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DEREGULATION OF UV-ACTIVATED INFLAMMASOME BY HPV 38 IN HUMAN KERATINOCYTES

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*"I have no special talent.
I am only passionately curious."
(Cit. Albert Einstein)*

Sommario

I carcinomi spinocellulari (o a cellule squamose) sono le malattie umane più comuni che si verificano negli adulti di carnagione chiara e rappresentano circa il 30% dei tumori totali nel mondo. L'eziologia di questo tipo di cancro è multifattoriale, dal momento che fattori genotipici, fenotipici e/o ambientali possono essere coinvolti nel suo sviluppo.

Diversi studi hanno messo in evidenza che l'esposizione ai raggi ultravioletti (UVB) rappresenta un fattore di rischio chiave nello sviluppo del cancro della pelle. Inoltre, gli agenti infettivi potrebbero costituire un ulteriore fattore di rischio, ipotesi sostenuta dal fatto che i pazienti immunocompromessi, quali ad esempio i soggetti che hanno subito un trapianto di organo (OTR), mostrano un rischio più elevato di sviluppare carcinomi spinocellulari rispetto agli individui immunocompetenti. Molti agenti infettivi sono in grado di colonizzare la pelle e in particolare un sottogruppo di beta papillomavirus umani a tropismo cutaneo (β -HPV) è considerato il fattore eziologico più probabile nello sviluppo del carcinoma a cellule squamose.

In particolare, la presenza di β -HPV è stata riportata nei pazienti affetti da un raro difetto dell'immunità cellulo-mediata, chiamato epidermodisplasia verruciformis (EV), che causa un'elevata suscettibilità allo sviluppo del carcinoma a cellule squamose della pelle (SCC). Questo costituisce la prova iniziale secondo la quale gli agenti infettivi, come β -HPV, rappresenterebbero un potenziale fattore di rischio supplementare nello sviluppo dei tumori spinocellulari.

Diversi studi hanno dimostrato le proprietà trasformanti delle oncoproteine virali, E6 e E7, sia in modelli sperimentali in vitro che in vivo. Entrambe le proteine, alterando le funzioni di proteine cellulari, quali p53 e pRb, sono in grado di deregolare diversi eventi cellulari chiave, come il ciclo cellulare, la riparazione del danno del DNA, l'apoptosi e la senescenza.

Sulla base di questi risultati in vitro, il nostro gruppo ha generato un modello animale di topi transgenici (Tg) che esprimono le proteine virali E6 e E7 di HPV38 nello strato basale della pelle e nella mucosa epiteliale. Questi topi hanno mostrato un'elevata suscettibilità alla radiazione UV; infatti, la loro esposizione ai raggi UVB causa lo sviluppo di lesioni attiniche simili alla cheratosi che sono considerati come precursori del carcinoma a cellule squamose della pelle nell'uomo, e alcune settimane dopo hanno sviluppato il carcinoma conclamato. Al contrario, i topi wild-type non hanno sviluppato alcun tipo di lesioni cutanee quando sono stati esposti alla stessa dose di radiazioni UV. Tuttavia, il meccanismo della cooperazione tra UV e HPV38 non è stato ancora chiarito e l'obiettivo di questo lavoro di tesi è stato quello di esaminare i meccanismi molecolari di tale sinergismo.

A tale scopo, abbiamo valutato se l'espressione degli oncogeni virali negli animali transgenici potesse alterare l'espressione genica indotta dalle radiazioni UV. L'analisi dell'intero trascrittoma ha rilevato che più di 300 geni sono deregolati nei topi transgenici dopo l'esposizione ai raggi UVB, rispetto agli animali wild-type. In particolare, le oncoproteine E6 e E7 sono in grado di deregolare l'espressione di proteine coinvolte nella formazione dei complessi dell'inflammasoma o dei loro effettori a valle, come IL-18. È interessante notare che molti studi condotti su linee cellulari di cheratinociti hanno dimostrato che le radiazioni UV attivano

l'inflammosoma, portando alla secrezione di specifiche citochine pro-infiammatorie, come IL-1 β e IL-18.

I risultati ottenuti tramite RT-qPCR hanno confermato i dati dell'analisi del trascrittoma, mostrando che l'espressione dell'IL-18 è regolata nei topi wild-type dopo l'esposizione a UV, mentre è fortemente inibita nei topi transgenici.

Successivamente, utilizzando i cheratinociti umani primari (HPKs) come modello sperimentale, abbiamo analizzato i meccanismi coinvolti nell'espressione dell'IL-18 indotta dai raggi UV, nonché il ruolo di HPV38 nella deregolazione di questi eventi.

I cheratinociti primari umani in seguito all'irradiazione con raggi UV mostravano un aumento dei livelli di espressione di mRNA dell'IL-18 e di altri componenti chiave dei complessi dell'inflammosoma, quali AIM2 e ASC; al contrario, la presenza delle proteine virali E6 e E7 riduceva fortemente l'espressione dell'IL-18 sia a livello di espressione di mRNA che di secrezione.

L'utilizzo di cheratinociti esprimenti il gene della telomerasi umano (hTERT), al fine di prolungare il ciclo vitale delle cellule, quale modello sperimentale in vitro alternativo, ha messo in evidenza un elevato livello basale di mRNA di IL-18 rispetto ai cheratinociti primari; inoltre, la presenza di E6 e E7 svolge un ruolo inibitorio su tale espressione.

L'espressione transiente di un vettore contenente il promotore isolato dell'IL-18 clonato davanti al gene reporter della luciferasi, in cheratinociti esprimenti il gene hTERT, ha confermato la capacità di E6 e E7 di HPV38 di reprimere l'attività del promotore dell'Interleuchina 18.

Inoltre, l'analisi del promotore dell' IL-18, mediante l'utilizzo del programma bioinformatico TFBIND, ha rivelato la presenza di 15 putativi siti di legame per p53, classicamente attivato dalle radiazioni UV. Eseguendo test di immunoprecipitazione della cromatina (CHIP), abbiamo dimostrato che p53 viene reclutato in due distinte regioni del promotore dell'IL-18, che comprendono quattro elementi responsivi per p53. Le proteine E6 e E7 di HPV38 impediscono in modo efficace il reclutamento di p53 su queste regioni del promotore dell'IL-18.

Questi risultati corroborano la nostra ipotesi circa la possibilità che β -HPV38 svolga un ruolo importante nell'inibizione della risposta dell'inflammosoma indotta dai raggi UV; in particolare, il virus potrebbe alterare l'espressione genica e la produzione di proteine coinvolte nella formazione del complesso dell'inflammosoma così come di citochine specifiche (quali ad esempio IL-18). Altresì, la riduzione della regolazione dell'IL-18 potrebbe compromettere la capacità dei cheratinociti di reclutare le cellule del sistema immunitario nell'area esposta ai raggi UV, causando un difetto nell'eliminazione delle cellule che hanno subito un danno al DNA.

In conclusione, tutte queste alterazioni potrebbero determinare l'accumulo di danni al DNA indotti dall'esposizione ai raggi UV e allo sviluppo del cancro a cellule squamose della pelle.

Abstract

Non-melanoma skin cancers (NMSC) are the most common human malignancies occurring in fair-skinned adult population, representing approximately 30% of total cancer. The etiology of this cancer is multifactorial, since genotypic, phenotypic and/or environmental factors can be involved in its development.

Several studies have reported that the exposure to ultraviolet irradiation (UVB) represents a key risk factor in the development of skin cancer. Furthermore, infectious agents could be an additional risk factor and this hypothesis is supported by the evidence that immunocompromised people, e.g. organ transplant recipients (OTRs), show a higher risk of developing NMSC compared with immunocompetent individuals. Several infectious agents are able to colonize the skin and especially a subgroup of beta cutaneous human papillomavirus (HPV) is considered the most likely additional etiological factors of NMSC.

In particular, the presence of β -HPV has been reported in patients with a rare cell-mediated immunity disorder, called epidermodysplasia verruciformis (EV), which causes high rates of susceptibility to develop skin squamous cell carcinoma (SCC) on sun-exposed areas. Thus, it provided the initial clues about infectious agents, such as β HPV, as potential additional risk factor for NMSC.

Several studies have demonstrated the transforming properties of the viral oncoproteins, E6 and E7, in in vitro and in vivo experimental models. Both proteins, altering the functions of tumour suppressor gene products, e.g. p53 and retinoblastoma (pRb), are able to deregulate key cellular events, such as cell cycle, DNA repair, apoptosis and senescence.

Based on these in vitro results, our group has generated a transgenic (Tg) mouse model that express E6 and E7 oncogenes from beta HPV38 in the basal level of the skin and mucosal epithelia. Those mice were highly susceptible to UV-radiation; in fact, the exposure of HPV38 E6/E7 Tg mice to UVB caused the development of actinic keratosis-like lesions that are considered as precursors of SCC in humans and a few week later SCC. Conversely, the wild-type mice did not develop any type of skin lesions when exposed to the same dose of UV radiations. However, the mechanism of the UV/HPV38 cooperation has not been elucidated yet. The aim of this work thesis was to dissect the molecular mechanisms of such cooperation.

We have first determined whether the expression of the viral oncogenes in the Tg animals alters the pattern of gene expression induced by UV radiation. The analysis of the entire transcriptome pointed out that more than 300 genes are deregulated in the Tg mice after UVB exposure, compared to the wild type animals. In particular, the HPV38 E6 and E7 oncoproteins were able to down regulate the expression of proteins involved in the inflammasome complexes or the downstream effectors, such as IL-18. Interestingly, many studies in keratinocyte cell lines provided evidence that UV radiation activate the inflammasome pathway, leading the secretion of specific pro-inflammatory cytokines, like IL-1 β and IL-18.

RT-qPCR experiments confirmed the transcriptome findings, showing that IL-18 expression is up-regulated in wild-type mice after UV exposure, while it is strongly inhibited in HPV38 E6/E7 Tg mice.

Using primary human keratinocytes (HPKs) as experimental models, we dissected the mechanisms involved in the UV-induced IL-18 expression as well as the role of HPV38 in the deregulation of these events. RT-qPCR analysis showed an UV-

induced upregulation of IL-18 mRNA expression as well as some key inflammasome components, like AIM2 and ASC, in HPKs cells; conversely, the presence of E6 and E7 oncoproteins strongly down regulated IL-18 both at the expression and secretion levels. Using another in vitro experimental model, such as keratinocytes expressing the human telomerase reverse transcriptase gene (hTERT), in order to prolong the life span of the cells, we found that this cell line presents a higher basal level of IL-18 compared to HPKs and the presence of E6 and E7 plays an inhibitory role.

Transient transfection experiments in hTERT HPKs using a vector containing the isolated IL-18 promoter cloned in front the luciferase reporter gene confirmed the ability of HPV38 E6 and E7 to repress the IL-18 promoter activity.

The analysis of IL-18 promoter by TFBIND bioinformatics tool revealed the presence of 15 putative binding sites for p53, which is well known to be activated by UV radiation. Performing chromatin immunoprecipitation assays (CHIP), we showed that p53 is recruited to two distinct regions of the IL-18 promoters that includes four p53 responsive elements. Importantly, HPV38 E6 and E7 efficiently prevent p53 recruitment to these IL-18 promoter regions.

Together these results corroborated our hypothesis that beta HPV38 play an important role in the inhibition UV-induce inflammasome response, by altering the gene expression and the production of proteins involved in the inflammasome pathways as well as of specific cytokines (e.g. IL-18). Most likely, the down regulation of IL-18 may impair the ability of the keratinocytes to recruit the immune cell population at the UV-exposed area, causing a defect in the elimination of cells harboring DNA damage. In conclusion, all these alterations may lead to the accumulation of UV-induced DNA damage and development of non-melanoma skin cancer.

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List of abbreviations

ADC adenocarcinoma

AIM2 absent in melanoma 2

ALRs absent in melanoma 2-like receptors

APAF-1 apoptotic-protease activating factor 1

ASC apoptosis-associated speck-like protein

BCC basal-cell carcinomas

CARDs Caspase activation and recruitment domains

CSS cervical squamous cell carcinoma

DAMP Danger-associated molecular pattern

DNA Deoxyribonucleic acid

DNMT1 DNA methyltransferase 1

ds Double-stranded

E Early

EBNA EBV nuclear antigen 1

EBV Epstein Barr Virus

ECM extracellular matrix

EV Epidermodysplasia verruciformis

EZH2 Enhancer of zeste homolog 2

FADD Fas-associated death domain

FKLC familial keratosis lichenoides chronica

HBsAg hepatitis B surface antigen

HBV Hepatitis B virus

HCV Hepatitis C virus

HFK/HPKs Human primary foreskin keratinocyte

HHV-8 Human herpes virus type 8

HIV Human immunodeficiency virus

HNSCC head-and-neck squamous cell carcinoma

HPV Human papillomaviruses

HR High-risk
HSV Human herpes simplex
HTERT Human telomerase reverse transcriptase
HTLV-1 Human T lymphotropic virus 1
IFN Interferon
IKK β I κ B kinase beta
IL Interleukin
IL-18BP IL-18 binding protein
INF- γ interferon gamma
IPAF IL-1 β -converting enzyme (ICE)-protease activating factor
IRAK Interleukin-1 receptor-associated kinase
IRF Interferon regulatory factor
KSHV Kaposi sarcoma-associated herpes virus
L Late
LANA latency-associated nuclear antigen
LCR Long control region
LGP2 laboratory of genetics and physiology-2
LR Low-risk
LRR Leucine-rich repeats
MAPK mitogen-activated protein kinase
MCR1 melanocortin-1 receptor
MDA5 melanoma differentiation-associated gene 5
MMP metalloproteinase
MSPC multiple self-healing palmoplantar carcinoma
MyD88 Myeloid differentiation primary response gene 88
NAIPs neuronal apoptosis inhibitor factors
NEK7 NIMA-related kinase7
NF- κ B Nuclear factor kappa-light-chain-enhancer of activated B cells
NK Natural killer

NLRs nucleotide oligomerization domain (NOD)-like receptors
NMSC Non-melanoma skin cancer
ORF Open Reading Frame
OTRs organ transplant recipients
PAMP Pathogen-associated molecular patterns
PBMCs peripheral blood mononuclear cells
pRb Tumour suppressor protein retinoblastoma
PRR Pattern recognition receptor
PYD Pyrin domain
RE Responsive element
RLRs retinoic acid-inducible gene I (RIG-I)-like receptors
RNA Ribonucleic acid
ROS Reactive oxygen species
RSV Rous sarcoma virus
SCC squamous cell carcinomas
SSC Skin squamous cell carcinoma
STING stimulator of IFN genes
TIR Toll/interleukin-1 receptor
TLR Toll-like receptor
TNF R1tumor necrosis factor receptor 1
TRAF6 Tumor necrosis factor receptor-associated factor 6
TRIF TIR-domain-containing adapter-inducing IFN- β
UVB Ultraviolet light

INTRODUCTION

1. GENERAL INTRODUCTION

Non-melanoma skin cancers (NMSCs) are the most common malignancies occurring in fair-skinned adult population, representing approximately 30% of total cancer [1], and their increased incidence has generated a great interest in the last few years, into understanding their pathogenesis in order to find new non-invasive treatments [2]–[4]. NMSC is also known as keratinocytes carcinoma and is mainly used to describe basal-cell (BCC) and squamous cell carcinomas (SCC), although their aetiopathogenesis and clinical course are different [5]. BCC is considered a hair follicle-derived tumor and is caused by few genetic alterations [6], while the development of SCC is caused by multiple factors and little is known about the causal alteration [7].

The etiology of this cancer is multifactorial, in fact immune suppression, genetic and environmental factors are strong risk factors for NMSC development. In particular, several studies have reported that the exposure to ultraviolet irradiation (UVB) represents a key risk factor in the increase in incidence of skin cancer [8]. Furthermore, infectious agents could be an additional risk factor for skin cancer. This hypothesis is supported by evidence that immunocompromised people, e.g. organ transplant recipients (OTRs), show a higher risk of developing NMSC compared with immunocompetent individuals [9].

The present work thesis will provide important insights into the molecular mechanism of the cooperation between two environmental, such as UV irradiation and HPV infection, in promoting skin carcinogenesis by the modulation of inflammasome response. To better understand the relationship between virus/NMSC/immune system responses, the

introduction is divided in 4 major parts that will provide a brief summary of current knowledge about these topics:

- Pathogenesis of NMSC
- Role of viruses in carcinogenesis, focusing on HPV infection and in particular on the β -type
- Innate immune response to viral infection, with specific reference to inflammasome pathway activation and IL-18 production
- Role of the immune system in cancer

1.1 Pathogenesis of non-melanoma skin cancer

Epidemiological data have reported that several factors (Table 1) and different pathways (Figure 1) could be involved in the pathogenesis of non-melanoma skin cancers.

Many studies show that NMSC occurs mainly on area of the body that are exposed to the sun and that the incidence in the development of skin cancer is associated with ambient solar irradiance. All this evidence provides further clues on the relation between this type of cancer and sun exposure [10]. Exposure to UVB radiation causes direct damage of DNA and RNA because it induces a covalent bond between adjacent pyrimidines with the formation [11]–[13] of cyclopyrimidine dimers (TT) or pyrimidine-pyrimidine (6-4) adducts, that are mutagenic. Furthermore, UVA is less mutagenic compared to UVB because the DNA damage is indirect and is caused by the generation of reactive oxygen species (ROS). To prevent the harmful effects of these premutagenic adducts, the cells need an efficient DNA repair system.

As mentioned above, another key risk factor for skin cancers is the immunosuppression. Literature data highlights that organ transplant

recipients, HIV-positive patients and with non-Hodgkin lymphoma patients have an elevated risk of developing NMSC in comparison to healthy individuals [14], [15]. The importance of the status of the immune system in skin carcinogenesis supports the possibility that an infectious agent is involved in this pathological condition. Many biological and epidemiological studies provide evidence that cutaneous β -HPV types can synergize with UV in promoting skin carcinogenesis. β -HPV types were first identified in patients with a rare cell-mediated immunity disorder, called epidermodysplasia verruciformis (EV). EV patients are highly susceptible to beta HPV infection and NMSC development in anatomical sites exposed to UV light. This finding provided the initial clues about infectious agents, such as β -HPV, as a potential additional risk factor for NMSC [16]. Furthermore, analysis of non-melanoma skin cancers in immunocompromised and immunocompetent individuals showed that both contain DNA from cutaneous human papillomavirus, although with different percentages [17]. How these viruses cooperate with UV irradiation in the development of skin cancer is not completely understood. One plausible hypothesis is that β -HPV, hampering the elimination of UV-damaged cells [18].

Other risk factors for NMSC include TYR and ASIP gene variants and of the melanocortin-1 receptor (MCR1) as well as the inactivation of the TP53 tumor suppressor gene by UV irradiation or the inactivation of CDKN2A locus.

	Type of non-melanoma skin cancer
Solar UV radiation ^{16,17,20-22}	BCC, SCC
Human papillomavirus ²⁸⁻³³	SCC, BCC
Iatrogenic immunosuppression ³⁴⁻³⁷	SCC, BCC
HIV/AIDS and non-Hodgkin lymphoma ³⁸⁻³⁹	BCC, SCC
PUVA therapy ^{40,41}	SCC, BCC
Photosensitising drugs (eg, fluoroquinolone antibiotics) ⁴²	SCC, BCC
UVB radiation ⁴³	BCC
Ionising radiation ⁴⁴	BCC
Occupational factors ^{45,46}	BCC, SCC
Arsenic ⁴⁷	SCC, BCC
Tobacco smoking ⁴⁸	SCC

UV=ultraviolet. BCC=basal-cell carcinoma. SCC=squamous-cell carcinoma.
PUVA=psoralen and UVA.

Table 1: Environmental risk factors for non-melanoma skin cancer. Image from [5].

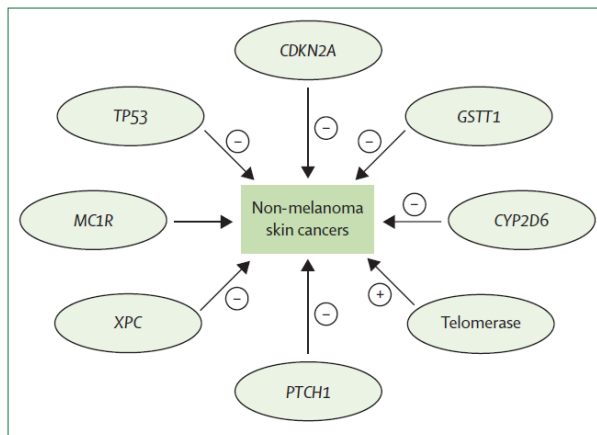


Figure 1: Major pathways involved in the pathogenesis of non-melanoma skin cancer. Image from [5].

1.2 Human cancers and viruses

In 1911, Peyton Rous described that a filterable agent in cell extracts of a chicken tumor could transmit the tumor into healthy chickens [19]. This discovery paved the way into the study of tumor virology.

Several decades later, two tumor viruses were discovered in mammals, suggesting that viruses may be risk factors in the development of some human cancers [20]. To date, approximately half of all human cancers are caused by infectious agents [21].

In 2009, the International Agency for Research on Cancer (IARC) Monographs program reviewed all the known infectious agents that have been classified as carcinogenic to human [22]. These viruses include HBV, HCV, HPV, EBV, KSHV (also known as human herpes virus type 8, HHV-8) and HTLV-1 (table 2).

Viruses can be classified as direct or indirect carcinogens. The former can express oncogenes that play a direct role in cellular transformation by the alteration of key cellular pathways. In contrast, viruses classified as indirect carcinogens cause chronic inflammation in the infected area, leading to the dysregulation of immune system responses as well as to the promotion of angiogenesis, that is important for tumor development and survival. KSHV, HTLV-1, EBV and several HPV types belong to the first group, while HBV and HCV to the second.

A vast number of studies have clearly demonstrated that viral oncoproteins display transforming activities in in vitro and in vivo experimental models. In EBV, LMP-1 induces the invasiveness and the production of metastasis factors, nuclear antigen 1 (EBNA-1) is able to bind DNA of the host regulating the transcription of cellular genes [23], and EBNA-2 plays a critical role in lymphocyte immortalization [24].

KSHV express a protein called latency-associated nuclear antigen (LANA) which is able to dysregulate several pathways linked to cellular proliferation [25].

HBV encodes HBx protein and hepatitis B surface antigen (HBsAg) that are important in transformation. Furthermore, integration of HBV genome causes disruption of key cellular gene that are involved in the regulation of proliferation [26].

<u>Virus</u>	<u>Oncoprotein</u>	<u>Cellular target/pathway</u>	<u>Cancer</u>
EBV	LAMP-1	NF-κB TRAFs	Burkitt's lymphoma, nasopharyngeal carcinoma
HTLV-1	TAX, p12, p8, p30, p13	CREB/ATF, NF-κB, AP-1 and SRF.	T-cell leukemia
HPV16 and 18	E6 E7	p53 pRb	Cervical cancer, anal carcinoma, penile carcinoma
KSHV, HHV8	LANA, vBCL2, vFLIP, vIL-6	Cell proliferation	Kaposi's sarcoma, primary effusion lymphoma, multicentric Castleman's disease
HBV	HBx	p53	Hepatocellular carcinoma
MCPyV	T antigen	pRb	Merkel cell carcinoma

Table 2: Human cancers viruses and their relative oncoproteins and cellular targets. Image modified from [21].

2. HUMAN PAPILLOMAVIRUSES

2.1 Classification

The papillomaviruses are small DNA viruses that cause warts (or papillomas) in several higher vertebrates, including humans. The first papillomavirus was studied by Richard Shope in 1933, who described CRPV (cottontail rabbit papillomavirus) as the etiologic agent responsible for the cutaneous papillomatosis in cottontail rabbit.

Historically, the papillomaviruses were grouped together with the polyomavirus in the papovavirus family because they shared similar properties such as small size, an icosahedral capsid and double-stranded circular DNA. However, further studies have highlighted differences in the genomic organization between papillomaviruses and polyomaviruses, which led to their reclassification as different virus family.

PaVe: Papillomavirus Episteme report more than 300 papillomavirus identified and completely sequenced, of which 200 are human.

Papillomaviruses classification is based on the DNA sequence of the L1 gene. To be considered as a new PV type the L1 nucleotide sequence must differ by >10% of the closest known type. Differences between 2% and 10% define a subtype and less than 2% a variant.

According with the phylogenetic tree (Figure 2), human papillomaviruses (HPVs) are divided in five genera identified with a Greek letter followed by a number that indicates the species. To date, the majority of HPV types are comprised in alpha, beta and gamma genera and are further subdivided into cutaneous or mucosal according to their tissue tropism. Genus alpha includes the mucosal HPV types that are sub-divided in high-risk (HR) and low-risk (LR) according to their ability to induce benign or malignant lesions. HR HPVs type are normally associated with development of cancer lesions, while the LR HPVs are linked with benign warts.

The genus beta includes several types of HPV that can be found on skin, which is why they are considered cutaneous type. The cutaneous types were isolated from patients with Epidermodysplasia verruciformis (EV), a rare autosomal recessive genetic disorder, and are also detected in non-melanoma skin cancers of EV patients [27]. Epidermodysplasia verruciformis causes a decrease in the cell-mediated immune response making patients more susceptible to HPV infection. This provides evidence about the role of HPV infection in the pathogenesis of skin cancer associated with EV [28]. Moreover, recent studies have suggested the involvement of cutaneous β -HPV type in the development of skin cancer in non-EV patients. For instance, β -type are frequently detected in immunocompromised patients, such as organ transplant recipients (OTRs) or HIV infected people, as well as in NMSC of both immunocompromised and immunocompetent individuals [27], [29].

The characteristics and the pathogenesis of β -type are described more in detail below.

Genus gamma includes more HPV types than the other genera [30]. Interestingly, three gamma HPV types, 101, 103, and 108, isolated from cervico-vaginal cells of healthy women or women with cervical lesions revealed an unusual genome organization [31], [32]. All three genomes lack the E6 open reading frame (ORF).

Currently, little is known about the biological properties of the gamma HPV types that can be found in high abundance on the skin of normal individuals [33].

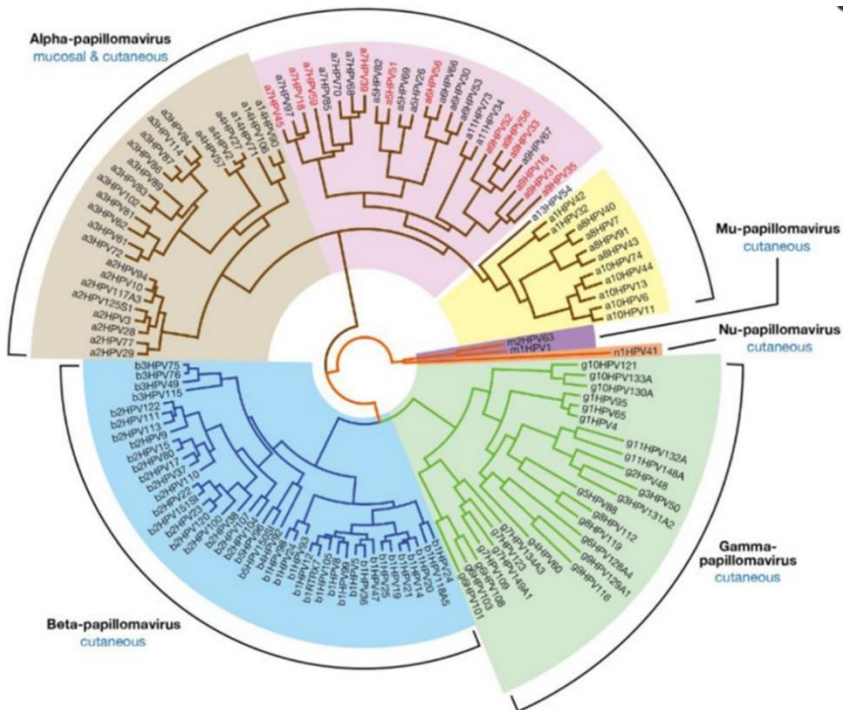


Figure 2: HPV phylogenetic tree. Alpha-, Beta- (blue) and Gamma-papillomavirus represent the largest groups. Alpha papillomavirus genus includes low-risk cutaneous (light brown), low-risk mucosal (yellow) and high-risk (pink). The high risk highlighted in red are confirmed as “human carcinogens” based on epidemiological data. The evolutionary tree is based on the alignment of the E1, E2, L1 and L2 genes. Image modified from [33].

2.2 Structure of the virion and genome organization

All HPVs are non-enveloped DNA viruses with an icosahedral structure, 50-60 nm diameter. The capsid contains 360 molecules of L1 protein arranged into 72 capsomeres. Each capsomer is composed of 5 L1 protein [32] that interact at the C-terminal [34]. The virion contains several L2 protein that are not fully exposed on the surface except for the N-terminal. During infection, L2 becomes exposed in order to bind the extracellular matrix [35]. Each virion contains a single copy of the genome, comprised of a double-stranded, circular DNA of around 7,5-8 kb in length. One of the two strands contain the Open Reading Frames (ORFs) which encode for the viral proteins. The genome can be divided into three major regions: an early, a late and a long non coding region (LCR or non-coding region, NCR), all of which are separated by two polyadenylated (pA) sites, that are called early pA (A_E) and late pA (A_L) sites (Figure 3). The LCR region, that represent around 10% of the genome, contains the origin of replication and the regulatory elements of the transcription [36].

Despite of the small size of the HPV genome, the number of encoded proteins is higher. This is due to the use of multiple promoters and complex patterns of splicing [37].

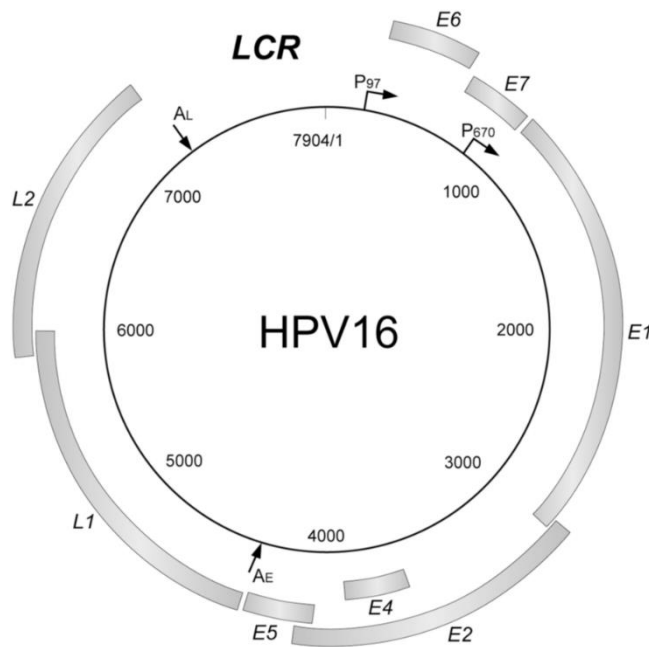


Figure 3: The genome organization of HPV16. The viral genes are transcribed in a single direction (clockwise). There are genes coding for non-structural proteins (E1, E2, E4, E5, E6, and E7) and structural proteins (L1, L2), and a transcriptional control region (long control region; LCR). LCR contains a DNA replication origin and functions as the regulator for the DNA replication. The major promoters and polyadenylation signals are indicated (P97, P670, AE, AL). Image from [38].

The early region occupies about 50% of the entire genome and contains six common ORFs that encode viral proteins involved in DNA replication, control of viral transcription and in pathogenesis:

- **E1** is highly conserved protein comprised of around 600 aa. It is a virus-specific DNA helicase that is involved in viral genome replication and amplification.
- **E2** is involved in viral transcription, replication and genome partitioning. It is conserved among the HPVs types in its N-term and C-terminal domains.
- **E4** is a small cytoplasmic protein that is involved in the remodeling of the cyokeratin, in cell cycle arrest and in virion assembly [39].
- **E5** controls cell growth and differentiation, and it is also involved in the modulation of the immune system. Interestingly, Beta HPVs type lack of E5 ORFs.
- **E6** and **E7** are the most studied and characterized proteins because of their role in oncogenesis. Their functions are described in more detail below.

The late region encodes for the protein that form the capsid of the virion particles, L1 (major capsid protein) and L2 (minor capsid protein) [40], [41].

2.3 Life cycle

HPV life cycle is tightly linked to the differentiation program of the host cells and the target of these viruses is the stratified epithelium (Figure 4). The life cycle can be divided in two stages:

- non-productive that occurs in the proliferating basal layers of the epithelium where the virus is present as a low copy-number episome

- productive that occurs in the terminally differentiated cells.

Human papillomavirus virions invade the epithelium through damaged areas in order to infect the basal cells. The entry of the virions has not yet been fully characterized. The virion attaches to heparan sulfate proteoglycan (HSPG) on the basal membrane through the major capsid protein, L1, and then transfers to the receptor expressed on keratinocytes moving on the basal membrane in the wound-healing process [35]. After the entry, the genome of the virus is transported into the nucleus where it is maintained in low-copy number (50-100 copies per cell). This step is essential for the establishment of the early phase of HPV life cycle (Figure 4). After this, the infected cells start the differentiation program. HPV does not encode for any DNA polymerase enzyme so the virus needs the DNA replication machinery of the host. Viral gene expression is strictly related to the differentiation stage of the infected cells. Expression of the viral gene required for viral genome expression occurs in the spinous layer of the stratified epithelium and can be found at high-copy per cell. The capsid proteins are synthesized and assembled to form the virions in the differentiated cells. Then the new progeny is released with peeled keratinocytes [38].

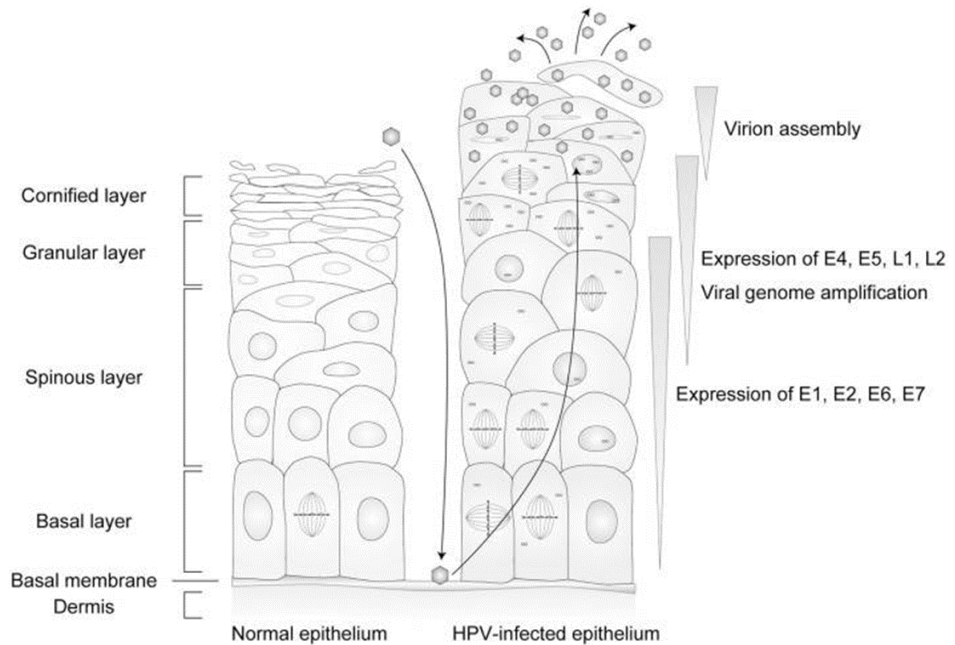


Figure 4: The lifecycle of human papillomaviruses (HPVs). HPVs specifically infect the cells in the basal layer of the stratified epithelium through lesions. Viral genomes are maintained as episomal DNA in the nuclei of infected cells. The viral lifecycle is strictly controlled by host cell differentiation, and the late lifecycle (productive lifecycle) occurs in upper layers of the epithelia that are terminally differentiated. The progenitor virions are released from the cornified keratinocytes. Image from [38].

2.4 Oncogenic properties of E6 and E7

The oncoproteins E6 and E7, encoded by human papillomaviruses, are directly responsible for the development of HPV-related cancer. They interact together by targeting different cellular pathways involved in regulation of cell cycle and apoptosis, in order to alter the host cell response. Unless otherwise stated, the description of the two viral oncoproteins refers to High Risk (HR) HPV, such as HPV16. Due to their importance in cellular immortalization and transformation, E6 and E7 are considered the bests target to develop new therapeutic and antiviral strategies.

2.4.1 E6

E6 is a small nuclear protein of about 16-19 kDa with two zinc finger domains formed by two pairs of CXXC motifs, that are conserved among the human papillomaviruses and their integrity is essential for the protein functions (Figure 5) [42], [43]. The most characterized target of E6 is the tumor suppressor protein, p53.

The main function of p53 is to arrange the response to cellular stresses leading to different outcomes, such as cell-cycle arrest, DNA repair or apoptosis. Under stress condition, p53 is rapidly stabilized by post-translational modifications and regulates the expression of different cellular genes in order to preserve genome integrity. During HPV infection p53 is inactivated and degraded via the proteasome pathway [44], [45].

E6 from HR HPV types interacts with the ubiquitin ligase E6AP inducing a conformational change that generates a cleft for p53 binding. The formation of this trimeric complex, E6/E6AP/p53, leads to the rapid degradation of p53 [46].

E6 from HR types inhibit the expression of p53-regulated genes through the interaction with CBP and p300, two histone acetyltransferases [47], [48].

E6 targets many other cellular pro-apoptotic proteins and one of the most studied is *Bak*. This protein could play a role in terminal differentiation because it is highly expressed in the upper part of the epithelium. Interestingly it has been described that after UV exposure, Bak is activated and stabilized independently of p53, in fact E6 binds Bak in order to degrade it via E6AP-dependent proteasome [44]–[47].

The viral oncoprotein interacts with other components of the apoptotic pathway. It acts indirectly by upregulating the activity of *survivin* promoter in order to block apoptosis [53]. HPV E6 also interacts with the tumor necrosis factor receptor 1 (TNF R1), the adaptor molecule Fas-associated death domain (FADD) and pro-caspase 8. The interaction between E6 and FADD or pro-caspase 8 mediates the degradation of both proteins [49]–[51].

E6 is also able to deregulate the cellular DNA replication machinery. It prolongs the life-span of the primary epithelial cells by increasing the telomerase activity, through the activation of the transcription of the human telomerase reverse transcriptase (hTERT) gene [57], [58]. This activation is in part due to E6/E6AP complex that causes the binding and the degradation of NFX1, a negative transcriptional regulator.

Moreover, other studies have shown that the hTERT activation requires the binding of E6 to Myc, but not to E6AP, which in turn is able to activate hTERT promoter [54]–[56].

However, to date the exact mechanism of hTERT gene activation is not fully clear although this event appears to be crucial for HPV-induced immortalization and transformation.

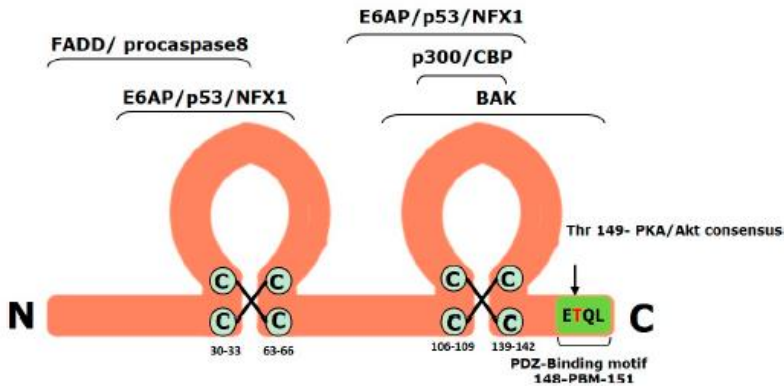


Figure 5: Schematic diagram of the high-risk HPV E6. The two zinc fingers are shown, together with regions that are involved in interacting with some of its cellular target proteins. The C-terminal PBM is shown and the PKA phosphorylation site is also indicated. Image from [62].

2.4.2 E7

E7 is protein of 10-14 kDa with a C-terminal zinc-binding domain and structural integrity is important for E7 activity [63], [64]. The viral oncoprotein protein is divided in three conserved domains, identified as CD1, CD2 and CD3 (Figure 6).

The ability of E7 to induce S-phase progression and cellular transformation is dependent on the CD1 domain [64], [65].

The CD2 domain contains a LXCXE motif that mediates the interaction with the retinoblastoma tumor suppressor protein (pRb). In many E7 proteins, this domain also contains a phosphorylation site of casein kinase 2 (CK2). Lastly is the CD3 domain, which contains four highly conserved cysteine residues and is involved in interaction with different cellular proteins, such as p21 and p27 CDK inhibitors. E7 abrogates the activity of those proteins in order to permit the cell cycle progression and overcoming the cell cycle

arrest due to DNA-damage [67], [68]. It can also interact with several transcription factors like AP-1(activator protein 1), TBP (TATA box binding protein) or c-Jun [69]. One of the most studied properties of E7 is its ability to target pRb, p107 and p130. pRb plays a key role in cell-cycle control; in particular, it negatively regulates the G1/S transition. pRb, via direct binding, inhibits the activity of the transcription factors E2F1–3, which are involved in the expression of many genes encoding positive cell-cycle regulators, such as cyclin E and A. In normal cells, cell-cycle progression is dependent on the formation and activation of the cyclin D1-3/cyclin-dependent kinase (CDK) 4 and 6 complexes, which in turn phosphorylate pRb. pRb hyperphosphorylation leads to the release of E2F1–3, which are active in promoting cell-cycle progression. HR HPV E7 proteins associate with hypophosphorylated pRb, mimicking the CDK-mediated phosphorylation and resulting in uncontrolled and constitutive activation of E2Fs. Similar to E6 with p53, HPV16 E7 protein is able to promote pRb degradation through the ubiquitin-proteasome pathway. Thus, HR HPV E7-expressing cells undergo unscheduled proliferation, which is independent of external stimuli.

E7 also binds other cellular proteins, such as p107 and p130, whose play a role in regulation of cellular proliferation, differentiation and apoptosis [70]. In particular, p107 is expressed in proliferating cells and inhibits E2F4, while p130 is present in non-proliferative cells and inhibits E2F5 activity [71], [72].

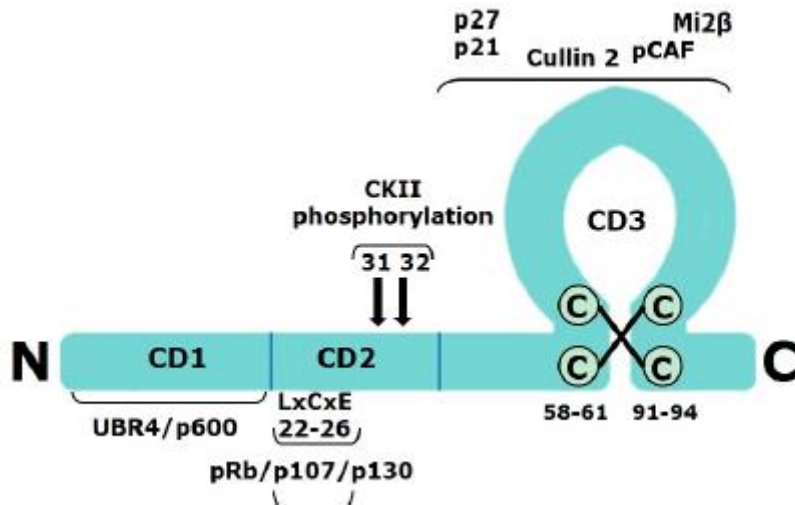


Figure 6: Schematic diagram of the high-risk HPV E7. Three regions of E7 that are homologous to adenovirus E1A conserved regions 1-3 (CD1-3) are shown. The zinc finger is also shown, together with the regions involved in pRb binding (LXCXE) and the two serine residues (31 and 32) that are susceptible to casein kinase II (CKII) phosphorylation. Image from [62].

2.5 Mucosal human papillomavirus infections

The association between HPV and cervical cancer is well known. Epidemiological studies reported that the detection of HPV increases with the disease severity with percentage of 50-70 % in CIN1/LSIL, 85% in CIN2 and around 90-100% in CIN3 lesions [73]–[75]. These viruses are sexually transmitted and their detection is higher in young women and men [76]. Twelve HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) are associated with the development of cancer in humans in different genital tracts, such as anal, vulvar, penile, vaginal and the well-known cervical tract. Among the HR HPV types, HPV16 and 18 are the most oncogenic viruses responsible for approximately 70% of all cervical squamous cell carcinomas (CSS) and adenocarcinomas (ADC). The immune system is able to clear the infection of HR HPV types in 12-14 months without clinical manifestation but in some cases the infection may persist and after a period of latency, can evolve into premalignant lesions which can lead to the development of invasive cervical carcinoma.

Recent studies have also demonstrated the association of HR HPV in a subset of head-and-neck cancers (HNSCC). Most of the HNSCCs positive for HPV are in the oropharynx, but a small percentage, 1-2, of oral and laryngeal cancers have been attributed to HPV infection. The percentage of HR HPV-positive oropharyngeal cancers varies according to geographical regions, ranging from approximately 10% in East European countries to 50% in north European countries and North America. In the oropharyngeal cavity there are the tonsillar crypts that are formed by a specialized squamous epithelium, which is infiltrated with lymphoid tissue. The susceptibility of this body site to HPV infection is probably due to the presence of “basaloid” cells, which permit virus entry, and could also be due to the special conformation of the crypts that are considered “immune-privileged zones” facilitating the virus immune evasion [77]. Epidemiological

analysis show that in these areas the HPV-related cancers are principally HPV-16 positive, with a low percentage of other HR-HPV types, such as HPV-18, HPV-31 and HPV-32 [78]–[81].

As regards the LR HPV types, they are responsible for genital warts or condylomata acuminata and the infections are cleared by the immune system within 2 years. Recurrence of the infections, however, can be observed in a small percentage of cases. HPV-6 and 11 are also associated with the rare laryngeal papillomatosis in children as well as in adults and the infection is acquired during the passage through an HPV-infected birth canal. Although , these tumors do not usually progress to malignancy [82]

2.6 Cutaneous human papillomavirus infections

The HPV types associated with cutaneous diseases belong to different genera. The most common are the alpha types HPV-2, 3, 10, 27 and 57, the gamma type HPV-4, 60 and 65, and the mu types HPV-1 and 63. These are normally present in common warts that can occur at many sites, such as the feet and hands. In most cases, these are benign lesions with a spontaneous immune regression of 80%, but they are highly productive with a number of particles around 10^{12} . The common warts generally show hypertrophy that leads to acanthosis or thickening of the epithelium. The transmission of the virions released from the infected epithelium can occur indirectly or directly from person to person [82]–[84].

Molecular analyses have also shown the presence of certain human papillomaviruses belonging to the β -genus, such as HPV-8, HPV-20 and HPV-38, in cutaneous epithelia.

2.7 β -HPV infections of cutaneous epithelia

Approximately 50 different β -HPV types have been characterized so far, but it is likely that many more exist. The first characterized β -HPV types, HPV-5 and 8, were isolated in the skin of individuals suffering from epidermodysplasia verruciformis (EV), as described above. Since the discovery of HPV-5 and 8, many more beta HPV types have been isolated, as a result of the development of highly sensitive polymerase chain reaction (PCR)-based methods [30]. They are subdivided into five different species: beta-1, -2, -3, -4, and -5 and can be detected on the skin, oral cavity and also in genitals area of healthy people, in fact the environment of β -types infection is significantly different from the one where the HR α -types infect the host cells. Commonly, they can be found in proximity to the hair follicles, nails, between the digits and also at the eccrine and apocrine sweat apparatus; the characteristic of these different district is the presence of different tissue close to each other. For example, the sweat glands present two different stem cells population both of which susceptible to the virus infection. It seems that the virus is able to reach the cells of these areas via hair follicles, via micro-injuries or via the eccrine ducts, depending on the zone [33].

However, biological and epidemiological studies support the role of β -HPV type in the development of non-melanoma skin cancer (NMSC), together with ultraviolet (UV) light [86]. For instance, it has been reported that people with a history of skin SCC show a higher positivity for viral DNA in the skin and/or antibodies against the L1 major capsid protein [87]–[92]. Moreover, the DNA load of the virus is higher in actinic keratosis lesions, which are considered the premalignant lesions of SCC, than in SCC [93], [94] Thus, as reported at the beginning, these findings support the hypothesis that β -HPV types play a role at early stages of carcinogenesis, facilitating the

accumulation of DNA damage induced by UV exposure, which is one of the most important risk factors for NMSC.

2.7.1 The β E6 and E7 proteins

The β -HPV E6 and E7 oncoproteins are able to interact with similar cellular proteins and relative pathways, as HR α -HPV oncoproteins, but functional studies have shown that there are some important differences between the two types.

One of the most relevant difference is the way in which they target the *tumor suppressor protein*, p53. As previously mentioned, α -HPV E6 targets p53 for proteasome-mediated degradation while the majority of β -HPV E6 proteins are not able to do this. Literature data shows that some β -types, such as HPV-49, target p53 for proteasome degradation via E6AP [95], while others, such as HPV-38 and HPV-92, bind p53 stabilizing the cellular protein [96]. The E6 proteins of β -types are also able to interfere with the expression of p53-related genes. Muench and colleagues reported that the E6 proteins of HPV-5, HPV-8 and HPV-38 interact with p300. In particular, HPV-8 E6 induces p300 degradation leading to the reduction of ATR protein levels, which is an important protein of the UV-induced damage signaling pathway [97]. The interaction between E6 and p300 blocks also p53 acetylation, causing the inhibition of its transcriptional functions [98]. Furthermore, HPV-23 E6 binds and inhibits HIPK2 kinase leading to the prevention of UV-induced p53 phosphorylation at residue serine 46 [99].

E6s of β -types also block p53-dependent and independent apoptosis, which is induced in cutaneous sites after UV exposure and DNA damage, similar to the α -HPV types [50]. They also inhibit and degrade the protein Bak via E6AP proteasome-mediated degradation; probably for this reason Bak protein levels are undetectable in HPV-positive cancers, compare to the ones HPV-negative [100]. Recently, Holloway and colleagues showed

that HPV-5 E6 protein requires HERC1 ubiquitin ligase for Bak degradation [101]. HPV-38 E6 also interacts with the ubiquitin ligase p600/UBR4 but the role of this interaction is still unclear [102].

Literature data shows that β -type E6 proteins interact with many other cellular targets, such as MAML-1 and hTERT. MAML-1 is a transcriptional activator of Notch-regulated genes but the strong binding between this cellular protein and E6 leads to the inhibition of the Notch signaling pathway. It is known that Notch inhibition slows epithelial differentiation and this event could contribute to viral oncogenesis. The binding of MAML-1 takes place at the LXXLL motif, which is the same motif used by the E6 from α -HPV type to bind E6AP [96], [103], [104]. It is likely that the differences between α and β -HPV types in binding preferences represent an evolutionary adaptation to the different tissue where they replicate.

Regarding hTERT, HPV-5, HPV-20, HPV-22 and HPV-38 activate it via E6AP, and the intensity of the telomerase activation depends on the strength of the bond between E6 and E6AP (Figure 7 and 8). In fact, E6 of HPV-38 binds E6AP strongly and in combination with E7 is able to immortalize human primary keratinocytes [105], [106].

The interactions between E7s of β -HPV types with cellular proteins have not been as well-characterized as those of α -type. They contain the LXCXE motif and interact with pRb, p107 and 130, but to date only E7 of HPV-38 is able to degrade pRb in rodent fibroblasts while in human keratinocytes E7 phosphorylates the retinoblastoma protein inhibiting its function[42], [95], [105], [107].

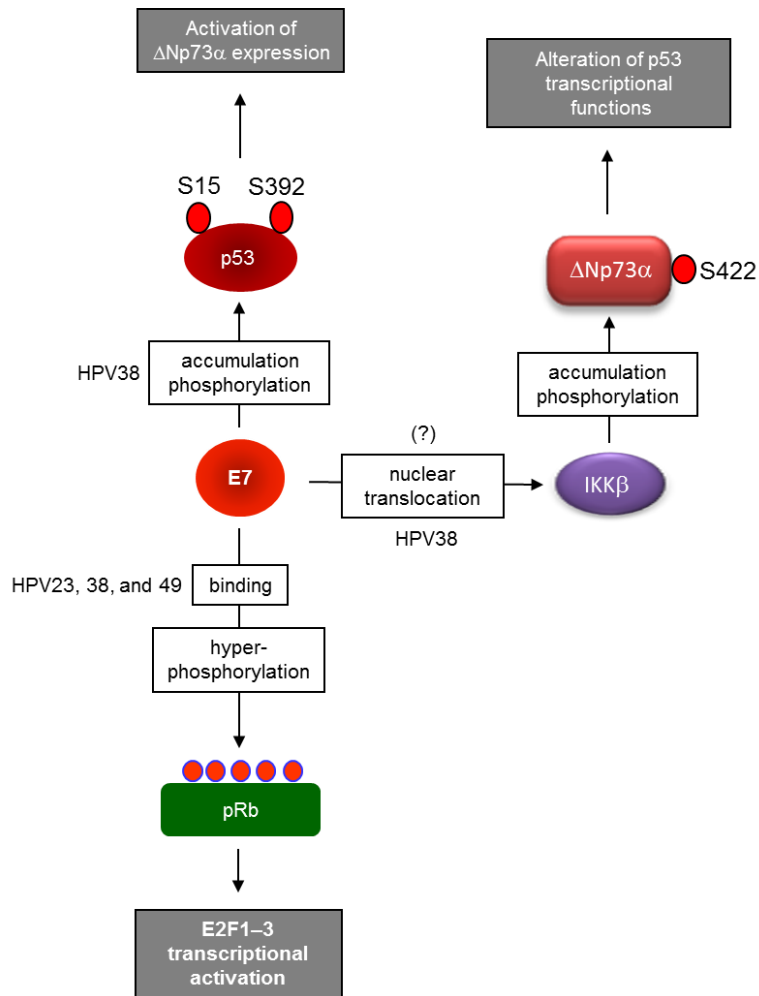


Figure 7: Biological properties of beta HPV E7. Image from [108]

An additional role of HPV-38 E7 is to induce changes in the regulation of p53 related genes. It has been reported that in the skin of transgenic mice, E7 cooperates with E6 to induce p53 stabilization that leads to the transcription of $\Delta Np73\alpha$ isoform of p73. The accumulation of $\Delta Np73\alpha$ blocks the transcription of p53-related genes involved in the regulation of cell cycle and apoptosis. Furthermore, E7 directly regulates IKK β nuclear translocation and promotes the formation of the complex $\Delta Np73\alpha$ /IKK β /DNMT1/EZH2. This complex binds different p5-related promoters in order to inhibit their activation. Finally, the immortalization of the keratinocytes mediated by E6 and E7 of HPV-38 is related to hTERT overexpression which, as previously mentioned, that in turn is partly due to $\Delta Np73\alpha$ accumulation (Figure 7 and 8).

To date, only HPV-8 and HPV-38 seem to promote tumorigenesis in transgenic mice [86], [112], [113]. The results obtained in mouse models show that when HPV-8 early region, HPV-8 E6 or HPV-38 E6/E7 are expressed from a K14 promoter epidermal hyperplasia forms and in some cases squamous cell carcinomas result. Furthermore, UV- exposure of these mice increases the numbers of squamous cell carcinoma while the wild mice do not develop any such lesions [113].

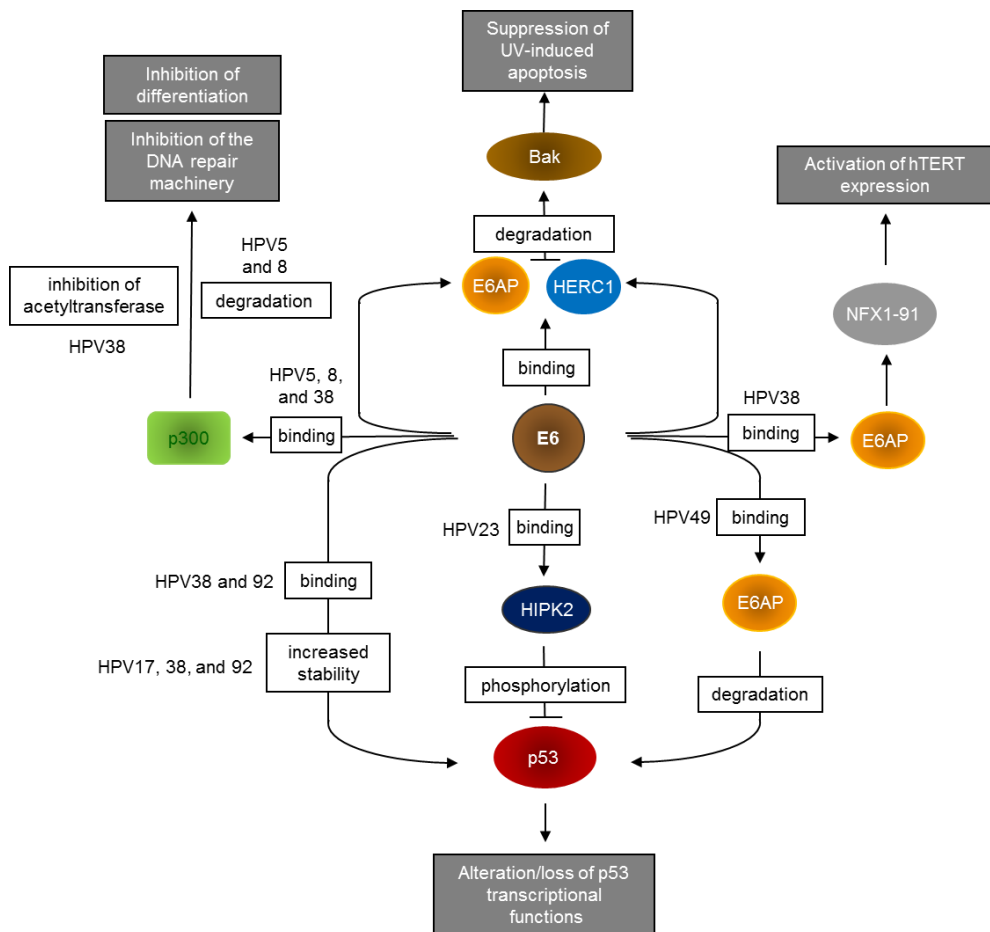


Figure 8: Biological properties of beta HPV E6. Image from [108].

3. INNATE IMMUNE RESPONSE TO VIRAL INFECTION

Vertebrates have developed different mechanisms of defense towards viral infections. Immune cells of the host are able to recognize the invasion of the pathogens and activate strong antiviral responses. Two distinct parts, the innate and the acquired immune system, compose the immune response of mammals, which work together in order to eliminate the pathogens. Initially, the presence of the virus activates the innate immune response, while the components of the acquired immune system play an important role in the response to re-infection.

The host recognizes specific viral components (pathogen-associated molecular patterns, or PAMPs), such as viral genomes or viral proteins, through pattern recognition receptors (PRRs) that are divided in three classes: Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) (Figure 9).

Among them, the best characterized are the TLRs [114], [115]. They are a group of highly conserved integral membrane glycoproteins found throughout the evolution, from *Drosophila* to humans, and their discovery was an important event for immunology [116]. The mammalian TLRs family is composed of 13 members, which are activated by different evolutionary-conserved molecular structures. They are expressed on different immune cells as well as on epithelial cells and fibroblasts [117]. The Toll-like receptors expressed on the cell surface recognize microbial membranes, whereas the TLRs expressed in intracellular compartments (such as TLR3, 7, 8 and 9) recognize the viral genome.

The RLRs are a family of cytoplasmatic proteins that include RIG-1, MDA5 (melanoma differentiation-associated gene 5) and LGP2 (laboratory of genetics and physiology-2), that recognize viral RNAs in the cytoplasm of the host cells [118], [119].

NLRs are soluble proteins that survey the cytoplasm in order to detect intracellular pathogens. The NLR family is composed by NODs, NALPs, IPAF (IL-1 β -converting enzyme (ICE)-protease activating factor), NAIPs (neuronal apoptosis inhibitor factors) and MHC class II transactivator (CIITA) [120]. NOD1 and NOD2 are the first NLRs to have been described and they drive the activation of MAPK (mitogen-activated protein kinase) and NF- κ B pathway [121]. The activation and oligomerization of NLRs proteins leads to the formation of multiprotein complexes called *inflammasomes*. The structure and the inflammasomes pathway will be described more in detail below.

Evasion of the host immune system is important for viruses as it allows them to persist inside the cells and to produce new viral progeny. To do this, viruses have developed over time several strategies in order to modulate the innate immune system suppressing or escaping from the host defense and establish a productive infection.

Lastly, an additional role of the PRRs is to recognize also endogenous danger by sensing danger-associated molecular patterns (DAMPs).

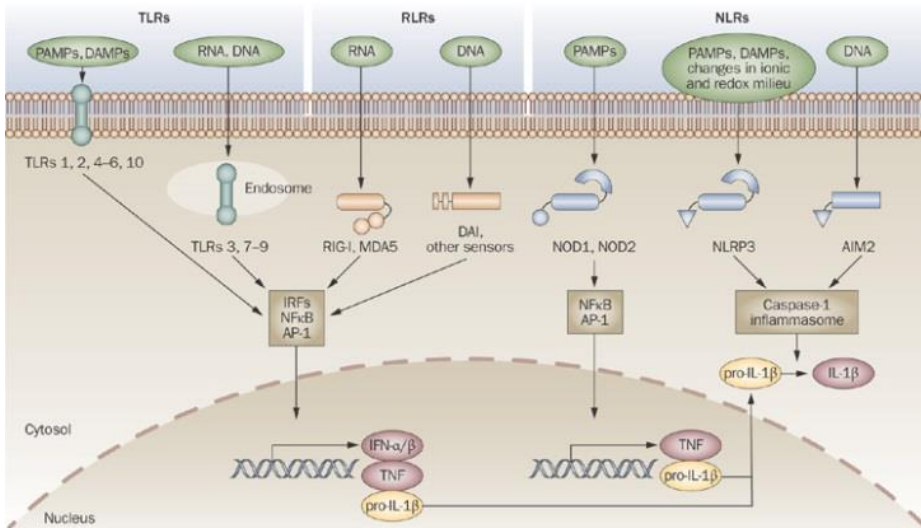


Figure 9: Innate immune sensors. TLRs, RLRs and NLRs are innate immune sensors that recognize danger signals derived from pathogens (PAMPs), damaged cells (DAMPs) or associated nucleic acids on the cell surface, in endolysosomes or in the cytoplasm. Signaling by these sensors promotes either the activation or nuclear translocation of transcription factors (IRFs, NFκB and AP-1) that drive expression of cytokines (IFN-α/β, TNF and pro-IL-1β), or the assembly of the caspase-1 inflammasome and subsequent maturation of IL-1β from pro-IL-1β. Image from [122].

3.1 Inflammasome

In 2002, Tschopp coined the term inflammasome to describe a high-molecular-weight complex, present in the cytoplasm of immune cells, responsible of the activation of inflammatory caspases [123]. Inflammasomes have been recognized for their important role in host defense against microorganisms and their assembly is due to unique

PAMPs or DAMPs, but it has also been reported that dysregulation of inflammasome activation is linked to the development of cancer or to autoimmune, metabolic or neurodegenerative diseases [124]. Due to their role, a tight control of inflammasome assembly and signaling is necessary in order to initiate antimicrobial and inflammatory responses, and to avoid tissue damage.

According to their structural components, inflammasome sensors are grouped into nucleotide-binding domain-like receptors (NLRs), absent in melanoma 2-like receptors (ALRs) and pyrin, which has been most recently identified. Most inflammasomes also use also an adaptor protein called ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain). Detection of the stimuli by the sensors leads to the aggregation of ASC to form specks inside cells, which in turn recruits caspase-1, inducing its autocatalytic cleavage. Finally, the active form of caspase-1 processes different cytokines, such as IL-1 β or IL-18.

Many other cellular signals are involved together with the inflammasome components in the defense against pathogens or cells damage.

3.1.1 NLR family

All the members of this family share the same structure: a central nucleotide-binding domain (NBD) with a C-terminal leucine-rich repeat (LRR) domain and a variable N-terminal domain, which may contain a pyrin or caspase activation and recruitment domain. Members of NLR family can be also subdivided into NLRP or NLRC depending of the N-terminal (Figure 10).

- NLRP1

NLRP1 was the first sensor identified by its ability to form an inflammasome complex [123] and human cells present only one NLRP1 protein while in rodents it has been found several paralogues.

In humans, NLRP1 is composed of the following domains: an N-terminal PYD, an NOD, LRRs, FIIND (function-to-find domain) and a C-terminal CARD. The specific regulatory mechanism of NLRP1 is still not very well described. Several studies have reported that mutations in this protein cause auto inflammatory diseases, such as vitiligo, Addison's disease, rheumatoid arthritis, Crohn's disease and systemic sclerosis . Moreover a recent study showed that NLRP1 is the most expressed inflammasome sensor in human skin and two Mendelian monogenic skin disorders, multiple self-healing palmoplantar carcinoma (MSPC) and familial keratosis lichenoides chronica (FKLC), are caused by distinct gain of function mutations in NLRP1 gene [128].

To date, nothing is known about microorganisms and NLRP1 activation in humans, while in mice it seems to be activated by a lethal toxin of *Bacillus anthracis*. Both rat NLRP1 and mice NLRP1b confer resistance to *Toxoplasma gondii* infection [129].

- NLRP3

NLRP3 is the most studied inflammasome and its domain structure is PYD-NACHT-LRRs (from N-terminal to C-terminal). When activated, NLRP3 forms a homo-oligomer via NACHT domain and interacts directly with ASC, via the PYD domain. ASC interacts with its CARD domain with pro-caspase-1 leading to the cleavage and the activation of caspase-1 [130]. NLRP3 is also known as cryopyrin because it was associated with hereditary auto inflammatory syndromes called cryopyrin-associated periodic syndromes, characterized by skin rushes and episode of fever [131]. It responds to different stimuli, both external and endogenous, and it is implicated in the pathogenesis of many auto inflammatory diseases, such as arthritis, diabetes, gout, obesity and Alzheimer's disease [132]. Despite several years of studies, the mechanism of NLRP3 activation is still unclear; it requires priming by external stimuli, which leads in the

transcriptional induction and post-translational modification of this sensor. However, three mechanisms of activation have been proposed. The first involves the ion channels. The increase of extracellular concentration of ATP leads to the stimulation of potassium ion efflux, through P2X7 ATP-gated ion channel, and the formation of membrane pannexin-1 pore. These events facilitate the influx of PAMPs and DAMPs [133]. The second mechanism involves the lysosomal rupture model and it is related to activators, such as silica or asbestos aggregates, which enter cells via phagocytosis. This induces lysosome collapse and the release of lysosomal contents, such as cathepsin B which leads to NLRP3 activation [134]. The last mechanism is related to reactive oxygen species (ROS) production where some stimuli induce the production of ROS by NADPH oxidase, which in turn activates NLRP3 [135], [136].

A different activation pathway has also been proposed for NLRP3 inflammasome, known as the non-canonical activation pathway (Figure 11) to differentiate it from the “canonical” pathway of activation described above. It results in caspase 11 activation, in mice, or caspase 4 and caspase 5 in humans. When the lipopolysaccharide (LPS) reaches the cytosol, it is directly bound by caspase 11, 5 or 4. This binding leads to the oligomerization and activation of the caspases, which in turn cleaves gasdermin D to induce pyroptosis. Thus, the activation of caspase 11 causes the non-canonical activation of NLRP3 inflammasome with the consequent activation of pro-caspase 1 and the cleavage and the release of pro-inflammatory cytokines [134]–[136].

How caspase 11, and more specifically human caspases 4 and 5, promote the non-canonical activation of NLRP3 needs to be better characterized.

As described above, several PAMPs from pathogens can activate NLRP3 and among them, the most common viral activator is *influenza virus*.

Infection by influenza virus induces caspase-1 cleavage and maturation [140]. It is likely that this activation occurs through the lysosomal rupture and ROS models [141], [142]. In addition, HCV also seems to activate NLRP3 through the ROS model, even if the detailed mechanism of action requires further study [143]. Many other viruses have been reported as NLRP3 activators, such as Herpes simplex 1, Vesicular stomatitis virus, West Nile virus and rabies virus. All of which are able to induce caspase-1 maturation and IL-1 β secretion. The exact mechanism of inflammasome activation, however, is still unclear.

Finally, three recent reports have shown the potential role of NIMA-related kinase7 (NEK7), a protein known to be involved in cell cycle progression, during NLRP3 activation. According to these studies, NEK7 acts downstream of potassium efflux and is able to activate the inflammasome complex in response to both canonical and non-canonical stimuli. The authors of the reports also demonstrated the ability of NEK7 to bind NLRP3 and controls its oligomerization, with NEK7 itself a component of the inflammasome complex. Because NEK7 plays a role in promoting mitotic spindle formation, when cells enter mitosis we assist to an attenuation of NLRP3 activation. This regulation is probably a safeguard cells employ to control inflammasomes during cell division, when there may be many endogenous ligands inside cell that could be misrepresented as cellular stress [143]–[145].

- NLRC4

Initially, NLRC4 was identified for its similarity to APAF-1 (apoptotic-protease activating factor 1). NLRC4 contains a winged-helix domain and together with NBD its domain stabilizes, the inflammasome in a closed conformation under basal conditions. On the other hand, LRR domain acts as a steric hindrance preventing the oligomerization of the complex. These

two mechanisms are important to maintain NLRC4 in an inactive state in absence of a ligand [147]. Unlike other NLR family members, NLRC4 does not interact directly with its activators but instead requires proteins, called NAIPs (NLR family, apoptosis inhibitory proteins), which act as a sensors that recognize the ligand [148], [149]. In humans, only one gene has been found that encodes NAIP [150]. Several PAMPs can activate NLRC4, such as bacterial flagellin or *Salmonella* infection. The activation of the inflammasome complex leads to procaspase-1 activation, which is mediated by the CARD domain. Man and colleagues showed that in macrophages infected with *Salmonella*, NLRC4 induces an actin polymerization response and also increases the intracellular level of ROS in order to kill the intracellular pathogen and to avoid bacterial dissemination.

Moreover, NLRC4 is also active in epithelial cell infected with *Salmonella*. This activation is important to control pathogen load and extra intestinal dissemination [151]. Finally, different human genetic studies have shown that autoinflammation and enterocolitis may be due to gain-of-function mutations in NLRC4 gene [152], [153]. Further studies are required to better elucidate the role and the mechanism of activation of this inflammasome complex in human patients.

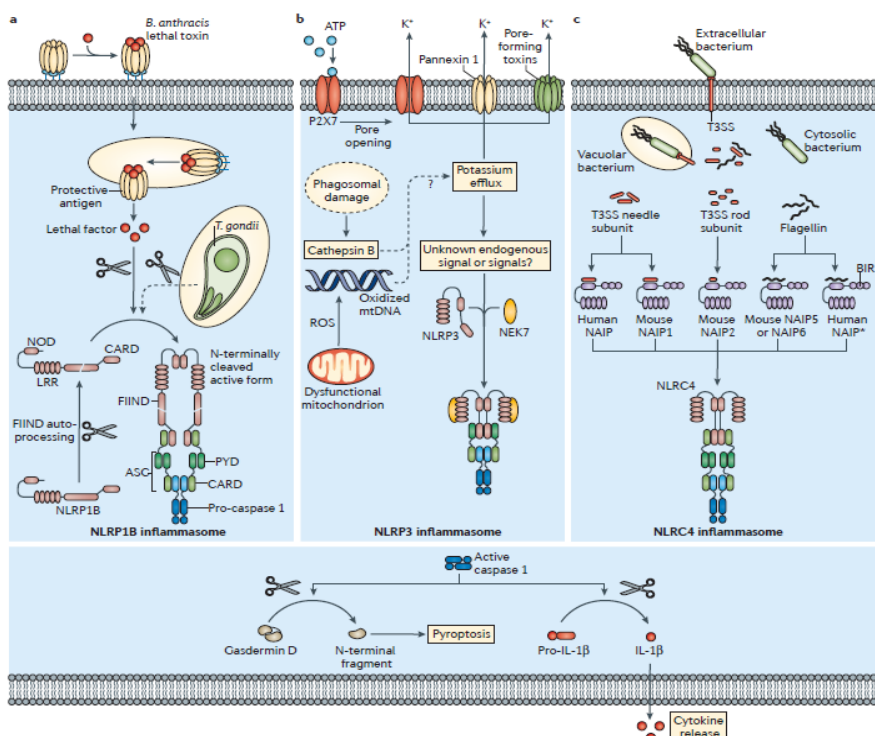


Figure 10 The NLR inflammasomes. Image from [154]

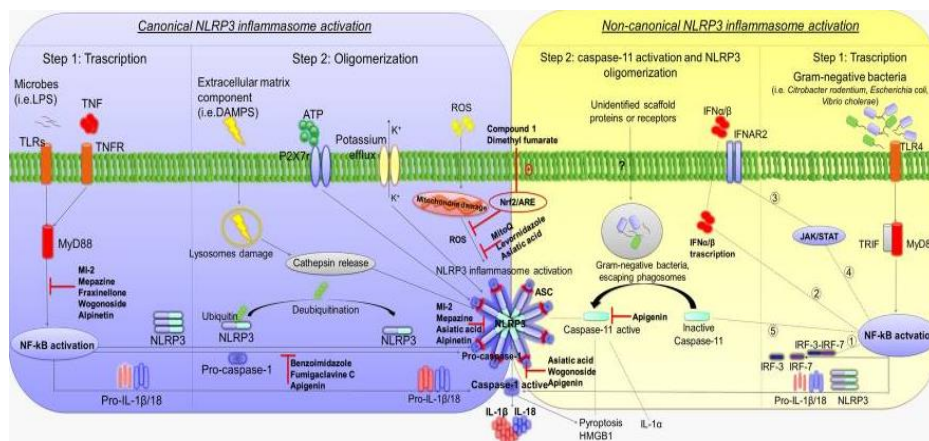


Figure 11: The canonical and non-canonical NLRP3 inflammasome activation. Image from [137].

3.1.2 ALR family

The expression of the members of this family is restricted to and conserved in mammals and the most well studied member is AIM2 (absent in melanoma 2) (Figure 12) [155]. All the members contain an N-terminal PYD domain and a C-terminal hematopoietic interferon-inducible nuclear protein with HIN200 (200-aa repeat) domain. The AIM2 PYD domain is different from other members of this family, in fact it is the only one able to interact with ASC to form an inflammasome complex. Another difference between AIM2 and other members of ALR family is the lack of the nuclear localization signal; which why AIM2 can be found only in the cytoplasm [156]. Kerur and colleagues showed that γ -INF-inducible protein 16, another human ALR, could be a putative inflammasome because of its ability to interact with the adaptor protein, ASC [157].

- AIM2

Initially identified improperly as a γ -INF-inducible protein, AIM2 is a nucleic acid sensor able to form an inflammasome complex in response to double-stranded DNA [157]–[159]. The recognition of the dsDNA is mediated by the HIN200 domain and is independent of sequence identity, although it does require around 80 bp of dsDNA [161]. Moreover, structural studies have revealed that HIN200 domain plays an auto inhibitory role because in absence of a ligand it interacts with the PYD domain to avoid homotypic interaction with ASC. In this complex, AIM2 and ASC form the center of the structure while caspase-1 constitutes the filaments [162] and has also been identified as a negative regulator of AIM2 inflammasome. It belongs to HIN200 family and it is known as p202. AIM2 lacks the PYD domain and most likely acts by sequestering dsDNA and/or mediating AIM2 heterodimerization [159], [163]. AIM2 is able to bind both bacterial and viral DNA but the signaling pathways for each are different. In fact, in response to bacterial stimuli type I IFN signaling is required to promote bacterial lysis

and the consequent release of the genome that is necessary for AIM2 activation. The same pathway is not involved during viral infection [164], [165]. Moreover, recent studies have shown that some bacteria, such as *Mycobacterium* or *Legionella*, produce several virulence factors that reduce the quantity of dsDNA release in order to evade AIM2 detection [165]–[167].

Finally, AIM2 can also detect host DNA released in response to cellular damage. This event occurs in various auto inflammatory diseases, such psoriasis or systemic lupus erythematosus. For this reason, AIM2 is considered as a possible therapeutic target for autoimmune disorders [169], [170].

3.1.3 The pyrin inflammasome

The gene MEFV encodes Pyrin, also known as marenostrin or TRIM20 (Figure 12). A PYD domain, two B-boxes and a coiled-coil domain compose this new inflammasome and only recently, its physiological function has been discovered. Pyrin is associated with an auto inflammatory disorder called familial Mediterranean fever and the inflammasome can be activated in response to bacterial toxins, such as *Clostridium difficile* protein B and *Clostridium botulinum* C3 toxin, or to effector proteins, likes VopS from *Vibrio parahaemolyticus* and IbpA from *Histophilus somni* [171]. Recently 14-3-3 proteins were identified as regulators of pyrin activity. When Pyrin is phosphorylated, under basal conditions, binds 14-3-3 proteins, while its dephosphorylation attenuates 14-3-3 binding. Moreover, a human mutation in this phosphosite causes pyrin activation also in the absence of stimuli [172]. However, the exact mechanism of interaction between pyrin and 14-3-3 protein needs to be clarified.

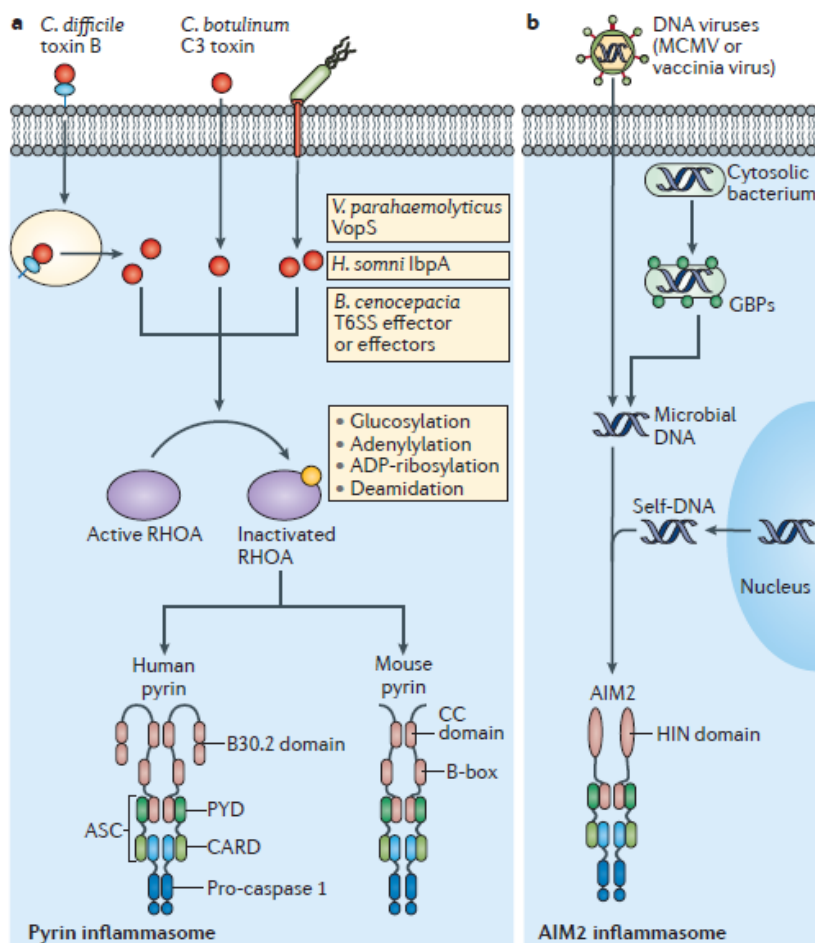


Figure 12: The pyrin and AIM2 inflammasomes. Image from [154].

3.1.4 Other inflammasome complexes

Several studies have shown that many other inflammasome complexes might yet exist. NLRP6 and IFI16 appear to be able to form multiprotein complexes with consequential maturation and release of pro-inflammatory cytokines.

It has been demonstrated that mice knock out for Nlrp6, Asc or Casp1 show an invasive dysbiotic microbiota with an increased susceptibility to chemically induced colitis and colitis induced tumorigenesis [173]. These mice show also a deficiency in IL-18 secretion from intestinal cells but, how NLRP6 controls this event is still unclear.

Regarding IFI16 and the mouse orthologue IFI204, they seem to play an interesting role as regulators of STING (stimulator of IFN genes)-dependent IFN production during infection [174], [175]. It has also reported that IFI16 interacts with ASC in the nucleus of cells infected with KHSV, and that it mediates caspase 1 activation during HIV infection [157], [176], [177].

Despite these studies, additional analysis are necessary to confirm unequivocally IFI16 as inflammasome.

3.2 Inflammasome assembly and structure

Inflammasome activation is a complex mechanism highly regulated that consists in three stages: stimulus, receptor activation and recruitment of the adaptor ASC and pro-caspase1.

The structure of inflammasome complexes is object of several study. Cryo-electron microscopy analysis demonstrated that its structure is similar to the apoptosome and it adopt a wheel- or disk-like architecture, with 10-12 spokes that correspond to the individuals promoters [178], [179]. Moreover, during NLRC4 activation there is a conformational change due to a rotation of 90°. This is important to promote the progressive oligomerization of the complex [180], [181]. On the contrary, AIM2 shows a different complex formation because of the lack of a NOD domain that normally mediates self-oligomerization. In this case, cytosolic DNA is bound by HIN domain forming an oligomerization template or scaffold [161].

After the assembly of the receptor complex, this structure recruits the adaptor ASC and also pro-caspase 1, through homotypic interactions PYD-

PYD or and CARD-CARD. When recruited, ASC forms a single macromolecular aggregate called ASC speck, which are composed by filaments of ASC [162], [182]. These filaments act as a recruitment point for pro-caspase 1. Even if ASC is essential for this signal, sometimes also CARD-containing receptors could directly recruit and activate pro-caspase 1. However, the lack of ASC leads to a reduction of IL-1 β release, in mice *Asc*^{-/-}. This indicates that the maturation and release of this cytokine is closely linked to ASC speck formation. This result is supported by evidence that single point mutation in ASC gene abrogates the ability of oligomerization of this adaptor protein, leading to a reduction of speck formation, pro-caspase processing and IL-1 β maturation after inflammasome activation.

Thus, ASC is important and acts as a signal amplification mechanism for inflammasome-mediated cytokines production.

3.3 Inflammasome effector mechanisms

Several study in mouse model have highlighted two important inflammasome effector mechanisms: pyroptosis and cytokine release.

The term pyroptosis refers to the pro-inflammatory cell death induced by inflammatory caspases, such as caspase 1 in humans and caspase 11 in mice. The term comes from the Greek “pyro”, that means fire, and “ptosis”, that means fall [183]. Pyroptosis is a particular kind of cell death characterized by cell swelling, lysis and release of cytoplasmic content, after the formation of membrane pores. Recently it has been demonstrated in mice models that pyroptosis requires gasdermin D to start. This protein is a substrate of inflammatory caspases by which it is cleaved generating N-terminal fragment necessary to drive pyroptosis cell death. Although gasdermin D is essential for caspase 11-induced pyroptosis, mice *Gsdmd*^{-/-} show pyroptosis cell death after a prolonged caspase 1 activation [184].

However, the prolonged activation of caspase 1 can also drive the cells to necrosis death.

On the other hand, Caspase 1 (also known as interleukin-converting enzyme - ICE) plays an important role in processing pro-IL-1 β and IL-18, in the canonical inflammasome activation, two cytokines that are known because of their role in inflammation and their coordination of innate adaptive immune response [185]. To date, the exact mechanism by which IL-1 β and IL-18 are secreted from the cells is unclear, in fact, their release is independent of the endoplasmic reticulum and Golgi. Different studies suggest that the release of these cytokines is due to cell lysis, at least in macrophages, but other cells type are able to secrete IL-1 β and IL-18, and neutrophils, for example, release IL-1 β without undergoing pyroptosis [186].

4. CYTOKINES

Cytokines are a group of small proteins secreted by cells with specific effect on the interactions and communications between cells. They can have autocrine, paracrine or endocrine action depending on where they act. Different cell types can secrete the same cytokine, which in turn can play different role, even if their activity are redundant, meaning that similar functions can be played by different cytokines. Moreover, they are named differently according to the role played in immune system or depending on the cells that produce them. Thus, they are divided in lymphokines, the ones made by the lymphocytes, monokines (made by monocytes), chemokines (that shows chemotactic activities) and finally interleukins (IL) that are produced by one leukocyte and act on other cells [187].

Among the Interleukins, IL-1 was the first to be discovered in 1977. The nomenclature of the members of this group of cytokines is continuously evolving and the new ILs are assigned to each family based on sequence homology, receptor chain similarities and also common biological properties. Thus, they are divided in five groups:

- IL-1 family composed of 11 members that share minimal sequence homology and differences in their localization, maturation and secretion. However, they show similar biological properties.
- IL-10 family composed of 9 members which show mainly anti-inflammatory properties.
- IL-12 family with its four members (IL-12, 23, 27 and 35) that share receptor and ligand chains but their functions are different depending on the cell type.
- IL-17 family includes only one member, IL-17, with its sub-type (IL-17A, B, C and D).
- All the others interleukins share common γ -chain, such as IL-2, 4, 7, 9, 15 and 21, or chemokine activity, such as IL-8 and IL-16.

Finally, some interleukins can exert both inflammatory and anti-inflammatory actions [188].

4.1 Interleukin 1 family

The interleukin 1 family, with its 11 members (Table 3), is the most studied group of cytokines because it is closely linked to the innate immune system, more than the other known cytokines. Nevertheless, most of the IL-1 members do not affect directly lymphocytes function but they act in an indirect manner. Virtually, the members of this family act on all the cells of the immune system promoting pro-inflammatory response. However, several study have also shown that some cytokines of this family can suppress inflammation, such as IL-18 binding protein or IL-1 receptor type 1.

The genes, which encode for IL-1 family members, are located on the long arm of chromosome 2, except IL-18 and IL-33 that are also closely related. The most characterized members of IL-1 family are IL-1 α and IL-1 β that are encoded by two distinct genes but they bind to the same receptor and share similar biological properties. The IL-1 α precursor is constitutively expressed in epithelial cells and it mediates, after the release, early phases of sterile inflammation, while IL-1 β is produced by hematopoietic cells. Unlike IL-1 α precursor, which is fully active, IL-1 β precursor needs to be cleaved to be released and become active [189]. Another important difference between them is their role in carcinogenesis. In fact, Krelin et colleagues have shown that mice deficient in IL-1 β are more susceptible to develop cancer compare to the mice IL-1 α knockout [190].

In the last years, different studies have shed light on the role of another member of this family, which is IL-18, during inflammation to better understand how it acts.

New Name	Other Name	Property
IL-1F1	IL-1 α	Agonist
IL-1F2	IL-1 β	Agonist
IL-1F3	IL-1Ra	Receptor antagonist
IL-1F4	IL-18; IFN- γ -inducing factor	Agonist
IL-1F5	FIL18	Anti-inflammatory
IL-1F6	FIL-1 ϵ	Agonist
IL-1F7	IL-1H4, IL-1 ζ	Anti-inflammatory
IL-1F8	IL-1H2	Agonist
IL-1F9	IL-1 ϵ	Agonist
IL-1F10	IL-1Hy2	Receptor antagonist (?)
IL-1F11	IL-33	Agonist

Table 3: IL-1 family members. Image from [185].

4.2 Interleukin 18

IL-18 was identified for the first time in 1989 as interferon- γ inducing factor, and for this it was firstly named IGIF, in mice suffering from endotoxin shock [191]. The human IL-18 gene is located on chromosome 11q22 and encode 193 amino acid protein [192]. hIL-18 shares 12% of amino acid sequence homologies with IL-1 α and 19% with IL-1 β . The regulation of IL-18 gene differs from most of other cytokines. Human PBMC and epithelial cells express a steady-state IL-18 mRNA and constitutively accumulate IL-18 precursor. The presence of the immature form of this cytokines ready to use allows the cell to rapidly produce the active protein in order to respond immediately to a stimulus [193]. As IL-1 β , also IL-18 is synthesized as a biologically inactive precursor of 24 kDa, without a signal peptide. After stimulation, the precursor is cleaved into an active mature molecule of 18 kDa by caspase-1. The important role of caspase-1 in this event has been

demonstrated in mice knockout for this converting enzyme, which showed a defect in IL-18 processing upon stimulation with lipopolysaccharide (LPS) [194], [195]. In addition, caspase-3 (CPP32) is also able to cleave both precursor and mature form of IL-18 generating biologically inactive degraded products. For this reason caspase-3 can be considered as a potential down-regulator of IL-18 [196] (Figure 13). Finally it has been identified a 29 kDa extracellular serine protease, called proteinase-3 (PR-3), that can also cleave proIL-18.

IL-18 is produced at high levels by activated macrophages and Kupffer cells. Literature data showed that also dendritic and Langerhans cells can produce this cytokine.

The genomic analysis of the hIL-18 showed that the gene is composed by six exons and five introns (Figure 14). Studies of the promoter activity in the 5' flanking region of the hIL-18 gene demonstrated a constitutive expression of the gene in human myeloid cell lines, such as THP-1 or U937, and DNA protein binding experiments revealed also a specific binding of STAT-5. In fact, the transfection of STAT-5 in THP-1 or U937 increases the promoter activity of IL-18 [197].

Moreover, in another study Kalina and colleagues showed that the consensus-binding site for PU.1 and a GC-rich region are the two important regulatory regions required for a basal IL-18 promoter activity in human myeloid cells [198].

In addition, it has been demonstrated that the human keratinocytes are the major producer of IL-18 that can be found inside the cells in the inactive precursor in huge amounts [199], [200]. Moreover, a recent study showed an increase of IL-18 secretion by keratinocytes after UVB exposure [201].

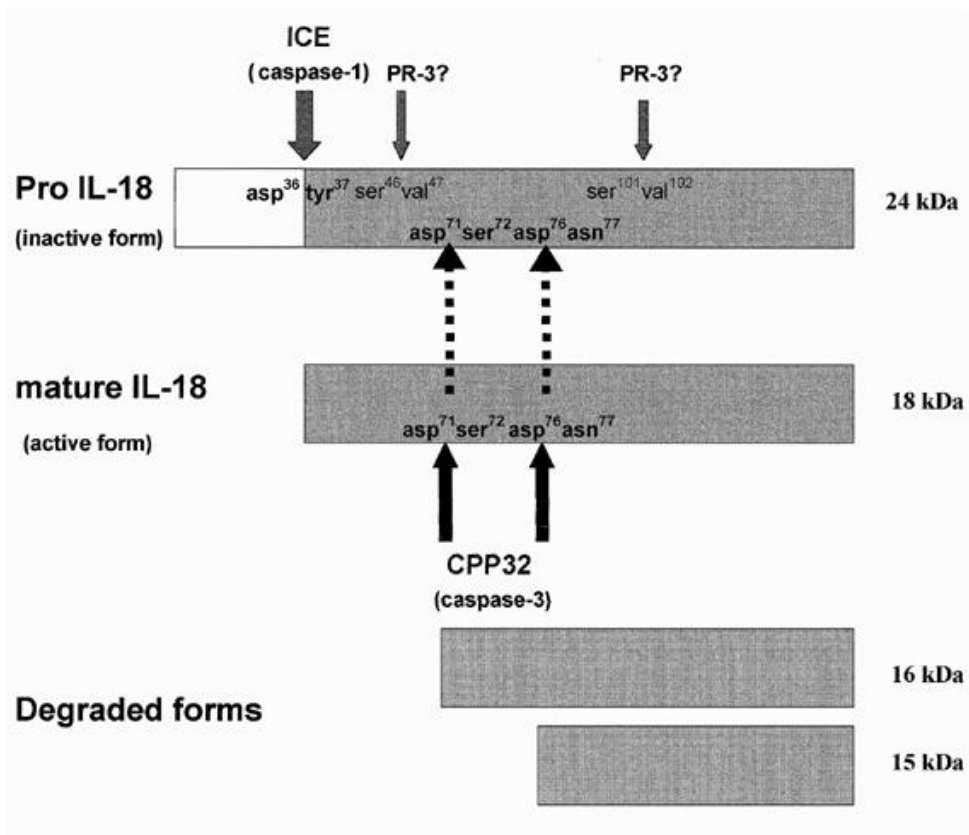


Figure 13: Processing of hIL-18. Maturation of proIL-18 (24 kDa) into an active mature molecule of 18 kDa requires the cleavage by the intracellular cysteine protease IL-1 β converting enzyme (ICE, caspase-1). The proteinase-3 (PR-3) may cleave extracellularly the proIL-18 into active form. CPP32 (caspase-3) cleaves both precursor and mature form into biologically inactive, degraded products. Image from [202].

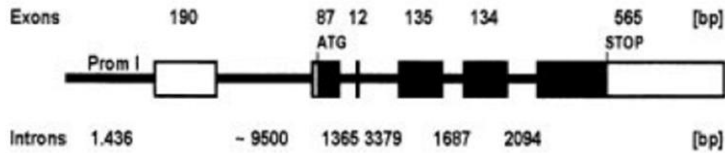


Figure 14 Genomic organization of human IL-18. The filled boxes indicate translated and the open boxes untranslated exon regions. Image from [203].

4.2.1 Biological properties

When mature IL-18 is released from the cells it binds to a heterodimer receptor complex, formed by IL-18R α and β chains. These two chains have a TIR domain in the intracellular portion and the binding of IL-18 to IL-18R α recruits also the β chain to initiate the signal transduction. The adaptor protein MyD88 binds to TIR domain and phosphorylates IRAK, which in turn recruits TRAF-6, leading to the activation of the transcription factors of NF- κ B and AP-1 (Figure 15) [204], [205].

IL-18 modulates the immune system functions together with other cytokines. It acts with IL-12 to promote the production of interferon- γ (INF- γ), Tumor necrosis factor alpha (TNF- α), CCL3, CCL4 and GM-CSF by CD4 Th1 and Natural Killer (NK) cells [206]. IL-18 can also play an important role in Th2 and Th17 responses inducing the naïve T cell to differentiate in Th2 cells and promoting IL-17 production by CD4⁺ in absence of TCR engagement [207], [208]. In addition, IL-18 is involved in allergic diseases, including asthma, as well as in autoimmune disorders, such as lupus erythematosus, arthritis, Crohn's disease [207]–[209].

IL-18 activity is neutralized by a natural inhibitor named IL-18 binding protein (IL-18BP). It was discovered in human urine [212] and it has high affinity for the active mature form of IL-18. It acts blocking the interaction between the cytokine and its receptor, preventing the dimerization of the receptor [213]. IL-18BP belongs to Ig superfamily, it is constitutively produced by monocytes and macrophages, and it can be found in the blood of healthy people [214]. INF- γ induces IL-18BP secretion with negative-feedback loop: it starts from IL-18-triggered INF- γ production that in turn induce IL-18BP gene expression to prevent excessive immune response. INF- γ , but also IL-27, induces STAT1 binding to IL-18BP promoter. These findings suggest a possible anti-inflammatory role of IL-18BP in skin diseases where there is an over production of IL-18 [215], [216].

Interestingly, the study carried out by Schwarz and colleagues provided evidence for the involvement of IL-18 in DNA repair induced by UVB irradiation. The injection of IL-18 in mice exposed to UVB reduced the amounts of apoptotic keratinocytes and DNA damage into the cells. The same results were not observed in DNA-repair deficient mice confirming the ability of IL-18 to play a role in UVB-induced DNA repair [217], [218].

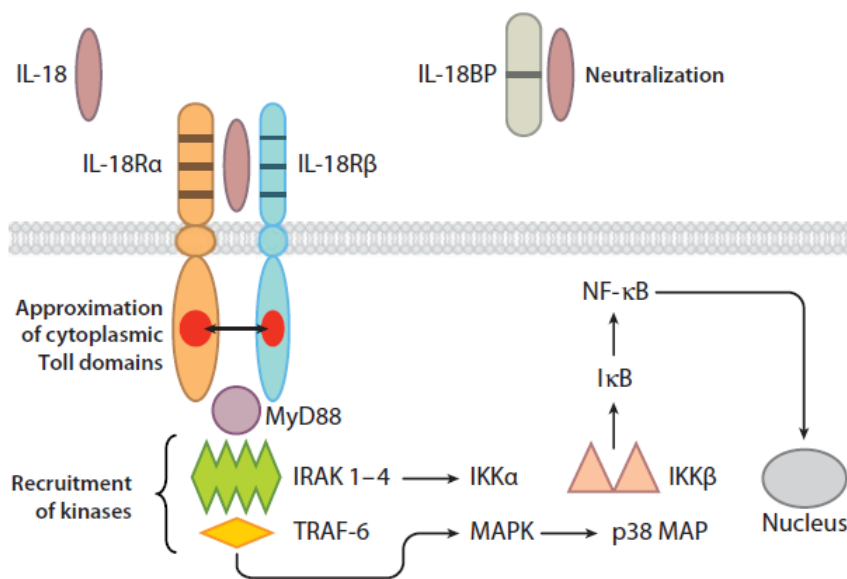


Figure 15: IL-18 signal transduction. It involves MyD88 and IL-1 receptor–associated kinases (IRAKs). TNF receptor–associated factor (TRAF)-6 is also a part of IL-18 signaling. TRAF-6 is required for the phosphorylation of mitogen-activated protein kinase (MAPK) p38. Image from [185].

4.2.2 Regulation of IL-18 expression during infection

Many literature data shows the role of IL-18 during infection, both bacterial and viral. For example, IL-18 and IL-18R deficient mice are not able to respond to bacterial invasion [219]. Moreover, mice infected with vaccinia virus, herpes simplex type 1 (HSV-1) or encephalomyocarditis virus and treated with IL-18 exogenous survive to infection [217]–[219]. Several studies, both in vitro and in vivo, have demonstrate that many viruses stimulate the production of IL-18 mature protein, which in turn act promoting antiviral activities. Moreover, viruses evolved different strategies

to evade and block IL-18 pathway and the regulation of IL-18 production after infection often occurs at post-translational level. During pulmonary adenovirus infection, the virus inhibit IL-18 activity through the production of a neutralizing anti-IL18 monoclonal antibody [223]. Poxviruses, such as *Molluscum contagiosum*, produce a protein similar to human IL-18BP, which acts blocking mammalian IL-18 activity [224].

In addition, E6 and E7 from HR HPV-16 inhibit the production of IL-18 in human peripheral blood mononuclear and NK cells. Moreover, studies conducted in primary human keratinocytes showed that E6 down regulates IL-18 expression, through the binding of the cytokines, while E7 oncoprotein leads to an increased production of IL-18BP that neutralize IL-18 activity [223]–[225].

Finally, there are some evidence that caspase-3 can be involved in the cleavage of IL-18 during virus infections. This enzyme is constitutively expressed in macrophage, such as caspase-1, and the infection can promote its activation that leads to IL-18 cleavage into two inactive fragments. Thus, caspase-3, acting in contrast to caspase-1, can promote viral immune evasion by the downregulation of IL-18 activity [196].

However, IL-18 can also promote viral infection. In vitro study have shown that IL-18 is able to enhance HIV-1 replication in monocyte and T cells [228], [229]. HIV-1 infection down regulates the endogenous levels of IL-18, thus PBMCs (peripheral blood mononuclear cells) from HIV-1⁺ patients are not able to respond to bacterial stimuli [230].

5. ROLE OF THE IMMUNE SYSTEM IN CANCER

In 1909, Paul Ehrlich was the first to propose a protective role of the immune system against cancer [231]. Not long after, Coley reported that some patients showed a spontaneous tumor regression after developing post-surgical infections. These findings and other experimental results let him to hypothesize to apply immunotherapy for cancer regression [232]. Many years later, the theory of immunosurveillance of Burnet and Thomas was supported by the discovery that syngeneic mice were able to reject tumors upon secondary challenge and that those mice could be also vaccinated [233]. To support the hypothesis of the interaction between immune system and cancer, Schreiber and colleagues proposed the term “cancer immunoediting” [234], meaning that tumors are continuously edited by the immune system. This editing progress requires three stages during which immunogenic cancer cells are progressively eliminated (Figure 16). The first stage is the elimination phase: innate and adaptive immune response work together to detect and destroy cancer cells. The second stage is the equilibrium that is establish when the tumor is not completely eliminated. During this phase immune system cells and cancer cells interact in order to promote the development of alterations in both cell populations. The last stage is the escape; at this point cancer cells are less immunogenic and start to develop mechanism of evasion or to actively immunosuppress the host [235], [236]. To date, there are not definitive evidence for these stages in human tumors progression, but this is likely a useful conceptual framework to explain cancer immunosurveillance. For example, the immunoediting could be apply to the development of skin cancer because of its long latency and also because of the apparent spontaneous resolution of pre-neoplastic lesions. Nevertheless, the progression of these stages during skin cancer need to be confirmed in-vivo.

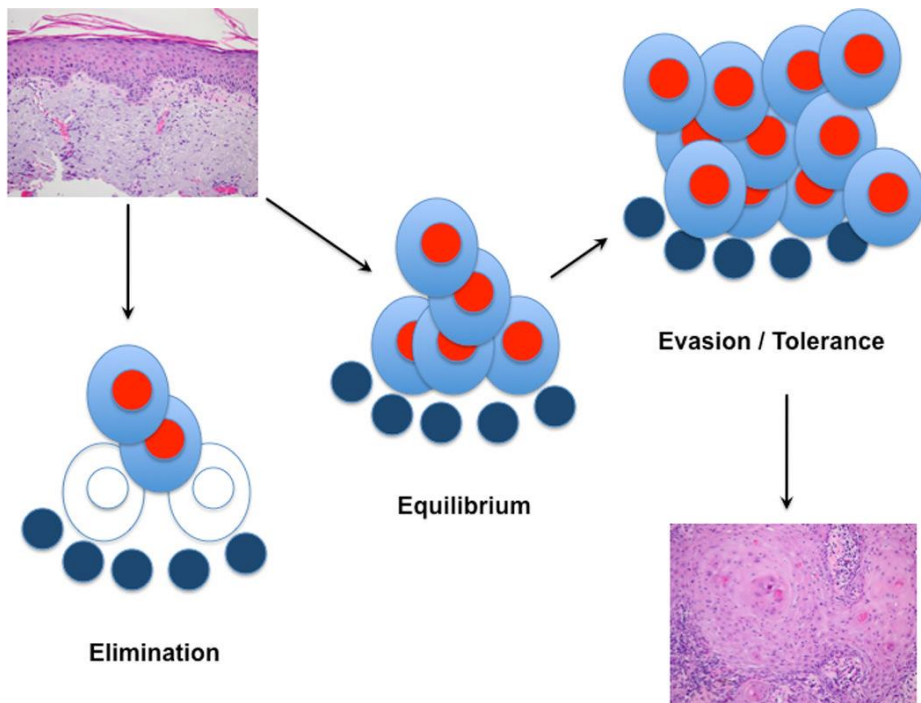


Figure 16: A Paradigm of Cancer Immunosurveillance. Initially, there are pre-cancerous lesions, such as the illustrated actinic keratosis (upper left), in which elimination may occur due to the killing of altered cells by elements of the immune system. Alternatively, a stage of equilibrium may result where tumor cells and immune cells interact during a period of stable tumor size. During this period, immune cells may select to more aggressive and/or less immunogenic tumor variants. Eventually, perhaps as a result of this process, the tumor expands and continues to grow despite the presence of an immune response. This continued growth can be observed in invasive squamous cell carcinoma which typically does not spontaneously regress (lower right). Image from [237].

As said at the beginning, UVB radiation represents one of the most important risk factor for skin cancer development. In fact, chronic UVB exposure leads to the formation of actin keratosis and as final stage development of SCC. On the other hand, intermittent high dose of UVB causes mostly BCC and then melanoma [238]. Moreover, many studies underline the dramatic effect of UV exposure on immune system. Kripke and colleagues showed the antigenicity and immunogenicity of UV-induced skin cancers because these tumors are rejected by syngeneic mice but not by mice exposed to UVB. A possible explanation could be that the exposure induces “suppressor T cells” that in turn might inhibit the ability of the mice to reject UV-induced tumor, compared to the non-exposed mice [239].

UVB immunosuppression can be local, with the depletion and/or downregulation of the capability of the Langerhans cells to present the antigens, and systemic, through the stimulation of keratinocytes and macrophages to produce cytokines with high impact on systemic immunosuppression.

DNA damage and the production of ROS and cytokines, which lead to chronic inflammation, cooperate to mutagenize epithelial cells in order to create an immunosuppressive environment that supports cancer formation and progression [239]–[241].

5.1 Inflammasome activation in cancer

Different studies support the hypothesis of a link between inflammation and cancer development. In fact, chronic inflammatory response are often associated with many types of cancer playing an important role during cancer development.

Literature data showed that different subsets of immune cells are involved in cancer progression and more often inflammasome complexes are

activated in response to danger signals that come from tumors but this activation can play a different role in cancer progression depending on the specific context. For instance, the etiological link between tumor development and chronic inflammation is well known and documented for cancers of the gastrointestinal tract. In this case, the chronic inflammation of the stomach, due to *Helicobacter pylori* infection or other causes, provokes a continuous activation of inflammasome complexes that in turn causes an up-regulation of pro-inflammatory cytokine secretion. To support this finding, Tu and colleagues showed that stomach-specific release of pro-inflammatory cytokines induces inflammation and high risk to develop cancer in mice [243]. Moreover, Jee and colleagues demonstrated that there is a decrease of caspase-1 expression in 19% of gastric cancer cases [244]. In addition, Allen et al. support this etiological study arguing that mice knockout for different inflammasome components, such as caspase-1, ASC or NLRP3, show increased and recurring acute colitis and tumorigenesis compared to wild type mice [245]. On the other hand, it has also been reported that inflammasome complexes, such as NLRP6, can suppress tumor progression in mouse models [246].

There are no direct evidence about the involvement of inflammasome in breast cancer, but different studies demonstrated that inflammasome activation could play an important role in hepatitis C-associated hepatocellular carcinoma. In fact, it is known that HCV infection activates NLRP3 inflammasome [247].

Furthermore, the involvement of inflammation in melanoma is also known. Okamoto reported that there is a constitutive activation of NLRP3 inflammasome in skin cancer [248] but the function of active inflammasomes can be different depending on the cell type, where the complex is activated, as well as the stage of the cancers. For example, in non-metastatic cells, ASC expression decrease the activity of NF- κ B

leading to a reduction of the tumor while the opposite behavior is shown in metastatic melanoma cells [249].

Recently, the analysis of the expression of inflammasome components in cSCC showed that there is a strong up-regulation of AIM2 expression, both at mRNA and protein levels, compared to normal human keratinocytes. In addition, the knockout of the inflammasome AIM2 in those cells leads to a reduction of the viability as well as of the invasiveness of the cSCC [250]. Inflammasome-mediated secretion of pro-inflammatory cytokines, such as IL-18, plays a critical role in innate immune response as well as in adaptive immune response. Thus, it is not surprising to think that inflammasome can modulate immune system response towards tumors development and therapy.

5.2 IL18 a double-edged sword in cancer

Interleukin 18 shows a dual role in cancer progression: it can promote tumor development and progression, or oppositely limit tumor growth.

High level of expression and secretion of IL-18 have been found in patients with cancers, such SCC, melanoma or others skin cancer cell lines [251]. IL-18 seems to promote tumor progression stimulating angiogenesis and metastasis. It is well known that angiogenesis is an important event not only during inflammatory response and wound healing, but also during tumor progression, because solid tumors require oxygen and nutrient to grow [252]. High level of IL-18 are detected in invasive tumor tissue of gastric cancer where the over-expression of the pro-inflammatory cytokine is associated with the formation of new blood vessel. Kim and colleagues demonstrated that the pretreatment of gastric cancer cells with JNK inhibitor lead to a reduction of thrombospondin-1 (a pro-angiogenic factor in gastric cancer) expression IL-18- mediated [253].

In addition, several studies, conducted in patient suffering of rheumatoid arthritis, demonstrated that IL-18 stimulates production of vascular endothelial grow factor (VEGF) both at mRNA and protein level, supporting the theory that IL-18 is a direct angiogenic stimulator [254]. Moreover, angiogenic factors can also induce IL-18 maturation suggesting a positive-feedback mechanism of regulation between the cytokine and VEGF production in cancer.

The ability to metastasize is important for cancer progression and malignancy and IL-18 can acts as a cell migration stimulator in gastric cancer as well as in melanoma. IL-18 increases the expression of the metalloproteinase 9 (MMP-9) both at mRNA and protein level in leukemia cell line, promoting the degradation of the ECM (extracellular matrix) surrounding the cells and the invasion ability [255], [256].

In addition, as previously mentioned, the immune system is able to detect and eliminate cancerous cells, however cancer cells have developed several mechanism to evade the immune response. For example, in melanoma cells IL-18 promotes the expression of Fas ligand giving them resistance to immune surveillance, because the cells of the immune system are not able to recognize cancer cells. Moreover, cancer cells induce apoptotic death of immune cells because of the expression of Fas ligand [257].

Finally, there are also several evidence of the anti-cancer effects of IL-18. During *Helicobacter pylori* infection of gastric mucosa, high level of IL-18 expression may contribute to Th1 activation and response [258]. Furthermore, Myd88 KO-mice with a defect in IL-18 production show more susceptibility to develop colorectal cancer [259] as well as mice IL-18^{-/-}. Nishio and colleagues reported also that the administration of recombinant IL-18 in syngeneic mice mediates the regression of melanoma or sarcoma through the activation of CD4⁺ and NK cells [260].

IL-18 may have anti-tumor effects in some human cancer. Nonetheless, ovarian and colon epithelia produce and release mature IL-18 in response to pathogens but during neoplastic transformation they are unable to process IL-18 because of a defect in caspase-1 expression [261], [262]. In addition, in prostatic and ovarian cancer, high levels of IL-18BP have been detected. This event may inhibit the therapeutic and anti-cancer effect of IL-18, representing a probable mechanism of immune escape [263].

More often, IL-18 does not act alone but in combination with other cytokines, such as IL-12, or costimulatory molecules in order to increase its anti-tumor effects.

In conclusion, the current literature underlines the important role of immune system in development, progression and elimination of cancers cells, emphasizing the double and divergent behavior of this system in different neoplastic conditions. Inflammasomes with its several components could be considered a promising target for prevention as well as for cancer therapy. However, additional studies are required to better shed light on the mechanisms involved in immune surveillance failure with the consequent development and progression of cancer.

AIM OF THE STUDY

UV-radiation represents a key risk factor for the development of non-melanoma skin cancer (NMSC) [5], [240]. Moreover, several studies have shown a greater tendency to develop skin cancer in immunocompromised individuals, emphasizing a potential role of infectious agents in these events [9]. Among them, the human beta papillomavirus types, that normally colonize the skin, are suspected to be involved in the development of NMSC, acting as a co-risk factor together with ultraviolet radiation. This hypothesis is mainly supported by the fact that people suffering of *epidermodysplasia verruciformis*, a skin disease due to an autosomal recessive disorder, are more susceptible to β -HPV infections and also to the development of skin squamous cell carcinoma (SCC) after sun-exposure [27]. Functional studies, both in *in vivo* and *in vitro* experimental models, have highlighted the oncogenic properties of E6 and E7 proteins of some HPV β -type and their ability to evade the immune response of the host, in order to efficiently produce new progeny. For instance, Caldeira and colleagues have characterized the transforming properties of E6 and E7 of β -HPV38, showing their ability to immortalize human primary keratinocytes and to inactivate the functions of the retinoblastoma protein, pRb, and the tumor suppressor factor, p53 [105]. In addition, previous studies conducted in our group on transgenic mouse model, which expresses of E6 and E7 of HPV38 in the skin under the control of K14 promoter, showed that the transgenic mice do not present any spontaneous formation of cancer lesion under physiological condition, while after UV-exposure they develop actinic keratosis-like lesions that are considered as a precursor of SCC in human. However, the wild type mice do not develop any kind of lesion after the irradiation [113].

Furthermore, independent studies carried out on keratinocyte cell lines provided evidence that the exposure to ultraviolet radiation causes the activation of pathways involved in the innate immune response, such as the

inflammasome pathway, which leads to the secretion of specific pro-inflammatory cytokines, like IL-1 β or IL-18 [201]. More important, it has been reported that in the presence of E6 and E7 from α -HPV16 as well as β -HPV38 the secretion of the pro-inflammatory cytokine IL-1 β is impaired after UVB exposure [264].

Lastly, it is also known the possible involvement of IL-18 in DNA repair after UV irradiation [217], [218], as well as its potential role in cancer development [259] .

Therefore, based on this literature data the aim of this work is to evaluate and analyze the mechanisms involved in the deregulation of UV-activated inflammasome by E6 and E7 from HPV38 in human keratinocytes in order to further corroborate the hypothesis of the cooperation between sun exposure and β -HPV infection in the development of non-melanoma skin cancer.

MATERIALS AND METHODS

1. CELL CULTURE AND TREATMENTS

Experiments were carried out in human primary foreskin keratinocytes (HPKs), HPKs expressing E6 and E7 from HPV38, HPKs hTERT and HPKs hTERT expressing E6 and E7 from HPV38.

Primary cultures of human epithelial keratinocytes and hTERT cell line were cultured together with NIH 3T3 feeder layers in FAD medium containing: 3 parts Ham's F12, 1 part DMEM, 2,5% fetal calf serum, insulin (5 µg/ml), epidermal growth factor (10 ng/ml), cholera toxin (8.4 ng/ml), adenine (24 µg/ml), hydrocortisone (0.4 µg/ml) and 1% of pen/strep preparation. Feeder layers were prepared by treating NIH 3T3 with mitomycin for 2h. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplement with 10% fetal calf serum and 1% pen/strep preparation. HPKs expressing E6 and E7 from HPV38 and HPKs hTERT, with or without E6 and E7 from HPV38, were generated and cultured as previously described [105].

Transient transfection experiments were performed using TransIT®-Keratinocyte Transfection Reagent (Mirus) according to the manufacturer protocols.

Cells were exposed to 50 mJ/cm² UVB dose with a thin layer of phosphate-buffered saline (PBS) using the BIO SUN UV Light Irradiation apparatus (Vilber Lourmat). Cells were then incubated at 37°C in a humidified chamber and harvested at specific time points.

2. TRANSCRIPTOME ANALYSIS

Wild-type (WT) and HPV38 E6/E7 Tg mice were UV-irradiated 5 times or not irradiated. After 24h, skin was collected and expression of cellular genes was determined by Illumina array system (Illumina, San Diego, CA). The heat map was generated by comparing the expression levels of cellular genes of UV-exposed wild-type and HPV38 E6/E7 Tg animals. Only the

transcripts that showed a standard deviation lower than 0.9 were used for the analysis, and only differences that were significant with $P < 0.05$ in a two-group test were considered.

3. RNA EXTRACTION AND RT-qPCR

Total RNA was isolated from keratinocytes using NucleoSpin RNA protocol (MACHEREY-NAGEL) and the cDNA generated with RevertAid H minus First Strand cDNA Synthesis Kit (Thermo Scientific).

The resulting cDNA was amplified using specific primers. The sequences of the primers are listed in the table 4 below. GAPDH was used as the internal control for each reaction.

4. SDS-PAGE AND IMMUNOBLOTTING

Cells were lysed using Ripa buffer (50mM TrisHCl, 150mM NaCl, 10mM $MgCl_2$, 0,5% Sodium Deoxycholate, 0.1% SDS, 1% Np-40) supplemented with cOmplete Protease Inhibitor mixture (Roche).

Proteins from the supernatant of the cell cultures were extracted according the following protocol:

Keep 500 μ L supernatant from the cells culture (after the treatment, cells were cultured in OptiMEM without serum medium to facilitate the protein extraction). To precipitate supernatant:

- add 100 μ L chloroform + 500 μ L methanol
- Vortex
- Centrifuge 3 min 13000 rpm 4°C
- Trash the aqueous phase without touching the white disc (precipitated proteins)
- Add 500 μ L methanol and vortex
- Centrifuge 3 min 13000 rpm 4°C.

- Carefully remove the solution with a tip
- Dry the pellet 5 min at RT
- Add 35 μ L Laemmli Buffer 4X (BioRad) + DTT (50 mM) or + β -mercaptoethanol (Sigma)
- Heat 10 min at 90°C and load a third of the total volume of the samples.

Samples were resolved by SDS-PAGE and transferred to membranes PVDF membrane (PERKIN ELMER). Membranes were blocked in 5% nonfat milk and incubated overnight at 4°C with the appropriate primary Ab. Samples were resolved by SDS-PAGE and transferred to membranes PVDF membrane (PERKIN ELMER). Membranes were blocked in 5% nonfat milk and incubated overnight at 4°C with the appropriate primary Ab. Membranes were probed with the following primary antibodies: Anti-pro-IL-18 (Human) mAb (MBL- M156-3), Anti-IL-18 (Human) pAb (MBL-PM014), AIM2 (D5X7K, Cell Signalling), anti-NLRP1/NALP1 (human) pAb (AL176, AdipoGen®, Life Sciences), p53 DO-1 (sc-126, Santa Cruz biotechnology, INC), β -actin (MP, Biomedicals) and GAPDH (6C5, sc-32233 Santa Cruz biotechnology, INC) were used as loading control. Membranes were then incubated with the appropriate HRP-conjugated secondary antibody (anti-rabbit and anti-mouse from Sigma). Proteins were detected using Clarity™ Western ECL Substrate (Biorad) and ChemiDoc XRS imaging system (Bio-Rad).

5. ELISA ASSAY

The concentration of IL-18 in the culture supernatant was measured by a commercially available ELISA (K151MCD-1, Meso Scale Discovery). After stimulation, cell culture supernatants were collected, centrifuged at 1500 rpm for 10 min at 4 °C and concentrated using Amicon Ultra-4 Centrifugal Filter 3 (Millipore) and then added to coated wells. Elisa assay was

performed following the protocol: <https://www.mesoscale.com/~media/files/product%20inserts/human%20il-18.pdf>. Relative absorbance was measured at 450 nm, and the IL-18 concentration was calculated using a standard curve. Each supernatant was analyzed in duplicate.

6. LUCIFERASE ASSAY

Transient transfection experiments were performed using TransIT®-Keratinocyte Transfection Reagent (Mirus) according to the manufacturer protocols. hTERT cells were transfected with pGL3 firefly luciferase IL-18 short or long promoter vectors (1 µg). A pRL-Renilla reporter vector (10 ng) was used as an internal control. After 24 hours the cells were harvested, lysed and luciferase activity was measured using a dual-luciferase reporter assay system (Promega). The expression of firefly luciferase relative to Renilla luciferase was expressed in relative luminescence units (RLU).

7. CHROMATIN IMMUNOPRECIPITATION

Chromatin immunoprecipitation (ChIP) was performed with Shearing ChIP and OneDay ChIP kits (Diagenode) according to the manufacturer's instruction. Briefly, cells were sonicated to obtain DNA fragments of 200–500 bp. Sheared chromatin was immunoprecipitated with isotype control IgG or with the indicated antibodies. The eluted DNA was used as a template for qPCR. Primers for qPCR are listed in Table 5 below.

8. STATISTICAL ANALYSIS

Statistical significance was determined by Student t-tests. Statistically significant p values of each experiment are indicated in the corresponding Figure legends, $P < 0.05$ (*); $P < 0.01$ (**); ns = non-significant. Error bars in the graphs represent the standard deviation.

Gene	Primer sequence
GAPDH	F: 5'-GCCAAAAGGGTCATCATC-3'
	R: 5'-TGCCAGTGAGCTTCCCCTTG-3'
IL-18	F: 5'-GGAATTGTCTCCAGTGCAT-3'
	R: 5'-ACTGGTTCAGCAGCCATCTT-3'
AIM2	F: 5'-AACTTTGGGATCAGCCTCCTG-3'
	R: 5'-TGAAGCCGTCCAGAAGTGTC-3'
ASC	F: 5'-TCTACCTGGAGACCTACGGC-3'
	R: 5'-TCCAGAGCCCTGGTGC-3'
NLRP1	F: 5'-CATGCTGCCTGTTCTCATGG-3'
	R: 5'-CCCCAACCACAATCTCCGAATG-3'

^aF, forward; R, reverse.

Table 4: Sequences of primers used for RT-qPCR analyses.

Promoter	Primer sequence
p53RE on IL-18 promoter region 1	F: 5'-ATAGTCACCACACCAA-3'
	R: 5'-GAACAAAATGCTTACATAA-3'
p53RE on IL-18 promoter region 2	F: 5'-ATTCCCTTCCTAGCAA-3'
	R: 5'-CAATTTAAGGATACATATAT-3'
p53RE on IL-18 promoter region 3	F: 5'-AATAAAATTCTAATGATG-3'
	R: 5'-TGCAAATATTTCTTTTA-3'
p53RE on IL-18 promoter region 4	F: 5'-AATAAGAAAAAGTTTTGTGT-3'
	R: 5'-TGAAATCTTTTCTCCGT-3'
p53RE on IL-18 promoter region 5	F: 5'-GGAAGAAAAGATTTTCATGA-3'
	R: 5'-AATGAGGAAGAAGGTGG-3'

^aF, forward; R, reverse.

Table 5: Sequences of primers used for ChIP analyses

RESULTS

1. EFFECT OF UVB IRRADIATION ON GENE EXPRESSION PROFILE OF HPV38 E6/E7 TRANSGENIC MICE

In our group a transgenic (Tg) mouse model has been generated that constitutively expresses E6 and E7 from HPV38 in the skin epithelium under the control of K14 promoter. This model has been used to explore the interaction between β -HPV38 E6 and E7 and the environmental risk factors, such as UVB exposure, that are involved in the development of NMSC. Previous results obtained by our group have shown that chronic exposure to UVB of HPV38 E6/E7 Tg mice primarily resulted in the development of actinic keratosis-like lesions that are considered precursors of SCC in humans and few weeks later the mice developed SCC. Conversely, the wild type mice did not develop any kind of lesions when exposed to the same UVB dose [113]. Similarly to the development of UV-induced skin lesions, HPV38 E6/E7 Tg mice accumulated thousands UVB-induced C>T mutations during the long-term UV irradiation, while only a small number of mutations were detected in the skin of the wild-type animals exposed to the same treatment (unpublished data).

To better understand the molecular mechanisms of the cooperation between HPV38 E6 and E7 and exposure to UVB, we analyzed the entire transcriptome profile in the skin of WT and HPV38 E6/E7 Tg mice that were either exposed or not-exposed to UV irradiation. The mice (3x Tg / 3x WT) were irradiated for 5 days with a daily dose of 450mJ/cm² or not irradiated and then the skin was collected to determine the cellular genes expression profile by Illumina array. The analysis pointed out that more than 300 genes were deregulated in the skin of the HPV38 E6/E7 Tg mice after UVB exposure, compared to WT animals. In particular, HPV38 E6 and E7 oncoproteins were able to downregulate the expression of genes involved in the inflammasome complexes or the downstream effector, such as the pro-inflammatory cytokine IL-18. The results are shown in the heat map in

Figure 17a. To confirm the transcriptome findings, RT-qPCR was performed. The skin of the mice, both WT and Tg, was irradiated with one dose of 50mj/cm² for 5 consecutive days and after 24h the IL-18 mRNA level was determined. Results are reported in figure 17b.

As we can observe in Figure 17b, RT-qPCR confirmed the transcriptome results showing that IL-18 expression was upregulated in wild-type mice after the exposure to UVB, while it was strongly inhibited in the skin of HPV38 E6/E7 Tg mice.

Interestingly, the study carried out by Schwarz and colleagues showed that the injection of recombinant IL-18 in mice exposed to UVB reduced the amounts of apoptotic keratinocytes and DNA damage into the cells, providing evidence for a link between IL-18 and the accumulation of damaged cells by UVB irradiation [217], [218]. Based on this literature data, we performed an experiment injecting recombinant IL-18 (rIL-18) subcutaneously in HPV38 E6/E7 Tg mice. The injection was performed twice per week during the well-established chronic UV-irradiation protocol for 30 weeks [113]. As shown in Figure 17c, the injection of rIL-18 into the mice caused a delay in UVB-induced skin lesions.

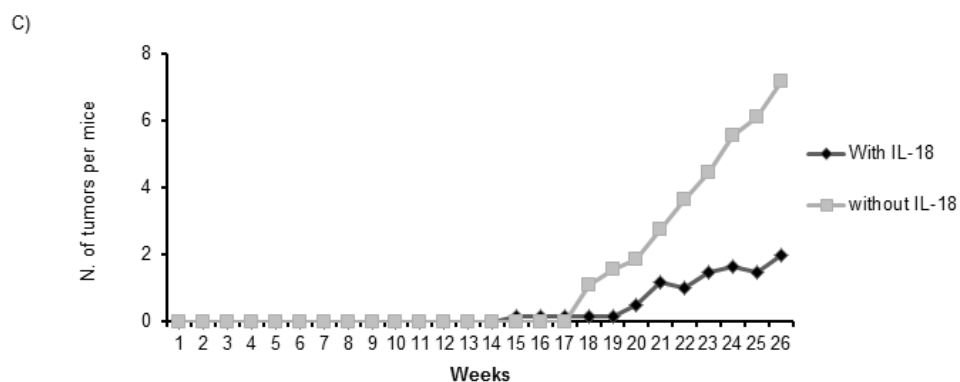
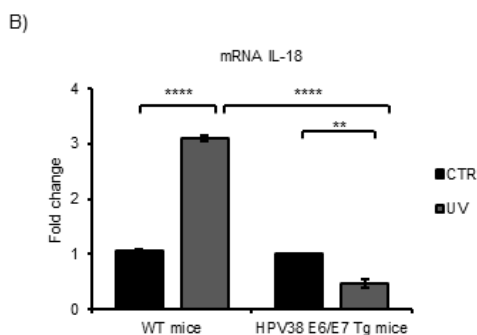
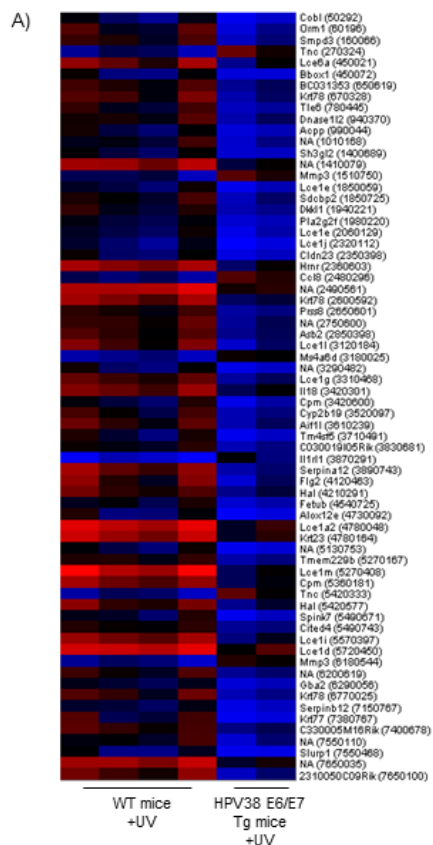


Figure 17. E6 and E7 of HPV38 alter the cellular gene expression induced by UVB. (A) The heat map was generated comparing the expression levels of cellular genes after the exposure to UVB of the wild type and Tg mice. (B) IL-18 mRNA level was determined by RT-qPCR. The amount of IL-18 mRNA extracted from non-treated Tg keratinocytes is taken as reference (=1). (C) **rIL-18 injection causes a delay in UVB-induced skin lesions.** Tg mice were UVB-irradiated for 30 weeks. One group received 300 ng injections of rIL-18 twice per week. The graph reports the number of skin lesions in each mouse, treated or non-treated with IL-18.

2. MODULATION OF IL-18 EXPRESSION AFTER UVB EXPOSURE IN HUMAN KERATINOCYTES EXPRESSING OR NOT HPV38 E6 AND E7

To gain further insight into the mechanism of UV-induced IL-18 expression and the role played by HPV38 E6 and E7 in these events, we used human primary keratinocytes (HPKs), expressing or not the two viral oncoproteins, as experimental model. Cells were irradiated with one dose of UVB (50mj/cm²) and collected after 4h. As observed in the animal model and accordingly to the literature data [201], RT-qPCR showed an up-regulation of IL-18 mRNA level (Figure 18a upper graph) in HPKs after 4h post irradiation. Conversely, the presence of E6 and E7 of HPV38 strongly downregulated this activation.

Interestingly, HPV38 E6 and E7 were also able to block the cleavage and the consequent release of IL-18 protein as shown in the western blot analysis (Figure 18a left) and the ELISA assay (Figure 18a right), indicating that HPV38 uses transcriptional and post-translational mechanisms to inhibit IL-18 production and secretion.

Due to limited life span of primary cells, several experiments were hampered using this in vitro experimental model. To overcome this problem, we have used alternative models such as human spontaneously immortalized keratinocytes, i.e. HaCat and NIKs cell lines, or we developed HPKs expressing the human telomerase reverse transcriptase (hTERT) gene, in order to prolong the life span of the primary cells. All the cell lines were stably transduced with a retrovirus expressing the two HPV38 oncogenes, E6 and E7. Cells were irradiated with one dose of UVB (50mj/cm²) and collected after 4h.

Western Blot analysis showed that after UVB exposure all the cell lines are able to cleave IL-18 and release the protein into the supernatant (Figure 18b). As observed in HPKs, the expression of HPV38 E6 and E7 genes strongly inhibited IL-18 secretion, indicating that the HPV38 mechanism in

altering the production of the mature form of IL-18 via post-translational mechanisms is conserved in several in vitro experimental models. Surprisingly, none of these three cell lines were able to modulate IL-18 mRNA expression when exposed to UVB radiation, as we can see in the RT-qPCR (Figure 18c). However, the expression of the two viral oncoproteins strongly downregulated the basal expression of IL-18 both at mRNA and protein levels (Figure 18).

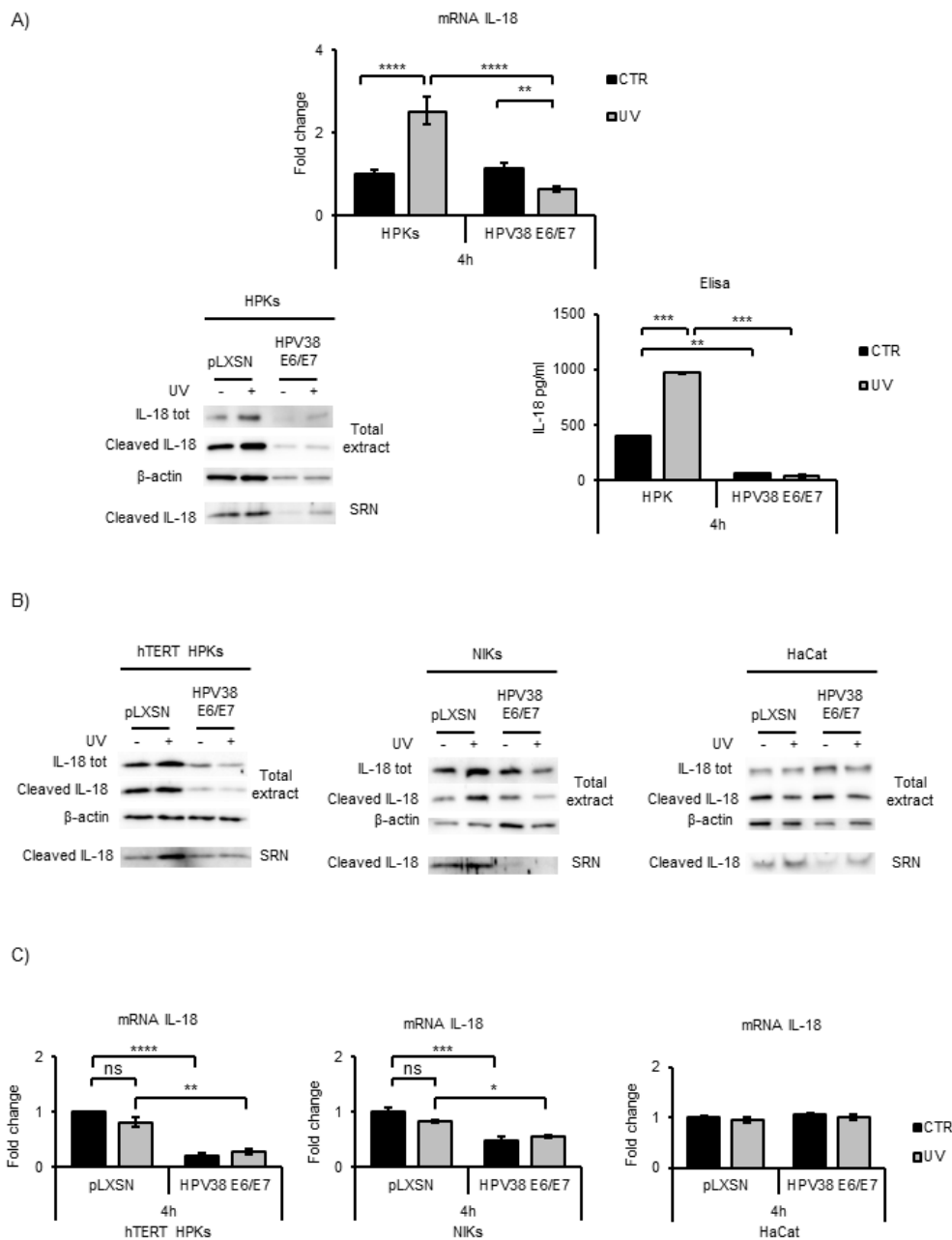


Figure 18: Human primary keratinocytes expressing HPV38 E6 and E7 inhibit IL-18 mRNA expression and secretion after UVB exposure.

(A) Total RNA and total protein were extracted and the supernatant was collected from HPKs irradiated with UVB or not. IL-18 expression levels were measured by RT-q PCR and using GAPDH gene as an internal control (upper panel). Proteins extracts were analyzed by immunoblot for the indicated antibody (left panel). The supernatant was collected and concentrated, and IL-18 concentration was measured by ELISA assay (right panel). Data shown are the mean of three independent experiments carried out on three different HPKs from healthy donors. *, $P<0.05$; **, $P<0.01$; ns, non-significant. **Human immortalized keratinocytes modulate IL-18 expression after UVB exposure only at protein level.**

(B, C) Total protein and total RNA were extracted from HaCat, NIKs and HPKs hTERT cell lines irradiated with UVB ($50\text{mj}/\text{cm}^2$) or not. Proteins extracts, from cell lysate and from the supernatant, were analyzed by immunoblot for the indicated antibody and bands intensity were quantified and normalized to β -actin (panel B). IL-18 expression levels were measured by RT-q PCR and using GAPDH gene as internal control (panel C). Data shown are the mean of three independent experiments. *, $P<0.05$; **, $P<0.01$; ns, non-significant.

3. ANALYSIS OF THE TRANSCRIPTIONAL REGULATION OF IL-18 GENE.

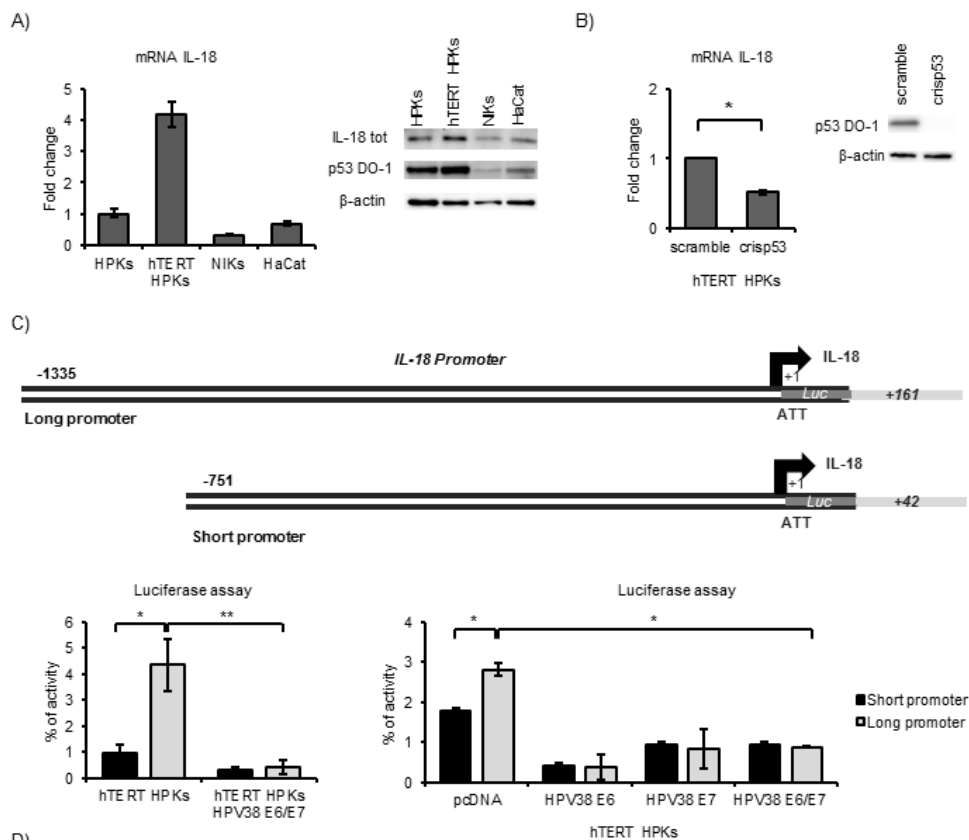
Based on our results that highlighted the loss of *IL-18* transcriptional activation by UV irradiation in immortalized keratinocytes (i.e. HaCaT, NIKs and hTERT HPKs), in comparison to HPKs, we hypothesized that immortalization of these cells correlated with alteration of the transcriptional functions of potential transcription factors involved in the UV-induced IL-18 expression. Moreover, the analysis of IL-18 mRNA expression of the immortalized keratinocytes showed a strong upregulation of the basal level of IL-18 compared with HPKs, an event strongly appreciate in hTERT HPKs.

It is well-known that UVB radiation causes an accumulation of p53 in HPKs [265], [266], thus it is possible that the induction of IL-18 in UV-exposed keratinocytes is mediated by p53. Interestingly, it has been reported that ectopic expression of hTERT in HPKs induces p53 accumulation [267], [268]. Based on this literature data, we evaluated whether in the immortalized keratinocytes the IL-18 basal mRNA levels correlate with p53 expression levels. As shown in Figure 19a, p53 levels tightly correlated with the basal levels of IL-18 expression. In particular, p53 and IL-18 were strongly accumulated in hTERT HPKS (Figure 19a).

To further corroborate the link of p53 with IL-18 expression, we generated p53 knockout hTERT HPKs using Crispr/Cas9 technology. Expression of p53 was efficiently silenced when the gene was deleted compared to the control cells transfected with Crispr/Cas9 vector containing the scramble sequence. IL-18 mRNA expression was evaluated by RT-qPCR that showed a downregulation of the cytokine expression after p53 knockdown (Figure 19b). These results support our hypothesis of the possible role played by p53 in this event.

To evaluate the ability of HPV38 E6 and E7 in the repression of IL-18 promoter activity, we performed a transient transfection experiment in hTERT HPKs cell line using a vector containing the isolated IL-18 promoter cloned in front of the luciferase reporter gene. In agreement with the RT-PCR data, IL-18 promoter is very active in hTERT HPKs (Figure 19c). A small deletion of approximately 500 nucleotides (short promoter) resulted in the loss of IL-18 promoter activity, indicating that this region contains key response elements. In hTERT HPKs, that were stably transduced or transiently transfected with E6 and E7, we observed a strong repression of IL-18 promoter. In addition, the results obtained with the transient transfection of HPV38 E6 or E7 alone showed that both are able to efficiently repress the activity of IL-18 promoter.

In order to clarify the role played by p53 in the modulation IL-18 mRNA expression, we investigated the IL-18 promoter by using TFBIND bioinformatics tools. The analysis revealed the presence of 15 putative binding sites for p53. Next, we divided the promoter in five regions and designed five different pairs of primers in order to cover all the putative binding sites (Figure 19d). We performed chromatin immunoprecipitation (ChIP) assays in hTERT HPKs and HPV38 E6/E7 hTERT HPKs cells. The results showed that p53 is mainly recruited in two distinct regions of the promoters, which includes four p53 putative responsive elements. Importantly, HPV38 E6 and E7 efficiently prevent p53 recruitment to these IL-18 promoter regions (Figure 19d).



D)

>IL18_promoter_region1:from3666to5000:chr11:112036175-112034841:len=1335:Strand-

ctacttgatccactctgctcttcattgtaattggcccaattggactctacagttggaagtgaaaacttactattcaactgagtcacgtatgtattcttcatatactcttaaggatcatattttttctctgatagtc
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>IL18_exon1:from5001to5212:chr11:112034840-112034629:len=212:Strand-

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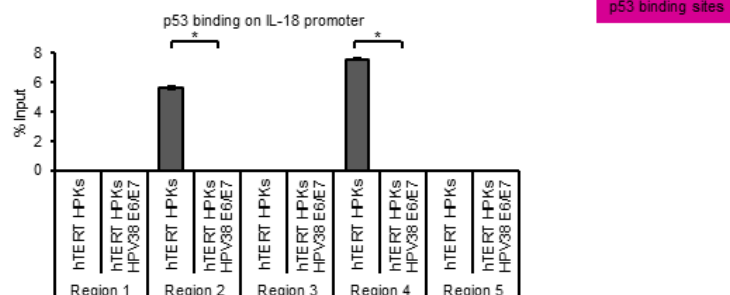


Figure 19: Possible role of p53 in the transcriptional regulation of IL-18 gene. (A) Total RNA and total protein were extracted from HPKs, HaCat, NIKs and hTERT HPKs cell lines expressing or not HPV38 E6 and E7. IL-18 expression levels were measured by RT-qPCR and using GAPDH gene as an internal control. Proteins extracts from cell lysate were analyzed by immunoblot for the indicated antibodies and bands intensity were quantified and normalized to β -actin. (B) Total RNA and total protein were extracted from hTERT HPKs expressing wt p53 (scramble) or Crispr/Cas9 deleted p53 (crisp p53). IL-18 expression levels were analyzed by RT-q PCR while the efficiency of p53 knockdown was determined by western blot analysis. Data shown are the mean of two independent experiments. *, $P < 0.05$; **, $P < 0.01$. (C) **The promoter activity of IL-18 gene is repressed in hTERT HPKs cell line expressing E6 and E7 of HPV38.** Scheme of IL-18 promoter luciferase constructs: the long promoter (-1335/+161) and the short promoter (-751/+42). hTERT HPKs and hTERT HPKs HPV38 E6/E7 cells were transfected with the two promoter constructs. After 24h cells were harvested and the promoter activity was measured (left panel). hTERT HPKs were co-transfected with IL-18 short and long promoters and with E6 and E7 together or alone. After 24 hours, the cells were harvested and the promoter activity was measured (right panel). Data are the average of three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$. (D) **p53 is recruited on IL-18 promoter.** IL-18 promoter region: exon 1 from 3666 to 5000 (left panel). ChIP was performed in hTERT HPKs and hTERT HPKs HPV38 E6/E7 cells using p53 antibody. Part of the total chromatin fraction (1/10) was used as input. RT-qPCR was performed using specific primers flanking the five regions of IL-18 promoter. The histogram shows the percentage of p53 binding to the promoter after the subtraction of the background of nonspecific IgG control (right panel). Data

*are expressed as percentage of input and represent the average of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.*

4. RESPONSES OF OTHER COMPONENTS OF THE INFLAMMASOME COMPLEXES AFTER UVB EXPOSURE IN HPKs.

Taking into account that IL-18 is the final downstream effector of the inflammasome activation, we further evaluated the relationship between HPV38 E6/E7 and UVB exposure in innate immune response activation. HPKs or HPV38 E6/E7 HPKs were irradiated with UVB (50mj/cm²) and collected 4 hours after irradiation. RT-qPCR and Western Blot analysis showed the capability of HPKs to increase the levels of AIM2 and the relative adaptor protein ASC after the exposure to UVB (Figure 20a), while NLRP1 inflammasome, which is typically associated to keratinocytes [128] appeared to be not involved in this event (Figure 20b). Interestingly, the expression of HPV38 E6 and E7 oncoproteins in hTERT HPKs abolished the UV-induced up-regulation of AIM2 and ASC.

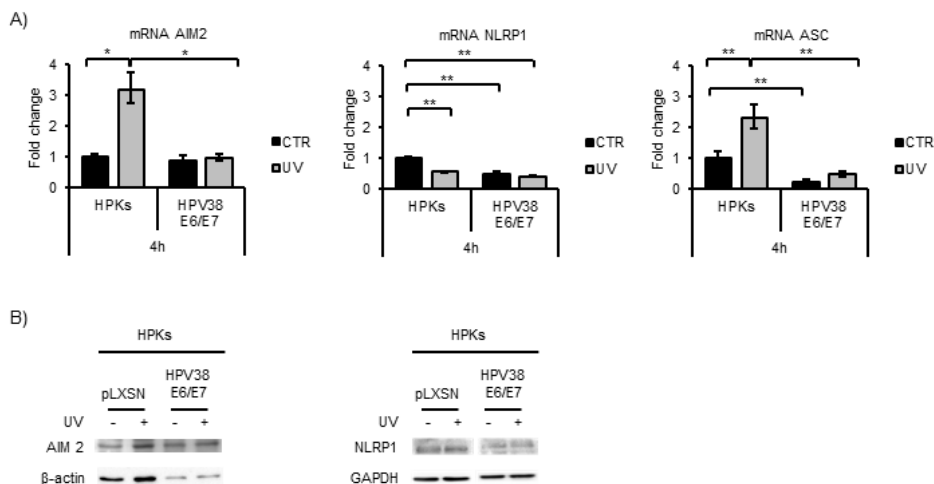


Figure 20: UVB exposure induces an upregulation of AIM2 and ASC inflammasome complex in HPKs. (A, B) Total RNA and total proteins were extracted from HPKs and HPKs HPV38E6/E7 irradiated with UVB (50mj/cm²) or not and collected after 4 hours. AIM2, ASC and NLRP1 expression levels were measured by RT-q PCR and using GAPDH gene as an internal control (upper panels). Protein extracts from cell lysates were analyzed by immunoblot for the indicated antibodies and bands intensity were quantified and normalized to β-actin (bottom panels). Data shown are the mean of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

DISCUSSIONS

Several epidemiological studies have reported that the incidence of non-melanoma skin cancers (NMSCs) in fair skinned adult populations has considerably increased in the last years, exceeding that of all other cancers [1], [2], [5]. This phenomenon has generated a great interest in understanding the pathogenesis of this type of cancer in order to find new non-invasive treatments. Many risk factors have been associated to the development of NMSCs but undoubtedly the most important is the sun exposure. Indeed, the ultraviolet (UV) radiation causes skin damage that after repeated exposure can lead to the formation of skin lesions and then develop into skin cancer [8], [10]. Moreover, several clues have indicated infectious agents as additional risk factor for skin carcinogenesis and this hypothesis is supported by the evidence that people with a compromised immune system, such as organ transplant recipients (OTRs), are much more susceptible to develop NMSC compared to the normal population [9]. In addition, our skin is colonized by different infectious agents and, among them, the cutaneous human papillomaviruses, belonging to the beta genus, are considered the most likely additional etiological factor for NMSC. In fact, β -HPV types were isolated for the first time in EV patients, who are highly susceptible to β -HPV infection and UV-induced skin carcinogenesis [27], [29], [105]. All these findings support the potential role of β -HPV types in the development of skin cancer, together with sun-exposure. Moreover, several functional studies carried out in *in vitro* as well as in *in vivo* experimental models, have also highlighted the transforming properties of the viral proteins E6 and E7 from some β -HPV types. For instance, the two viral proteins are able to deregulate key molecular pathways related to cell survival, proliferation, apoptosis and DNA repair [108]. To date, only E6 and E7 from β -HPV 38 and 49 have demonstrated the ability to immortalize human primary keratinocytes, like the two oncoproteins of HPV16. HPV38 E7 associates with pRb, promoting its degradation, while E6 is not able to

promote p53 degradation, as it has been shown for HPV16. Moreover, HPV38 is able to alter the deregulation of the p53 network by inducing the accumulation of a p53 antagonist, $\Delta Np73\alpha$, which in turn inhibits the expression of several p53-regulated-genes encoding positive regulators of apoptosis [109], [110].

Based on these *in vitro* results, our group generated a mouse model to further investigate the oncogenic properties of HPV38 and the interaction between E6/E7 and the environmental risk factors potentially involved in skin carcinogenesis, such as UV radiation. In this mouse model, HPV38 E6 and E7 are expressed in the undifferentiated basal level of the epithelia under the control of the keratin 14 (K14) promoter.

The experiments carried out using this mouse model showed that Tg mice were highly susceptible to UV irradiation since their exposure to UVB caused the development of skin pre-malignant lesions and subsequently squamous cell carcinoma. In contrast, wild type mice exposed to the same doses of UV irradiation did not develop any lesions [113]. These findings indicated that HPV38 has developed mechanisms to alter the response of normal skin keratinocytes to UV irradiation, creating a favorable environment for the development of skin cancer.

However, the mechanism of this synergism has not yet been elucidated and our ongoing studies are now focused on better understanding the events involved in UV-induced NMSC, both in *in vivo* and in *in vitro* models expressing E6 and E7 from HPV38.

Our findings obtained in HPV38 E6/E7 Tg mice showed that the development of skin cancer tightly correlated with the accumulation of the typical C>T mutations induced by UV irradiation. In addition, the analysis of the entire transcriptome profile in wild-type and Tg mice non-exposed or exposed to UV irradiation revealed that more than 300 genes were deregulated in the Tg mice after the UV exposure, compared to the wild-

type animals. In particular, the viral oncoproteins were able to downregulate the expression of proteins involved in the inflammasome complexes or the downstream effectors, such as pro inflammatory cytokines belonging to IL-1 family, e.g. IL-18.

Literature data provided evidence that the exposure of keratinocytes cell lines to ultraviolet radiation resulted in the activation of pathways involved in the innate immune response, such as the inflammasome pathway, which leads to the secretion of specific pro-inflammatory cytokines, like IL-1 β or IL-18 [201]. Interestingly, Dell'Oste and colleagues have demonstrated that HPV38, as well as HPV16, were able to block IL-1 β secretion [264]. As observed in the transcriptome profile, RT-qPCR showed that the expression of the pro-inflammatory cytokine IL-18 was strongly inhibited in HPV38 E6/E7 Tg mice when exposed to UVB radiation. In contrast, the wild-type mice showed an up-regulation of cytokine expression when exposed to the same dose.

More interestingly, in agreement with the published data obtained by Schwarz and colleagues, that provided evidence of the involvement of IL-18 in DNA repair damage induced by the UV irradiation [217], [218] our preliminary results showed that the injection of exogenous rIL-18 into HPV38 E6/E7 Tg mice caused a significant delay in the appearance of UVB-induced skin lesions. Most likely, the injection of the exogenous cytokine into these mice also causes a decrease in the accumulation of DNA damage UV-induced, but more experiments are necessary to corroborate this hypothesis. This result was in line with the data obtained by Trinchieri and colleagues that showed the highest susceptibility of IL18 $^{-/-}$ and IL18r1 $^{-/-}$ mice to develop colitis and polyp compared to wild-type mice [259], further corroborating the role played by IL-18 in cancer development.

The results obtained with the *in vivo* mouse model were confirmed also using human primary keratinocytes (HPKs) as an experimental model. Even under *in vitro* condition, we observed an UV-induced upregulation of IL-18 mRNA expression in HPKs, as well as of some key inflammasome components, such as AIM2 and the adaptor protein ASC, but not of NLRP1, which is known to be the most expressed inflammasome in keratinocytes [128]. Interestingly, experiments in other cell lines, such as immortalized keratinocytes HaCat and NIKs as well as primary keratinocytes with extended life span due to the overexpression of hTERT, showed high basal levels of IL-18 mRNA levels that were not further increased by UV irradiation. However, the presence of the two viral oncoproteins significantly down regulated the basal level of IL-18 expression in HPKs hTERT and NIKs cell lines. Of note, it is known that in the cell lines that we used as experimental models p53 status is altered; in fact, HPKs hTERT and NIKs [269] showed an accumulation of wild type p53 while in HaCat this protein is mutated [270]. Furthermore, it is also well-known that UVB radiation caused an accumulation of p53 in HPKs as well as the an over-expression of the hTERT gene [265], [266], [271]. In order to correlate p53 expression with IL-18 modulation at basal level in those cell lines we observed that in our systems high level of p53 expression corresponded to IL-18 accumulation, an event that was strongly appreciated at the mRNA level in HPKs hTERT cell line when compared with human primary keratinocytes.

More interestingly, we found a reduction of IL-18 expression after the knockdown of p53 from HPKs hTERT cell line. These findings supported our hypothesis that p53 may play a role in the regulation of these events, miming the effect of UVB exposure in the up-regulation of the transcription of IL-18.

It is also well known about the ability of p53 to control the expression of target genes by either activating or inhibiting the activity of p53 responsive promoters [272]. For instance, p53 can contribute to immune response by the direct transactivation of key components of immune signaling pathways, i.e. TLR3 or TLR9 in response to cellular stress [273], [274]. Our bioinformatics analysis of the IL-18 promoter revealed the presence of several putative p53 responsive elements and ChIP experiments confirmed that p53 is recruited to IL-18 promoter. Furthermore, this event was completely abolished in the *hTERT* HPKs cell line expressing E6 and E7 of HPV38.

In addition, also the analysis of the promoter activity, through use of a vector containing the isolated IL-18 promoter cloned in front of the luciferase reporter gene, showed a strong activation of IL-18 promoter in *hTERT* HPKs only in absence of the two viral oncoproteins.

Interestingly, the analysis of the protein level of IL-18 showed that the cytokine was cleaved and released into the supernatant after UVB irradiation in all the experimental models used in this study, in accordance with the literature data [201], while HPV38 E6 and E7 were able to block the cleavage and the consequent release of the protein.

In conclusion, our data clearly demonstrated that E6 and E7 were able to interfere with the inflammasome pathway at different levels, leading to a complete block of the transcription as well as of the secretion of the pro-inflammatory cytokine, such as IL-18. In particular, our study provides new possible explanations for the cooperation between UV irradiation and β -HPV types in cellular transformation.

However, future studies are necessary to further elucidate the mechanisms of maturation and secretion of IL-18 and how the virus blocks this event.

CONCLUSIONS

To date, almost 20% of human cancer worldwide are attributed to the viral infection [20]–[22] and in particular the association between HPV and the development of cancer has been broadly studied. As reported above, E6 and E7 are the main players for cancer development since they are able to deregulate many cellular events that favor the transformation of infected cells [86], [108]. In particular, the evasion of the immune system response by the virus is another important key event in cancer progression. In fact, many studies have demonstrated the ability of the mucosal HR HPV types to inhibit the innate and adaptive immune response but very little is known about the impact of viral oncoproteins from β -HPV types on the immune surveillance. The inflammasome complexes are important players in the innate immune response against viruses but also in this case there is limited published data regarding the activation of the immune system in keratinocytes [154].

In the present work, we have provided evidence that the expression of E6 and E7 oncoproteins from β -HPV38 in human primary keratinocytes causes the inhibition of UV-induced inflammasome response, by the alteration of the gene expression profile of the host. We demonstrated that the two oncoproteins are able to impair the UV-mediated production of protein involved in the inflammasome pathway, such as through the downstream effector IL-18.

Here, we also showed a novel mechanism of transcriptional regulation of the promoter region of IL-18 mediated by p53. The structure of this promoter has been previously characterized and has revealed the presence of several putative responsive elements for different transcription factors [203], but not for p53. Our findings also exhibited the recruitment of p53 on the promoter region, which is completely abolished in presence of the viral oncoproteins. Moreover, further studies will need to better understand the role of p53 in the regulation of IL-18 promoter.

Based on these findings, we could hypothesize that the down regulation of IL-18, due to the presence of E6 and E7, may impair the ability of keratinocytes to recruit the cells of the immune system to the UV-exposed area, causing a defect in the elimination of keratinocytes harboring DNA damage. These alterations also may cause an accumulation of UV-induced DNA damage and subsequently the development of skin cancer. Together these findings provide further lines of evidence for cutaneous β -HPV38 in the development of NMSC after UV-exposure.

Furthermore, more study are necessary to better characterize the molecular mechanisms involved in the deregulation of these events mediated by HPV38 and possible other β -HPV types.

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SCIENTIFIC PRODUCTS

List of scientific products produced during the PhD Course (November 2014-October 2017)

Publications

The manuscript describing the results presented in this thesis is in preparation.

- Laura Pacini, Maria Grazia Ceraolo, Assunta Venuti, Giusi Melita, Uzma A. Hasan, Rosita Accardi, Massimo Tommasino; “*UV Radiation Activates Toll-like Receptor 9 Expression in Primary Human Keratinocytes, an Event Inhibited by Human Papillomavirus Type 38 E6 and E7 Oncoproteins.*” J Virol. 2017 Jul 19. pii: JVI.01123-17. doi: 10.1128/JVI.01123-17

Abstracts

- Laura PACINI, Maria Grazia CERAOLO, Gennaro ALTAMURA, Rosita ACCARDI, Massimo TOMMASINO, “*E6 and E7 of human papillomavirus 38 alters the UV-induced cellular response by inhibiting the expression of toll-like receptor 9*”. 4th Workshop EMERGING ISSUES IN ONCOGENIC VIRUS RESEARCH, 15-16 June 2016, San Pietro in Bevagna, Manduria, Italy.
- Lucia MINONI, Cécilia SIRAND, Maria Grazia CERAOLO, Gennaro ALTAMURA, Daniele VIARISIO, Martin MÜLLER, Rosita ACCARDI, Massimo TOMMASINO, “*In vitro assays classify the HPV beta-3 species in low-, intermediate-, and high risk type*”. 4th Workshop EMERGING ISSUES IN ONCOGENIC VIRUS RESEARCH, 15-16 June 2016, San Pietro in Bevagna, Manduria, Italy.
- Rosita ACCARDI, Cécilia SIRAND, Maria Carmen ROMERO MEDINA, Mariafrancesca SCALISE, Maria Grazia CERAOLO,

Ingunn Tjelde FAUSKERUND, Gennaro ALTAMURA, Hector HERNANDEZ-VARGAS, Roberto FERRARI, Cesare INDIVIERI, Massimo TOMMASINO, *“p53 plays a key role in regulation of cellular adhesion and migration in HPV38 E6/E7- expressing keratinocytes”*. 4th Workshop EMERGING ISSUES IN ONCOGENIC VIRUS RESEARCH, 15-16 June 2016, San Pietro in Bevagna, Manduria, Italy.

- Shankhadeep DUTTA, Lucia MINONI, Davide D. ESPOSTI, Laura Pacini, Maria Grazia CERAOLO, Maria Carmen ROMERO MEDINA, Cécilia SIRAND, Sandrine MCKAY-CHOPIN, Rosita Maria ACCARDI, Tarik GHEIT, Massimo TOMMASINO, *“Alterations of microRNA expression in keratinocytes containing E6 and E7 of beta-3 HPV types”*. 4th Workshop EMERGING ISSUES IN ONCOGENIC VIRUS RESEARCH, 15-16 June 2016, San Pietro in Bevagna, Manduria, Italy.

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*There is only one thing that makes a
dream impossible to achieve: the **fear of failure**...*
*...but, when you want something,
all the universe conspires in helping you to achieve it."*
(Cit. Paulo Coelho – **The Alchemist**)

*"Don't give up.
Normally it is the last key on the ring which opens the door."*
(Cit. Paulo Coelho - **Manuscript Found in Accra**)