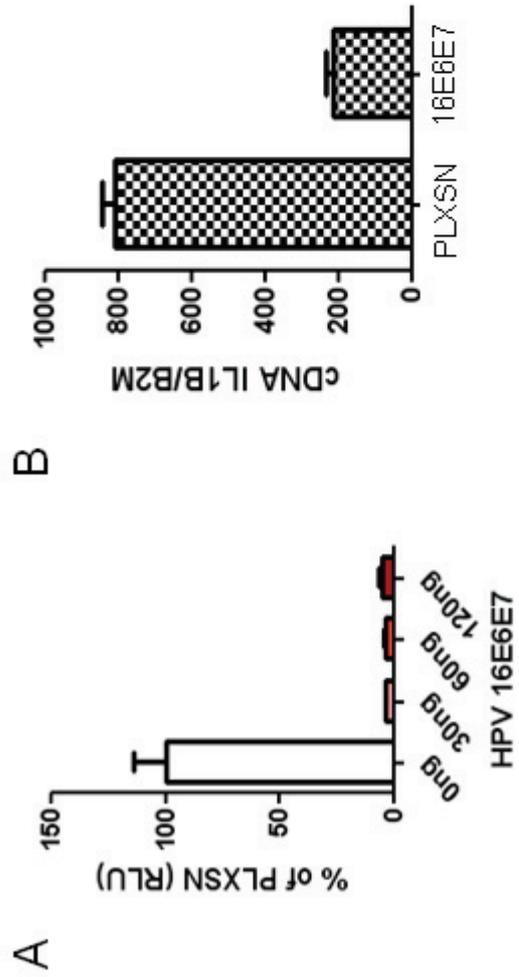
While CpG increase EBV-mediated B cell proliferation, EBV was shown to inhibit CpG-induced B cell proliferation within the first 80 hours post EBV infection [558]. Moreover, half of the CpG-mediated LCL outgrowth increase was lost when CpG were added 12 hours after infection [759]. This could be due to the LMP1 dependent TLR9 down-regulation that we have reported [381]. In conclusion, while CpG increase EBV-mediated B cell proliferation and LCL outgrowth, EBV decreases CpG mediated responses. One might wonder how to reconcile those two pieces of information. In that respect considering that CpG stimulation induced an inhibition of TLR9 expression [761], it might also favor the transformation. We could argue that in the conditions where CpG and EBV are co-administered *tlr9* expression is more quickly and/or strongly down-regulated, when compared to EBV alone, thus leading to an increase in proliferation and B cell transformation. This could be confirmed by checking *tlr9* expression in those set of experiments.

As TLR9 triggering favors proliferation and EBV+ B cell transformation, one might wonder as well what would be the benefit for the virus of deregulating *tlr9*. Our main hypothesis is illustrated by the fact that *tlr9* expression might limit cell proliferation (paper 2). Thus, it is possible that, as previously discussed, TLR9 down-regulation would favor the transformation of newly infected B cells.

Furthermore, TLR9 down-regulation might impact on the immune sensing of the newly produced virus. Data indeed showed the down-regulation of TLR9 in B cells EBV infected during the lytic cycle [452]. The inhibition of TLR9 might be a mechanism of immune escape to avoid the detection of EBV by the infected B cell.



Supplementary Figure 3: IL1 β production by HPV16 virions in bone marrow derived macrophages.



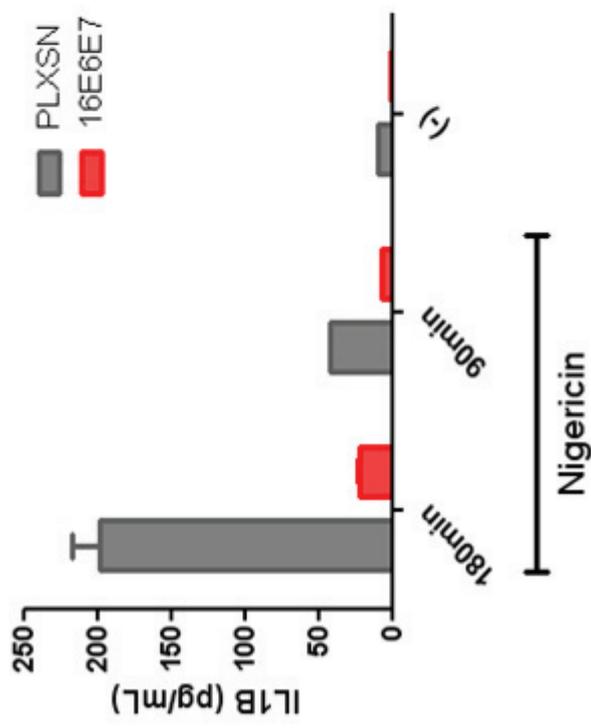
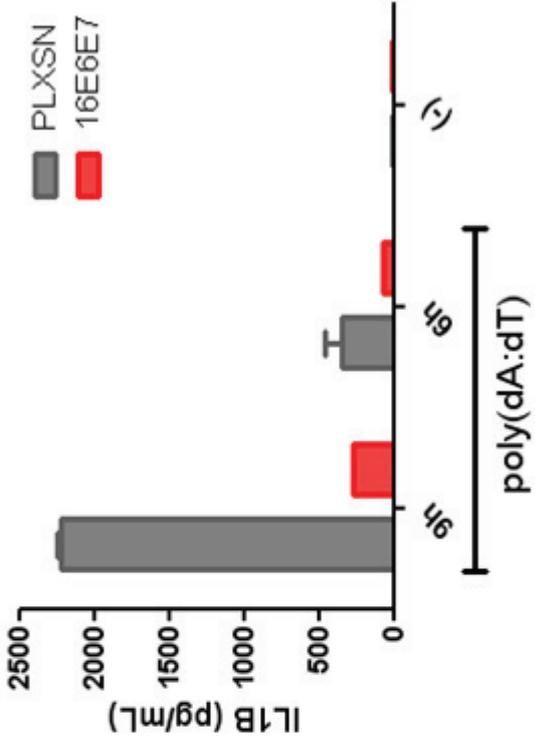
Supplementary Figure 4: (A) IL1 β reporter assay in Hek transfected with increasing concentration of HPV 16E6E7 plasmid. (B) mRNA levels of IL1 β over b2-microglobulin of keratinocytes stably expressing HPV 16E6E7 or a control plasmid.

3. MECHANISM OF LMP1 INDUCED SUPPRESSION OF TLR9

We have shown that LMP1 was mediating TLR9 suppression *via* NF κ B. However the precise mechanisms behind TLR9 suppression by EBV during primo-infection remain to be elucidated. It would be interesting to know which NF- κ B cis-elements bind to the TLR9 promoter and are responsible for the EBV-induced TLR9 transcriptional repression. The analysis of the DNA sequence of this region of TLR9 promoter revealed the presence of four NF- κ B binding sequences [761]. To identify the precise cis-element(s) that is/are responsible for the inhibition of TLR9 expression induced by EBV, the NF- κ B binding sites could be individually mutated and the activity of mutated TLR9 promoters could be tested in transient transfection experiments in presence or absence of EBV- infected or LMP-1-expressing cells. This finding would be confirmed by chromatin immunoprecipitation experiments (ChIP) in normal or EBV positive B cells versus primary B cells using specific anti antibodies for the different NF- κ B subunits. We could also investigate whether the transcription factors regulating TLR9 promoter may also be silenced by promoter region methylation using for example methylation-sensitive restriction enzymes and pyrosequencing. We could also look for histone modifications that are important in gene regulation. Histone acetylation and methylation at H4 and H3K4 respectively, are positive marks associated with transcriptionally active chromatin, whereas deacetylated and demethylated histones are found in closed, inactive chromatin. We could use ChIP to compare EBV infected and non-infected human B cells. We could monitor by qPCR the expression level of cis-elements regulating the TLR9 promoter and correlate these data to their respective level of their histone acetylation and methylation.

4. INNATE IMMUNITY TO ONCOVIRUSES AND REDUNDANCY OF PRRS

Because PRRs are redundant we wonder whether EBV or HPV that inhibit *tlr9* to escape innate recognition might trigger other DNA receptors. It would be interesting to see if HPV and EBV could trigger the cytosolic receptor AIM2 or the cytosolic / nuclear receptor IFI16. Keratinocytes and epithelial cells express those two receptors, as for B cells the expression of AIM2 has not been assessed [762-765]. Activation of those receptors was shown to lead to IL1 β or type I IFN production [501-503, 766]. We have preliminary data showing that early after infection keratinocytes infected with HPV16 viruses produced and released a high amount of mature IL1 β , as early as 4h post infection (Supplementary Figure 1). This production might be important to alert the immune system that an infection is going on. We demonstrated that this induction is caspase 1 dependent using chemical caspase 1 inhibitors. However, as shown using the monocytic thp1 cell lines knock down for asc (with 2 independent shRNA sequences), this IL1 β induction is ASC independent, which rather uncommon (Supplementary Figure 2). We have also seen that HPV 16 viruses were able to stimulate bone marrow derived macrophages to produce IL1 β (Supplementary Figure 3). However we do not know yet which inflammasome sensor is triggered. It is indeed possible that HPV16 genomic DNA would be recognized by AIM2 in the cytoplasm, IFI16 in the nucleus or that the lysosomal damage caused by HPV16 would activate NLRP3. We are currently screening BL6/C57 bone-marrow derived macrophages and DCs that are knock-out for different genes of the inflammasome pathway for their ability to produce IL1 β in response to HPV16 viruses (Collaboration O.Gross). In the meantime we will assess the effect of IFI16 AIM2 and Nalp3 knock down on IL1 β induction in keratinocytes using shRNA approaches. We will also determine if the viral replication is required for this IL1 β production using UV inactivated viruses. Finally, we do not know yet if EBV infection triggers IL1 β production by B cells. We are planning to do some preliminary experiments to answer this question.



Supplementary Figure 5: (A) IL1β production in response to NLRP3 (A) or AIM2 (B) ligands in keratinocytes expressing HPV 16E6E7 or control plasmid.

However despite the eventual stimulation of PRRs upon cell infection, EBV and HPV16 display several proteins that impede production of antiviral cytokines. Our preliminary data show that the oncoproteins E6 and E7 from HPV high risk type 16 but not from HPV low risk type 6 are able to inhibit *il1 β* transcription (Supplementary Figure 4). Upon stimulation with AIM2 or NLRP3 ligands, pdAdT and nigericin respectively, immortalized NIK keratinocytes expressing HPV16 oncoproteins were almost unable to produce IL1 β (Supplementary Figure 5). This failure is mostly due to high decrease in IL1 β protein level as seen by western blotting in conditions where the oncoproteins are expressed. We are currently analyzing the *IL1 β* promoter to look for binding sites for transcription factor known to be modulated by HPV 16. The binding of candidate transcription factors will be validated with a CHIP approach and the effect on *IL1B* transcription by over-expression studies and qPCR approach.

Overall our studies implicate that innate sensor regulation is essential for oncoviral clearance as our models of viral induced cancers severely impede their expression and regulation. We have in addition addressed the role of innate sensors in their ability to control the cell cycle, indicating that they have dual activities. This point highlights that sensing by PRRs are crucial for host cell activity in combating viral infection.

V. REFERENCES

1. Parkin, D.M., et al., *Global cancer statistics, 2002*. CA Cancer J Clin, 2005. **55**(2): p. 74-108.
2. Zur Hausen, H., *The search for infectious causes of human cancers: where and why*. Virology, 2009. **392**(1): p. 1-10.
3. Parkin, D.M. and F. Bray, *Chapter 2: The burden of HPV-related cancers*. Vaccine, 2006. **24 Suppl 3**: p. S3/11-25.
4. Smyth, M.J., G.P. Dunn, and R.D. Schreiber, *Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity*. Adv Immunol, 2006. **90**: p. 1-50.
5. Rakoff-Nahoum, S. and R. Medzhitov, *Innate immune recognition of the indigenous microbial flora*. Mucosal Immunol, 2008. **1 Suppl 1**: p. S10-4.
6. Rakoff-Nahoum, S. and R. Medzhitov, *Role of toll-like receptors in tissue repair and tumorigenesis*. Biochemistry (Mosc), 2008. **73**(5): p. 555-61.
7. Rakoff-Nahoum, S. and R. Medzhitov, *Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88*. Science, 2007. **317**(5834): p. 124-7.
8. Rakoff-Nahoum, S. and R. Medzhitov, *Prostaglandin-secreting cells: a portable first aid kit for tissue repair*. J Clin Invest, 2007. **117**(1): p. 83-6.
9. El-Omar, E.M., M.T. Ng, and G.L. Hold, *Polymorphisms in Toll-like receptor genes and risk of cancer*. Oncogene, 2008. **27**(2): p. 244-52.
10. Xu, C.J., et al., *Association study of a single nucleotide polymorphism in the exon 2 region of toll-like receptor 9 (TLR9) gene with susceptibility to systemic lupus erythematosus among Chinese*. Mol Biol Rep, 2009. **36**(8): p. 2245-8.
11. Pine, S.O., M.J. McElrath, and P.Y. Bochud, *Polymorphisms in toll-like receptor 4 and toll-like receptor 9 influence viral load in a seroincident cohort of HIV-1-infected individuals*. AIDS, 2009. **23**(18): p. 2387-95.
12. Chang, Y., et al., *Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma*. Science, 1994. **266**(5192): p. 1865-9.
13. Feng, H., et al., *Clonal integration of a polyomavirus in human Merkel cell carcinoma*. Science, 2008. **319**(5866): p. 1096-100.
14. Bouvard, V., et al., *A review of human carcinogens--Part B: biological agents*. Lancet Oncol, 2009. **10**(4): p. 321-2.
15. Epstein, M.A., B.G. Achong, and Y.M. Barr, *Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma*. Lancet, 1964. **1**(7335): p. 702-3.
16. Henle, G., W. Henle, and V. Diehl, *Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis*. Proc Natl Acad Sci U S A, 1968. **59**(1): p. 94-101.
17. Lunemann, J.D. and C. Munz, *EBV in MS: guilty by association?* Trends Immunol, 2009. **30**(6): p. 243-8.
18. Tugizov, S.M., J.W. Berline, and J.M. Palefsky, *Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells*. Nat Med, 2003. **9**(3): p. 307-14.
19. Yachie, A., H. Kanegane, and Y. Kasahara, *Epstein-Barr virus-associated T-natural killer cell lymphoproliferative diseases*. Semin Hematol, 2003. **40**(2): p. 124-32.
20. Tugizov, S., et al., *Epstein-Barr virus (EBV)-infected monocytes facilitate dissemination of EBV within the oral mucosal epithelium*. J Virol, 2007. **81**(11): p. 5484-96.
21. Jiang, R., et al., *Laser-capture microdissection of oropharyngeal epithelium indicates restriction of Epstein-Barr virus receptor/CD21 mRNA to tonsil epithelial cells*. J Oral Pathol Med, 2008. **37**(10): p. 626-33.
22. Babcock, G.J., D. Hochberg, and A.D. Thorley-Lawson, *The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell*. Immunity, 2000. **13**(4): p. 497-506.
23. Fingerroth, J.D., et al., *Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2*. Proc Natl Acad Sci U S A, 1984. **81**(14): p. 4510-4.
24. Li, Q., et al., *Epstein-Barr virus uses HLA class II as a cofactor for infection of B lymphocytes*. J Virol, 1997. **71**(6): p. 4657-62.
25. Molesworth, S.J., et al., *Epstein-Barr virus gH is essential for penetration of B cells but also plays a role in attachment of virus to epithelial cells*. J Virol, 2000. **74**(14): p. 6324-32.
26. Oda, T., et al., *Epstein-Barr virus lacking glycoprotein gp85 cannot infect B cells and epithelial cells*. Virology, 2000. **276**(1): p. 52-8.
27. McShane, M.P. and R. Longnecker, *Cell-surface expression of a mutated Epstein-Barr virus glycoprotein B allows fusion independent of other viral proteins*. Proc Natl Acad Sci U S A, 2004. **101**(50): p. 17474-9.
28. Joseph, A.M., G.J. Babcock, and D.A. Thorley-Lawson, *Cells expressing the Epstein-Barr virus growth program are present in and restricted to the naive B-cell subset of healthy tonsils*. J Virol, 2000. **74**(21): p. 9964-71.
29. Babcock, G.J., E.M. Miyashita-Lin, and D.A. Thorley-Lawson, *Detection of EBV infection at the single-cell level. Precise quantitation of virus-infected cells in vivo*. Methods Mol Biol, 2001. **174**: p. 103-10.
30. Babcock, G.J. and D.A. Thorley-Lawson, *Tonsillar memory B cells, latently infected with Epstein-Barr virus, express the restricted pattern of latent genes previously found only in Epstein-Barr virus-associated tumors*. Proc Natl Acad Sci U S A, 2000. **97**(22): p. 12250-5.
31. Babcock, G.J., et al., *EBV persistence in memory B cells in vivo*. Immunity, 1998. **9**(3): p. 395-404.
32. Thorley-Lawson, D.A. and G.J. Babcock, *A model for persistent infection with Epstein-Barr virus: the stealth virus of human B cells*. Life Sci, 1999. **65**(14): p. 1433-53.
33. Hochberg, D., et al., *Demonstration of the Burkitt's lymphoma Epstein-Barr virus phenotype in dividing latently infected memory cells in vivo*. Proc Natl Acad Sci U S A, 2004. **101**(1): p. 239-44.
34. Ehlin-Henriksson, B., J. Gordon, and G. Klein, *B-lymphocyte subpopulations are equally susceptible to Epstein-Barr virus infection, irrespective of immunoglobulin isotype expression*. Immunology, 2003. **108**(4): p. 427-30.
35. Yates, J.L., N. Warren, and B. Sugden, *Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells*. Nature, 1985. **313**(6005): p. 812-5.
36. Kennedy, G. and B. Sugden, *EBNA-1, a bifunctional transcriptional activator*. Mol Cell Biol, 2003. **23**(19): p. 6901-8.
37. Levitskaya, J., et al., *Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1*. Nature, 1995. **375**(6533): p. 685-8.
38. Kieff, E., *Fields virology*, in *Fields virology*. 1996, Lippincott-Raven: Philadelphia.
39. Sinclair, A.J., et al., *EBNA-2 and EBNA-LP cooperate to cause G0 to G1 transition during immortalization of resting human B lymphocytes by Epstein-Barr virus*. EMBO J, 1994. **13**(14): p. 3321-8.
40. Ling, P.D., J.J. Ryon, and S.D. Hayward, *EBNA-2 of herpesvirus papio diverges significantly from the type A and type B EBNA-2 proteins of Epstein-Barr virus but retains an efficient transactivation domain with a conserved hydrophobic motif*. J Virol, 1993. **67**(6): p. 2990-3003.
41. Yalamanchili, R., et al., *Genetic and biochemical evidence that EBNA 2 interaction with a 63-kDa cellular GTG-binding protein is essential for B lymphocyte growth transformation by EBV*. Virology, 1994. **204**(2): p. 634-41.
42. Harada, S. and E. Kieff, *Epstein-Barr virus nuclear protein LP stimulates EBNA-2 acidic domain-mediated transcriptional activation*. J Virol, 1997. **71**(9): p. 6611-8.
43. Peng, C.W., et al., *Direct interactions between Epstein-Barr virus leader protein LP and the EBNA2 acidic domain underlie coordinate transcriptional regulation*. Proc Natl Acad Sci U S A, 2004. **101**(4): p. 1033-8.
44. Le Roux, A., et al., *The Epstein-Barr virus determined nuclear antigens EBNA-3A, -3B, and -3C repress EBNA-2-mediated transactivation of the viral terminal protein 1 gene promoter*. Virology, 1994. **205**(2): p. 596-602.

45. Robertson, E.S., J. Lin, and E. Kieff, *The amino-terminal domains of Epstein-Barr virus nuclear proteins 3A, 3B, and 3C interact with RBP1(kappa)*. *J Virol*, 1996. **70**(5): p. 3068-74.
46. Wang, D., D. Liebowitz, and E. Kieff, *An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells*. *Cell*, 1985. **43**(3 Pt 2): p. 831-40.
47. Kulwichit, W., et al., *Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice*. *Proc Natl Acad Sci U S A*, 1998. **95**(20): p. 11963-8.
48. Kaye, K.M., K.M. Izumi, and E. Kieff, *Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation*. *Proc Natl Acad Sci U S A*, 1993. **90**(19): p. 9150-4.
49. Banchemereau, J., et al., *The CD40 antigen and its ligand*. *Annu Rev Immunol*, 1994. **12**: p. 881-922.
50. Kilger, E., et al., *Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor*. *EMBO J*, 1998. **17**(6): p. 1700-9.
51. Webster-Cyriaque, J., J. Middeldorp, and N. Raab-Traub, *Hairy leukoplakia: an unusual combination of transforming and permissive Epstein-Barr virus infections*. *J Virol*, 2000. **74**(16): p. 7610-8.
52. Dukers, D.F., et al., *Quantitative immunohistochemical analysis of cytokine profiles in Epstein-Barr virus-positive and -negative cases of Hodgkin's disease*. *J Pathol*, 2000. **190**(2): p. 143-9.
53. Houali, K., et al., *A new diagnostic marker for secreted Epstein-Barr virus encoded LMP1 and BARF1 oncoproteins in the serum and saliva of patients with nasopharyngeal carcinoma*. *Clin Cancer Res*, 2007. **13**(17): p. 4993-5000.
54. Liebowitz, D., *Epstein-Barr virus and a cellular signaling pathway in lymphomas from immunosuppressed patients*. *N Engl J Med*, 1998. **338**(20): p. 1413-21.
55. Lee, D.Y. and B. Sugden, *The latent membrane protein 1 oncogene modifies B-cell physiology by regulating autophagy*. *Oncogene*, 2008. **27**(20): p. 2833-42.
56. Aviel, S., et al., *Degradation of the Epstein-Barr virus latent membrane protein 1 (LMP1) by the ubiquitin-proteasome pathway. Targeting via ubiquitination of the N-terminal residue*. *J Biol Chem*, 2000. **275**(31): p. 23491-9.
57. Lo, A.K., et al., *Modulation of LMP1 protein expression by EBV-encoded microRNAs*. *Proc Natl Acad Sci U S A*, 2007. **104**(41): p. 16164-9.
58. Flanagan, J., J. Middeldorp, and T. Sculley, *Localization of the Epstein-Barr virus protein LMP 1 to exosomes*. *J Gen Virol*, 2003. **84**(Pt 7): p. 1871-9.
59. Dukers, D.F., et al., *Direct immunosuppressive effects of EBV-encoded latent membrane protein 1*. *J Immunol*, 2000. **165**(2): p. 663-70.
60. Raposo, G., et al., *B lymphocytes secrete antigen-presenting vesicles*. *J Exp Med*, 1996. **183**(3): p. 1161-72.
61. Skokos, D., et al., *Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes*. *J Immunol*, 2001. **166**(2): p. 868-76.
62. Skokos, D., et al., *Nonspecific B and T cell-stimulatory activity mediated by mast cells is associated with exosomes*. *Int Arch Allergy Immunol*, 2001. **124**(1-3): p. 133-6.
63. Zitvogel, L., et al., *Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes*. *Nat Med*, 1998. **4**(5): p. 594-600.
64. Caldwell, R.G., et al., *Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals*. *Immunity*, 1998. **9**(3): p. 405-11.
65. Maruyama, M., K.P. Lam, and K. Rajewsky, *Memory B-cell persistence is independent of persisting immunizing antigen*. *Nature*, 2000. **407**(6804): p. 636-42.
66. Burkhardt, A.L., et al., *An Epstein-Barr virus transformation-associated membrane protein interacts with src family tyrosine kinases*. *J Virol*, 1992. **66**(8): p. 5161-7.
67. Fruehling, S. and R. Longnecker, *The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR-mediated signal transduction*. *Virology*, 1997. **235**(2): p. 241-51.
68. Miller, C.L., et al., *An integral membrane protein (LMP2) blocks reactivation of Epstein-Barr virus from latency following surface immunoglobulin crosslinking*. *Proc Natl Acad Sci U S A*, 1994. **91**(2): p. 772-6.
69. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function*. *Cell*, 2004. **116**(2): p. 281-97.
70. Pfeffer, S., et al., *Identification of virus-encoded microRNAs*. *Science*, 2004. **304**(5671): p. 734-6.
71. Feederle, R., et al., *A viral microRNA cluster strongly potentiates the transforming properties of a human herpesvirus*. *PLoS Pathog*. **7**(2): p. e1001294.
72. Seto, E., et al., *Micro RNAs of Epstein-Barr virus promote cell cycle progression and prevent apoptosis of primary human B cells*. *PLoS Pathog*. **6**(8).
73. Tovey, M.G., G. Lenoir, and J. Begon-Lours, *Activation of latent Epstein-Barr virus by antibody to human IgM*. *Nature*, 1978. **276**(5685): p. 270-2.
74. Fahmi, H., et al., *Transforming growth factor beta 1 stimulates expression of the Epstein-Barr virus BZLF1 immediate-early gene product ZEBRA by an indirect mechanism which requires the MAPK kinase pathway*. *J Virol*, 2000. **74**(13): p. 5810-8.
75. Fu, Z. and M.J. Cannon, *Functional analysis of the CD4(+) T-cell response to Epstein-Barr virus: T-cell-mediated activation of resting B cells and induction of viral BZLF1 expression*. *J Virol*, 2000. **74**(14): p. 6675-9.
76. Zalani, S., E. Holley-Guthrie, and S. Kenney, *Epstein-Barr viral latency is disrupted by the immediate-early BRLF1 protein through a cell-specific mechanism*. *Proc Natl Acad Sci U S A*, 1996. **93**(17): p. 9194-9.
77. Rooney, C.M., et al., *The spliced BZLF1 gene of Epstein-Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle*. *J Virol*, 1989. **63**(7): p. 3109-16.
78. Holley-Guthrie, E.A., et al., *The Epstein-Barr virus (EBV) BMRF1 promoter for early antigen (EA-D) is regulated by the EBV transactivators, BRLF1 and BZLF1, in a cell-specific manner*. *J Virol*, 1990. **64**(8): p. 3753-9.
79. Khan, G., et al., *Is EBV persistence in vivo a model for B cell homeostasis?* *Immunity*, 1996. **5**(2): p. 173-9.
80. Blaskovic, D., et al., *Isolation of five strains of herpesviruses from two species of free living small rodents*. *Acta Virol*, 1980. **24**(6): p. 468.
81. Flano, E., D.L. Woodland, and M.A. Blackman, *A mouse model for infectious mononucleosis*. *Immunol Res*, 2002. **25**(3): p. 201-17.
82. Nash, A.A. and N.P. Sunil-Chandra, *Interactions of the murine gammaherpesvirus with the immune system*. *Curr Opin Immunol*, 1994. **6**(4): p. 560-3.
83. Sunil-Chandra, N.P., et al., *Virological and pathological features of mice infected with murine gamma-herpesvirus 68*. *J Gen Virol*, 1992. **73** (Pt 9): p. 2347-56.
84. Moghaddam, A., et al., *An animal model for acute and persistent Epstein-Barr virus infection*. *Science*, 1997. **276**(5321): p. 2030-3.
85. Rivallier, P., et al., *Experimental rhesus lymphocryptovirus infection in immunosuppressed macaques: an animal model for Epstein-Barr virus pathogenesis in the immunosuppressed host*. *Blood*, 2004. **104**(5): p. 1482-9.
86. Walboomers, J.M., et al., *Human papillomavirus is a necessary cause of invasive cervical cancer worldwide*. *J Pathol*, 1999. **189**(1): p. 12-9.
87. Harwood, C.A. and C.M. Proby, *Human papillomaviruses and non-melanoma skin cancer*. *Curr Opin Infect Dis*, 2002. **15**(2): p. 101-14.
88. Pfister, H., *Chapter 8: Human papillomavirus and skin cancer*. *J Natl Cancer Inst Monogr*, 2003(31): p. 52-6.
89. Stark, L.A., et al., *Prevalence of human papillomavirus DNA in cutaneous neoplasms from renal allograft recipients sup-*

- ports a possible viral role in tumour promotion. *Br J Cancer*, 1994. **69**(2): p. 222-9.
90. Zheng, Z.M. and C.C. Baker, *Papillomavirus genome structure, expression, and post-transcriptional regulation*. *Front Biosci*, 2006. **11**: p. 2286-302.
 91. Ishii, Y., et al., *Human papillomavirus 16 minor capsid protein L2 helps capsomeres assemble independently of intercapsomeric disulfide bonding*. *Virus Genes*, 2005. **31**(3): p. 321-8.
 92. Okun, M.M., et al., *L1 interaction domains of papillomavirus L2 necessary for viral genome encapsidation*. *J Virol*, 2001. **75**(9): p. 4332-42.
 93. Kamper, N., et al., *A membrane-destabilizing peptide in capsid protein L2 is required for egress of papillomavirus genomes from endosomes*. *J Virol*, 2006. **80**(2): p. 759-68.
 94. Florin, L., et al., *Assembly and translocation of papillomavirus capsid proteins*. *J Virol*, 2002. **76**(19): p. 10009-14.
 95. Florin, L., et al., *Reorganization of nuclear domain 10 induced by papillomavirus capsid protein L2*. *Virology*, 2002. **295**(1): p. 97-107.
 96. Roberts, S., et al., *The ND10 component promyelocytic leukemia protein relocates to human papillomavirus type 1 E4 intranuclear inclusion bodies in cultured keratinocytes and in warts*. *J Virol*, 2003. **77**(1): p. 673-84.
 97. Bryan, J.T. and D.R. Brown, *Association of the human papillomavirus type 11 E1(E4) protein with cornified cell envelopes derived from infected genital epithelium*. *Virology*, 2000. **277**(2): p. 262-9.
 98. Roberts, S., et al., *Mutational analysis of human papillomavirus E4 proteins: identification of structural features important in the formation of cytoplasmic E4/cytokeratin networks in epithelial cells*. *J Virol*, 1994. **68**(10): p. 6432-45.
 99. Raj, K., et al., *E1 empty set E4 protein of human papillomavirus type 16 associates with mitochondria*. *J Virol*, 2004. **78**(13): p. 7199-207.
 100. Stenlund, A., *E1 initiator DNA binding specificity is unmasked by selective inhibition of non-specific DNA binding*. *EMBO J*, 2003. **22**(4): p. 954-63.
 101. Van Tine, B.A., et al., *Human papillomavirus (HPV) origin-binding protein associates with mitotic spindles to enable viral DNA partitioning*. *Proc Natl Acad Sci U S A*, 2004. **101**(12): p. 4030-5.
 102. Disbrow, G.L., J.A. Hanover, and R. Schlegel, *Endoplasmic reticulum-localized human papillomavirus type 16 E5 protein alters endosomal pH but not trans-Golgi pH*. *J Virol*, 2005. **79**(9): p. 5839-46.
 103. Crusius, K., I. Rodriguez, and A. Alonso, *The human papillomavirus type 16 E5 protein modulates ERK1/2 and p38 MAP kinase activation by an EGFR-independent process in stressed human keratinocytes*. *Virus Genes*, 2000. **20**(1): p. 65-9.
 104. Huibregtse, J.M., M. Scheffner, and P.M. Howley, *Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins*. *Mol Cell Biol*, 1993. **13**(8): p. 4918-27.
 105. Spanos, W.C., et al., *Deletion of the PDZ motif of HPV16 E6 preventing immortalization and anchorage-independent growth in human tonsil epithelial cells*. *Head Neck*, 2008. **30**(2): p. 139-47.
 106. Boyer, S.N., D.E. Wazer, and V. Band, *E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway*. *Cancer Res*, 1996. **56**(20): p. 4620-4.
 107. Giroglou, T., et al., *Human papillomavirus infection requires cell surface heparan sulfate*. *J Virol*, 2001. **75**(3): p. 1565-70.
 108. Roberts, J.N., et al., *Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan*. *Nat Med*, 2007. **13**(7): p. 857-61.
 109. Sapp, M. and M. Bienkowska-Haba, *Viral entry mechanisms: human papillomavirus and a long journey from extracellular matrix to the nucleus*. *FEBS J*, 2009. **276**(24): p. 7206-16.
 110. Elenius, K., et al., *Induced expression of syndecan in healing wounds*. *J Cell Biol*, 1991. **114**(3): p. 585-95.
 111. Gallo, R.L., et al., *Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds*. *Proc Natl Acad Sci U S A*, 1994. **91**(23): p. 11035-9.
 112. Selinka, H.C., et al., *Inhibition of transfer to secondary receptors by heparan sulfate-binding drug or antibody induces noninfectious uptake of human papillomavirus*. *J Virol*, 2007. **81**(20): p. 10970-80.
 113. Day, P.M., et al., *Mechanisms of human papillomavirus type 16 neutralization by L2 cross-neutralizing and L1 type-specific antibodies*. *J Virol*, 2008. **82**(9): p. 4638-46.
 114. Spoden, G., et al., *Clathrin- and caveolin-independent entry of human papillomavirus type 16--involvement of tetraspanin-enriched microdomains (TEMs)*. *PLoS One*, 2008. **3**(10): p. e3313.
 115. Day, P.M., D.R. Lowy, and J.T. Schiller, *Papillomaviruses infect cells via a clathrin-dependent pathway*. *Virology*, 2003. **307**(1): p. 1-11.
 116. Day, P.M., et al., *Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression*. *Proc Natl Acad Sci U S A*, 2004. **101**(39): p. 14252-7.
 117. Florin, L., et al., *Identification of a dynein interacting domain in the papillomavirus minor capsid protein L2*. *J Virol*, 2006. **80**(13): p. 6691-6.
 118. Sherman, L., et al., *Inhibition of serum- and calcium-induced differentiation of human keratinocytes by HPV16 E6 oncoprotein: role of p53 inactivation*. *Virology*, 1997. **237**(2): p. 296-306.
 119. Kukimoto, I., T. Takeuchi, and T. Kanda, *CCAAT/enhancer binding protein beta binds to and activates the P670 promoter of human papillomavirus type 16*. *Virology*, 2006. **346**(1): p. 98-107.
 120. Buck, C.B., et al., *Efficient intracellular assembly of papillomaviral vectors*. *J Virol*, 2004. **78**(2): p. 751-7.
 121. Lin, B.Y., et al., *Chaperone proteins abrogate inhibition of the human papillomavirus (HPV) E1 replicative helicase by the HPV E2 protein*. *Mol Cell Biol*, 2002. **22**(18): p. 6592-604.
 122. Doorbar, J., et al., *Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network*. *Nature*, 1991. **352**(6338): p. 824-7.
 123. Wang, Q., et al., *Functional analysis of the human papillomavirus type 16 E1-E4 protein provides a mechanism for in vivo and in vitro keratin filament reorganization*. *J Virol*, 2004. **78**(2): p. 821-33.
 124. Asselineau, D. and M. Prunieras, *Reconstruction of 'simplified' skin: control of fabrication*. *Br J Dermatol*, 1984. **111 Suppl 27**: p. 219-22.
 125. Campo, M.S., *Animal models of papillomavirus pathogenesis*. *Virus Res*, 2002. **89**(2): p. 249-61.
 126. Arbeit, J.M., et al., *Progressive squamous epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice*. *J Virol*, 1994. **68**(7): p. 4358-68.
 127. Olsson, S.E., et al., *Evaluation of quadrivalent HPV 6/11/16/18 vaccine efficacy against cervical and anogenital disease in subjects with serological evidence of prior vaccine type HPV infection*. *Hum Vaccin*, 2009. **5**(10): p. 696-704.
 128. Romanowski, B., et al., *Sustained efficacy and immunogenicity of the human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine: analysis of a randomised placebo-controlled trial up to 6.4 years*. *Lancet*, 2009. **374**(9706): p. 1975-85.
 129. Shi, L., et al., *GARDASIL: prophylactic human papillomavirus vaccine development--from bench top to bed-side*. *Clin Pharmacol Ther*, 2007. **81**(2): p. 259-64.
 130. Monie, A., et al., *Therapeutic HPV DNA vaccines*. *Expert Rev Vaccines*, 2009. **8**(9): p. 1221-35.
 131. Watts, S.L., et al., *Free cottontail rabbit papillomavirus DNA persists in warts and carcinomas of infected rabbits and in cells in culture transformed with virus or viral DNA*. *Virology*, 1983. **125**(1): p. 127-38.
 132. Eddy, B.E., et al., *Identification of the oncogenic substance in*

- rhesus monkey kidney cell culture as simian virus 40*. *Virology*, 1962. **17**: p. 65-75.
133. Fruchter, R.G., et al., *Multiple recurrences of cervical intra-epithelial neoplasia in women with the human immunodeficiency virus*. *Obstet Gynecol*, 1996. **87**(3): p. 338-44.
134. Nakanishi, M., M. Shimada, and H. Niida, *Genetic instability in cancer cells by impaired cell cycle checkpoints*. *Cancer Sci*, 2006. **97**(10): p. 984-9.
135. Lindahl, T., *Instability and decay of the primary structure of DNA*. *Nature*, 1993. **362**(6422): p. 709-15.
136. Hoeijmakers, J.H., *Genome maintenance mechanisms for preventing cancer*. *Nature*, 2001. **411**(6835): p. 366-74.
137. Peltomaki, P., *DNA mismatch repair and cancer*. *Mutat Res*, 2001. **488**(1): p. 77-85.
138. Zhou, B.B. and S.J. Elledge, *The DNA damage response: putting checkpoints in perspective*. *Nature*, 2000. **408**(6811): p. 433-9.
139. Alarcon-Vargas, D. and Z. Ronai, *p53-Mdm2--the affair that never ends*. *Carcinogenesis*, 2002. **23**(4): p. 541-7.
140. Dumaz, N. and D.W. Meek, *Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2*. *EMBO J*, 1999. **18**(24): p. 7002-10.
141. Lohr, K., et al., *p21/CDKN1A mediates negative regulation of transcription by p53*. *J Biol Chem*, 2003. **278**(35): p. 32507-16.
142. Shats, I., et al., *p53-dependent down-regulation of telomerase is mediated by p21waf1*. *J Biol Chem*, 2004. **279**(49): p. 50976-85.
143. Moldovan, G.L., B. Pfander, and S. Jentsch, *PCNA, the maestro of the replication fork*. *Cell*, 2007. **129**(4): p. 665-79.
144. Devgan, V., et al., *p21WAF1/Cip1 is a negative transcriptional regulator of Wnt4 expression downstream of Notch1 activation*. *Genes Dev*, 2005. **19**(12): p. 1485-95.
145. Coqueret, O. and H. Gascan, *Functional interaction of STAT3 transcription factor with the cell cycle inhibitor p21WAF1/CIP1/SDI1*. *J Biol Chem*, 2000. **275**(25): p. 18794-800.
146. Kitaura, H., et al., *Reciprocal regulation via protein-protein interaction between c-Myc and p21(cip1/waf1/sdi1) in DNA replication and transcription*. *J Biol Chem*, 2000. **275**(14): p. 10477-83.
147. Snowden, A.W., et al., *A novel transcriptional repression domain mediates p21(WAF1/CIP1) induction of p300 transactivation*. *Mol Cell Biol*, 2000. **20**(8): p. 2676-86.
148. Fritah, A., et al., *p21WAF1/CIP1 selectively controls the transcriptional activity of estrogen receptor alpha*. *Mol Cell Biol*, 2005. **25**(6): p. 2419-30.
149. Sheikh, M.S., H. Rochefort, and M. Garcia, *Overexpression of p21WAF1/CIP1 induces growth arrest, giant cell formation and apoptosis in human breast carcinoma cell lines*. *Oncogene*, 1995. **11**(9): p. 1899-905.
150. Ping, B., et al., *Cytoplasmic expression of p21CIP1/WAF1 is correlated with IKKbeta overexpression in human breast cancers*. *Int J Oncol*, 2006. **29**(5): p. 1103-10.
151. Winters, Z.E., et al., *Subcellular localisation of cyclin B, Cdc2 and p21(WAF1/CIP1) in breast cancer. association with prognosis*. *Eur J Cancer*, 2001. **37**(18): p. 2405-12.
152. Xia, W., et al., *Phosphorylation/cytoplasmic localization of p21Cip1/WAF1 is associated with HER2/neu overexpression and provides a novel combination predictor for poor prognosis in breast cancer patients*. *Clin Cancer Res*, 2004. **10**(11): p. 3815-24.
153. Dotto, G.P., *p21(WAF1/Cip1): more than a break to the cell cycle?* *Biochim Biophys Acta*, 2000. **1471**(1): p. M43-56.
154. Roninson, I.B., *Oncogenic functions of tumour suppressor p21(Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts*. *Cancer Lett*, 2002. **179**(1): p. 1-14.
155. Suzuki, A., et al., *Mitochondrial regulation of cell death: mitochondria are essential for procaspase 3-p21 complex formation to resist Fas-mediated cell death*. *Mol Cell Biol*, 1999. **19**(5): p. 3842-7.
156. Asada, M., et al., *Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation*. *EMBO J*, 1999. **18**(5): p. 1223-34.
157. Dankort, D., et al., *A new mouse model to explore the initiation, progression, and therapy of BRAFV600E-induced lung tumors*. *Genes Dev*, 2007. **21**(4): p. 379-84.
158. Sarkisian, C.J., et al., *Dose-dependent oncogene-induced senescence in vivo and its evasion during mammary tumorigenesis*. *Nat Cell Biol*, 2007. **9**(5): p. 493-505.
159. Gartel, A.L. and A.L. Tyner, *Transcriptional regulation of the p21(WAF1/CIP1) gene*. *Exp Cell Res*, 1999. **246**(2): p. 280-9.
160. Black, A.R., J.D. Black, and J. Azizkhan-Clifford, *Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer*. *J Cell Physiol*, 2001. **188**(2): p. 143-60.
161. Ito, G., et al., *Kruppel-like factor 6 is frequently down-regulated and induces apoptosis in non-small cell lung cancer cells*. *Cancer Res*, 2004. **64**(11): p. 3838-43.
162. Kremer-Tal, S., et al., *Frequent inactivation of the tumor suppressor Kruppel-like factor 6 (KLF6) in hepatocellular carcinoma*. *Hepatology*, 2004. **40**(5): p. 1047-52.
163. Zhao, W., et al., *Identification of Kruppel-like factor 4 as a potential tumor suppressor gene in colorectal cancer*. *Oncogene*, 2004. **23**(2): p. 395-402.
164. Li, D., et al., *p150(Sal2) is a p53-independent regulator of p21(WAF1/CIP1)*. *Mol Cell Biol*, 2004. **24**(9): p. 3885-93.
165. Kohlhasse, J., et al., *SALL3, a new member of the human spalt-like gene family, maps to 18q23*. *Genomics*, 1999. **62**(2): p. 216-22.
166. Kohlhasse, J., et al., *Isolation, characterization, and organ-specific expression of two novel human zinc finger genes related to the Drosophila gene spalt*. *Genomics*, 1996. **38**(3): p. 291-8.
167. Kohlhasse, J., et al., *Cloning and expression analysis of SALL4, the murine homologue of the gene mutated in Okihiro syndrome*. *Cytogenet Genome Res*, 2002. **98**(4): p. 274-7.
168. Parrish, M., et al., *Loss of the Sall3 gene leads to palate deficiency, abnormalities in cranial nerves, and perinatal lethality*. *Mol Cell Biol*, 2004. **24**(16): p. 7102-12.
169. Karantzali, E., et al., *Sall1 regulates embryonic stem cell differentiation in association with nanog*. *J Biol Chem*. **286**(2): p. 1037-45.
170. Li, D., et al., *A tumor host range selection procedure identifies p150(sal2) as a target of polyoma virus large T antigen*. *Proc Natl Acad Sci U S A*, 2001. **98**(25): p. 14619-24.
171. Liu, H., et al., *A transcriptional program mediating entry into cellular quiescence*. *PLoS Genet*, 2007. **3**(6): p. e91.
172. Scheffner, M., et al., *The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53*. *Cell*, 1993. **75**(3): p. 495-505.
173. Scheffner, M., et al., *The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53*. *Cell*, 1990. **63**(6): p. 1129-36.
174. Matsuoka, S., et al., *Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro*. *Proc Natl Acad Sci U S A*, 2000. **97**(19): p. 10389-94.
175. Maya, R., et al., *ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage*. *Genes Dev*, 2001. **15**(9): p. 1067-77.
176. Brugarolas, J., et al., *Radiation-induced cell cycle arrest compromised by p21 deficiency*. *Nature*, 1995. **377**(6549): p. 552-7.
177. Deng, C., et al., *Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control*. *Cell*, 1995. **82**(4): p. 675-84.
178. McDonald, E.R., 3rd and W.S. El-Deiry, *Cell cycle control as a basis for cancer drug development (Review)*. *Int J Oncol*, 2000. **16**(5): p. 871-86.
179. Motokura, T., et al., *A novel cyclin encoded by a bcl1-linked candidate oncogene*. *Nature*, 1991. **350**(6318): p. 512-5.

180. Weisenburger, D.D., et al., *Intermediate lymphocytic lymphoma: immunophenotypic and cytogenetic findings*. *Blood*, 1987. **69**(6): p. 1617-21.
181. Hall, M. and G. Peters, *Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer*. *Adv Cancer Res*, 1996. **68**: p. 67-108.
182. Hunter, T. and J. Pines, *Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age*. *Cell*, 1994. **79**(4): p. 573-82.
183. Keyomarsi, K., et al., *Deregulation of cyclin E in breast cancer*. *Oncogene*, 1995. **11**(5): p. 941-50.
184. Leach, F.S., et al., *Amplification of cyclin genes in colorectal carcinomas*. *Cancer Res*, 1993. **53**(9): p. 1986-9.
185. Scuderi, R., et al., *Cyclin E overexpression in relapsed adult acute lymphoblastic leukemias of B-cell lineage*. *Blood*, 1996. **87**(8): p. 3360-7.
186. Dobashi, Y., et al., *Active cyclin A-CDK2 complex, a possible critical factor for cell proliferation in human primary lung carcinomas*. *Am J Pathol*, 1998. **153**(3): p. 963-72.
187. Nilsson, I. and I. Hoffmann, *Cell cycle regulation by the Cdc25 phosphatase family*. *Prog Cell Cycle Res*, 2000. **4**: p. 107-14.
188. Galaktionov, K., X. Chen, and D. Beach, *Cdc25 cell-cycle phosphatase as a target of c-myc*. *Nature*, 1996. **382**(6591): p. 511-7.
189. Galaktionov, K., C. Jessup, and D. Beach, *Raf1 interaction with Cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation*. *Genes Dev*, 1995. **9**(9): p. 1046-58.
190. Kamb, A., *Cyclin-dependent kinase inhibitors and human cancer*. *Curr Top Microbiol Immunol*, 1998. **227**: p. 139-48.
191. Harper, J.W. and S.J. Elledge, *Cdk inhibitors in development and cancer*. *Curr Opin Genet Dev*, 1996. **6**(1): p. 56-64.
192. Alessandrini, A., D.S. Chiaur, and M. Pagano, *Regulation of the cyclin-dependent kinase inhibitor p27 by degradation and phosphorylation*. *Leukemia*, 1997. **11**(3): p. 342-5.
193. Loda, M., et al., *Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas*. *Nat Med*, 1997. **3**(2): p. 231-4.
194. Esposito, F., et al., *Redox-mediated regulation of p21(waf1/cip1) expression involves a post-transcriptional mechanism and activation of the mitogen-activated protein kinase pathway*. *Eur J Biochem*, 1997. **245**(3): p. 730-7.
195. Lapointe, J., et al., *A p18 mutant defective in CDK6 binding in human breast cancer cells*. *Cancer Res*, 1996. **56**(20): p. 4586-9.
196. Shi, Y., et al., *Evidence of gene deletion of p21 (WAF1/CIP1), a cyclin-dependent protein kinase inhibitor, in thyroid carcinomas*. *Br J Cancer*, 1996. **74**(9): p. 1336-41.
197. Caffo, O., et al., *Prognostic value of p21(WAF1) and p53 expression in breast carcinoma: an immunohistochemical study in 261 patients with long-term follow-up*. *Clin Cancer Res*, 1996. **2**(9): p. 1591-9.
198. Lu, X., et al., *Expression of p21WAF1/CIP1 in adenocarcinoma of the uterine cervix: a possible immunohistochemical marker of a favorable prognosis*. *Cancer*, 1998. **82**(12): p. 2409-17.
199. Ogawa, M., et al., *A combination analysis of p53 and p21 in gastric carcinoma as a strong indicator for prognosis*. *Int J Mol Med*, 2001. **7**(5): p. 479-83.
200. Anttila, M.A., et al., *p21/WAF1 expression as related to p53, cell proliferation and prognosis in epithelial ovarian cancer*. *Br J Cancer*, 1999. **79**(11-12): p. 1870-8.
201. Kapranos, N., et al., *p53, p21 and p27 protein expression in head and neck cancer and their prognostic value*. *Anticancer Res*, 2001. **21**(1B): p. 521-8.
202. McKenzie, K.E., et al., *Altered WAF1 genes do not play a role in abnormal cell cycle regulation in breast cancers lacking p53 mutations*. *Clin Cancer Res*, 1997. **3**(9): p. 1669-73.
203. Patino-Garcia, A., E. Sotillo-Pineiro, and L. Sierrasesumaga-Ariznabarreta, *p21WAF1 mutation is not a predominant alteration in pediatric bone tumors*. *Pediatr Res*, 1998. **43**(3): p. 393-5.
204. Shiohara, M., et al., *Absence of WAF1 mutations in a variety of human malignancies*. *Blood*, 1994. **84**(11): p. 3781-4.
205. Martin-Caballero, J., et al., *Tumor susceptibility of p21(Waf1/Cip1)-deficient mice*. *Cancer Res*, 2001. **61**(16): p. 6234-8.
206. Jackson, R.J., et al., *Loss of the cell cycle inhibitors p21(Cip1) and p27(Kip1) enhances tumorigenesis in knockout mouse models*. *Oncogene*, 2002. **21**(55): p. 8486-97.
207. Philipp, J., et al., *Tumor suppression by p27Kip1 and p21Cip1 during chemically induced skin carcinogenesis*. *Oncogene*, 1999. **18**(33): p. 4689-98.
208. Poole, A.J., et al., *Tumor suppressor functions for the Cdk inhibitor p21 in the mouse colon*. *Oncogene*, 2004. **23**(49): p. 8128-34.
209. Topley, G.I., et al., *p21(WAF1/Cip1) functions as a suppressor of malignant skin tumor formation and a determinant of keratinocyte stem-cell potential*. *Proc Natl Acad Sci U S A*, 1999. **96**(16): p. 9089-94.
210. Carnero, A. and D.H. Beach, *Absence of p21WAF1 cooperates with c-myc in bypassing Ras-induced senescence and enhances oncogenic cooperation*. *Oncogene*, 2004. **23**(35): p. 6006-11.
211. Shen, K.C., et al., *ATM and p21 cooperate to suppress aneuploidy and subsequent tumor development*. *Cancer Res*, 2005. **65**(19): p. 8747-53.
212. Knudson, A.G., Jr., *Mutation and cancer: statistical study of retinoblastoma*. *Proc Natl Acad Sci U S A*, 1971. **68**(4): p. 820-3.
213. Greenblatt, M.S., et al., *Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis*. *Cancer Res*, 1994. **54**(18): p. 4855-78.
214. Miller, C. and H.P. Koeffler, *P53 mutations in human cancer*. *Leukemia*, 1993. **7 Suppl 2**: p. S18-21.
215. Hollstein, M., et al., *p53 mutations in human cancers*. *Science*, 1991. **253**(5015): p. 49-53.
216. Varley, J.M., *Germline TP53 mutations and Li-Fraumeni syndrome*. *Hum Mutat*, 2003. **21**(3): p. 313-20.
217. Bueso-Ramos, C.E., et al., *Multiple patterns of MDM-2 deregulation in human leukemias: implications in leukemogenesis and prognosis*. *Leuk Lymphoma*, 1995. **17**(1-2): p. 13-8.
218. Bueso-Ramos, C.E., et al., *Abnormal expression of MDM-2 in breast carcinomas*. *Breast Cancer Res Treat*, 1996. **37**(2): p. 179-88.
219. Moller, M.B., et al., *Disrupted p53 function as predictor of treatment failure and poor prognosis in B- and T-cell non-Hodgkin's lymphoma*. *Clin Cancer Res*, 1999. **5**(5): p. 1085-91.
220. Meijers-Heijboer, H., et al., *Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations*. *Nat Genet*, 2002. **31**(1): p. 55-9.
221. Kaiser, C., et al., *The proto-oncogene c-myc is a direct target gene of Epstein-Barr virus nuclear antigen 2*. *J Virol*, 1999. **73**(5): p. 4481-4.
222. Kohlhof, H., et al., *Notch1, Notch2, and Epstein-Barr virus-encoded nuclear antigen 2 signaling differentially affects proliferation and survival of Epstein-Barr virus-infected B cells*. *Blood*, 2009. **113**(22): p. 5506-15.
223. Maier, S., et al., *Cellular target genes of Epstein-Barr virus nuclear antigen 2*. *J Virol*, 2006. **80**(19): p. 9761-71.
224. Mohan, J., et al., *Epstein-Barr virus nuclear antigen 2 induces FcRH5 expression through CBF1*. *Blood*, 2006. **107**(11): p. 4433-9.
225. Pegman, P.M., et al., *Epstein-Barr virus nuclear antigen 2 trans-activates the cellular antiapoptotic bfl-1 gene by a CBF1/RBPJ kappa-dependent pathway*. *J Virol*, 2006. **80**(16): p. 8133-44.
226. Wang, F., et al., *Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23*. *J Virol*, 1990. **64**(5): p. 2309-18.
227. Li, M., et al., *Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization*. *Nature*, 2002. **416**(6881): p. 648-53.
228. Yi, F., et al., *Epstein-Barr virus nuclear antigen 3C targets p53*

- and modulates its transcriptional and apoptotic activities. *Virology*, 2009. **388**(2): p. 236-47.
229. Saha, A., et al., *Epstein-Barr virus nuclear antigen 3C augments Mdm2-mediated p53 ubiquitination and degradation by deubiquitinating Mdm2*. *J Virol*, 2009. **83**(9): p. 4652-69.
230. Saha, A., et al., *EBNA3C attenuates the function of p53 through interaction with inhibitor of growth family proteins 4 and 5*. *J Virol*. **85**(5): p. 2079-88.
231. Kashuba, E., et al., *Epstein-Barr virus-encoded EBNA-5 forms trimolecular protein complexes with MDM2 and p53 and inhibits the transactivating function of p53*. *Int J Cancer*. **128**(4): p. 817-25.
232. Saha, A., et al., *Epstein-Barr virus nuclear antigen 3C facilitates G1-S transition by stabilizing and enhancing the function of cyclin D1*. *PLoS Pathog*. **7**(2): p. e1001275.
233. Knight, J.S. and E.S. Robertson, *Epstein-Barr virus nuclear antigen 3C regulates cyclin A/p27 complexes and enhances cyclin A-dependent kinase activity*. *J Virol*, 2004. **78**(4): p. 1981-91.
234. Song, X., et al., *Epstein-Barr virus-encoded latent membrane protein 1 modulates cyclin D1 by c-Jun/Jun B heterodimers*. *Sci China C Life Sci*, 2005. **48**(4): p. 385-93.
235. Parker, G.A., R. Touitou, and M.J. Allday, *Epstein-Barr virus EBNA3C can disrupt multiple cell cycle checkpoints and induce nuclear division divorced from cytokinesis*. *Oncogene*, 2000. **19**(5): p. 700-9.
236. Bajaj, B.G., et al., *Epstein-Barr virus nuclear antigen 3C interacts with and enhances the stability of the c-Myc oncoprotein*. *J Virol*, 2008. **82**(8): p. 4082-90.
237. Krauer, K.G., et al., *The EBNA-3 gene family proteins disrupt the G2/M checkpoint*. *Oncogene*, 2004. **23**(7): p. 1342-53.
238. Kashuba, E., et al., *EBV-encoded EBNA-6 binds and targets MRS18-2 to the nucleus, resulting in the disruption of pRb-E2F1 complexes*. *Proc Natl Acad Sci U S A*, 2008. **105**(14): p. 5489-94.
239. Snopok, B., et al., *SPR-based immunocapture approach to creating an interfacial sensing architecture: Mapping of the MRS18-2 binding site on retinoblastoma protein*. *Anal Bioanal Chem*, 2006. **386**(7-8): p. 2063-73.
240. Holowaty, M.N., et al., *Protein profiling with Epstein-Barr nuclear antigen-1 reveals an interaction with the herpesvirus-associated ubiquitin-specific protease HAUSP/USP7*. *J Biol Chem*, 2003. **278**(32): p. 29987-94.
241. Shiseki, M., et al., *p29ING4 and p28ING5 bind to p53 and p300, and enhance p53 activity*. *Cancer Res*, 2003. **63**(10): p. 2373-8.
242. Knight, J.S., N. Sharma, and E.S. Robertson, *SCFSkp2 complex targeted by Epstein-Barr virus essential nuclear antigen*. *Mol Cell Biol*, 2005. **25**(5): p. 1749-63.
243. Knight, J.S., N. Sharma, and E.S. Robertson, *Epstein-Barr virus latent antigen 3C can mediate the degradation of the retinoblastoma protein through an SCF cellular ubiquitin ligase*. *Proc Natl Acad Sci U S A*, 2005. **102**(51): p. 18562-6.
244. Maruo, S., et al., *Epstein-Barr virus nuclear protein EBNA3C is required for cell cycle progression and growth maintenance of lymphoblastoid cells*. *Proc Natl Acad Sci U S A*, 2006. **103**(51): p. 19500-5.
245. Akpogheneta, O.J., et al., *Duration of naturally acquired antibody responses to blood-stage Plasmodium falciparum is age dependent and antigen specific*. *Infect Immun*, 2008. **76**(4): p. 1748-55.
246. Pasqualucci, L., et al., *AID is required for germinal center-derived lymphomagenesis*. *Nat Genet*, 2008. **40**(1): p. 108-12.
247. Allday, M.J., *How does Epstein-Barr virus (EBV) complement the activation of Myc in the pathogenesis of Burkitt's lymphoma?* *Semin Cancer Biol*, 2009. **19**(6): p. 366-76.
248. Dyson, N., et al., *The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product*. *Science*, 1989. **243**(4893): p. 934-7.
249. He, W., et al., *Direct activation of cyclin-dependent kinase 2 by human papillomavirus E7*. *J Virol*, 2003. **77**(19): p. 10566-74.
250. Nguyen, C.L. and K. Munger, *Direct association of the HPV16 E7 oncoprotein with cyclin A/CDK2 and cyclin E/CDK2 complexes*. *Virology*, 2008. **380**(1): p. 21-5.
251. Nguyen, D.X., T.F. Westbrook, and D.J. McCance, *Human papillomavirus type 16 E7 maintains elevated levels of the cd-c25A tyrosine phosphatase during deregulation of cell cycle arrest*. *J Virol*, 2002. **76**(2): p. 619-32.
252. Brehm, A., et al., *The E7 oncoprotein associates with Mi2 and histone deacetylase activity to promote cell growth*. *EMBO J*, 1999. **18**(9): p. 2449-58.
253. Antinore, M.J., et al., *The human papillomavirus type 16 E7 gene product interacts with and trans-activates the AP1 family of transcription factors*. *EMBO J*, 1996. **15**(8): p. 1950-60.
254. Funk, J.O., et al., *Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein*. *Genes Dev*, 1997. **11**(16): p. 2090-100.
255. Zerfass-Thome, K., et al., *Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein*. *Oncogene*, 1996. **13**(11): p. 2323-30.
256. Noya, F., et al., *p21cip1 Degradation in differentiated keratinocytes is abrogated by costabilization with cyclin E induced by human papillomavirus E7*. *J Virol*, 2001. **75**(13): p. 6121-34.
257. Lechner, M.S. and L.A. Laimins, *Inhibition of p53 DNA binding by human papillomavirus E6 proteins*. *J Virol*, 1994. **68**(7): p. 4262-73.
258. Kumar, A., et al., *Human papillomavirus oncoprotein E6 inactivates the transcriptional coactivator human ADA3*. *Mol Cell Biol*, 2002. **22**(16): p. 5801-12.
259. Patel, D., et al., *The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300*. *EMBO J*, 1999. **18**(18): p. 5061-72.
260. Jha, S., et al., *Destabilization of TIP60 by human papillomavirus E6 results in attenuation of TIP60-dependent transcriptional regulation and apoptotic pathway*. *Mol Cell*. **38**(5): p. 700-11.
261. Kiyono, T., et al., *Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells*. *Nature*, 1998. **396**(6706): p. 84-8.
262. Liu, Y., et al., *Multiple functions of human papillomavirus type 16 E6 contribute to the immortalization of mammary epithelial cells*. *J Virol*, 1999. **73**(9): p. 7297-307.
263. Nguyen, M.L., et al., *The PDZ ligand domain of the human papillomavirus type 16 E6 protein is required for E6's induction of epithelial hyperplasia in vivo*. *J Virol*, 2003. **77**(12): p. 6957-64.
264. Thomas, M. and L. Banks, *Human papillomavirus (HPV) E6 interactions with Bak are conserved amongst E6 proteins from high and low risk HPV types*. *J Gen Virol*, 1999. **80** (Pt 6): p. 1513-7.
265. Gardiol, D., et al., *Oncogenic human papillomavirus E6 proteins target the discs large tumour suppressor for proteasome-mediated degradation*. *Oncogene*, 1999. **18**(40): p. 5487-96.
266. James, M.A., J.H. Lee, and A.J. Klingelutz, *Human papillomavirus type 16 E6 activates NF-kappaB, induces cIAP-2 expression, and protects against apoptosis in a PDZ binding motif-dependent manner*. *J Virol*, 2006. **80**(11): p. 5301-7.
267. Garnett, T.O., M. Filippova, and P.J. Duerksen-Hughes, *Accelerated degradation of FADD and procaspase 8 in cells expressing human papilloma virus 16 E6 impairs TRAIL-mediated apoptosis*. *Cell Death Differ*, 2006. **13**(11): p. 1915-26.
268. Borbely, A.A., et al., *Effects of human papillomavirus type 16 oncoproteins on survivin gene expression*. *J Gen Virol*, 2006. **87**(Pt 2): p. 287-94.
269. Pedroza-Saavedra, A., et al., *The human papillomavirus type 16 E5 oncoprotein synergizes with EGF-receptor signaling to enhance cell cycle progression and the down-regulation of p27(Kip1)*. *Virology*. **400**(1): p. 44-52.

270. Oh, J.M., et al., *Human papillomavirus type 16 E5 protein inhibits hydrogen-peroxide-induced apoptosis by stimulating ubiquitin-proteasome-mediated degradation of Bax in human cervical cancer cells*. *Carcinogenesis*, **31**(3): p. 402-10.
271. Peitsaro, P., B. Johansson, and S. Syrjänen, *Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique*. *J Clin Microbiol*, 2002. **40**(3): p. 886-91.
272. Yu, T., et al., *The role of viral integration in the development of cervical cancer*. *Cancer Genet Cytogenet*, 2005. **158**(1): p. 27-34.
273. Arbeit, J.M., P.M. Howley, and D. Hanahan, *Chronic estrogen-induced cervical and vaginal squamous carcinogenesis in human papillomavirus type 16 transgenic mice*. *Proc Natl Acad Sci U S A*, 1996. **93**(7): p. 2930-5.
274. Pater, M.M., et al., *Glucocorticoid-dependent oncogenic transformation by type 16 but not type 11 human papilloma virus DNA*. *Nature*, 1988. **335**(6193): p. 832-5.
275. Duensing, S. and K. Munger, *Mechanisms of genomic instability in human cancer: insights from studies with human papillomavirus oncoproteins*. *Int J Cancer*, 2004. **109**(2): p. 157-62.
276. Apetoh, L., et al., *Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy*. *Nat Med*, 2007. **13**(9): p. 1050-9.
277. Bogdan, C., *The function of type I interferons in antimicrobial immunity*. *Curr Opin Immunol*, 2000. **12**(4): p. 419-24.
278. Diaz, M.O., et al., *Structure of the human type-I interferon gene cluster determined from a YAC clone contig*. *Genomics*, 1994. **22**(3): p. 540-52.
279. LaFleur, D.W., et al., *Interferon-kappa, a novel type I interferon expressed in human keratinocytes*. *J Biol Chem*, 2001. **276**(43): p. 39765-71.
280. Plataniias, L.C., *Mechanisms of type-I and type-II-interferon-mediated signalling*. *Nat Rev Immunol*, 2005. **5**(5): p. 375-86.
281. Swann, J.B., et al., *Type I IFN contributes to NK cell homeostasis, activation, and antitumor function*. *J Immunol*, 2007. **178**(12): p. 7540-9.
282. Luft, T., et al., *Type I IFNs enhance the terminal differentiation of dendritic cells*. *J Immunol*, 1998. **161**(4): p. 1947-53.
283. Montoya, M., et al., *Type I interferons produced by dendritic cells promote their phenotypic and functional activation*. *Blood*, 2002. **99**(9): p. 3263-71.
284. Ito, T., et al., *Differential regulation of human blood dendritic cell subsets by IFNs*. *J Immunol*, 2001. **166**(5): p. 2961-9.
285. Le Bon, A., et al., *Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon*. *Nat Immunol*, 2003. **4**(10): p. 1009-15.
286. Kurts, C., B.W. Robinson, and P.A. Knolle, *Cross-priming in health and disease*. *Nat Rev Immunol*. **10**(6): p. 403-14.
287. Rogge, L., et al., *The role of Stat4 in species-specific regulation of Th cell development by type I IFNs*. *J Immunol*, 1998. **161**(12): p. 6567-74.
288. Le Bon, A., et al., *Direct stimulation of T cells by type I IFN enhances the CD8+ T cell response during cross-priming*. *J Immunol*, 2006. **176**(8): p. 4682-9.
289. Dhib-Jalbut, S.S. and E.P. Cowan, *Direct evidence that interferon-beta mediates enhanced HLA-class I expression in measles virus-infected cells*. *J Immunol*, 1993. **151**(11): p. 6248-58.
290. Kikuta, H., et al., *Interferon production by Epstein-Barr virus in human mononuclear leukocytes*. *J Gen Virol*, 1984. **65** (Pt 4): p. 837-41.
291. Lotz, M., et al., *Release of lymphokines after Epstein Barr virus infection in vitro. I. Sources of and kinetics of production of interferons and interleukins in normal humans*. *J Immunol*, 1986. **136**(10): p. 3636-42.
292. Lotz, M., et al., *Regulation of Epstein-Barr virus infection by recombinant interferons. Selected sensitivity to interferon-gamma*. *Eur J Immunol*, 1985. **15**(5): p. 520-5.
293. Kochs, G., et al., *Antivirally active MxA protein sequesters La Crosse virus nucleocapsid protein into perinuclear complexes*. *Proc Natl Acad Sci U S A*, 2002. **99**(5): p. 3153-8.
294. Goodbourn, S., L. Didcock, and R.E. Randall, *Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures*. *J Gen Virol*, 2000. **81**(Pt 10): p. 2341-64.
295. Silverman, R.H., *Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response*. *J Virol*, 2007. **81**(23): p. 12720-9.
296. Pindel, A. and A. Sadler, *The role of protein kinase R in the interferon response*. *J Interferon Cytokine Res*. **31**(1): p. 59-70.
297. Ruvoilo, V.R., et al., *PKR regulates B56(alpha)-mediated BCL2 phosphatase activity in acute lymphoblastic leukemia-derived REH cells*. *J Biol Chem*, 2008. **283**(51): p. 35474-85.
298. McAllister, C.S. and C.E. Samuel, *The RNA-activated protein kinase enhances the induction of interferon-beta and apoptosis mediated by cytoplasmic RNA sensors*. *J Biol Chem*, 2009. **284**(3): p. 1644-51.
299. Schulz, O., et al., *Protein kinase R contributes to immunity against specific viruses by regulating interferon mRNA integrity*. *Cell Host Microbe*. **7**(5): p. 354-61.
300. Gilfoy, F.D. and P.W. Mason, *West Nile virus-induced interferon production is mediated by the double-stranded RNA-dependent protein kinase PKR*. *J Virol*, 2007. **81**(20): p. 11148-58.
301. Zampronio, A.R., et al., *Interleukin-8 induces fever by a prostaglandin-independent mechanism*. *Am J Physiol*, 1994. **266**(5 Pt 2): p. R1670-4.
302. Ferreira, S.H., et al., *Interleukin-1 beta as a potent hyperalgesic agent antagonized by a tripeptide analogue*. *Nature*, 1988. **334**(6184): p. 698-700.
303. Gabay, C., et al., *Production of IL-1 receptor antagonist by hepatocytes is regulated as an acute-phase protein in vivo*. *Eur J Immunol*, 2001. **31**(2): p. 490-9.
304. Stassen, M., et al., *Murine bone marrow-derived mast cells as potent producers of IL-9: costimulatory function of IL-10 and kit ligand in the presence of IL-1*. *J Immunol*, 2000. **164**(11): p. 5549-55.
305. Hsu, L.C., et al., *IL-1beta-driven neutrophilia preserves anti-bacterial defense in the absence of the kinase IKKbeta*. *Nat Immunol*. **12**(2): p. 144-50.
306. Hultner, L., et al., *In activated mast cells, IL-1 up-regulates the production of several Th2-related cytokines including IL-9*. *J Immunol*, 2000. **164**(11): p. 5556-63.
307. Watson, R.W., et al., *The IL-1 beta-converting enzyme (caspase-1) inhibits apoptosis of inflammatory neutrophils through activation of IL-1 beta*. *J Immunol*, 1998. **161**(2): p. 957-62.
308. Faccioli, L.H., et al., *Recombinant interleukin-1 and tumor necrosis factor induce neutrophil migration "in vivo" by indirect mechanisms*. *Agents Actions*, 1990. **30**(3-4): p. 344-9.
309. Chaix, J., et al., *Cutting edge: Priming of NK cells by IL-18*. *J Immunol*, 2008. **181**(3): p. 1627-31.
310. Dinarello, C.A., *Biologic basis for interleukin-1 in disease*. *Blood*, 1996. **87**(6): p. 2095-147.
311. Nielsen, B.W., et al., *Macrophages as producers of chemotactic proinflammatory cytokines*. *Immunol Ser*, 1994. **60**: p. 131-42.
312. Dinarello, C.A., *IL-18: A TH1-inducing, proinflammatory cytokine and new member of the IL-1 family*. *J Allergy Clin Immunol*, 1999. **103**(1 Pt 1): p. 11-24.
313. Allen, I.C., et al., *The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA*. *Immunity*, 2009. **30**(4): p. 556-65.
314. Ghiringhelli, F., et al., *Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors*. *Nat Med*, 2009. **15**(10): p. 1170-8.
315. Matsushima, N., et al., *Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors*. *BMC Genomics*, 2007. **8**: p. 124.
316. Beutler, B., et al., *Genetic analysis of host resistance: Toll-like*

- receptor signaling and immunity at large. *Annu Rev Immunol*, 2006. **24**: p. 353-89.
317. Bell, J.K., et al., *Leucine-rich repeats and pathogen recognition in Toll-like receptors*. *Trends Immunol*, 2003. **24**(10): p. 528-33.
318. Liu, L., et al., *Structural basis of toll-like receptor 3 signaling with double-stranded RNA*. *Science*, 2008. **320**(5874): p. 379-81.
319. O'Neill, L.A. and A.G. Bowie, *The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling*. *Nat Rev Immunol*, 2007. **7**(5): p. 353-64.
320. Jiang, Z., et al., *Details of Toll-like receptor:adapter interaction revealed by germ-line mutagenesis*. *Proc Natl Acad Sci U S A*, 2006. **103**(29): p. 10961-6.
321. Tsan, M.F. and B. Gao, *Pathogen-associated molecular pattern contamination as putative endogenous ligands of Toll-like receptors*. *J Endotoxin Res*, 2007. **13**(1): p. 6-14.
322. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity*. *Cell*, 2006. **124**(4): p. 783-801.
323. Urbonaviciute, V., et al., *Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE*. *J Exp Med*, 2008. **205**(13): p. 3007-18.
324. Bianchi, M.E., *DAMPs, PAMPs and alarmins: all we need to know about danger*. *J Leukoc Biol*, 2007. **81**(1): p. 1-5.
325. Muller, S., L. Ronfani, and M.E. Bianchi, *Regulated expression and subcellular localization of HMGB1, a chromatin protein with a cytokine function*. *J Intern Med*, 2004. **255**(3): p. 332-43.
326. Pisetsky, D.S. and W. Jiang, *Role of Toll-like receptors in HMGB1 release from macrophages*. *Ann N Y Acad Sci*, 2007. **1109**: p. 58-65.
327. Lamkanfi, M., et al., *Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia*. *J Immunol*. **185**(7): p. 4385-92.
328. Rovere-Querini, P., et al., *HMGB1 is an endogenous immune adjuvant released by necrotic cells*. *EMBO Rep*, 2004. **5**(8): p. 825-30.
329. Kazama, H., et al., *Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein*. *Immunity*, 2008. **29**(1): p. 21-32.
330. Thorburn, J., et al., *Autophagy regulates selective HMGB1 release in tumor cells that are destined to die*. *Cell Death Differ*, 2009. **16**(1): p. 175-83.
331. Bonaldi, T., et al., *Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion*. *EMBO J*, 2003. **22**(20): p. 5551-60.
332. Ito, I., J. Fukazawa, and M. Yoshida, *Post-translational methylation of high mobility group box 1 (HMGB1) causes its cytoplasmic localization in neutrophils*. *J Biol Chem*, 2007. **282**(22): p. 16336-44.
333. Tang, D., et al., *HMGB1 release and redox regulates autophagy and apoptosis in cancer cells*. *Oncogene*. **29**(38): p. 5299-310.
334. Straino, S., et al., *High-mobility group box 1 protein in human and murine skin: involvement in wound healing*. *J Invest Dermatol*, 2008. **128**(6): p. 1545-53.
335. Limana, F., et al., *Exogenous high-mobility group box 1 protein induces myocardial regeneration after infarction via enhanced cardiac C-kit+ cell proliferation and differentiation*. *Circ Res*, 2005. **97**(8): p. e73-83.
336. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene*. *Science*, 1998. **282**(5396): p. 2085-8.
337. Miyake, K., et al., *Innate recognition of lipopolysaccharide by Toll-like receptor 4/MD-2 and RP105/MD-1*. *J Endotoxin Res*, 2000. **6**(5): p. 389-91.
338. Youn, J.H., et al., *High mobility group box 1 protein binding to lipopolysaccharide facilitates transfer of lipopolysaccharide to CD14 and enhances lipopolysaccharide-mediated TNF-alpha production in human monocytes*. *J Immunol*, 2008. **180**(7): p. 5067-74.
339. Tanimura, N., et al., *Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling*. *Biochem Biophys Res Commun*, 2008. **368**(1): p. 94-9.
340. Gewirtz, A.T., et al., *Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression*. *J Immunol*, 2001. **167**(4): p. 1882-5.
341. Andersen-Nissen, E., et al., *Cutting edge: Tlr5-/- mice are more susceptible to Escherichia coli urinary tract infection*. *J Immunol*, 2007. **178**(8): p. 4717-20.
342. Brinkmann, M.M., et al., *The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling*. *J Cell Biol*, 2007. **177**(2): p. 265-75.
343. Tabet, K., et al., *The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9*. *Nat Immunol*, 2006. **7**(2): p. 156-64.
344. Kiyokawa, T., et al., *A single base mutation in the PRAT4A gene reveals differential interaction of PRAT4A with Toll-like receptors*. *Int Immunol*, 2008. **20**(11): p. 1407-15.
345. Ivanov, S., et al., *A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA*. *Blood*, 2007. **110**(6): p. 1970-81.
346. Asagiri, M., et al., *Cathepsin K-dependent toll-like receptor 9 signaling revealed in experimental arthritis*. *Science*, 2008. **319**(5863): p. 624-7.
347. Ewald, S.E., et al., *The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor*. *Nature*, 2008. **456**(7222): p. 658-62.
348. Park, B., et al., *Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9*. *Nat Immunol*, 2008. **9**(12): p. 1407-14.
349. Sepulveda, F.E., et al., *Critical role for asparagine endopeptidase in endocytic Toll-like receptor signaling in dendritic cells*. *Immunity*, 2009. **31**(5): p. 737-48.
350. Ewald, S.E., et al., *Nucleic acid recognition by Toll-like receptors is coupled to stepwise processing by cathepsins and asparagine endopeptidase*. *J Exp Med*. **208**(4): p. 643-51.
351. Barton, G.M. and J.C. Kagan, *A cell biological view of Toll-like receptor function: regulation through compartmentalization*. *Nat.Rev.Immunol.*, 2009. **9**(8): p. 535-542.
352. Hornung, V., et al., *Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides*. *J Immunol*, 2002. **168**(9): p. 4531-7.
353. Hayashi, F., T.K. Means, and A.D. Luster, *Toll-like receptors stimulate human neutrophil function*. *Blood*, 2003. **102**(7): p. 2660-9.
354. Muzio, M., et al., *Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells*. *J.Immunol.*, 2000. **164**(11): p. 5998-6004.
355. Grote, K., H. Schuett, and B. Schieffer, *Toll-like receptors in angiogenesis*. *ScientificWorldJournal*. **11**: p. 981-991.
356. Hwa, C.H., Y.C. Bae, and J.S. Jung, *Role of toll-like receptors on human adipose-derived stromal cells*. *Stem Cells*, 2006. **24**(12): p. 2744-2752.
357. Bsibsi, M., et al., *Broad expression of Toll-like receptors in the human central nervous system*. *J Neuropathol Exp Neurol*, 2002. **61**(11): p. 1013-21.
358. Miller, L.S. and R.L. Modlin, *Human keratinocyte Toll-like receptors promote distinct immune responses*. *J Invest Dermatol*, 2007. **127**(2): p. 262-3.
359. Alexopoulou, L., et al., *Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3*. *Nature*, 2001. **413**(6857): p. 732-738.
360. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr., *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity*. *Nature*, 1997. **388**(6640): p. 394-7.

361. Kollisch, G., et al., *Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes*. *Immunology*, 2005. **114**(4): p. 531-541.
362. Gribar, S.C., et al., *The role of epithelial Toll-like receptor signaling in the pathogenesis of intestinal inflammation*. *J Leukoc Biol*, 2008. **83**(3): p. 493-8.
363. Heil, F., et al., *Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8*. *Science*, 2004. **303**(5663): p. 1526-9.
364. Lund, J.M., et al., *Recognition of single-stranded RNA viruses by Toll-like receptor 7*. *Proc.Natl.Acad.Sci.U.S.A*, 2004. **101**(15): p. 5598-5603.
365. Jurk, M., et al., *Immunostimulatory Potential of Silencing RNAs Can Be Mediated by a Non-Uridine-Rich Toll-Like Receptor 7 Motif*. *Oligonucleotides*.
366. Ganguly, D., et al., *Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8*. *J Exp Med*, 2009. **206**(9): p. 1983-94.
367. Forsbach, A., et al., *Identification of RNA sequence motifs stimulating sequence-specific TLR8-dependent immune responses*. *J.Immunol.*, 2008. **180**(6): p. 3729-3738.
368. Girart, M.V., et al., *Engagement of TLR3, TLR7, and NKG2D regulate IFN-gamma secretion but not NKG2D-mediated cytotoxicity by human NK cells stimulated with suboptimal doses of IL-12*. *J Immunol*, 2007. **179**(6): p. 3472-9.
369. Komiya, A., et al., *Expression and function of toll-like receptors in human basophils*. *Int.Arch.Allergy Immunol.*, 2006. **140 Suppl 1**: p. 23-27.
370. Mansson, A. and L.O. Cardell, *Role of atopic status in Toll-like receptor (TLR)7- and TLR9-mediated activation of human eosinophils*. *J.Leukoc.Biol.*, 2009. **85**(4): p. 719-727.
371. Hasan, U.A., et al., *TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16*. *J Immunol*, 2007. **178**(5): p. 3186-97.
372. Edwards, A.D., et al., *Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines*. *Eur.J.Immunol.*, 2003. **33**(4): p. 827-833.
373. Pedersen, G., et al., *Expression of Toll-like receptor 9 and response to bacterial CpG oligodeoxynucleotides in human intestinal epithelium*. *Clin.Exp.Immunol.*, 2005. **141**(2): p. 298-306.
374. Platz, J., et al., *Microbial DNA induces a host defense reaction of human respiratory epithelial cells*. *J.Immunol.*, 2004. **173**(2): p. 1219-1223.
375. Lebre, M.C., et al., *Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9*. *J.Invest Dermatol.*, 2007. **127**(2): p. 331-341.
376. Hemmi, H., et al., *A Toll-like receptor recognizes bacterial DNA*. *Nature*, 2000. **408**(6813): p. 740-5.
377. Lund, J., et al., *Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells*. *J Exp Med*, 2003. **198**(3): p. 513-20.
378. Sasai, M., M.M. Linehan, and A. Iwasaki, *Bifurcation of Toll-like receptor 9 signaling by adaptor protein 3*. *Science*. **329**(5998): p. 1530-1534.
379. Krug, A., et al., *TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function*. *Immunity.*, 2004. **21**(1): p. 107-119.
380. Zhu, J., X. Huang, and Y. Yang, *Innate immune response to adenoviral vectors is mediated by both Toll-like receptor-dependent and -independent pathways*. *J.Virol.*, 2007. **81**(7): p. 3170-3180.
381. Fathallah, I., et al., *EBV latent membrane protein 1 is a negative regulator of TLR9*. *J Immunol*. **185**(11): p. 6439-47.
382. Andersen, J.M., D. Al-Khairy, and R.R. Ingalls, *Innate immunity at the mucosal surface: role of toll-like receptor 3 and toll-like receptor 9 in cervical epithelial cell responses to microbial pathogens*. *Biol.Reprod.*, 2006. **74**(5): p. 824-831.
383. Kandimalla, E.R., et al., *Immunomodulatory oligonucleotides containing a cytosine-phosphate-2'-deoxy-7-deazaguanosine motif as potent toll-like receptor 9 agonists*. *Proc.Natl.Acad.Sci.U.S.A*, 2005. **102**(19): p. 6925-6930.
384. Jarrossay, D., et al., *Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells*. *Eur.J.Immunol.*, 2001. **31**(11): p. 3388-3393.
385. Kadowaki, N., et al., *Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens*. *J.Exp.Med.*, 2001. **194**(6): p. 863-869.
386. Choe, J., M.S. Kelker, and I.A. Wilson, *Crystal structure of human toll-like receptor 3 (TLR3) ectodomain*. *Science*, 2005. **309**(5734): p. 581-5.
387. Daffis, S., et al., *Toll-like receptor 3 has a protective role against West Nile virus infection*. *J Virol*, 2008. **82**(21): p. 10349-58.
388. Zhang, S.Y., et al., *TLR3 deficiency in patients with herpes simplex encephalitis*. *Science*, 2007. **317**(5844): p. 1522-7.
389. Koyama, S., et al., *Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination*. *J Immunol*, 2007. **179**(7): p. 4711-20.
390. Jung, A., et al., *Lymphocytoid choriomeningitis virus activates plasmacytoid dendritic cells and induces a cytotoxic T-cell response via MyD88*. *J Virol*, 2008. **82**(1): p. 196-206.
391. Kane, M., et al., *Innate immune sensing of retroviral infection via Toll-like receptor 7 occurs upon viral entry*. *Immunity*. **35**(1): p. 135-45.
392. Mancuso, G., et al., *Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells*. *Nat Immunol*, 2009. **10**(6): p. 587-94.
393. Krug, A., et al., *Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9*. *Blood*, 2004. **103**(4): p. 1433-7.
394. Krug, A., et al., *Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells*. *Eur J Immunol*, 2001. **31**(7): p. 2154-63.
395. Krieg, A.M., et al., *CpG motifs in bacterial DNA trigger direct B-cell activation*. *Nature*, 1995. **374**(6522): p. 546-549.
396. Hartmann, E., et al., *Identification and functional analysis of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer*. *Cancer Res*, 2003. **63**(19): p. 6478-6487.
397. Samulowitz, U., et al., *A novel class of immune-stimulatory CpG oligodeoxynucleotides unifies high potency in type I interferon induction with preferred structural properties*. *Oligonucleotides*. **20**(2): p. 93-101.
398. Verthelyi, D., et al., *Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs*. *J Immunol*, 2001. **166**(4): p. 2372-7.
399. Kerkmann, M., et al., *Spontaneous formation of nucleic acid-based nanoparticles is responsible for high interferon-alpha induction by CpG-A in plasmacytoid dendritic cells*. *J Biol Chem*, 2005. **280**(9): p. 8086-93.
400. Vollmer, J., et al., *Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities*. *Eur.J.Immunol.*, 2004. **34**(1): p. 251-262.
401. Poeck, H., et al., *Plasmacytoid dendritic cells, antigen, and CpG-C license human B cells for plasma cell differentiation and immunoglobulin production in the absence of T-cell help*. *Blood*, 2004. **103**(8): p. 3058-64.
402. Hartmann, G., et al., *Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-alpha induction in plasmacytoid dendritic cells*. *Eur J Immunol*, 2003. **33**(6): p. 1633-41.
403. Guiducci, C., et al., *Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation*. *J.Exp.Med.*, 2006.
404. Gursel, M., D. Verthelyi, and D.M. Klinman, *CpG oligodeoxynucleotides induce human monocytes to mature into functional dendritic cells*. *Eur J Immunol*, 2002. **32**(9): p. 2617-

- 22.
405. Gursel, M., et al., *Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide*. J Leukoc Biol, 2002. **71**(5): p. 813-20.
406. Bauer, M., et al., *Bacterial CpG-DNA triggers activation and maturation of human CD11c-, CD123+ dendritic cells*. J Immunol., 2001. **166**(8): p. 5000-5007.
407. Bauer, M., et al., *Bacterial CpG-DNA triggers activation and maturation of human CD11c-, CD123+ dendritic cells*. J Immunol, 2001. **166**(8): p. 5000-7.
408. Ishii, K.J., et al., *Host innate immune receptors and beyond: making sense of microbial infections*. Cell Host Microbe, 2008. **3**(6): p. 352-63.
409. McGettrick, A.F. and L.A. O'Neill, *Toll-like receptors: key activators of leucocytes and regulator of haematopoiesis*. Br J Haematol, 2007. **139**(2): p. 185-93.
410. Krieg, A.M., *CpG motifs in bacterial DNA and their immune effects*. Annu Rev Immunol, 2002. **20**: p. 709-60.
411. Sester, D.P., et al., *Phosphorothioate backbone modification modulates macrophage activation by CpG DNA*. J Immunol, 2000. **165**(8): p. 4165-73.
412. Bird, A.P., *CpG-rich islands and the function of DNA methylation*. Nature, 1986. **321**(6067): p. 209-13.
413. Yasuda, K., et al., *CpG motif-independent activation of TLR9 upon endosomal translocation of "natural" phosphodiester DNA*. Eur J Immunol, 2006. **36**(2): p. 431-6.
414. Haas, T., et al., *The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation*. Immunity, 2008. **28**(3): p. 315-23.
415. Lande, R., et al., *Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide*. Nature, 2007. **449**(7162): p. 564-9.
416. Barrat, F.J., et al., *Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus*. J Exp Med, 2005. **202**(8): p. 1131-9.
417. Marshak-Rothstein, A., *Toll-like receptors in systemic autoimmune disease*. Nat Rev Immunol, 2006. **6**(11): p. 823-35.
418. Abdulahad, D.A., et al., *HMGB1 in systemic lupus Erythematosus: Its role in cutaneous lesions development*. Autoimmun Rev. **9**(10): p. 661-5.
419. Tian, J., et al., *Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE*. Nat Immunol, 2007. **8**(5): p. 487-96.
420. Kawai, T. and S. Akira, *TLR signaling*. Cell Death Differ, 2006. **13**(5): p. 816-25.
421. Lee, M.S. and Y.J. Kim, *Signaling pathways downstream of pattern-recognition receptors and their cross talk*. Annu Rev Biochem, 2007. **76**: p. 447-80.
422. Blander, J.M. and R. Medzhitov, *Regulation of phagosome maturation by signals from toll-like receptors*. Science, 2004. **304**(5673): p. 1014-8.
423. West, A.P., et al., *TLR signalling augments macrophage bactericidal activity through mitochondrial ROS*. Nature. **472**(7344): p. 476-80.
424. Nathan, C. and M.U. Shiloh, *Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens*. Proc Natl Acad Sci U S A, 2000. **97**(16): p. 8841-8.
425. Remer, K.A., M. Brcic, and T.W. Jungi, *Toll-like receptor-4 is involved in eliciting an LPS-induced oxidative burst in neutrophils*. Immunol Lett, 2003. **85**(1): p. 75-80.
426. Gerondakis, S., R.J. Grumont, and A. Banerjee, *Regulating B-cell activation and survival in response to TLR signals*. Immunol Cell Biol, 2007. **85**(6): p. 471-5.
427. Reynolds, J.M., et al., *Toll-like receptor 2 signaling in CD4(+) T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease*. Immunity. **32**(5): p. 692-702.
428. Pasare, C. and R. Medzhitov, *Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells*. Science, 2003. **299**(5609): p. 1033-6.
429. Grote, K., H. Schutt, and B. Schieffer, *Toll-like receptors in angiogenesis*. ScientificWorldJournal. **11**: p. 981-91.
430. Hwa Cho, H., Y.C. Bae, and J.S. Jung, *Role of toll-like receptors on human adipose-derived stromal cells*. Stem Cells, 2006. **24**(12): p. 2744-52.
431. Sawa, Y., et al., *LPS-induced IL-6, IL-8, VCAM-1, and ICAM-1 expression in human lymphatic endothelium*. J Histochem Cytochem, 2008. **56**(2): p. 97-109.
432. Galli, R., et al., *TLR stimulation of prostate tumor cells induces chemokine-mediated recruitment of specific immune cell types*. J Immunol. **184**(12): p. 6658-69.
433. Gaudreault, E., et al., *Epstein-Barr virus induces MCP-1 secretion by human monocytes via TLR2*. J Virol, 2007. **81**(15): p. 8016-24.
434. Ariza, M.E., et al., *The EBV-encoded dUTPase activates NF-kappa B through the TLR2 and MyD88-dependent signaling pathway*. J Immunol, 2009. **182**(2): p. 851-9.
435. Chang, S., A. Dolganiuc, and G. Szabo, *Toll-like receptors 1 and 6 are involved in TLR2-mediated macrophage activation by hepatitis C virus core and NS3 proteins*. J Leukoc Biol, 2007. **82**(3): p. 479-87.
436. Barbalat, R., et al., *Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands*. Nat Immunol, 2009. **10**(11): p. 1200-7.
437. Hoffmann, M., et al., *Toll-like receptor 2 senses hepatitis C virus core protein but not infectious viral particles*. J Innate Immun, 2009. **1**(5): p. 446-54.
438. Lagos, D., et al., *Toll-like receptor 4 mediates innate immunity to Kaposi sarcoma herpesvirus*. Cell Host Microbe, 2008. **4**(5): p. 470-83.
439. Yan, M., et al., *Activation of dendritic cells by human papillomavirus-like particles through TLR4 and NF-kappaB-mediated signalling, moderated by TGF-beta*. Immunol Cell Biol, 2005. **83**(1): p. 83-91.
440. Yang, R., et al., *B lymphocyte activation by human papillomavirus-like particles directly induces Ig class switch recombination via TLR4-MyD88*. J Immunol, 2005. **174**(12): p. 7912-9.
441. Machida, K., et al., *Hepatitis C virus induces toll-like receptor 4 expression, leading to enhanced production of beta interferon and interleukin-6*. J Virol, 2006. **80**(2): p. 866-74.
442. Eksioglu, E.A., et al., *Characterization of HCV interactions with Toll-like receptors and RIG-I in liver cells*. PLoS One. **6**(6): p. e21186.
443. Wang, N., et al., *Toll-like receptor 3 mediates establishment of an antiviral state against hepatitis C virus in hepatoma cells*. J Virol, 2009. **83**(19): p. 9824-34.
444. Moriyama, M., et al., *Interferon-beta is activated by hepatitis C virus NS5B and inhibited by NS4A, NS4B, and NS5A*. Hepatol Int, 2007. **1**(2): p. 302-10.
445. Iwakiri, D., et al., *Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from Toll-like receptor 3*. J Exp Med, 2009. **206**(10): p. 2091-9.
446. Colisson, R., et al., *Free HTLV-1 induces TLR7-dependent innate immune response and TRAIL relocalization in killer plasmacytoid dendritic cells*. Blood. **115**(11): p. 2177-85.
447. Rahman, S., et al., *Murine FLT3 ligand-derived dendritic cell-mediated early immune responses are critical to controlling cell-free human T cell leukemia virus type 1 infection*. J Immunol. **186**(1): p. 390-402.
448. Guggemoos, S., et al., *TLR9 contributes to antiviral immunity during gammaherpesvirus infection*. J Immunol, 2008. **180**(1): p. 438-43.
449. West, J.A., et al., *Activation of plasmacytoid dendritic cells by Kaposi's sarcoma-associated herpesvirus*. J Virol. **85**(2): p. 895-904.
450. Fiola, S., et al., *TLR9 contributes to the recognition of EBV by primary monocytes and plasmacytoid dendritic cells*. J Immunol. **185**(6): p. 3620-31.
451. Lim, W.H., et al., *Human plasmacytoid dendritic cells regulate*

- immune responses to Epstein-Barr virus (EBV) infection and delay EBV-related mortality in humanized NOD-SCID mice. *Blood*, 2007. **109**(3): p. 1043-50.
452. van Gent, M., et al., *EBV lytic-phase protein BGLF5 contributes to TLR9 downregulation during productive infection*. *J Immunol*. **186**(3): p. 1694-702.
453. Yanai, H., et al., *HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses*. *Nature*, 2009. **462**(7269): p. 99-103.
454. Ishikawa, H. and G.N. Barber, *STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling*. *Nature*, 2008. **455**(7213): p. 674-8.
455. Ishikawa, H., Z. Ma, and G.N. Barber, *STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity*. *Nature*, 2009. **461**(7265): p. 788-92.
456. Yan, N., et al., *The cytosolic exonuclease TREX1 inhibits the innate immune response to human immunodeficiency virus type 1*. *Nat Immunol*. **11**(11): p. 1005-13.
457. Takaoka, A., et al., *DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response*. *Nature*, 2007. **448**(7152): p. 501-5.
458. Lippmann, J., et al., *IFNbeta responses induced by intracellular bacteria or cytosolic DNA in different human cells do not require ZBP1 (DLM-1/DAI)*. *Cell Microbiol*, 2008. **10**(12): p. 2579-88.
459. Yang, P., et al., *The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway*. *Nat Immunol*. **11**(6): p. 487-94.
460. Kato, H., et al., *Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses*. *Nature*, 2006. **441**(7089): p. 101-105.
461. Pichlmair, A., et al., *RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates*. *Science*, 2006. **314**(5801): p. 997-1001.
462. Hornung, V., et al., *5'-Triphosphate RNA is the ligand for RIG-I*. *Science*, 2006. **314**(5801): p. 994-997.
463. Pichlmair, A., et al., *Activation of MDA5 requires higher-order RNA structures generated during virus infection*. *J Virol*, 2009. **83**(20): p. 10761-9.
464. Schlee, M., et al., *Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus*. *Immunity*, 2009. **31**(1): p. 25-34.
465. Satoh, T., et al., *LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses*. *Proc Natl Acad Sci U S A*. **107**(4): p. 1512-7.
466. Rothenfusser, S., et al., *The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I*. *J Immunol*, 2005. **175**(8): p. 5260-8.
467. Melchjorsen, J., et al., *Early innate recognition of herpes simplex virus in human primary macrophages is mediated via the MDA5/MAVS-dependent and MDA5/MAVS/RNA polymerase III-independent pathways*. *J Virol*. **84**(21): p. 11350-8.
468. Chiu, Y.H., J.B. Macmillan, and Z.J. Chen, *RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway*. *Cell*, 2009. **138**(3): p. 576-91.
469. Poeck, H., et al., *Recognition of RNA virus by RIG-I results in activation of CARD9 and inflammasome signaling for interleukin 1 beta production*. *Nat Immunol*. **11**(1): p. 63-9.
470. Saito, T., et al., *Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA*. *Nature*, 2008. **454**(7203): p. 523-7.
471. Samanta, M., et al., *EB virus-encoded RNAs are recognized by RIG-I and activate signaling to induce type I IFN*. *EMBO J*, 2006. **25**(18): p. 4207-14.
472. Li, X.D., et al., *Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity*. *Proc Natl Acad Sci U S A*, 2005. **102**(49): p. 17717-22.
473. Seth, R.B., et al., *Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3*. *Cell*, 2005. **122**(5): p. 669-682.
474. Samanta, M., D. Iwakiri, and K. Takada, *Epstein-Barr virus-encoded small RNA induces IL-10 through RIG-I-mediated IRF-3 signaling*. *Oncogene*, 2008. **27**(30): p. 4150-60.
475. Ablasser, A., et al., *RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate*. *Nat Immunol*, 2009. **10**(10): p. 1065-72.
476. Perrot, I., et al., *TLR3 and Rig-like receptor on myeloid dendritic cells and Rig-like receptor on human NK cells are both mandatory for production of IFN-gamma in response to double-stranded RNA*. *J Immunol*. **185**(4): p. 2080-8.
477. Parolini, S., et al., *X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells*. *J Exp Med*, 2000. **192**(3): p. 337-46.
478. Strowig, T., et al., *Tonsillar NK cells restrict B cell transformation by the Epstein-Barr virus via IFN-gamma*. *PLoS Pathog*, 2008. **4**(2): p. e27.
479. Kim, T., et al., *Aspartate-glutamate-alanine-histidine box motif (DEAH)/RNA helicase A helicases sense microbial DNA in human plasmacytoid dendritic cells*. *Proc Natl Acad Sci U S A*. **107**(34): p. 15181-6.
480. Zhang, Z., et al., *DDX1, DDX21, and DHX36 helicases form a complex with the adaptor molecule TRIF to sense dsRNA in dendritic cells*. *Immunity*. **34**(6): p. 866-78.
481. Zhang, Z., et al., *The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells*. *Nat Immunol*. **12**(10): p. 959-65.
482. Duewell, P., et al., *NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals*. *Nature*. **464**(7293): p. 1357-61.
483. Hornung, V., et al., *Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization*. *Nat Immunol*, 2008. **9**(8): p. 847-56.
484. Gregory, S.M., et al., *Discovery of a viral NLR homolog that inhibits the inflammasome*. *Science*. **331**(6015): p. 330-4.
485. Sabbah, A., et al., *Activation of innate immune antiviral responses by Nod2*. *Nat Immunol*, 2009. **10**(10): p. 1073-80.
486. Fritz, J.H., et al., *Nod-like proteins in immunity, inflammation and disease*. *Nat Immunol*, 2006. **7**(12): p. 1250-7.
487. Divangahi, M., et al., *NOD2-deficient mice have impaired resistance to Mycobacterium tuberculosis infection through defective innate and adaptive immunity*. *J Immunol*, 2008. **181**(10): p. 7157-65.
488. Faustin, B., et al., *Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation*. *Mol Cell*, 2007. **25**(5): p. 713-24.
489. Kanneganti, T.D., et al., *Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA*. *J Biol Chem*, 2006. **281**(48): p. 36560-8.
490. Muruve, D.A., et al., *The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response*. *Nature*, 2008. **452**(7183): p. 103-7.
491. Rajan, J.V., et al., *The NLRP3 inflammasome detects encephalomyocarditis virus and vesicular stomatitis virus infection*. *J Virol*. **85**(9): p. 4167-72.
492. Warren, S.E., et al., *Multiple Nod-like receptors activate caspase 1 during Listeria monocytogenes infection*. *J Immunol*, 2008. **180**(11): p. 7558-64.
493. Craven, R.R., et al., *Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells*. *PLoS One*, 2009. **4**(10): p. e7446.
494. Halle, A., et al., *The NALP3 inflammasome is involved in the innate immune response to amyloid-beta*. *Nat Immunol*, 2008. **9**(8): p. 857-65.
495. Mariathasan, S., et al., *Cryopyrin activates the inflammasome in response to toxins and ATP*. *Nature*, 2006. **440**(7081): p. 228-32.
496. Yamasaki, K., et al., *NLRP3/cryopyrin is necessary for interleukin-1beta (IL-1beta) release in response to hyaluronan, an*

- endogenous trigger of inflammation in response to injury. *J Biol Chem*, 2009. **284**(19): p. 12762-71.
497. Zhou, R., et al., *Thioredoxin-interacting protein links oxidative stress to inflammasome activation*. *Nat Immunol*. **11**(2): p. 136-40.
498. Kanneganti, T.D., et al., *Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3*. *Nature*, 2006. **440**(7081): p. 233-6.
499. Zhou, R., et al., *A role for mitochondria in NLRP3 inflammasome activation*. *Nature*. **469**(7329): p. 221-5.
500. Gross, O., et al., *Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence*. *Nature*, 2009. **459**(7245): p. 433-6.
501. Unterholzner, L., et al., *IFI16 is an innate immune sensor for intracellular DNA*. *Nat Immunol*. **11**(11): p. 997-1004.
502. Kerur, N., et al., *IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi Sarcoma-associated herpesvirus infection*. *Cell Host Microbe*. **9**(5): p. 363-75.
503. Hornung, V., et al., *AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC*. *Nature*, 2009. **458**(7237): p. 514-8.
504. Rathinam, V.A., et al., *The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses*. *Nat Immunol*. **11**(5): p. 395-402.
505. Arnaud, N., et al., *Hepatitis C virus controls interferon production through PKR activation*. *PLoS One*. **5**(5): p. e10575.
506. Garaigorta, U. and F.V. Chisari, *Hepatitis C virus blocks interferon effector function by inducing protein kinase R phosphorylation*. *Cell Host Microbe*, 2009. **6**(6): p. 513-22.
507. Lin, S.S., et al., *A role for protein kinase PKR in the mediation of Epstein-Barr virus latent membrane protein-1-induced IL-6 and IL-10 expression*. *Cytokine*. **50**(2): p. 210-9.
508. Scala, G., et al., *Expression of an exogenous interleukin 6 gene in human Epstein Barr virus B cells confers growth advantage and in vivo tumorigenicity*. *J Exp Med*, 1990. **172**(1): p. 61-8.
509. Scala, G., et al., *Induction of tumorigenicity and plasmacytoid differentiation in EBV-B cells by expression of exogenous interleukin-6 or IL-6 receptor genes*. *Leukemia*, 1992. **6 Suppl 3**: p. 26S-29S.
510. Park, I.H., et al., *PKR-dependent mechanisms of interferon-alpha for inhibiting hepatitis B virus replication*. *Mol Cells*.
511. Matloubian, M., R.J. Concepcion, and R. Ahmed, *CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection*. *J Virol*, 1994. **68**(12): p. 8056-63.
512. Mi, J.Q., et al., *Development of autologous cytotoxic CD4+ T clones in a human model of B-cell non-Hodgkin follicular lymphoma*. *Br J Haematol*, 2006. **135**(3): p. 324-35.
513. Rocha, N. and J. Neeffjes, *MHC class II molecules on the move for successful antigen presentation*. *EMBO J*, 2008. **27**(1): p. 1-5.
514. Rudensky, A. and C. Beers, *Lysosomal cysteine proteases and antigen presentation*. *Ernst Schering Res Found Workshop*, 2006(56): p. 81-95.
515. Csencsits, K.L. and D.K. Bishop, *Contrasting alloreactive CD4+ and CD8+ T cells: there's more to it than MHC restriction*. *Am J Transplant*, 2003. **3**(2): p. 107-15.
516. Shen, L. and K.L. Rock, *Priming of T cells by exogenous antigen cross-presented on MHC class I molecules*. *Curr Opin Immunol*, 2006. **18**(1): p. 85-91.
517. Sigal, L.J., et al., *Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen*. *Nature*, 1999. **398**(6722): p. 77-80.
518. Paludan, C., et al., *Endogenous MHC class II processing of a viral nuclear antigen after autophagy*. *Science*, 2005. **307**(5709): p. 593-6.
519. Carter, J.J., et al., *Comparison of human papillomavirus types 16, 18, and 6 capsid antibody responses following incident infection*. *J Infect Dis*, 2000. **181**(6): p. 1911-9.
520. af Geijerstam, V., et al., *Stability over time of serum antibody levels to human papillomavirus type 16*. *J Infect Dis*, 1998. **177**(6): p. 1710-4.
521. Roden, R.B., et al., *Minor capsid protein of human genital papillomaviruses contains subdominant, cross-neutralizing epitopes*. *Virology*, 2000. **270**(2): p. 254-7.
522. Welters, M.J., et al., *Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter*. *Cancer Res*, 2003. **63**(3): p. 636-41.
523. Evans, E.M., et al., *Infiltration of cervical cancer tissue with human papillomavirus-specific cytotoxic T-lymphocytes*. *Cancer Res*, 1997. **57**(14): p. 2943-50.
524. Nakagawa, M., et al., *Cytotoxic T lymphocyte responses to E6 and E7 proteins of human papillomavirus type 16: relationship to cervical intraepithelial neoplasia*. *J Infect Dis*, 1997. **175**(4): p. 927-31.
525. Nakagawa, M., et al., *CD4-positive and CD8-positive cytotoxic T lymphocytes contribute to human papillomavirus type 16 E6 and E7 responses*. *Clin Diagn Lab Immunol*, 1999. **6**(4): p. 494-8.
526. Nakagawa, M., et al., *Persistence of human papillomavirus type 16 infection is associated with lack of cytotoxic T lymphocyte response to the E6 antigens*. *J Infect Dis*, 2000. **182**(2): p. 595-8.
527. Laffort, C., et al., *Severe cutaneous papillomavirus disease after haemopoietic stem-cell transplantation in patients with severe combined immune deficiency caused by common gamma cytokine receptor subunit or JAK-3 deficiency*. *Lancet*, 2004. **363**(9426): p. 2051-4.
528. Apple, R.J., et al., *HLA DR-DQ associations with cervical carcinoma show papillomavirus-type specificity*. *Nat Genet*, 1994. **6**(2): p. 157-62.
529. Bickham, K., et al., *Dendritic cells initiate immune control of epstein-barr virus transformation of B lymphocytes in vitro*. *J Exp Med*, 2003. **198**(11): p. 1653-63.
530. Hislop, A.D., et al., *Cellular responses to viral infection in humans: lessons from Epstein-Barr virus*. *Annu Rev Immunol*, 2007. **25**: p. 587-617.
531. Callan, M.F., et al., *Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus In vivo*. *J Exp Med*, 1998. **187**(9): p. 1395-402.
532. Blake, N., et al., *The importance of exogenous antigen in priming the human CD8+ T cell response: lessons from the EBV nuclear antigen EBNA1*. *J Immunol*, 2000. **165**(12): p. 7078-87.
533. Blake, N., et al., *Human CD8+ T cell responses to EBV EBNA1: HLA class I presentation of the (Gly-Ala)-containing protein requires exogenous processing*. *Immunity*, 1997. **7**(6): p. 791-802.
534. Lee, S.P., et al., *CD8 T cell recognition of endogenously expressed epstein-barr virus nuclear antigen 1*. *J Exp Med*, 2004. **199**(10): p. 1409-20.
535. Bennett, S.R., et al., *Help for cytotoxic-T-cell responses is mediated by CD40 signalling*. *Nature*, 1998. **393**(6684): p. 478-80.
536. Cardin, R.D., et al., *Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells*. *J Exp Med*, 1996. **184**(3): p. 863-71.
537. Ridge, J.P., F. Di Rosa, and P. Matzinger, *A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell*. *Nature*, 1998. **393**(6684): p. 474-8.
538. Schoenberger, S.P., et al., *T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions*. *Nature*, 1998. **393**(6684): p. 480-3.
539. Marshall, N.A., M.A. Vickers, and R.N. Barker, *Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1*. *J Immunol*, 2003. **170**(12): p. 6183-9.
540. Amyes, E., et al., *Characterization of the CD4+ T cell response to Epstein-Barr virus during primary and persistent infection*. *J Exp Med*, 2003. **198**(6): p. 903-11.
541. Nikiforow, S., et al., *Cytolytic CD4(+)-T-cell clones reactive to*

- EBNA1 inhibit Epstein-Barr virus-induced B-cell proliferation. *J Virol*, 2003. **77**(22): p. 12088-104.
542. Fu, T., K.S. Voo, and R.F. Wang, *Critical role of EBNA1-specific CD4+ T cells in the control of mouse Burkitt lymphoma in vivo*. *J Clin Invest*, 2004. **114**(4): p. 542-50.
543. Leung, C.S., et al., *Nuclear location of an endogenously expressed antigen, EBNA1, restricts access to macroautophagy and the range of CD4 epitope display*. *Proc Natl Acad Sci U S A*. **107**(5): p. 2165-70.
544. Hoffman, G.J., S.G. Lazarowitz, and S.D. Hayward, *Monoclonal antibody against a 250,000-dalton glycoprotein of Epstein-Barr virus identifies a membrane antigen and a neutralizing antigen*. *Proc Natl Acad Sci U S A*, 1980. **77**(5): p. 2979-83.
545. Thorley-Lawson, D.A. and K. Geilinger, *Monoclonal antibodies against the major glycoprotein (gp350/220) of Epstein-Barr virus neutralize infectivity*. *Proc Natl Acad Sci U S A*, 1980. **77**(9): p. 5307-11.
546. Gu, S.Y., et al., *First EBV vaccine trial in humans using recombinant vaccinia virus expressing the major membrane antigen*. *Dev Biol Stand*, 1995. **84**: p. 171-7.
547. Bentz, G.L., et al., *Epstein-Barr virus BRLF1 inhibits transcription of IRF3 and IRF7 and suppresses induction of interferon-beta*. *Virology*. **402**(1): p. 121-8.
548. Wang, J.T., et al., *Epstein-Barr virus BGLF4 kinase suppresses the interferon regulatory factor 3 signaling pathway*. *J Virol*, 2009. **83**(4): p. 1856-69.
549. Wu, L., et al., *Epstein-Barr virus LF2: an antagonist to type I interferon*. *J Virol*, 2009. **83**(2): p. 1140-6.
550. Michaud, F., et al., *Epstein-Barr virus interferes with the amplification of IFN α secretion by activating suppressor of cytokine signaling 3 in primary human monocytes*. *PLoS One*. **5**(7): p. e11908.
551. Shah, K.M., et al., *The EBV-encoded latent membrane proteins, LMP2A and LMP2B, limit the actions of interferon by targeting interferon receptors for degradation*. *Oncogene*, 2009. **28**(44): p. 3903-14.
552. Geiger, T.R. and J.M. Martin, *The Epstein-Barr virus-encoded LMP-1 oncoprotein negatively affects Tyk2 phosphorylation and interferon signaling in human B cells*. *J Virol*, 2006. **80**(23): p. 11638-50.
553. Nanbo, A., H. Yoshiyama, and K. Takada, *Epstein-Barr virus-encoded poly(A)- RNA confers resistance to apoptosis mediated through Fas by blocking the PKR pathway in human epithelial intestine 407 cells*. *J Virol*, 2005. **79**(19): p. 12280-5.
554. Ruf, I.K., et al., *Protection from interferon-induced apoptosis by Epstein-Barr virus small RNAs is not mediated by inhibition of PKR*. *J Virol*, 2005. **79**(23): p. 14562-9.
555. Aman, P. and A. von Gabain, *An Epstein-Barr virus immortalization associated gene segment interferes specifically with the IFN-induced anti-proliferative response in human B-lymphoid cell lines*. *EMBO J*, 1990. **9**(1): p. 147-52.
556. Kanda, K., et al., *The EBNA2-related resistance towards alpha interferon (IFN-alpha) in Burkitt's lymphoma cells affects induction of IFN-induced genes but not the activation of transcription factor ISGF-3*. *Mol Cell Biol*, 1992. **12**(11): p. 4930-6.
557. Rowe, M., et al., *Upregulation of bcl-2 by the Epstein-Barr virus latent membrane protein LMP1: a B-cell-specific response that is delayed relative to NF-kappa B activation and to induction of cell surface markers*. *J Virol*, 1994. **68**(9): p. 5602-12.
558. Younesi, V., et al., *Epstein Barr virus inhibits the stimulatory effect of TLR7/8 and TLR9 agonists but not CD40 ligand in human B lymphocytes*. *Microbiol Immunol*. **54**(9): p. 534-41.
559. Kitagawa, N., et al., *Epstein-Barr virus-encoded poly(A)(-) RNA supports Burkitt's lymphoma growth through interleukin-10 induction*. *EMBO J*, 2000. **19**(24): p. 6742-50.
560. Yang, L., et al., *Epstein-Barr virus (EBV)-encoded RNA promotes growth of EBV-infected T cells through interleukin-9 induction*. *Cancer Res*, 2004. **64**(15): p. 5332-7.
561. Iwakiri, D., et al., *Epstein-Barr virus-encoded small RNA induces insulin-like growth factor 1 and supports growth of nasopharyngeal carcinoma-derived cell lines*. *Oncogene*, 2005. **24**(10): p. 1767-73.
562. Savard, M., et al., *Infection of primary human monocytes by Epstein-Barr virus*. *J Virol*, 2000. **74**(6): p. 2612-9.
563. Gosselin, J., et al., *Inhibition of tumor necrosis factor-alpha transcription by Epstein-Barr virus*. *Eur J Immunol*, 1991. **21**(1): p. 203-8.
564. Jabs, W.J., et al., *Inhibition of macrophage inflammatory protein-1 alpha production by Epstein-Barr virus*. *Blood*, 2002. **99**(5): p. 1512-6.
565. Savard, M., et al., *EBV suppresses prostaglandin E2 biosynthesis in human monocytes*. *J Immunol*, 2000. **164**(12): p. 6467-73.
566. Tardif, M., et al., *Impaired protein kinase C activation/translocation in Epstein-Barr virus-infected monocytes*. *J Biol Chem*, 2002. **277**(27): p. 24148-54.
567. Strockbine, L.D., et al., *The Epstein-Barr virus BARP1 gene encodes a novel, soluble colony-stimulating factor-1 receptor*. *J Virol*, 1998. **72**(5): p. 4015-21.
568. Nanbo, A., et al., *Epstein-Barr virus RNA confers resistance to interferon-alpha-induced apoptosis in Burkitt's lymphoma*. *EMBO J*, 2002. **21**(5): p. 954-65.
569. Morrison, T.E., et al., *Inhibition of IFN-gamma signaling by an Epstein-Barr virus immediate-early protein*. *Immunity*, 2001. **15**(5): p. 787-99.
570. Mahot, S., et al., *A novel function for the Epstein-Barr virus transcription factor EB1/Zta: induction of transcription of the hIL-10 gene*. *J Gen Virol*, 2003. **84**(Pt 4): p. 965-74.
571. Lee, C.H., et al., *Epstein-Barr virus Zta-induced immunomodulators from nasopharyngeal carcinoma cells upregulate interleukin-10 production from monocytes*. *J Virol*. **85**(14): p. 7333-42.
572. Salek-Ardakani, S., J.R. Arrand, and M. Mackett, *Epstein-Barr virus encoded interleukin-10 inhibits HLA-class I, ICAM-1, and B7 expression on human monocytes: implications for immune evasion by EBV*. *Virology*, 2002. **304**(2): p. 342-51.
573. Vieira, P., et al., *Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRF1*. *Proc Natl Acad Sci U S A*, 1991. **88**(4): p. 1172-6.
574. Devergne, O., et al., *A novel interleukin-12 p40-related protein induced by latent Epstein-Barr virus infection in B lymphocytes*. *J Virol*, 1996. **70**(2): p. 1143-53.
575. Nieuwenhuis, E.E., et al., *Disruption of T helper 2-immune responses in Epstein-Barr virus-induced gene 3-deficient mice*. *Proc Natl Acad Sci U S A*, 2002. **99**(26): p. 16951-6.
576. Horst, D., et al., *Specific targeting of the EBV lytic phase protein BNLF2a to the transporter associated with antigen processing results in impairment of HLA class I-restricted antigen presentation*. *J Immunol*, 2009. **182**(4): p. 2313-24.
577. Zuo, J., et al., *The Epstein-Barr virus G-protein-coupled receptor contributes to immune evasion by targeting MHC class I molecules for degradation*. *PLoS Pathog*, 2009. **5**(1): p. e1000255.
578. Li, D., et al., *Down-regulation of MHC class II expression through inhibition of CIITA transcription by lytic transactivator Zta during Epstein-Barr virus reactivation*. *J Immunol*, 2009. **182**(4): p. 1799-809.
579. Rowe, M., et al., *Host shutoff during productive Epstein-Barr virus infection is mediated by BGLF5 and may contribute to immune evasion*. *Proc Natl Acad Sci U S A*, 2007. **104**(9): p. 3366-71.
580. Levitskaya, J., et al., *Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1*. *Proc Natl Acad Sci U S A*, 1997. **94**(23): p. 12616-21.
581. Yin, Y., B. Manoury, and R. Fahraeus, *Self-inhibition of synthesis and antigen presentation by Epstein-Barr virus-encoded EBNA1*. *Science*, 2003. **301**(5638): p. 1371-4.
582. Crotzter, V.L., et al., *Immunodominance among EBV-derived*

- epitopes restricted by HLA-B27 does not correlate with epitope abundance in EBV-transformed B-lymphoblastoid cell lines. *J Immunol*, 2000. **164**(12): p. 6120-9.
583. Jilg, W., et al., Expression of class I major histocompatibility complex antigens in Epstein-Barr virus-carrying lymphoblastoid cell lines and Burkitt lymphoma cells. *Cancer Res*, 1991. **51**(1): p. 27-32.
584. Koppers-Lalic, D., et al., The UL41-encoded virion host shutoff (vhs) protein and vhs-independent mechanisms are responsible for down-regulation of MHC class I molecules by bovine herpesvirus 1. *J Gen Virol*, 2001. **82**(Pt 9): p. 2071-81.
585. Smiley, J.R., Herpes simplex virus virion host shutoff protein: immune evasion mediated by a viral RNase? *J Virol*, 2004. **78**(3): p. 1063-8.
586. Tigges, M.A., et al., Human herpes simplex virus (HSV)-specific CD8+ CTL clones recognize HSV-2-infected fibroblasts after treatment with IFN-gamma or when virion host shutoff functions are disabled. *J Immunol*, 1996. **156**(10): p. 3901-10.
587. Roucard, C., et al., In vivo and in vitro modulation of HLA-DM and HLA-DO is induced by B lymphocyte activation. *J Immunol*, 2001. **167**(12): p. 6849-58.
588. de Waal Malefyt, R., H. Yssel, and J.E. de Vries, Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells. Specific inhibition of IL-2 production and proliferation. *J Immunol*, 1993. **150**(11): p. 4754-65.
589. Taga, K. and G. Tosato, IL-10 inhibits human T cell proliferation and IL-2 production. *J Immunol*, 1992. **148**(4): p. 1143-8.
590. Taylor, A., et al., IL-10 suppresses CD2-mediated T cell activation via SHP-1. *Mol Immunol*, 2009. **46**(4): p. 622-9.
591. Elenkov, I.J. and G.P. Chrousos, Stress hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity. *Ann N Y Acad Sci*, 2002. **966**: p. 290-303.
592. Fiorentino, D.F., et al., IL-10 inhibits cytokine production by activated macrophages. *J Immunol*, 1991. **147**(11): p. 3815-22.
593. Fiorentino, D.F., et al., IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol*, 1991. **146**(10): p. 3444-51.
594. Moore, K.W., et al., Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*, 2001. **19**: p. 683-765.
595. Corinti, S., et al., Regulatory activity of autocrine IL-10 on dendritic cell functions. *J Immunol*, 2001. **166**(7): p. 4312-8.
596. Couper, K.N., D.G. Blount, and E.M. Riley, IL-10: the master regulator of immunity to infection. *J Immunol*, 2008. **180**(9): p. 5771-7.
597. Gasser, O., et al., HIV patients developing primary CNS lymphoma lack EBV-specific CD4+ T cell function irrespective of absolute CD4+ T cell counts. *PLoS Med*, 2007. **4**(3): p. e96.
598. Whittle, H.C., et al., T-cell control of Epstein-Barr virus-infected B cells is lost during *P. falciparum* malaria. *Nature*, 1984. **312**(5993): p. 449-50.
599. Li, J., et al., Functional inactivation of EBV-specific T-lymphocytes in nasopharyngeal carcinoma: implications for tumor immunotherapy. *PLoS One*, 2007. **2**(11): p. e1122.
600. Lau, K.M., et al., Increase in circulating Foxp3+CD4+CD25(high) regulatory T cells in nasopharyngeal carcinoma patients. *Br J Cancer*, 2007. **96**(4): p. 617-22.
601. Herbst, H., et al., Frequent expression of interleukin-10 by Epstein-Barr virus-harboring tumor cells of Hodgkin's disease. *Blood*, 1996. **87**(7): p. 2918-29.
602. Gandhi, M.K., et al., Expression of LAG-3 by tumor-infiltrating lymphocytes is coincident with the suppression of latent membrane antigen-specific CD8+ T-cell function in Hodgkin lymphoma patients. *Blood*, 2006. **108**(7): p. 2280-9.
603. Marshall, N.A., et al., Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood*, 2004. **103**(5): p. 1755-62.
604. Gandhi, M.K., et al., Galectin-1 mediated suppression of Epstein-Barr virus specific T-cell immunity in classic Hodgkin lymphoma. *Blood*, 2007. **110**(4): p. 1326-9.
605. Heller, K.N., et al., Patients with Epstein Barr virus-positive lymphomas have decreased CD4(+) T-cell responses to the viral nuclear antigen 1. *Int J Cancer*, 2008. **123**(12): p. 2824-31.
606. Kupper, T.S. and R.C. Fuhlbrigge, Immune surveillance in the skin: mechanisms and clinical consequences. *Nat Rev Immunol*, 2004. **4**(3): p. 211-22.
607. Crum, C.P., et al., In situ hybridization analysis of HPV 16 DNA sequences in early cervical neoplasia. *Am J Pathol*, 1986. **123**(1): p. 174-82.
608. Greenfield, I., et al., Human papillomavirus 16 E7 protein is associated with the nuclear matrix. *Proc Natl Acad Sci U S A*, 1991. **88**(24): p. 11217-21.
609. Stoler, M.H., et al., Human papillomavirus type 16 and 18 gene expression in cervical neoplasias. *Hum Pathol*, 1992. **23**(2): p. 117-28.
610. Cason, J., et al., Identification of immunogenic regions of the major coat protein of human papillomavirus type 16 that contain type-restricted epitopes. *J Gen Virol*, 1989. **70** (Pt 11): p. 2973-87.
611. Rudolf, M.P., et al., Induction of HPV16 capsid protein-specific human T cell responses by virus-like particles. *Biol Chem*, 1999. **380**(3): p. 335-40.
612. Zhou, J., et al., Papillomavirus capsid protein expression level depends on the match between codon usage and tRNA availability. *J Virol*, 1999. **73**(6): p. 4972-82.
613. Liu, W.J., et al., Codon modified human papillomavirus type 16 E7 DNA vaccine enhances cytotoxic T-lymphocyte induction and anti-tumour activity. *Virology*, 2002. **301**(1): p. 43-52.
614. Schwartz, S., Regulation of human papillomavirus late gene expression. *Ups J Med Sci*, 2000. **105**(3): p. 171-92.
615. Oldstone, M.B., Molecular mimicry and immune-mediated diseases. *FASEB J*, 1998. **12**(13): p. 1255-65.
616. Natale, C., et al., Computer-assisted analysis of molecular mimicry between human papillomavirus 16 E7 oncoprotein and human protein sequences. *Immunol Cell Biol*, 2000. **78**(6): p. 580-5.
617. O'Donovan, A. and R.D. Wood, Identical defects in DNA repair in xeroderma pigmentosum group G and rodent ERCC group 5. *Nature*, 1993. **363**(6425): p. 185-8.
618. Scherly, D., et al., Complementation of the DNA repair defect in xeroderma pigmentosum group G cells by a human cDNA related to yeast RAD2. *Nature*, 1993. **363**(6425): p. 182-5.
619. Lai, A., et al., RBP1 recruits both histone deacetylase-dependent and -independent repression activities to retinoblastoma family proteins. *Mol Cell Biol*, 1999. **19**(10): p. 6632-41.
620. Nees, M., et al., Papillomavirus type 16 oncogenes down-regulate expression of interferon-responsive genes and up-regulate proliferation-associated and NF-kappaB-responsive genes in cervical keratinocytes. *J Virol*, 2001. **75**(9): p. 4283-96.
621. Arany, I., A. Goel, and S.K. Tyring, Interferon response depends on viral transcription in human papillomavirus-containing lesions. *Anticancer Res*, 1995. **15**(6B): p. 2865-9.
622. Barnard, P. and N.A. McMillan, The human papillomavirus E7 oncoprotein abrogates signaling mediated by interferon-alpha. *Virology*, 1999. **259**(2): p. 305-13.
623. Barnard, P., E. Payne, and N.A. McMillan, The human papillomavirus E7 protein is able to inhibit the antiviral and anti-growth functions of interferon-alpha. *Virology*, 2000. **277**(2): p. 411-9.
624. Park, J.S., et al., Inactivation of interferon regulatory factor-1 tumor suppressor protein by HPV E7 oncoprotein. Implication for the E7-mediated immune evasion mechanism in cervical carcinogenesis. *J Biol Chem*, 2000. **275**(10): p. 6764-9.
625. Um, S.J., et al., Abrogation of IRF-1 response by high-risk HPV E7 protein in vivo. *Cancer Lett*, 2002. **179**(2): p. 205-12.
626. Ronco, L.V., et al., Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. *Genes Dev*, 1998. **12**(13): p. 2061-72.
627. Li, S., et al., The human papilloma virus (HPV)-18 E6 onco-

- protein physically associates with Tyk2 and impairs Jak-STAT activation by interferon- α . *Oncogene*, 1999. **18**(42): p. 5727-37.
628. Daud, I.I., et al., Association between toll-like receptor expression and human papillomavirus type 16 persistence. *Int.J.Cancer*. **128**(4): p. 879-886.
629. Georgopoulos, N.T., J.L. Proffitt, and G.E. Blair, Transcriptional regulation of the major histocompatibility complex (MHC) class I heavy chain, TAP1 and LMP2 genes by the human papillomavirus (HPV) type 6b, 16 and 18 E7 oncoproteins. *Oncogene*, 2000. **19**(42): p. 4930-5.
630. Campo, M.S., et al., HPV-16 E5 down-regulates expression of surface HLA class I and reduces recognition by CD8 T cells. *Virology*. **407**(1): p. 137-42.
631. Kleine-Lowinski, K., et al., Monocyte-chemo-attractant-protein-1 (MCP-1)-gene expression in cervical intra-epithelial neoplasias and cervical carcinomas. *Int J Cancer*, 1999. **82**(1): p. 6-11.
632. Kleine-Lowinski, K., et al., Selective suppression of monocyte chemoattractant protein-1 expression by human papillomavirus E6 and E7 oncoproteins in human cervical epithelial and epidermal cells. *Int J Cancer*, 2003. **107**(3): p. 407-15.
633. Huang, S.M. and D.J. McCance, Down regulation of the interleukin-8 promoter by human papillomavirus type 16 E6 and E7 through effects on CREB binding protein/p300 and P/CAF. *J Virol*, 2002. **76**(17): p. 8710-21.
634. Cho, Y.S., et al., Down modulation of IL-18 expression by human papillomavirus type 16 E6 oncogene via binding to IL-18. *FEBS Lett*, 2001. **501**(2-3): p. 139-45.
635. Lee, S.J., et al., Both E6 and E7 oncoproteins of human papillomavirus 16 inhibit IL-18-induced IFN- γ production in human peripheral blood mononuclear and NK cells. *J Immunol*, 2001. **167**(1): p. 497-504.
636. Giannini, S.L., et al., Influence of the mucosal epithelium microenvironment on Langerhans cells: implications for the development of squamous intraepithelial lesions of the cervix. *Int J Cancer*, 2002. **97**(5): p. 654-9.
637. Jacobs, N., et al., Inverse modulation of IL-10 and IL-12 in the blood of women with preneoplastic lesions of the uterine cervix. *Clin Exp Immunol*, 1998. **111**(1): p. 219-24.
638. Bais, A.G., et al., A shift to a peripheral Th2-type cytokine pattern during the carcinogenesis of cervical cancer becomes manifest in CIN III lesions. *J Clin Pathol*, 2005. **58**(10): p. 1096-100.
639. Sheu, B.C., et al., Predominant Th2/Tc2 polarity of tumor-infiltrating lymphocytes in human cervical cancer. *J Immunol*, 2001. **167**(5): p. 2972-8.
640. Hess, S., et al., Loss of IL-6 receptor expression in cervical carcinoma cells inhibits autocrine IL-6 stimulation: abrogation of constitutive monocyte chemoattractant protein-1 production. *J Immunol*, 2000. **165**(4): p. 1939-48.
641. Lopez-Ocejo, O., et al., Oncogenes and tumor angiogenesis: the HPV-16 E6 oncoprotein activates the vascular endothelial growth factor (VEGF) gene promoter in a p53 independent manner. *Oncogene*, 2000. **19**(40): p. 4611-20.
642. Matthews, K., et al., Depletion of Langerhans cells in human papillomavirus type 16-infected skin is associated with E6-mediated down regulation of E-cadherin. *J Virol*, 2003. **77**(15): p. 8378-85.
643. Fahey, L.M., et al., A major role for the minor capsid protein of human papillomavirus type 16 in immune escape. *J Immunol*, 2009. **183**(10): p. 6151-6.
644. Baggiolini, M., P. Loetscher, and B. Moser, Interleukin-8 and the chemokine family. *Int J Immunopharmacol*, 1995. **17**(2): p. 103-8.
645. Nakamura, K., I.R. Williams, and T.S. Kupper, Keratinocyte-derived monocyte chemoattractant protein 1 (MCP-1): analysis in a transgenic model demonstrates MCP-1 can recruit dendritic and Langerhans cells to skin. *J Invest Dermatol*, 1995. **105**(5): p. 635-43.
646. Larsen, C.G., et al., The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. *Science*, 1989. **243**(4897): p. 1464-6.
647. Oppenheim, J.J., et al., Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu Rev Immunol*, 1991. **9**: p. 617-48.
648. Azar, K.K., et al., Increased secretion patterns of interleukin-10 and tumor necrosis factor- α in cervical squamous intraepithelial lesions. *Hum Pathol*, 2004. **35**(11): p. 1376-84.
649. Kobayashi, A., et al., Functional attributes of mucosal immunity in cervical intraepithelial neoplasia and effects of HIV infection. *Cancer Res*, 2004. **64**(18): p. 6766-74.
650. Bolpetti, A., et al., Interleukin-10 production by tumor infiltrating macrophages plays a role in Human Papillomavirus 16 tumor growth. *BMC Immunol*. **11**: p. 27.
651. El-Sherif, A.M., et al., Decreased synthesis and expression of TGF- β 1, β 2, and β 3 in epithelium of HPV 16-positive cervical precancer: a study by microdissection, quantitative RT-PCR, and immunocytochemistry. *J Pathol*, 2000. **192**(4): p. 494-501.
652. Derynck, R., R.J. Akhurst, and A. Balmain, TGF- β signaling in tumor suppression and cancer progression. *Nat Genet*, 2001. **29**(2): p. 117-29.
653. Braun, L., et al., Regulation of growth and gene expression in human papillomavirus-transformed keratinocytes by transforming growth factor- β : implications for the control of papillomavirus infection. *Mol Carcinog*, 1992. **6**(2): p. 100-11.
654. Woodworth, C.D., V. Notario, and J.A. DiPaolo, Transforming growth factors β 1 and 2 transcriptionally regulate human papillomavirus (HPV) type 16 early gene expression in HPV-immortalized human genital epithelial cells. *J Virol*, 1990. **64**(10): p. 4767-75.
655. Fattorossi, A., et al., Lymphocyte composition of tumor draining lymph nodes from cervical and endometrial cancer patients. *Gynecol Oncol*, 2004. **92**(1): p. 106-15.
656. Chomarat, P., et al., IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol*, 2000. **1**(6): p. 510-4.
657. Menetrier-Caux, C., et al., Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor. *Blood*, 1998. **92**(12): p. 4778-91.
658. Nefedova, Y., et al., Hyperactivation of STAT3 is involved in abnormal differentiation of dendritic cells in cancer. *J Immunol*, 2004. **172**(1): p. 464-74.
659. Hegde, S., J. Pahne, and S. Smola-Hess, Novel immunosuppressive properties of interleukin-6 in dendritic cells: inhibition of NF- κ B binding activity and CCR7 expression. *FASEB J*, 2004. **18**(12): p. 1439-41.
660. Wei, L.H., et al., Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. *Oncogene*, 2003. **22**(10): p. 1517-27.
661. Ohm, J.E. and D.P. Carbone, VEGF as a mediator of tumor-associated immunodeficiency. *Immunol Res*, 2001. **23**(2-3): p. 263-72.
662. Dobbs, S.P., et al., Angiogenesis is associated with vascular endothelial growth factor expression in cervical intraepithelial neoplasia. *Br J Cancer*, 1997. **76**(11): p. 1410-5.
663. Guidi, A.J., et al., Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in cervical neoplasia. *J Natl Cancer Inst*, 1995. **87**(16): p. 1237-45.
664. Tang, A., et al., Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature*, 1993. **361**(6407): p. 82-5.
665. Hubert, P., et al., E-cadherin-dependent adhesion of dendritic and Langerhans cells to keratinocytes is defective in cervical human papillomavirus-associated (pre)neoplastic lesions. *J Pathol*, 2005. **206**(3): p. 346-55.
666. Merad, M., et al., Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat Immunol*, 2002. **3**(12): p. 1135-41.

667. Dieu-Nosjean, M.C., et al., *Macrophage inflammatory protein 3alpha is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors*. J.Exp.Med., 2000. **192**(5): p. 705-718.
668. Liao, F., et al., *CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha*. J.Immunol., 1999. **162**(1): p. 186-194.
669. Larregina, A.T., et al., *Dermal-resident CD14+ cells differentiate into Langerhans cells*. Nat Immunol, 2001. **2**(12): p. 1151-8.
670. Ostor, A.G., *Natural history of cervical intraepithelial neoplasia: a critical review*. Int J Gynecol Pathol, 1993. **12**(2): p. 186-92.
671. Brown, S.L., et al., *Myd88-dependent positioning of Ptgs2-expressing stromal cells maintains colonic epithelial proliferation during injury*. J Clin Invest, 2007. **117**(1): p. 258-69.
672. Fukata, M., et al., *Cox-2 is regulated by Toll-like receptor-4 (TLR4) signaling: Role in proliferation and apoptosis in the intestine*. Gastroenterology, 2006. **131**(3): p. 862-77.
673. Kim, D., et al., *A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity*. J Biol Chem, 2007. **282**(20): p. 14975-83.
674. Yu, L. and S. Chen, *Toll-like receptors expressed in tumor cells: targets for therapy*. Cancer Immunol Immunother, 2008. **57**(9): p. 1271-8.
675. Higgins, S.C., et al., *Toll-like receptor 4-mediated innate IL-10 activates antigen-specific regulatory T cells and confers resistance to Bordetella pertussis by inhibiting inflammatory pathology*. J Immunol, 2003. **171**(6): p. 3119-27.
676. Ng, M.T., et al., *Increase in NF-kappaB binding affinity of the variant C allele of the toll-like receptor 9 -1237T/C polymorphism is associated with Helicobacter pylori-induced gastric disease*. Infect Immun. **78**(3): p. 1345-52.
677. Nieters, A., et al., *Gene polymorphisms in Toll-like receptors, interleukin-10, and interleukin-10 receptor alpha and lymphoma risk*. Genes Immun, 2006. **7**(8): p. 615-24.
678. Vaisanen, M.R., et al., *Expression of toll-like receptor-9 is increased in poorly differentiated prostate tumors*. Prostate. **70**(8): p. 817-24.
679. Gonzalez-Reyes, S., et al., *Study of TLR3, TLR4 and TLR9 in breast carcinomas and their association with metastasis*. BMC Cancer. **10**: p. 665.
680. Salaun, B., et al., *TLR3 can directly trigger apoptosis in human cancer cells*. J.Immunol., 2006. **176**(8): p. 4894-4901.
681. Salaun, B., et al., *TLR3 as a biomarker for the therapeutic efficacy of double-stranded RNA in breast cancer*. Cancer Res. **71**(5): p. 1607-1614.
682. Kuninaka, N., et al., *Expression of Toll-like receptor 9 in bone marrow cells of myelodysplastic syndromes is down-regulated during transformation to overt leukemia*. Exp Mol Pathol. **88**(2): p. 293-8.
683. Salaun, B., P. Romero, and S. Lebecque, *Toll-like receptors' two-edged sword: when immunity meets apoptosis*. Eur J Immunol, 2007. **37**(12): p. 3311-8.
684. Fischer, S.F., et al., *Toll-like receptor 9 signaling can sensitize fibroblasts for apoptosis*. Immunol Lett, 2005. **97**(1): p. 115-22.
685. El Andaloussi, A., et al., *Stimulation of TLR9 with CpG ODN enhances apoptosis of glioma and prolongs the survival of mice with experimental brain tumors*. Glia, 2006. **54**(6): p. 526-35.
686. He, L., et al., *Toll-like receptor 9 is required for opioid-induced microglia apoptosis*. PLoS One. **6**(4): p. e18190.
687. Chen, L., et al., *Critical role of toll-like receptor 9 in morphine and Mycobacterium tuberculosis-Induced apoptosis in mice*. PLoS One. **5**(2): p. e9205.
688. Liang, X., et al., *Toll-like receptor 9 signaling by CpG-B oligodeoxynucleotides induces an apoptotic pathway in human chronic lymphocytic leukemia B cells*. Blood. **115**(24): p. 5041-52.
689. Jozsef, L., T. Khreiss, and J.G. Filep, *CpG motifs in bacterial DNA delay apoptosis of neutrophil granulocytes*. FASEB J, 2004. **18**(14): p. 1776-8.
690. Kuo, C.C., S.M. Liang, and C.M. Liang, *CpG-B oligodeoxynucleotide promotes cell survival via up-regulation of Hsp70 to increase Bcl-xL and to decrease apoptosis-inducing factor translocation*. J Biol Chem, 2006. **281**(50): p. 38200-7.
691. Jego, G., et al., *Pathogen-associated molecular patterns are growth and survival factors for human myeloma cells through Toll-like receptors*. Leukemia, 2006. **20**(6): p. 1130-7.
692. Chiron, D., et al., *Phosphorothioate-modified TLR9 ligands protect cancer cells against TRAIL-induced apoptosis*. J Immunol, 2009. **183**(7): p. 4371-7.
693. Gekeler, V., et al., *G3139 and other CpG-containing immunostimulatory phosphorothioate oligodeoxynucleotides are potent suppressors of the growth of human tumor xenografts in nude mice*. Oligonucleotides, 2006. **16**(1): p. 83-93.
694. Wang, H., et al., *Chemotherapy and chemosensitization of non-small cell lung cancer with a novel immunomodulatory oligonucleotide targeting Toll-like receptor 9*. Mol Cancer Ther, 2006. **5**(6): p. 1585-92.
695. Rayburn, E.R., et al., *Experimental therapy for colon cancer: anti-cancer effects of TLR9 agonism, combination with other therapeutic modalities, and dependence upon p53*. Int J Oncol, 2007. **30**(6): p. 1511-9.
696. Damiano, V., et al., *Novel toll-like receptor 9 agonist induces epidermal growth factor receptor (EGFR) inhibition and synergistic antitumor activity with EGFR inhibitors*. Clin.Cancer Res., 2006. **12**(2): p. 577-583.
697. Ilvesaro, J.M., et al., *Toll like receptor-9 agonists stimulate prostate cancer invasion in vitro*. Prostate, 2007. **67**(7): p. 774-81.
698. Ilvesaro, J.M., et al., *Toll-like receptor 9 mediates CpG oligonucleotide-induced cellular invasion*. Mol Cancer Res, 2008. **6**(10): p. 1534-43.
699. Sandholm, J., et al., *Estrogen receptor-alpha and sex steroid hormones regulate Toll-like receptor-9 expression and invasive function in human breast cancer cells*. Breast Cancer Res Treat. **100**: p. 437-47.
700. Merrell, M.A., et al., *Toll-like receptor 9 agonists promote cellular invasion by increasing matrix metalloproteinase activity*. Mol Cancer Res, 2006. **4**(7): p. 437-47.
701. Mittal, D., et al., *TLR4-mediated skin carcinogenesis is dependent on immune and radioresistant cells*. EMBO J. **29**(13): p. 2242-52.
702. Uronis, J.M., et al., *Modulation of the intestinal microbiota alters colitis-associated colorectal cancer susceptibility*. PLoS One, 2009. **4**(6): p. e6026.
703. Coste, I., et al., *Dual function of MyD88 in RAS signaling and inflammation, leading to mouse and human cell transformation*. J Clin Invest. **120**(10): p. 3663-7.
704. Ngo, V.N., et al., *Oncogenically active MYD88 mutations in human lymphoma*. Nature. **470**(7332): p. 115-9.
705. Salcedo, R., et al., *MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18*. J Exp Med. **207**(8): p. 1625-36.
706. Whiteside, T.L., *The tumor microenvironment and its role in promoting tumor growth*. Oncogene, 2008. **27**(45): p. 5904-12.
707. Li, H., et al., *Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1*. J Immunol, 2009. **182**(1): p. 240-9.
708. Strauss, L., C. Bergmann, and T.L. Whiteside, *Human circulating CD4+CD25highFoxp3+ regulatory T cells kill autologous CD8+ but not CD4+ responder cells by Fas-mediated apoptosis*. J Immunol, 2009. **182**(3): p. 1469-80.
709. Dolganiuc, A., et al., *Hepatitis C virus (HCV) core protein-induced, monocyte-mediated mechanisms of reduced IFN-alpha and plasmacytoid dendritic cell loss in chronic HCV infection*. J.Immunol., 2006. **177**(10): p. 6758-6768.
710. Labidi-Galy, S.I., et al., *Quantitative and Functional Altera-*

- tions of Plasmacytoid Dendritic Cells Contribute to Immune Tolerance in Ovarian Cancer. *Cancer Res.*
711. Woltman, A.M., et al., *Hepatitis B virus lacks immune activating capacity, but actively inhibits plasmacytoid dendritic cell function.* *PLoS One.* **6**(1): p. e15324.
712. Cao, W., et al., *Regulation of TLR7/9 responses in plasmacytoid dendritic cells by BST2 and ILT7 receptor interaction.* *J Exp Med,* 2009. **206**(7): p. 1603-14.
713. Cao, W., et al., *Plasmacytoid dendritic cell-specific receptor ILT7-Fc epsilonRI gamma inhibits Toll-like receptor-induced interferon production.* *J.Exp.Med.,* 2006. **203**(6): p. 1399-1405.
714. Cai, D., et al., *Up-regulation of bone marrow stromal protein 2 (BST2) in breast cancer with bone metastasis.* *BMC Cancer,* 2009. **9**: p. 102.
715. Cao, W., *Molecular characterization of human plasmacytoid dendritic cells.* *J Clin Immunol,* 2009. **29**(3): p. 257-64.
716. Ohtomo, T., et al., *Molecular cloning and characterization of a surface antigen preferentially overexpressed on multiple myeloma cells.* *Biochem Biophys Res Commun,* 1999. **258**(3): p. 583-91.
717. Wainwright, D.A., et al., *The expression of BST2 in human and experimental mouse brain tumors.* *Exp Mol Pathol.* **91**(1): p. 440-6.
718. Wenzel, J., et al., *Type I interferon-associated recruitment of cytotoxic lymphocytes: a common mechanism in regressive melanocytic lesions.* *Am J Clin Pathol,* 2005. **124**(1): p. 37-48.
719. Dunn, G.P., C.M. Koebel, and R.D. Schreiber, *Interferons, immunity and cancer immunoediting.* *Nat Rev Immunol,* 2006. **6**(11): p. 836-48.
720. Peng, G., et al., *Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function.* *Science,* 2005. **309**(5739): p. 1380-4.
721. Crellin, N.K., et al., *Human CD4+ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells.* *J Immunol,* 2005. **175**(12): p. 8051-9.
722. Tsuji, S., et al., *Maturation of human dendritic cells by cell wall skeleton of Mycobacterium bovis bacillus Calmette-Guerin: involvement of toll-like receptors.* *Infect Immun,* 2000. **68**(12): p. 6883-90.
723. Uehori, J., et al., *Dendritic cell maturation induced by muramyl dipeptide (MDP) derivatives: monoacylated MDP confers TLR2/TLR4 activation.* *J Immunol,* 2005. **174**(11): p. 7096-103.
724. Krieg, A.M., *Toll-like receptor 9 (TLR9) agonists in the treatment of cancer.* *Oncogene,* 2008. **27**(2): p. 161-167.
725. Okamoto, A., et al., *Indoleamine 2,3-dioxygenase serves as a marker of poor prognosis in gene expression profiles of serous ovarian cancer cells.* *Clin.Cancer Res.,* 2005. **11**(16): p. 6030-6039.
726. Smits, E.L., et al., *The use of TLR7 and TLR8 ligands for the enhancement of cancer immunotherapy.* *Oncologist,* 2008. **13**(8): p. 859-75.
727. Schon, M., et al., *Tumor-selective induction of apoptosis and the small-molecule immune response modifier imiquimod.* *J Natl Cancer Inst,* 2003. **95**(15): p. 1138-49.
728. Leonard, J.P., et al., *Phase I trial of toll-like receptor 9 agonist PF-3512676 with and following rituximab in patients with recurrent indolent and aggressive non Hodgkin's lymphoma.* *Clin Cancer Res,* 2007. **13**(20): p. 6168-74.
729. Zent, C.S., et al., *A Phase I Clinical Trial of CpG Oligonucleotide 7909 (PF-03512676) in Patients with Previously Treated Chronic Lymphocytic Leukemia.* *Leuk Lymphoma.*
730. Kim, Y.H., et al., *Phase I trial of a Toll-like receptor 9 agonist, PF-3512676 (CPG 7909), in patients with treatment-refractory, cutaneous T-cell lymphoma.* *J Am Acad Dermatol.* **63**(6): p. 975-83.
731. Hofmann, M.A., et al., *Phase 1 evaluation of intralesionally injected TLR9-agonist PF-3512676 in patients with basal cell carcinoma or metastatic melanoma.* *J Immunother,* 2008. **31**(5): p. 520-7.
732. Murad, Y.M. and T.M. Clay, *CpG oligodeoxynucleotides as TLR9 agonists: therapeutic applications in cancer.* *BioDrugs,* 2009. **23**(6): p. 361-75.
733. Spaner, D.E. and A. Masellis, *Toll-like receptor agonists in the treatment of chronic myelophocytic leukemia.* *Leukemia,* 2007. **21**(1): p. 53-60.
734. Holtick, U., et al., *Toll-like receptor 9 agonists as cancer therapeutics.* *Expert Opin Investig Drugs.* **20**(3): p. 361-72.
735. Krieg, A.M., *Therapeutic potential of Toll-like receptor 9 activation.* *Nat Rev Drug Discov,* 2006. **5**(6): p. 471-84.
736. van Ojik, H.H., et al., *CpG-A and B oligodeoxynucleotides enhance the efficacy of antibody therapy by activating different effector cell populations.* *Cancer Res,* 2003. **63**(17): p. 5595-600.
737. Damiano, V., et al., *Novel toll-like receptor 9 agonist induces epidermal growth factor receptor (EGFR) inhibition and synergistic antitumor activity with EGFR inhibitors.* *Clin Cancer Res,* 2006. **12**(2): p. 577-83.
738. Malanchi, I., et al., *Human papillomavirus type 16 E6 promotes retinoblastoma protein phosphorylation and cell cycle progression.* *J Virol,* 2004. **78**(24): p. 13769-78.
739. Malanchi, I., et al., *Identification of a novel activity of human papillomavirus type 16 E6 protein in deregulating the G1/S transition.* *Oncogene,* 2002. **21**(37): p. 5665-72.
740. Jegerlehner, A., et al., *TLR9 signaling in B cells determines class switch recombination to IgG2a.* *J Immunol,* 2007. **178**(4): p. 2415-20.
741. Jiang, W., et al., *TLR9 stimulation drives naive B cells to proliferate and to attain enhanced antigen presenting function.* *Eur J Immunol,* 2007. **37**(8): p. 2205-13.
742. Huang, B., et al., *Toll-like receptors on tumor cells facilitate evasion of immune surveillance.* *Cancer Res.,* 2005. **65**(12): p. 5009-5014.
743. Szulc, J., et al., *A versatile tool for conditional gene expression and knockdown.* *Nat Methods,* 2006. **3**(2): p. 109-16.
744. Allen-Hoffmann, B.L., et al., *Normal growth and differentiation in a spontaneously immortalized near-diploid human keratinocyte cell line, NIKS.* *J Invest Dermatol,* 2000. **114**(3): p. 444-55.
745. Parroche, P., et al., *Human papillomavirus type 16 E6 inhibits p21(WAF1) transcription independently of p53 by inactivating p150(Sal2).* *Virology.*
746. Pincheira, R., et al., *Sall2 is a novel p75NTR-interacting protein that links NGF signalling to cell cycle progression and neurite outgrowth.* *EMBO J,* 2009. **28**(3): p. 261-73.
747. Huang, S.Y., et al., *Epstein-Barr virus Rta-mediated transactivation of p21 and 14-3-3{sigma} arrests cells at G1/S transition by reducing cyclin E/CDK2 activity.* *J Gen Virol.*
748. Chen, Y.L., et al., *The Epstein-Barr virus replication and transcription activator, Rta/BRLF1, induces cellular senescence in epithelial cells.* *Cell Cycle,* 2009. **8**(1): p. 58-65.
749. Liu, T.Y., et al., *EBV-positive Hodgkin lymphoma is associated with suppression of p21cip1/waf1 and a worse prognosis.* *Mol Cancer.* **9**: p. 32.
750. Shibui, S., et al., *Double labeling with iodo- and bromodeoxyuridine for cell kinetics studies.* *J Histochem Cytochem,* 1989. **37**(7): p. 1007-11.
751. Millar, B.C., et al., *Activation of murine 'T' lymphomas in the presence of a human myeloma cell line, RPMI-8226, in vivo.* *Br J Cancer,* 1988. **57**(3): p. 290-2.
752. Martin, H.J., et al., *Manipulation of the toll-like receptor 7 signaling pathway by Epstein-Barr virus.* *J Virol,* 2007. **81**(18): p. 9748-58.
753. Quan, T.E., et al., *Epstein-Barr virus promotes interferon-alpha production by plasmacytoid dendritic cells.* *Arthritis Rheum.* **62**(6): p. 1693-701.
754. Chiron, D., et al., *Toll-like receptors: lessons to learn from normal and malignant human B cells.* *Blood,* 2008. **112**(6): p. 2205-13.

755. Bernasconi, N.L., N. Onai, and A. Lanzavecchia, *A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells*. *Blood*, 2003. **101**(11): p. 4500-4.
756. Ruprecht, C.R. and A. Lanzavecchia, *Toll-like receptor stimulation as a third signal required for activation of human naive B cells*. *Eur J Immunol*, 2006. **36**(4): p. 810-6.
757. Gires, O., et al., *Latent membrane protein 1 of Epstein-Barr virus mimics a constitutively active receptor molecule*. *EMBO J*, 1997. **16**(20): p. 6131-40.
758. Hartmann, G. and A.M. Krieg, *Mechanism and function of a newly identified CpG DNA motif in human primary B cells*. *J Immunol*, 2000. **164**(2): p. 944-53.
759. Iskra, S., et al., *Toll-like receptor agonists synergistically increase proliferation and activation of B cells by epstein-barr virus*. *J Virol*. **84**(7): p. 3612-23.
760. Traggiai, E., et al., *An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus*. *Nat Med*, 2004. **10**(8): p. 871-5.
761. Takeshita, F., et al., *Transcriptional regulation of the human TLR9 gene*. *J Immunol*, 2004. **173**(4): p. 2552-61.
762. Dombrowski, Y., et al., *Cytosolic DNA triggers inflammasome activation in keratinocytes in psoriatic lesions*. *Sci Transl Med*. **3**(82): p. 82ra38.
763. Costa, S., et al., *Redistribution of the nuclear protein IFI16 into the cytoplasm of ultraviolet B-exposed keratinocytes as a mechanism of autoantigen processing*. *Br J Dermatol*. **164**(2): p. 282-90.
764. Lee, J., et al., *Absent in Melanoma 2 (AIM2) is an important mediator of interferon-dependent and -independent HLA-DRA and HLA-DRB gene expression in colorectal cancers*. *Oncogene*.
765. Wei, W., et al., *Expression of IFI 16 in epithelial cells and lymphoid tissues*. *Histochem Cell Biol*, 2003. **119**(1): p. 45-54.
766. Fernandes-Alnemri, T., et al., *AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA*. *Nature*, 2009. **458**(7237): p. 509-513.

RESUME en français

Le cancer représente la deuxième cause de mortalité dans les pays industrialisés. Il a été démontré que 20% des cancers sont d'origine infectieuse. Nous nous sommes intéressés à deux oncovirus HPV (virus du papillome humain) et EBV (Epstein-Barr Virus) responsable du cancer de l'utérus et de divers lymphome B réciproquement. Les événements clés pour le développement d'un cancer viro-induit sont la persistance du virus *via* la dérégulation des réponses immunitaires et l'induction d'une instabilité génomique *via* une dérégulation du cycle cellulaire. Nous avons donc cherché si EBV était capable d'altérer la réponse immunitaire innée. Nous avons montré que EBV était capable d'inhiber TLR9 un acteur clef de la réponse immunitaire innée. Comme TLR9 est inhibé dans un certain nombre de cancers, nous nous sommes demandé si ce récepteur pouvait également, avoir un rôle dans l'oncogénèse. Nous avons montré que la réexpression de TLR9 induisait un ralentissement transitoire de la prolifération cellulaire. Nous nous sommes par la suite intéressés aux mécanismes de dérégulation du cycle cellulaire induits par E6 une oncoprotéine de HPV16. Nous avons trouvé un nouveau mécanisme d'inhibition de l'inhibiteur du cycle cellulaire, p21. HPV16E6 se lie et inhibe les fonctions de du facteur de transcription p150^{Sal2}, ce qui induit une inhibition de p21 dans un contexte p53 indépendant.

TITRE en anglais

Modulation of the innate immune response by the oncoviruses EBV and HPV

RESUME en anglais

Cancer represents the second most common cause of death in industrialized countries. Epidemiological and biological studies have now conclusively proved that a variety of infectious agents constitute one of the main causes of cancer worldwide. It has been pointed out that more than 20% of cancers are from infectious origin. HPV high-risk mucosal types are associated to 98% of all cervical cancer cases. Regarding EBV, over 90% of the world's population is infected and can give rise to malignancies such as Burkitt lymphoma or Hodgkin disease. (Young and Rickinson 2004) Key features for oncoviruses to induce cancer are firstly to persist by dampening host immune responses and to induce genomic instability in the host by altering the regulation of the cell cycle leading the infected cells to an uncontrolled proliferation. The purpose of this thesis was to find new mechanisms by which EBV and HPV can promote carcinogenesis. We have shown that EBV can alter the regulation and expression of TLRs, the key effectors molecules of the innate immune response. EBV infection of human primary B cells resulted in the inhibition of TLR9 functionality. Our study described a mechanism used by EBV to suppress the host immune response by deregulating the TLR9 transcript through LMP1-mediated NF- κ B activation. As TLR was found deregulated in many cancers, we hypothesized that TLR9 may also a direct role in the process of cell cycle control and that loss of its expression may lead to transformation of the cell. Our overall objective here was to study the role of TLR9 in suppressing the events that initiates transformation of epithelial cells in the setting of cervical cancer (virus-associated) and in head and neck cancer (non-virus-associated). A third project dealt with the mechanism cell cycle deregulation by the oncoprotein E6 which expressed during infection with HPV16. We reported that HPV16E6 targets the cellular factor p150^{Sal2}, which positively regulates p21 transcription. HPV16E6 associates with p150^{Sal2}, inducing its functional inhibition by preventing its binding to cis elements on the p21 promoter. These data described a novel mechanism by which HPV16E6 induces cell cycle deregulation with a p53-independent pathway preventing G1/S arrest and allowing cellular proliferation and efficient viral DNA replication.

DISCIPLINE

Viral oncogenesis

MOTS-CLES

Innate immune response, TLR9, carcinogenesis, EBV, HPV

INTITULE ET ADRESSE DE L'U.F.R. OU DU LABORATOIRE :

Infections and Cancer Biology Group

International Agency for Research on Cancer-World Health Organization

150 Cours Albert Thomas, 69372 Lyon, France